Regulatory branch points affecting protein and lipid biosynthesis in the diatom \textit{Phaeodactylum tricornutum}

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\textbf{Abstract}

It is widely established that nutritional nitrogen deprivation increases lipid accumulation but severely decreases growth rate in microalgae. To understand the regulatory branch points that determine the partitioning of carbon among its potential sinks, we analyzed metabolite and transcript levels of central carbon metabolic pathways and determined the average fluxes and quantum requirements for the synthesis of protein, carbohydrates and fatty acid in the diatom \textit{Phaeodactylum tricornutum}. Under nitrate-starved conditions, the carbon fluxes into all major sinks decrease sharply; the largest decrease was into proteins and smallest was into lipids. This reduction of carbon flux into lipids together with a significantly lower growth rate is responsible for lower overall FA productivities implying that nitrogen starvation is not a bioenergetically feasible strategy for increasing biodiesel production. The reduction in these fluxes was accompanied by an 18-fold increase in $\alpha$-ketoglutarate (AKG), 3-fold increase in NADPH/NAD$^+$, and sharp decreases in glutamate (GLU) and glutamine (GLN) levels. Additionally, the mRNA level of acetyl-CoA carboxylase and two type II diacylglycerol-acyltransferases were increased. Partial suppression of nitrate reductase by tungstate resulted in similar trends at lower levels as for nitrate starvation. These results reveal that the GS/GOGAT pathway is the main regulation site for nitrate dependent control of carbon partitioning between protein and lipid biosynthesis, while the AKG/GL(N/U) metabolite ratio is a transcriptional signal, possibly related to redox poise of intermediates in the photosynthetic electron transport system.

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\textbf{Abbreviations:} GS, glutamine synthetase; GOGAT, glutamine oxoglutarate aminotransferase; GDH, glutamate dehydrogenase; GLN, glutamine; GLU, glutamate; AKG, $\alpha$-ketoglutarate.

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1. Introduction

The appropriation of photosynthetically fixed carbon in microalgae is strongly affected by environmental conditions. Under optimal growth conditions, most fixed carbon is allocated to proteins [1]. In contrast, under suboptimal conditions, especially nitrogen limitation, cells divert most of the fixed carbon into nitrogen deficient compounds such as lipids and/or carbohydrates, at the expense of growth [2]. The tradeoff between growth rate and lipid content has been one of the biggest factors preventing the industrial production of algal biodiesel at prices competitive with fossil sources [3]. Hence, considerable effort has gone into understanding the molecular mechanisms that determine the fate of photosynthetically fixed carbon under stress conditions [2]. In this paper we use a metabolic analysis to identify the regulatory branch points in the ‘carbon decision tree’ under nitrogen limitation in a marine diatom.

Inspection of the intermediate metabolism of eukaryotic algae (Fig. 1) reveals several potential regulatory branch points that could control the fate of carbon. Pyruvate (Pyr) is the first key intermediate in this process. This 3-carbon molecule can take several alternative paths in the metabolic network which will determine if the carbon is deposited into amino or fatty acids (Fig. 1). Pyruvate can be directly aminated to generate alanine and thus proteins (denoted as step (1) in Fig. 1). More commonly however, pyruvate undergoes decarboxylation to...
form acetyl-CoA (AcCoA), (denoted as step (2) in Fig. 1). AcCoA, the second key intermediate, can either undergo successive oxidation in the TCA cycle or be used by acetyl-CoA carboxylase (ACCase) to form malonyl-CoA (MacCoA), which is the first committed intermediate in the synthesis of lipids [4]. If it enters the TCA cycle it produces α-ketoglutarate (AKG), which can be used for assimilation of ammonium via the glutamate synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle, yielding glutamine (GLN) and glutamate (GLU), (step 3) in Fig. 1) [5]. GLU, the third intermediate, can also be produced by the NAD(P)H-dependent amination of AKG via the reversible glutamate dehydrogenase (GDH), step (4) in Fig. 1. However the contribution of this pathway for assimilation of ammonium was shown to be minor in relation to the GS/GOGAT cycle as its Michaelis constant $K_m$ for ammonium is 1000-fold higher than GS’s [5]. Thus, AKG is an essential carbon source for the synthesis of glutamate (GLN) and glutamate (GLU), from which many other amino acids and cofactors are produced. Finally, two anapleurotic pathways exist to replenish AKG in the TCA cycle: pyruvate may undergo carboxylation to form oxaloacetate via step (5) in Fig. 1, or AKG can be derived from the deamination of glutamic acid by the catabolism of proteins via GDH in step (4). In conclusion, there are at least five key carbon branch points that can influence the supply of intermediates, and hence the fate of carbon, all of which intersect in a small part of the metabolic processes centered on pyruvate and the TCA cycle.

