ALINE DUARTE BATISTA

METABOLIC RESPONSES OF *Chlamydomonas reinhardtii* CC125 UNDER DIFFERENT PROPORTIONS OF UREA AND AMMONIUM

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Botânica, para obtenção do título de *Magister Scientiae*.

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APROVADA: 10 de fevereiro de 2017.

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Adriano Nunes Nesi
(Orientador)

Dedico aos meus pais, Paulo e Rita, ao Maurílio,
à Júlia e ao Altiére; pessoas que sempre me
inspiram e fazem os meus dias mais felizes!

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BIOGRAFIA

Aline Duarte Batista, filha de Paulo Estevam Batista e Rita de Cássia Duarte Batista, nasceu em 11 de janeiro de 1991, na cidade de Viçosa, Minas Gerais. Completou o ensino médio no Colégio de Aplicação da Universidade Federal de Viçosa em 2009.

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ABSTRACT

BATISTA, Aline Duarte, M.Sc., Universidade Federal de Viçosa, February, 2017. **Metabolic responses of *Chlamydomonas reinhardtii* CC125 under different proportions of urea and ammonium.** Adviser: Adriano Nunes Nesi.

Chlamydomonas reinhardtii (phylum Chlorophyta) as a model organism for nitrogen (N) starvation studies in order to evaluate lipid accumulation, changes in bioenergetics and the regulation of photosynthesis. Chlorophytes can incorporate N in inorganic or organic form and the source of N influences the accumulation of lipids. The most frequently available inorganic sources are Ammonium (NH_4^+), Nitrate (NO_3^-) and Nitrite (NO_2^-) and the most common organic sources are purines, urea and amino acids. Use of urea in others chlorophytes usually promotes enhancement in both growth, lipid production and biomass when compared to NH_4^+. However, for *C. reinhardtii* there is a lack of more specific information on how their metabolism responds to the assimilation of N organic sources. Thus, in this work we studied the metabolism and growth of *C. reinhardtii* CC125 in urea as the only N source as well as combined with NH_4^+. Aliquots of *C. reinhardtii* CC125 were maintained in mixotrophic growth in TAP (Tris-Acetate-Phosphate) medium under temperature between 24±2 ° C, photoperiod of 16:8 h (light: dark), 90 μmol photons m^{-2} s^{-1} and constant shaking of 110 rpm. Five treatments were carried out: (i) 100% NH_4^+ (0,4 g L^{-1} NH_4Cl); (ii) 25% urea (0,3 g L^{-1} NH_4Cl and 0,11 g L^{-1} urea); (iii) 50% urea (0,2 g L^{-1} NH_4Cl and 0,21 g L^{-1} urea); (iv) 75% urea (0,1 g L^{-1} NH_4Cl and 0,32 g L^{-1} urea); and (v) 100% urea (0,42 g L^{-1} urea). The treatments were evaluated in the logarithmic phase (LOG), after 50 hours of growth and in the stationary phase (STA), after 240 hours of growth. The number of cells, cell area, ash-free dry weight, chlorophyll *a* and *b* contents, amino acids, starch, proteins, total carbohydrates and lipids were determined at LOG and STA phases. The metabolic profile and fatty acids profile was determined at STA phase. The growth observed in medium with urea was similar to 100% NH_4^+ by comparing growth curves and kinetic growth. The number of cells after in LOG and STA phase was higher in treatments with lower percentage of urea. Determination of total chlorophyll *a* and *b*, free amino acids and proteins showed no differences between treatments in the LOG phase. At STA phase level of total chlorophyll as well as chlorophyll *a* were higher in 75 and 100% urea.