In eukaryotic microalgae, nitrate reductase (NR) is responsible for the NAD(P)H dependent reduction of nitrate to nitrite, and is sequentially coupled to ferredoxin-dependent nitrite reductase (NiR) for production of ammonium which is used in amino acid and protein synthesis (Fig. 1) [6]. Hence, coordinated regulation of NR by several factors including the cellular redox poise provides the initial mechanism for controlling the conversion of intracellular nitrate for downstream biosynthesis reactions [7]. The cellular redox poise has also been implicated in controlling pathways of carbon metabolism such as lipid biosynthesis [8].

Although phylogenetically distant from diatoms, cyanobacteria use AKG and GLN or GLU to report the C:N status of the cell in real time [9,10]. The levels of these metabolites are sensed by NtcA, a global nitrogen regulator that can function as both a transcriptional activator and a repressor. For instance, NtcA binds and activates the promoters of glnA (encoding glutamine synthetase), the nir operon (nitrate uptake) and icd (isocitrate dehydrogenase) while it also represses transcription of gifA and gifB (encoding inactivating factors of glutamine synthetase) [10].

Based on the logic of intermediate metabolism of acetyl-CoA, Dunahay and coworkers (1996) tried to increase carbon flow towards lipid biosynthesis by increasing the activity of ACCCase. In the first genetic transformation of a diatom, these researchers overexpressed ACCCase in Cyclotella cryptica [11]. The transformant strain had increased ACCCase activity, but the phenotype did not show any significant change in lipid content [2]. Similar results were achieved when ACCase of Arabidopsis thaliana was expressed in Brassica napus and Solanum tuberosum [12,13]. These results indicate that ACCCase does not control the flux of carbon into lipids, thus, leaving the regulatory branch point for lipid biosynthesis poorly defined.

In the present study we sought to understand the mechanism of carbon partitioning under nitrate depletion in the marine diatom Phaeodactylum tricornutum. We probed changes in intracellular metabolite pools and transcripts of genes involved in central carbon metabolism. For this, we compared cells grown under nitrate replete (control), nitrate starved (−NO$_3$), and nitrate replete with tungstate ($W$). In the absence or limitation for molybdenum, tungsten can substitute in the active site of nitrate reductase, rendering the enzyme catalytically inactive [14]. Our results on the influence of nitrate assimilation/deprivation on terminal products in P. tricornutum reveal that regulation occurs at the level of the TCA/GS/GOGAT/GDH pathways, similar to cyanobacteria, and is consistent with the multi-functional role of AKG in microbial phototrophs.

2. Materials and methods

2.1. Culturing system and sampling

P. tricornutum was obtained from the National Center for Marine Algae and Microbiota (NCMA, formerly CCMP) accession Pt1 8.6 (clone CCMP 632) was maintained in an artificial sea water medium [15] supplemented with F/2 nutrients [16] and buffered with 2 mmol L$^{-1}$ Tris to pH 8 (hereafter called F/2). Cultures were grown at 18 °C, with air bubbling, under continuous white LED light at a photon flux density of 200 µmol m$^{-2}$ s$^{-1}$. Pre-inocula were grown with NaN$_3$ (0.88 mmol L$^{-1}$) as the sole nitrogen source. Exponentially growing pre-inocula were centrifuged (5500 g, 10 min) and washed twice with nitrate-free F/2 medium and inoculated in triplicate at a concentration of 2.5 × 10$^5$ cells mL$^{-1}$ into fresh F/2 medium with nitrate (0.88 mmol L$^{-1}$, control), F/2 medium without any nitrogen source (−NO$_3$) and F/2 medium with nitrate (0.88 mmol L$^{-1}$) and sodium tungstate (0.88 mmol L$^{-1}$, $W$) replacing molybdate in the trace metal mixture. All cultures were grown for 3 days in the same conditions as above. Known numbers of cells were then filtered, flash frozen in liquid nitrogen, and kept at −80 °C until analysis. The only exception was the metabolite extraction which was performed on fresh samples.

2.2. Cell number, cell volume and chlorophyll determination

Cell numbers were measured with a Coulter counter multisizer 3 (Beckman Coulter Inc, Fullerton, CA, USA). After 3 days of growth, cell volume (approximated to a prolate spheroid) was determined by measuring the length and width of 100 cells' fatty acids to get fatty acids methyl esters (FAMEs). 5 × 10$^7$
cells were filtered onto 25 mm GF/F filters (Whatman) and subsequently extracted and transesterified in a single step as reported previously [19]. Analysis of the hexane fraction containing the FAMEs was performed by gas chromatography as in Ref. [14]. Helium was used as the carrier gas at 179 kPa.

Triacylglycerols (TAGs) were extracted with methyl tert-butyl ether from 5 × 10⁷ cells collected on GF/F filters [20]. TAG estimation was performed by thin layer chromatography (TLC). Briefly, after resuspension of dried samples in a known volume of chloroform:methanol:water mix (60 mL:30 mL:4.5 mL), samples were spotted on silica gel 60 10 cm × 20 cm TLC plates (Millipore) with hexane:diethyl ether:acetic acid mix (80 mL:20 mL:2 mL) as running solvent. The plates were sprayed with 50 mg L⁻¹ primuline in an 80% volume fraction acetone solution, dried for several hours and the TAGs were visualized under a UV lamp. ImageJ [17] was used to quantify the intensity of the spots which were compared to spots of known amounts of a commercial mono-, di-, and triglyceride standard mix (1787, Sigma–Aldrich).

2.4. Carbohydrate content determination

5 × 10⁷ cells were collected in 1.2 µm pore size polycarbonate filters (Millipore, Billerica, MA, USA). Total carbohydrate levels (intracellular reducing pentoses and hexoses) were measured by reaction with anthrone [21] with a procedure adapted to diatoms [22].

2.5. Total protein determination

For total protein determination, 2 × 10⁷ cells were filtered onto 1.2 µm pore size polycarbonate filters and re-suspended in 1× denaturing extraction buffer (300 µL), containing 140 mmol L⁻¹ Tris base, 105 mmol L⁻¹ Tris–HCl, 0.5 mmol L⁻¹ EDTA, 20 g L⁻¹ lithium dodecyl sulfate, 10% volume fraction glycerol, and protease inhibitor (1 mL:200 mL, P2714, Sigma–Aldrich). After re-suspension, the samples underwent two cycles of freezing in liquid nitrogen and thawing with a Microson ultrasonic cell disruptor XL (Qsonica, Newtown, CT, USA). After pelleting insoluble cell debris total protein concentration was measured using a modified Lowry assay (Bio-Rad DC 500-0111, Hercules, CA, USA). Bovine gamma globulin was used as the protein standard.

2.6. Metabolite extraction and LC-MS/MS

Intracellular metabolite extraction and analysis was made via liquid chromatography mass spectrometry (LC-MS/MS). 2 × 10⁷ cells were filtered onto a 0.45 µm pore size nylon filter (Pall Corporation, Port Washington, NY, USA) and immediately cold extracted [23]. Samples were then analyzed on a 1200-series Agilent LC system coupled to an Agilent 6410 Triple Quadrupole (QQQ) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), using previously optimized Selective Reaction Monitoring parameters [23]. Ion-pairing chromatography was employed using a Pursuit XR3 C18 (50 mm × 2 mm, Varian, Palo Alto, CA, USA.). Mobile phase A consisted of 10 mmol L⁻¹ tributylamine (pairing agent), 11 mmol L⁻¹ CH₃OH while mobile phase B was pure methanol. The chromatography program of mixture of mobile phase B into mobile phase A was as follows: 0 min (Volume fraction of B: 0%), 8 min (Volume fraction of B: 40%), 10 min (Volume fraction of B: 40%), 12.5 min (Volume fraction of B: 90%), 18 min (Volume fraction of B: 90%), 18.1 min (Volume fraction of B: 0%). A 6 min post-run equilibration period at 0% B was used. Intracellular metabolite pools were quantified by comparison to a calibration curve established by spiking a standard mixture of the target compounds at 10 mmol L⁻¹~10,000 nmol L⁻¹ into a sample background and determining the per-metabolite linear response of the instrument signals to the expected concentrations.

2.7. Metabolic analysis and real time qPCR

Genes that are involved in lipid biosynthesis and central carbon pathways, were selected from the literature [24] and from both the genome (http://genome.jgi-psf.org/Phatr2/Phatr2.home.html) [25] and the expressed sequence tag database (http://www.diatomics.biologie.ens.fr/EST3/seq.php) [26,27]. The prediction of localization of target genes was made using Mitoprot [28], TargetP1.1 [29] and signalP4.0 [30] online tools. Total RNA was extracted from 1 × 10⁸ cells collected on 1.2 µm pore size polycarbonate filters with an RNAeasy plant mini kit (Qiagen, Venlo, Netherlands). Ambion™ Turbo™ DNase (Life Technologies, Carlsbad, CA, USA) was used to remove DNA contamination. PCR was used before cDNA generation to confirm that there was no DNA contamination. Total RNA quantification and quality assessment was made spectrophotometrically with a Nanodrop 1000 (Thermo Scientific). cDNA generation was done using oligo(dT) as primers and the SuperScript™ III reverse transcriptase kit (Life Technologies). The resulting cDNA-generation reaction mixture (1 µL) was directly used as the template for qPCR. Primers for target genes were constructed with NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), designed to anneal near to the 3’ end of the mRNA (Table S1). Standard molecules of known molecular weight, for copy number calculation, were generated by cloning each amplicon onto a TOPO™ TA (Life Technologies) cloning kit. qPCR was made using the Applied Biosystems SYBR® Green PCR Master mix (Life Technologies) on a Mx3000P QPCR system (Agilent Technologies). All standard curves had at least 5 points, covered 5 orders of magnitude and had an R² > 0.94. Each qPCR amplification on biological samples was then compared to its specific calibration curve to calculate the gene copy number present. Calculated copy numbers were normalized to total RNA extracted.