The levels of chlorophyll *b* and total soluble proteins increased with increasing urea. The levels of carbohydrates, in the LOG phase, were higher in 100% urea treatment. In the STA phase, the highest values were observed for 100% urea and 100% NH_4^+ treatments. 100% urea treatment produced more lipids than other treatments in the two growth phases. Quantification of sugars indicated that disaccharides increased in treatments with more than 75% urea. Out of 14 quantified organic acids, 11 decreased in the treatment with 25% of urea as well many amino acids. Four intermediates of TCA cycle (citrate, isocitrate, succinate and malate) increased in the treatment with 100% urea. The FAMEs profile of *C. reinhardtii* was altered by concentration of NH_4^+ and urea: Total saturated fatty acids (ΣSFA) increase with amount of urea; however, total monounsaturated fatty acids (ΣMUFA) decrease with amount of urea. The most abundant fatty acid observed was palmitic acid (C16:0) which there is a tendency to increased with the amount of urea in the medium. The percentage of oleic acid (C18:1 w8) decreased with amount of urea, while percentages of linoleic acid (C18:2 w6) doubled in treatments containing urea. Thus, our data indicate that higher the availability of urea, higher are the Carbon (C) and N metabolism changes, without, however, promoting drastic changes in growth. In addition, our results suggest that urea might also provide additional C, altering C:N ratio in medium and lead changes in lipids and total fatty acid production and profile.

RESUMO

BATISTA, Aline Duarte, M.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Respostas metabólicas de *Chlamydomonas reinhardtii* CC125 sob diferentes proporções de ureia e amônio.** Orientador: Adriano Nunes Nesi.

Chlamydomonas reinhardtii (filo Chlorophyta) é um organismo modelo amplamente utilizado para estudos de carência de nitrogênio (N), no intuito de avaliar a acumulação de lipídeos, alterações na bioenergética e a regulação da fotossíntese. As clorófitas podem incorporar N inorgânico e orgânico, e a fonte de N utilizada influencia no padrão de acúmulo de lipídeos. As fontes inorgânicas mais frequentemente disponíveis são amônio (NH_4^+), nitrato (NO_3^-) e nitrito (NO_2^-); enquanto as fontes orgânicas mais comuns são purinas, ureia e aminoácidos. O uso de ureia por outras clorófitas usualmente promove melhoras no crescimento, na produção de lipídeos e biomassa quando comparado ao NH_4^+. Contudo, para *C. reinhardtii* faltam informações mais específicas de como o seu metabolismo responde à assimilação de ureia. Assim, neste trabalho estudou-se o metabolismo e o crescimento de *C. reinhardtii* CC125 em ureia como única fonte nitrogenada, bem como diferentes proporções de ureia combinada com NH_4^+. Alíquotas de *C. reinhardtii* CC125 foram mantidas em crescimento mixotrófico em meio TAP (Tris-Acetato-Fosfato), sob temperatura de 24±2 ° C, fotoperíodo de 16:8 h (luz: escuro), 90 μmol fótons m^{-2} s^{-1} e constante agitação a 110 rpm. Cinco tratamentos foram testados: (i) 100% NH_4^+ (0,4 g L^{-1} NH_4Cl); (ii) 25% ureia (0,3 g L^{-1} NH_4Cl e 0,11 g L^{-1} ureia); (iii) 50% ureia (0,2 g L^{-1} NH_4Cl e 0,21 g L^{-1} ureia); (iv) 75% ureia (0,1 g L^{-1} NH_4Cl e 0,32 g L^{-1} ureia); e (v) 100% ureia (0,42 g L^{-1} ureia). Os tratamentos foram avaliados na fase logarítmica (LOG) após 50 h de cultivo e na fase estacionária (STA), após 240 h de crescimento. O número de células, área celular, biomassa livre de cinzas, os conteúdos de clorofila *a* e *b*, aminoácidos, amido, proteínas, carboidratos e lipídeos foram determinados nas fases LOG e STA. O perfil metabólico e de ácidos graxos foram determinados na fase STA. O crescimento observado nos meios com ureia foi similar ao 100% NH_4^+, quando comparadas as curvas e os parâmetros de crescimento. O número de células foi superior nos tratamentos com menos ureia, tanto na fase LOG quanto STA. Na fase LOG não houve diferença nos totais de clorofila *a* e *b*, aminoácidos e proteínas. Na fase STA