2.8. Quantum requirement and fluxes calculations

Carbon fluxes (µmol cell⁻¹ d⁻¹) into proteins, carbohydrates and fatty acids were calculated according to the equation

\[ N \times \frac{\mu}{dN/dt} \]

where \( N \) is moles of carbon in protein, carbohydrate or fatty acids measured after 3 days, \( \mu \) the cellular specific growth rate and dN/dt the average flux. The average carbon mass fraction in proteins (44%) was calculated using the mass of carbon per mass of amino acid normalized by the relative abundance of each amino acid according to the genetic code. The mass fraction of carbon in carbohydrates (40%) was calculated using glucose as a standard. The mass fraction
of carbon in fatty acids (76%) was calculated using the mass of carbon per mass of fatty acid normalized by the abundance of each fatty acid measured in this study. The quantum requirement for each pool (\(a'\)) was calculated according to [31] using the values for \(a'\) and \(E_e\) values reported in Ref. [14].

### 3. Results

#### 3.1. Physiological responses to nitrate limitation

The control condition presented a growth rate of 0.86 \(d^{-1} \pm 0.04 \, d^{-1}\), while the growth rate of the \(-NO_3\) and the W cultures were 0.36 and 0.76-fold of the control cultures, respectively (Table 1). Chlorophyll \(a\) (Chl \(a\)) content, which generally reflects total biosynthetic nitrogen accumulation at a constant irradiance, was lower for both \(-NO_3\) and W cultures by 0.83 and 0.50-fold, respectively, vs. the control condition (0.12 pg \(\pm\) 0.02 pg cell \^{-1}\) (Table 1). The volume of the cells in the \(-NO_3\) cultures did not change significantly in relation to the control, while the ones in the W condition increased their volume by approximately 1.18-fold (\(p\)-value < 0.01).

Regarding the main carbon sinks, the \(-NO_3\) cultures, had a 1.21-fold increase in levels of FA per cell accompanied by a 0.74-fold decrease in proteins and a 0.29-fold decrease in the carbohydrates relative to the control. In the W condition, the intracellular metabolite pools were measured by LC-MS/MS (Fig. 2). The \(-NO_3\) condition produced an overall decrease of several metabolites in comparison to the control. Most significantly, the nitrogen carriers for biosynthesis, GLU and GLN were reduced 7 and 55-fold, respectively, in the \(-NO_3\) case compared to the control condition. As these metabolites are formed from AKG and ammonium, we looked at intermediates of the TCA cycle. The AKG level increased by 18-fold compared to the control condition, while other TCA metabolites downstream such as succinate increased nearly 3-fold, while fumarate and malate remained unchanged. These data provide definitive evidence for control occurring at (GS/GOGAT)/GDH (steps (3) and (4) in Fig. 1).

The large increase in AKG suggests either increased flux into AKG from the upstream portion of the TCA cycle, greater input through amino acid catabolism via GDH, or decreased output of AKG into the GS/GOGAT pathway. To further constrain the source of AKG, we examined the levels of the TCA metabolites upstream prior to the typically irreversible step at isocitrate dehydrogenase. This analysis revealed a 3-fold decrease in levels of citrate, acetyl-CoA (AcCoA) and pyruvate under \(-NO_3\) condition, suggestive of either faster depletion or reduced input into these pools. AcCoA is the immediate precursor to malonyl-CoA (MaCoA) for FA biosynthesis. MaCoA was significantly decreased (10-fold), in the \(-NO_3\) condition.

As expected, the total pool sizes of adenosine phosphates and both pyridine nucleotide pairs were also highly decreased in the \(-NO_3\) case (Fig. 2). However, the adenylate cell energy charge (CEC) decreased, from 0.74 \(\pm\) 0.03 in the control to 0.63 \(\pm\) 0.06 in the \(-NO_3\) condition (\(p\)-value < 0.05) (Table 2). Regarding the cellular pyridine nucleotide redox poise, the NADPH/NADP\(^+\) ratio was unchanged, while the NADPH/NADP\(^+\) ratio increased 3-fold in the \(-NO_3\) condition vs. the control (\(p\)-value < 0.01) (Table 2). This significant increase in the ratio of NADPH/NADP\(^+\) is consistent with lower nitrate reduction to ammonium and lower activity of other sinks such as the GS/GOGAT cycle and carbon fixation.

In general, the W condition presented fewer changes in the metabolite pools than the \(-NO_3\) condition in comparison to the control (Fig. 2). The levels of AKG, AcCoA, MaCoA, adenosine phosphates, NADH and NAD\(^+\) were unchanged, while the level of NADP\(^+\) decreased by about 60% relative to the control. The CEC and the NADPH/NADP\(^+\) redox poise, were also unchanged for the W condition in comparison to the control. The GLU and GLN levels decreased approximately 1.7 and 12-fold, respectively, indicating some degree of nitrogen limitation. The NADPH/NADP\(^+\) redox poise showed a statistically significant increase, albeit much smaller in magnitude than that of the \(-NO_3\) condition (Table 2).

#### 3.2. Changes in intracellular metabolite pools in response to nitrate limitation

In order to obtain further insight into the metabolic state of cells, the intracellular metabolite pools were measured by LC-MS/MS (Fig. 2). The \(-NO_3\) condition produced an overall decrease of several metabolites in comparison to the control. Most significantly, the nitrogen carriers for biosynthesis, GLU and GLN were reduced 7 and 55-fold, respectively, in the \(-NO_3\) case compared to the control condition. As these metabolites are formed from AKG and ammonium, we looked at intermediates of the TCA cycle. The AKG level increased by 18-fold compared to the control condition, while other TCA metabolites downstream such as succinate increased nearly 3-fold, while fumarate and malate remained unchanged. These data provide definitive evidence for control occurring at (GS/GOGAT)/GDH (steps (3) and (4) in Fig. 1).

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#### 3.3. mRNA abundance of genes of the central carbon metabolism in response to nitrate limitation

In order to relate the metabolite data to the genetic response of cells, the mRNA abundance of key genes involved in the key branch points were measured by real-time RT-qPCR (Fig. 3). The total RNA (ribosomal + messenger + transfer) measured in the \(-NO_3\) condition, was only 44% of that of the control (\(p\)-value < 0.01, \(n = 6\)). In contrast, the total RNA levels extracted from the W condition were 2.3-fold higher than...
those of the control condition (p-value < 0.01, n = 6) (Table 1). For all 18 genes tested, there was a general increase in the abundance of their mRNA levels in both conditions compared to the control. In the eNO/C03 condition, genes involved in TCA cycle were amongst the ones that presented highest increases. Specifically, the highest increase (65-fold) was observed in isocitrate dehydrogenase (Isocitrate DH) mRNA. In respect to lipid biosynthesis genes, a 35-fold increase in the ACCase mRNA abundance was observed in the eNO/C03 condition, while the mRNA levels of diacylglycerol-acyltransferase (DGAT)2B and DGAT2D were increased 3- and 8-fold, respectively, in comparison to the control. The W condition generally had higher fold changes than those of the nitrogen-starved condition. The mRNA levels of isocitrate dehydrogenase increased 90-fold, ACCase increased 28-fold, while the DGAT2B and DGAT2D increased 8 and 20-fold respectively. A very large increase in mRNA abundance of nitrate reductase (NR) was also observed in response to nitrogen starvation (26-fold) and W conditions (248-fold).

4. Discussion

4.1. GS/GOGAT/GDH are important regulatory branch points

The results of this study strongly suggest that the key branch point in lipid biosynthesis in P. tricornutum centers on the metabolism of AKG (Fig. 1). In the eNO/C03 condition, the general decrease in metabolite pools is consistent with lower overall metabolic activity due to the global translational limitation this nitrogen deprivation inevitably imposes. Nonetheless, the changes in the levels of glutamate (GLU), glutamine (GLN), and a-ketoglutarate (AKG) indicate that the GS/GOGAT/GDH branch points, steps (3) and (4) of Fig. 1, the shift the flux of carbon from protein into lipid biosynthesis. The observed large decrease in the pool sizes of GLU and GLN, in parallel with the 18-fold increase in AKG and 3-fold increase in NADPH/NADP+ ratio, are consistent with an arrest of the GS/GOGAT cycle due to ammonium depletion at step (3). The 18-fold increase in AKG is likely also due to recycling of amino acids from protein catabolism, including chlorophyll associated proteins [a large decrease in chlorophyll a and protein levels were observed in this condition, (Table 1)] through the GDH pathway. This pathway is readily reversible and, because