os níveis de clorofila total bem como clorofila *a* foram maiores nos tratamentos 75% e 100% ureia. Os totais de clorofila *b* e proteínas solúveis totais aumentaram com o aumento de ureia. Os níveis de carboidratos, na fase LOG, foram maiores no tratamento 100% ureia. Na fase STA, os maiores valores obtidos foram dos tratamentos 100% ureia e 100% NH_4^+. O tratamento 100% ureia produziu maior quantidade de lipídeos nas duas fases de crescimento analisadas. A quantificação de açúcares indicou que alguns dissacarídeos aumentaram nos tratamentos com mais de 75% de ureia. Dos 14 ácidos orgânicos quantificados, 11 diminuíram no tratamento com 25% de ureia, assim como muitos aminoácidos. Quatro intermediários do ciclo TCA (citrato, isocitrato, succinato e malato) aumentaram no tratamento 100% ureia. O perfil de ácidos graxos foi modificado pelas concentrações de NH_4^+ e ureia em *C. reinhardtii*: O total de ácidos graxos saturados (ΣSFA) aumentou com o aumento de ureia, porém o total de ácidos graxos monoinsaturados diminuiu com o aumento de ureia. O ácido graxo mais abundante foi o ácido palmítico (C16:0) e apresentou uma tendência ao aumento com o aumento de ureia no meio. A porcentagem de ácido oleico (C18:1 w8) decresceu com o amento de ureia, enquanto as porcentagens do ácido linoleico (C18:2 w6) dobrou nos tratamentos que continha ureia. Logo, nossos dados indicam que quanto maior a disponibilidade de ureia, maiores são as mudanças no metabolismo de Carbono (C) e N, sem, contudo, promover drásticas mudanças no crescimento. Além disso, nossos resultados sugerem que a ureia pode fornecer C adicional para a biossíntese, alterando a razão C:N no meio e promovendo mudanças no total de lipídeos e no perfil de ácidos graxos produzidos.

TITLE: Metabolic responses of *Chlamydomonas reinhardtii* CC125 under different proportions of urea and ammonium

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ABSTRAT

In *Chlamydomonas reinhardtii* nitrogen (N) is assimilated either in inorganic or organic form. The most frequently available inorganic sources in natural environment are ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-), and the most common organic sources are purines, urea, urate and amino acids. Use of urea in others chlorophytes usually promotes enhancement in both growth, lipid production and biomass when compared to NH_4^+. However, for *C. reinhardtii* there is a lack of more specific information on how their metabolism responds to the assimilation of N organic sources. We studied the metabolism and growth of *C. reinhardtii* CC125 in medium containing urea as N source solely as well as combined with NH_4^+. Aliquots of *C. reinhardtii* CC125 were maintained in mixotrophic growth and five treatments were carried out: (i) 100% NH_4^+ (0,4 g L^{-1} NH_4Cl); (ii) 25% urea (0,3 g L^{-1} NH_4Cl and 0,11 g L^{-1} urea); (iii) 50% urea (0,2 g L^{-1} NH_4Cl and 0,21 g L^{-1} urea); (iv) 75% urea (0,1 g L^{-1} NH_4Cl and 0,32 g L^{-1} urea); and (v) 100% urea (0,42 g L^{-1} urea). The treatments were evaluated in terms of growth, biochemical and metabolic parameters

in logarithmic phase (LOG) and in stationary phase (STA). The growth observed in medium with urea was similar to 100% NH_4^+ by comparing growth curves and kinetic growth. The number of cells after in LOG and STA phase was higher in treatments with lower percentage of urea. Total chlorophyll *a* and *b*, free amino acids and proteins levels did not differ between treatments at LOG phase. However at STA phase the levels of chlorophyll *a* were higher in medium containing 75 and 100% urea. The levels of chlorophyll *b* and proteins also increased with the increase of urea. The treatment 100% urea promoted the production of higher levels of carbohydrates, lipids and intermediates of TCA cycle (citrate, isocitrate, succinate and malate). Of all treatments, the 25% urea obtained the greatest reductions in the levels of organic acids and amino acids. The FAMEs profile of *C. reinhardtii* was altered by concentration of NH_4^+ and urea: The most abundant fatty acid observed was palmitic acid (C16:0) has a minor increased with the amount of urea in the medium. Oleic acid (C18:1 w8) decreased with amount of urea, while percentages of linoleic acid (C18:2 w6) doubled in treatments containing urea. Thus, our data indicate that higher the availability of urea display several adjustments in C and N metabolism without drastic changes in growth. In addition, our results suggest that urea might also provide additional C, altering C:N ratio in medium and lead changes in lipids and total fatty acid production and profile.