<table>
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Less abundant

More abundant

Fig. 2 – Ratio of abundance of key cellular metabolites at steady state growth determined by LC-MS/MS. The abundance of each metabolite in the control condition was used as reference and set to 1. *Student t-test p-value < 0.05, **Student t-test p-value < 0.01. AA = amino-acids; FA = fatty acids; CEC = adenylate cell energy charge; Pyr Nuc = Pyridine nucleotides; UDP-Glc = UDP-glucose; G1P = Glucose-1-phosphate; R5P = Ribulose-5-phosphate; X5P = Xilulose-5-Phosphate; F6P = Fructose-6-Phosphate; FBP = Fructose bis-phosphate; DHAP = Dihydroxyacetone; GAP = Glyceraldehyde-3-phosphate; 3 PG = 3-phosphoglycerate; PEP = Phosphoenolpyruvate; AcCoA = Acetyl-CoA; AKG = a-ketoglutarate; GLN = Glutamine; GLU = Glutamate; MaCoA = Malonyl-Co; AMP, ADP, ATP – adenosyl-(mono,di,tri)phosphate; NAD+, NADH – Nicotinamide adenine dinucleotide, NADP+ - Nicotinamide adenine dinucleotide phosphate.
Fig. 3 – mRNA abundances of target genes as measured by real-time RT-qPCR. Logarithm of the ratio of change of mRNA abundance of target genes in the nitrate depleted and tungstate conditions relative to the control condition whose copy number was set to 1. The calculated copy number for each gene was normalized to total RNA. Gene name abbreviations are given in Table S1.

of the high $K_m$ for ammonium, it operates primarily to convert GLU to AKG. Analogously, this pathway was suggested to have a mainly catabolic role in *Chlamydomonas reinhardii* [32].

Interestingly, the mRNA level of the *icd* gene, which codes for isocitrate DH (responsible for synthesizing AKG) was increased 65-fold under nitrate deprivation. Yet, it remains to be clarified if the higher mRNA levels observed in our experiments are translated into higher protein levels. Our results strongly suggest that blockage of the GS/GOGAT branch point due to low ammonium availability is responsible for the decrease in protein content observed. The system attempts to compensate for this by elevating the *icd* gene abundance, attempting to make more AKG, which is not in shortage. As a consequence, the system redirects newly fixed carbon into lipids (via AcCoA and MaCoA) which requires no nitrogen for its biosynthesis and, in addition, is a more effective way to relieve the stronger reducing intracellular environment [3].

The observed redirection of carbon metabolism into FA and TAGs under $\sim \text{NO}_3^-$ conditions is consistent with the increases in the mRNA levels of ACCase, DGAT2B and DGAT2D, which are key steps for FA and TAG biosynthesis pathways [33,34]. Increased mRNA levels of the chloroplast ACCase were also recently reported in nitrogen depleted *P. tricornutum* cultures, albeit with a lower magnitude [35].

The replacement of Mo by W generated an intermediary response between the control and the $\sim \text{NO}_3^-$ condition, likely due to the incomplete inactivation of NR activity. Nitrogen limitation in this condition was apparent by the decrease in GLN and GLU levels which are in agreement with the 40% lower proteins per cell, relative to the control. The AKG level was, however similar to that of the control. This result is consistent with an incomplete inactivation of NR activity by W, which would still allow some nitrogen assimilation by the GS/GOGAT cycle, preventing the accumulation of AKG.

The FA and TAGs per cell were also increased in the W conditions, but not as much as in nitrogen-starved. This is consistent with the slight but significant increase in the NADPH/NADP$^+$ ratio observed and with an incomplete NR inactivation by W. Lastly, the changes in mRNA levels of isocitrate DH, NR, ACCase, DGAT2B and DGAT2D were, in general, larger than what was observed in nitrogen starved conditions. This result is somewhat unexpected, as W causes only a mild nitrogen limitation. Together with the fact that we observed a 2.3-fold increase in total RNA in the W condition and a 1.18-fold increase in cell volume, relative to the control, this indicates that there are unspecific effects of tungstate in cells. Furthermore, a specific deregulation of nitrate reductase mRNA by tungstate was previously reported in the higher plant *Nicotiana tabacum* [36]. RNAi NR knock-downs could further clarify the importance of this enzyme on the fate of carbon, circumventing the unspecific effects of tungstate in cellular physiology.

4.2. *Putative nitrogen sensing mechanisms in diatoms*

Taking the $\sim \text{NO}_3^-$ and the W conditions together, we suggest that the cell senses the ratio of AKG to GLN or GLU (GL(N/U)) in addition to its overall redox state. FA and TAG synthesis have a higher NADPH requirement in comparison with carbohydrates and proteins. Consistent with this requirement, the ratio of NADPH/NADP$^+$, which is generally tightly regulated, was increased 3-fold relative to the control, implying a significantly more reducing intracellular environment, including a more reduced plastoquinone (PQ) pool. The control of microalgal nuclear genes involved in photosynthesis, carbon fixation, carbohydrate metabolism and nitrogen assimilation (including nitrate reductase) has been previously proposed to be controlled, at least in part, by the redox state of the PQ pool [7,37,38]. The increase in the NADPH/NADP$^+$ ratio observed could then, not only facilitate lipid biosynthesis as a substrate, but also be a sign of a reduced PQ pool that can regulate nuclear genes for lipid biosynthesis.

Under mild nitrate limitation the decrease in the pools of GLN and GLU are responsible for the AKG/GL(N/U) ratio increase. However, under nitrate starvation both the increase of AKG and the decrease of GL(N/U) are responsible for the large ratio increase. In *N. tabacum* leaves, it was suggested that the transcription level of NR was antagonistically controlled by AKG (activator) and GLN (repressor). The ratio of these
metabolites was proposed to be more important for nitrogen sensing than their individual concentrations [39]. In cyanobacteria, similar changes in AKG, GLU and GLN concentration were reported to take place upon nitrogen limitation [9]. These metabolites signal the nitrogen availability and start a cascade of responses, through the global nitrogen regulator NtcA, that leads to the increase in expression of several genes including isocitrate DH, NR, GS/GOGAT and several nitrate transporters [10]. Interestingly, both isocitrate DH and NR mRNA were also over-represented in our experimental conditions, and there is expressed sequence tag indication that the mRNAs levels for nitrate transporters and GS/GOGAT genes are also increased under nitrogen limitation (Table S2). Our results suggest that a similar nitrogen sensing mechanism that responds to the AKG/GLN(U) ratio may exist in P. tricornutum. A similar conclusion was reached by analyzing the protein expression levels in the diatom Thalassiosira pseudonana in response to nitrogen starvation [40]. However, no ortholog of NtcA could be found in the genome of P. tricornutum, implying that the signal transduction between AKG/GLN(U) and gene expression must use a different protein or mechanism.

The general increase in mRNA abundance of nitrogen assimilation genes seems contradictory to the arrest of the GS/GOGAT cycle observed here under −NO\textsubscript{3} conditions, although it is unclear if the increased mRNA levels are translated into higher protein levels or increased enzymatic activity (untested). In the diatom T. pseudonana these proteins were observed to be increased in response to nitrogen starvation and it was suggested that they could serve, together with the urea cycle, to more efficiently recycle nitrogen rich compounds from catabolic processes [40]. This is similar to what is observed in cyanobacteria which also have an urea cycle [41]. The physiological result of this putative sensing mechanism is however, different – nitrate starvation leads to accumulation of high levels of carbohydrates in cyanobacteria [42], and to high lipid accumulation in diatoms.

4.3. Fluxes and quantum requirements of the main carbon pools

The average flux into each major carbon pool as well as the quantum requirement for placing carbon into each pool was calculated as described in the methods section. These values allow the quantification of the carbon that is placed into each sink per unit time and the energetic cost of that flux. The flux into each pool decreases exponentially with the increase of its quantum requirements (Table 3, Fig. 4). This robust relationship does not differentiate between carbon pools (protein, carbohydrate or FA) or growth conditions (control, −NO\textsubscript{3} or W). In the control condition, protein has the largest influx of carbon and the lowest quantum requirement, while FA has the highest quantum requirement and the lowest carbon influx. In the −NO\textsubscript{3} condition, the quantum requirement for all three carbon pools increased significantly relative to the control: 7-fold for proteins, 2.6-fold for carbohydrates and 1.5-fold for FA. This translates into a quantitatively related reduction of total carbon flux into these pools: 10-fold for proteins, 3.7-fold for carbohydrates and 2-fold for FA. This overall lower total carbon flux is in agreement with the lower growth rate and general lower levels of metabolite intermediates measured by LC-MS/MS. Despite the measured increase in the mass fraction of carbon deposited into FA in the −NO\textsubscript{3} condition relative to the control (41% vs. 20%, respectively) the total carbon flux into FA is still 2-fold lower than in the control. This lower total flux can also explain the 2.7 and 10-fold lower levels of AcCoA and MaCoA relative to the control. Nonetheless, it is possible that the decrease of AcCoA and MaCoA is being overestimated as the local concentration of AcCoA and MaCoA inside the chloroplast may be considerably higher than the average cellular concentration.