Key word: *Chlamydomonas reinhardtii*, nitrogen metabolism, nitrogen sources.

1- INTRODUCTION

Chlamydomonas reinhardtii (phylum Chlorophyta) as a model organism for physiological and molecular studies. Basic studies related to the biology of chloroplasts, the photosynthetic process and both structure and the function of eukaryotic flagella were developed based on this organism [1–4]. In addition, *Chlamydomonas* and others microalgae can be used as producers of recombinant proteins for industrial purposes, pharmaceutically applied products and biofuels, such as hydrogen or biodiesel [5–8].

The production of microalgae with high biomass yield and high percentage of lipids are desirable in a short period of time [9, 10]. This aim can be reached based on medium manipulation which leads to changes in central energy metabolism [11–13]. *C. reinhardtii* has been used as a model organism for N starvation studies in

order to evaluate lipid accumulation, changes in bioenergetics and the regulation of photosynthesis [14–18].

Although, nutrient starvation can inhibit mitosis and photosynthesis, impairing biomass production [34, 35], higher production of lipids in chlorophytes occurs in response to starvation of several nutrients, such as for N [19–28], sulfur [29] and zinc [30]. Under nutrient limited conditions some species of chlorophytes can accumulate nearly 80% of cellular dry weight as lipids [31–33]. Thus, to obtain high lipid productivity maintaining high biomass is essential to recognize and understand the regulatory mechanisms controlling lipid accumulation in N-starved algal cells [36].

The molecular basis of triacylglycerol (TAG) accumulation in *C. reinhardtii* has been extensively investigated [37–40]. It was observed that N limiting conditions lead to dramatic changes in the transcriptome and in the regulation of the algae metabolism directing carbon flow from photosynthesis into glyoxylate pathways and gluconeogenesis producing lipids [41]. Under N starvation, the levels of metabolites such as amino acids, intermediates of ribosome biosynthesis, phosphate pentose pathway and Calvin-Benson cycle are reduced [14]. The opposite occurs in the biosynthesis of fatty acids and proteins related to N assimilation, which increase during stress [14, 42]. In addition to N starvation, extra carbon (C) supplement in the form of acetate (mixotrophic growth) also increases the production of lipid in *C. reinhardtii*, suggesting that the C:N ratio influences the reserve accumulation pattern [43].

Chlorophytes can incorporate N in inorganic or organic form and the source of N might affect lipids accumulation. The most frequently available inorganic sources of N are Ammonium (NH_4^+), Nitrate (NO_3^-) and Nitrite (NO_2^-) [44]. NO_2^- and NO_3^- in general promote growth and accumulation of lipids concomitantly [45, 46]. In turn, the use of NH_4^+ decreases accumulation of TAG [45]. Concerning organic sources of N, the most common are purines, urea and amino acids. Urea (H_2NCONH_2) is a highly soluble organic compound containing a carbonyl (C=O) group attached to two amine groups ($-NH_2$). Urea can be produced by excretion of animals and can be used as N source by photosynthetic organisms and thus contributes with at least 50% of total N used by phytoplankton in coastal and oceanic ecosystems [47, 48]. In many algae species urea promotes growth, lipid and biomass

production when compared to NH_4^+. This accumulation is variable according with the C: N ratios in the medium [45, 49, 50].

Analysis of the genome of *C. reinhardtii* revealed the potential for use of inorganic and organic N sources. There are many NH_4^+, NO_3^- and NO_2 transporters anchored in plasma membrane and in chloroplast membrane [51]. There are four glutamine synthase (GS) -two cytosolic and two plastid-targeted - and two plastidic glutamine: 2-oxoglutarate aminotransferase (GOGAT), one NADH and another Fd $_{red}$.-dependent [4, 44]. The reduction of NO_3^- to NO_2^- is done by the enzyme nitrate reductase (NR) in the cytosol. The reduction of NO_2^- to NH_4^+ is catalyzed by nitrite reductase (NiR) and occurs in the stroma of the chloroplast. *C. reinhardtii* presents single isoforms of NR and NiR enzymes [44, 52].

C. reinhardtii has three DUR3 genes, which encode a high-affinity urea active transporter [50]. Urea transport uses sodium gradient energy and is less active or absent when other N sources are used, especially NH_4^+ [53–56]. In *Chlamydomonas* urea catabolism is catalyzed by urea amidolyase (UAL-ase), which requires ATP, bicarbonate, magnesium ions (Mg^{+2}), biotin and univalent cations (K^+, Na^+, or NH_4^+) for its activity. UAL-ase presents urea carboxylase (UC) activity, which catalyzes the ATP-dependent condensation of urea and bicarbonate, producing allophanate, and allophanate hydrolase (A^c) activity which produces ammonia and bicarbonate ion (HCO_3^-) [47, 53, 57–59]. This enzyme is a protein complex encoded by two genes (*DUR1* and *DUR2*), activated in the absence of NH_4^+ and presence of urea or acetamide. After being converted to NH_3 the urea-derived N can be assimilated via GS-GOGAT. CO_2, resulting from the hydrolysis of allophanate, can be reduced to HCO_3^- by carbonic anhydrase enzyme. Thus, this can be used both in the photosynthetic pathway and can remain in the cytosol and contribute to the maintenance of intracellular pH [43, 59].

Many studies related to N limitation in a culture medium for *C. reinhardtii* have been described [15, 37, 51, 60–62]. Schmollinger et al. [51] evaluated the transcriptome and proteome of *C. reinhardtii* growing in acetate, before and after N limitation in the medium. The prevalence of respiratory metabolism in relation to photosynthesis and a reduction of transcripts and proteins of the Calvin-Benson cycle were observed. However, genes related to metabolism of alternative N sources have

increased in abundance. This indicates the existence of specific mechanism for metabolic adaptation allowing the use of different N sources.

The evaluation of NO_3^- and urea as alternative N sources for microalgae have been performed for green algae and diatoms groups [37, 47, 54, 63–65]. In chlorophytes such as *Chlorella* and *Scenedesmus* urea experiments were performed to evaluate the production and accumulation of triacylglycerol (TAG) with routes to biodiesel production [47, 63, 66]. However, for *C. reinhardtii* there is a lack of more specific information on how their metabolism responds to the assimilation of N organic sources. Thus, in this work we studied the metabolism and growth of *C. reinhardtii* CC125 in urea as the only N source as well as combined with NH_4^+. The results obtained are discussed in the context of the efficiency of urea as N source for cultivation and impacts on primary metabolism of *C. reinhardtii*.

2- MATERIAL AND METHODS

2.1- Microorganisms and culture media

Aliquots of *C. reinhardtii* CC125 mt+ were maintained in mixotrophic growth in Tris-Acetate-Phosphate (TAP) medium with N (Supplemental Table 1) [67], under temperature of 24±2 ° C, 16:8 h (light: dark) photoperiod, 90 μmol photons m^{-2} s^{-1} and constant shaking at 110 rpm. Five treatments were carried out: (i) 100% NH_4^+ (0,4 g L^{-1} NH_4Cl); (ii) 25% urea (0,3 g L^{-1} NH_4Cl and 0,11 g L^{-1} urea); (iii) 50% urea (0,2 g L^{-1} NH_4Cl and 0,21 g L^{-1} urea); (iv) 75% urea (0,1 g L^{-1} NH_4Cl and 0,32 g L^{-1} urea); and (v) 100% urea (0,42 g L^{-1} urea). In urea treatments were used TAP medium without N (TAP -N) [67] and the concentration of NH_4Cl in solution was adjusted to provide the desired amount of N. In treatments (ii) to (v), the urea (Sigma, V900119) solution was filtered on membranes of 0.22 μm (Millipore) and added to the culture medium after sterilization.