This means that the global productivity of FA in a −NO\textsubscript{3} culture is severely diminished relative to the control, since both the flux of carbon to FA per cell and the number of cells is decreased. Thus, this treatment is not a good option for biotechnological production of biodiesel.

The addition of W to the media affects mainly protein biosynthesis and consequently cell growth. The quantum requirement for protein synthesis increased 1.8-fold and leads to a 2-fold decrease of carbon flux into proteins. The quantum requirements for FA and carbohydrates were similar to the control and thus the carbon fluxes into those pools were also indistinguishable from those of the control condition. This is consistent with the levels of AcCoA and MaCoA that remained stable in this treatment, while GLU and GLN levels decreased sharply as described above. Similarly to the −NO\textsubscript{3} case, the lower levels of proteins and the lower

| Table 3 – Quantum requirements and flux of carbon into for protein, carbohydrates and fatty acids in the control, −NO\textsubscript{3} and W conditions. The fluxes and ϕ⁻¹ was calculated as described in the methods section using the μ, protein, carbohydrate or lipid data from Table 1 |
|-----------------|-----------------|-----------------|-----------------|
|                 | Control         | −NO\textsubscript{3} | W               |
| Protein         |                 |                 |                 |
| ϕ⁻¹ (mol⁻¹)     | 0.43 ± 0.02     | 0.04 ± 0.01     | 0.2 ± 0.02      |
| Flux C\textsubscript{protein} (pmol cell⁻¹ d⁻¹) | 53              | 25              | 38              |
| Mass fraction flux\textsubscript{protein} |                 |                 |                 |
| Carbohydrate    |                 |                 |                 |
| ϕ⁻¹ (mol⁻¹)     | 0.22 ± 0.02     | 0.06 ± 0.01     | 0.18 ± 0.02     |
| Flux C\textsubscript{carbohydrate} (pmol cell⁻¹ d⁻¹) | 27              | 34              | 35              |
| Mass fraction flux\textsubscript{carbohydrate} |                 |                 |                 |
| Fatty acid      |                 |                 |                 |
| ϕ⁻¹ (mol⁻¹)     | 0.16 ± 0.01     | 0.07 ± 0.01     | 0.14 ± 0.01     |
| Flux C\textsubscript{fatty acid} (pmol cell⁻¹ d⁻¹) | 20              | 41              | 28              |
| Mass fraction flux\textsubscript{fatty acid} |                 |                 |                 |

* Assumes that the sum of fluxes of carbon for protein, carbohydrate and fatty acid is equal to 100.
increasing the NADPH/NADP⁺ expression in conjunction with DGATs, while biosynthesis. This potentially could be achieved by over-neously promoting higher fluxes of carbon towards lipid or proteins in the GDH/GS/GOGAT pathways, while simulta-

Fig. 4 – Relationship between carbon fluxes and quantum requirements. Values of quantum requirements and fluxes are indicated in Table 3. An exponential decay equation was fitted to the points with a correlation coefficient of 0.97. The markers used for each condition are given in the inset table.

growth rates will limit the productivity of cultures treated with W in the presence of nitrate, even if the flux of carbon to FA remains unchanged. The trends observed here for FAs fluxes and accumulation are consistent with the ones calculated previously under similar conditions [14]. However, in that study these parameters were not calculated for pro-
tein or carbohydrates.

5. Conclusions

In conclusion, here we demonstrated that under nitrogen starvation all fluxes of carbon into the major carbon sinks are considerably decreased. The addition of tungstate mimicked mild nitrogen limitation. Our metabolite data strongly sug-
gests that the GS/GOGAT/GDH pathways are key branch points controlling the allocation of carbon and the recycling of proteins, in P. tricornutum. The ratio of AKG/GL(N/U) likely re-
ports the nitrogen availability in the cell triggering the gene expression responses observed, by an unknown signal pathway. The high NADPH/NADP⁺ ratio may facilitate lipid biosynthesis and implies a more reduced cellular environ-
ment, which could trigger previously proposed nuclear gene expression mechanisms. These putative signal transduction cascades (both AKG/GL(N/U) sensitive and the redox sensitive) deserve further investigation in diatoms, as they may provide new targets for inducing lipid biosynthesis in actively growing cells. Thus, future studies may focus on controlling the AKG/ GL(N/U) ratio by genetically knocking down nitrate reductase or proteins in the GDH/GS/GOGAT pathways, while simulta-
neously promoting higher fluxes of carbon towards lipid biosynthesis. This potentially could be achieved by over expressing ACCase in conjunction with DGATs, while increasing the NADPH/NADP⁺ ratio by over expressing the ferredoxin:NADPH oxidoreductase.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biombioe.2013.10.007.

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