2.2- Culture conditions and Growth curves

The cultures were carried out in Erlenmeyer flasks of 125 mL with 50 mL of useful volume in culture room, under temperature 24±2 °C and photoperiods of 16:8 h (light:dark). The cultures were aerated by constant shaking of 110 rpm. The light intensity was maintained at 90 ± 10 μmol photons m^{-2} s^{-1}. The initial density of the experimental units was $1x10^5$ cel mL^{-1}, being 4 replicates per treatment. The

experiment lasted ten days. Five growth curves were constructed, one for each treatment. The growth was followed based on absorbance measurements at 750 nm in spectrophotometer (UVM 340, AsysHitech) [68] and cell counts under light microscopy (Olympus CX40, U.S.A) with a hemocytometer (New Optics). The data were used to determine the growth phases, kinetic growth parameters such as specific growth rate ($\mu_{máx}$) and generation time (g), based on the Ln optical density (O.D.) versus time curve [69].

2.3- Characterization of growth, dry weight and ash-free mass

The treatments were evaluated in the logarithmic phase (LOG), after 50 hours of growth and in the stationary phase (STA), after 240 hours of growth. The number of cells, cell area and ash-free dry weight were determined at LOG and STA phases. An aliquot of the culture was photographed under light microscope (Olympus CKX41, U.S.A) in 40X objective (LCAchN), using the SC30 capture system and the Olympus Cell F software. The generated images were used to calculate the average cell area, considering the circular cells.

Dry weight determination was performed according to Griffiths et al. [68]. At each collection time point, 10 mL of the culture was filtered into cellulose membranes with porosity of 0.45 μm (Millipore), previously dried and weighed. After filtration, the membranes were oven dried at 60 °C and weighed until constant weight was obtained. For the determination of ash-free dry weight, the pre-weighed dry matter membranes were calcined in a muffle oven (Fornos Jung Ltda, LF 02312, Brazil) at 550 °C for 30 minutes. After cooling, the porcelain crucibles with the ashes were weighed. The ash-free dry weight was determined by subtraction of the dry weight by ash weight after calcination. The result was expressed in mg ash-free weight per mL of culture.

2.4- Biochemical characterization

For the biochemical analyzes, samples in LOG and STA phase were collected and centrifuged at 10,000 g for 10 minutes. These were frozen in liquid nitrogen and kept at -80 °C until analysis. The samples submitted to the hot ethanol extraction, the chlorophyll *a* and *b* contents [70] and total soluble amino acids were determined in ethanol-soluble fraction [71]. In the ethanol-insoluble fraction, the amounts of starch [72] and total water-soluble proteins were quantified [73, 74]. For the determination

of total carbohydrates, 2 mL of the culture were used. Extraction was performed according to Teoh et al. [75], substituting HCl for H_2SO_4. Quantification was performed according to Masuko et al. [76] using glucose as standard. For the quantification of total lipids, the NileRed dye was used, according to a methodology adapted by Chen et al. [77]. The results for proteins, chlorophyll *a* and *b*, neutral carbohydrates and total lipids were expressed in μg number of cells $^{-1}$. The amino acid results were expressed as μmol number of cells $^{-1}$ and those of starch as μmol NADPH number of cells $^{-1}$.

2.5- Metabolic profile

At the STA phase, 20 mg of lyophilized material were destined to determine the metabolic profile. The metabolites were extracted and derivatized following the protocol description of Roessener et al. [78]. The metabolites were determined in a TruTOF GC-MS system, according to Lisec et al [79]. Chromatograms were exported from ChromaTof software (version 3.25) for processing in the program R. Peak detection, retention time alignment and library matching were performed using the TagSearch R package from Bioconductor [80]. Each analyte peak was normalized to the peak of the internal standard (ribitol) and cell count in sample, as previously described by Machado et al. [81].

2.6- Analysis of fatty acid profile

For the extraction and derivatization of the fatty acids, 30 mg of lyophilized culture from stationary phase were used. The procedure consisted in adding to the samples 800 μL of toluene, 400 μL of methanol and 1800 μL of HCl in 8% (v v^{-1}) methanol. The tubes were incubated at 65 °C for 12 h. After that time, 6 mL of hexane was added. 0.5 mL of the non-polar fraction was removed for analysis on Agilent 7890 gas chromatograph (Santa Clara, CA, USA). The MIDI Sherlock version 6.2 (Sherlock Microbial Identification MIDI System of Inc. Newark, Delaware, USA) software was used to adjust the operational parameters and for recognition, quantification and comparison with the reference libraries. The result is expressed as a percentage (%) in relation to the total response obtained in the chromatogram.

2.7- Statistical analysis

The experiments were carried out in a completely randomized design with four replicates for each treatment. The observed values for all variables were submitted to analysis of variance (ANOVA). In addition, Tukey's test ($p \leq 0.05$) using the SISVAR 5.6 software [82] was used to study the effect of urea concentration variation on response variables. In metabolic profile and fatty acid profile Student't tests was performed for compare treatments with urea to 100% NH_4^+ treatment.

3- RESULTS

3.1- Growth and accumulation of biomass

The growth performance observed in urea containing medium was similar to the one with 100% NH_4^+ (Figure 1A, 1B) and analyzing the obtained growth parameters no variations were observed (Figure 1C, 1D). In the LOG phase the cells were more homogeneous in size and arrangement between the treatments (Supplemental Figure 1 A-E). However, in the stationary phase (STA), increasing urea amount lead to higher cell aggregation proportionally (Supplemental Figure 1 J).

Concerning the cell number after 50 hours of culture, higher number in the treatments with lower percentage of urea was observed (Figure 2A). The cell area in the LOG phase was higher in the urea-free treatment and there were no statistical differences between treatments with 25, 50, 75 and 100% urea (Figure 2C). In the STA phase, the treatments with up to 50% of urea promoted higher number of cells. While treatments with 75 and 100% of urea presented approximately 40 and 60%, respectively, less cells than the 25% urea treatment (Figure 2B). In contrast, the cell area increased in the treatment with 100% urea (Figures 2D).

The ash-free dry biomass produced did not differ between the treatments in the LOG phase (Figure 2E). However, in the STA phase the treatments of 0, 50 and 100% urea promoted higher ash-free dry matter production (Figure 2F).

3.2- Biochemical analyzes

The levels of total chlorophyll *a* and *b* did not differs between treatments in the LOG phase (Figure 3A, 3B). In contrast, at STA phase the levels of total chlorophyll and chlorophyll *a* increased in 75 and 100% urea (Figure 3E, 3G).

Chlorophyll *b* levels increase with increasing availability of urea in the medium (Figure 3F). The chlorophyll *a*/*b* ratio remained similar between treatments in both growth phases (Figure 3D, 3H). At LOG phase, the levels of free amino acids and total soluble proteins were not altered with urea addition (Figure 4A, 4B). At the STA phase, a tendency of decrease in amino acids levels with increasing urea in the medium was observed. However, the levels of total soluble proteins increased proportionally with the amount of urea in the medium (Figure 4C, 4D). In STA phase, protein/amino acids ratio was progressively increased in urea treatments (Figure 5A), while the protein/chlorophyll ratio was higher only in medium with 100% urea (Figure 5B).

Carbohydrates levels were higher in the treatment with 100% urea in the LOG phase (Figure 6A). Similarly, in the STA phase, the highest values were observed for 100% urea and also for 100% NH_4^+ treatments (Figure 6B). There were no consistent changes in starch levels between treatments in any of the analyzed phases, with exception of the treatment with 100% of urea, which was significantly higher in LOG phase (Figure 6C, 6D). The 100% urea treatment promoted higher lipid production in comparison with other treatments in the two growth phases (Figure 6E, 6F).

3.3- Metabolic profile

Sugars measurements by GCMS indicated that disaccharides, such as isomaltose, sucrose and galactinol, increased in treatments with more than 75% urea. In the same way, fructose levels were increased in 100 % urea treatment. Surprisingly, glucose levels were decreased in all treatments containing urea, similarly to glycerol, a sugar alcohol, which decreased in 25 % urea treatment (Figure 7).

Out of 14 quantified organic acids, 11 decreased in the treatment with 25% of urea. Between them, four TCA cycle intermediates (citrate, isocitrate, succinate and malate) increased in the treatment with 100% urea. Isocitrate and malate also increased in the treatments with 50 and 75% of urea (Figure 7). Despite the fact that there were no differences in the total amount of free amino acids in the STA phase (Figure 4C), several amino acids decreased in the medium containing 25% urea

(Figure 7). Glycil-proline and hydroxyproline and cell wall components decreased in all treatments containing urea (Figure 7 and Supplemental Table 2).

3.4- Analysis of fatty acid profile

Interestingly the treatments containing NH_4^+ and urea altered the profile of FAMES (Figure 8 and Supplemental Table 3). The most abundant fatty acid observed between all five treatments was palmitic acid (C16:0) which displayed a mild increased with the amount of urea in the medium. At 100 % of NH_4^+ palmitic acid represented approximately 36% of the total FAMEs content, while in 100% urea it corresponded about 46.5% of the total. The second most abundant fatty acid was oleic acid (C18:1 w8c). Its percentage decreased with the amount of urea, ranging from 39.6% (100% NH_4^+) to 28.4% (100% urea). The percentage of linoleic acid (C18:2 w6c) doubled in treatments containing urea, and myristic acid (C14:0) was only detected in treatments with 75% and 100% urea. The total amount of saturated fatty acids (ΣSFA) increased with the amount of urea in the medium; however, the amount of total monounsaturated fatty acids (ΣMUFA) decreased with amount of urea. The amount of total polyunsaturated fatty acids (ΣPUFA) did not differ between treatments.

4- DISCUSSION

Under our conditions *C. reinhardtii* CC125 was able to grow in medium containing NH_4^+ combined with urea in different proportions as well as in medium containing urea as N source solely. Many algal species have been able to grow on urea containing medium and some species of diatoms and chlorophytes showed better growth performance on urea than NO_3^- and NH_4^+ [49, 50]. The preference for urea by some algal species might not be related to the use of urea as N source only, but also as carbon (C) source. It has been shown that urea, after being transported and metabolized by the urea carboxylase and allophanate hydrolase, is converted to NH_3 and HCO_3^- in the cytosol [53, 59]. The presence of carbon concentrating mechanisms in *C. reinhardtii*, using carbonic anhydrase in different cell compartments, suggest that the carbon derived from urea catabolism can be used by cells [83, 84]. Thus, the HCO_3^- released by the catabolism of urea may be assimilated and contributing to biomass production. If it is considered that all C present in the

urea molecule is available for biosynthesis, it is possible to estimate the increment of C from the N source. In the mixotrophic growth used here, all treatments present 35 mmol of C from acetate [67], as C organic. The treatments 25, 50, 75 and 100% urea represent an increase of 1.75; 3.5; 5.25 and 7.00 mmol of organic C, respectively. This indicates a considerable increase in the C:N ratio and can be influence cell metabolism.

In this study, it was observed that addition of higher proportions of urea/NH_4^+ reduced the number of cells in the STA phase. However, increase in cell area guaranteed that 100% NH_4^+ and 50% of urea produced the same amount of biomass in the LOG phase. When *Scenedesmus* sp. was cultivated in medium with urea the growth rate was 0.5 times higher and the biomass productivity increased 26% in comparison to NO_3^- as N source [49]. Interestingly, in the present work, the cell area did not change in the LOG phase. In addition the biomass produced was reduced and without differences between treatments. This results might reflect higher metabolic activity since cell reproduction is extremely high in the log phase, and thus cells reduce the accumulation of reserves [81, 85].

By increasing urea contents in medium higher amounts of protein and total chlorophylls amounts were observed (Figure 3 and 4). This result may be related to changes in uptake rates and catabolism of urea following the NH_4^+ concentration. Indeed, urea uptake rates increase by decreasing NH_4^+ concentrations or after N-starvation in the medium [50]. It has been shown in *Chlamydomonas* that increasing the amount of urea and reducing the NH_4^+ availability in the medium leads to increased transcription of urea transporters [50, 51]. Based on the obtained data, we hypothesize that there is an initial response to the absence or reduced amount of NH_4^+ in the medium that is similar to starved-N cell responses. It allows that the cell modulates its central metabolism and increases the activity of transporters and enzymes related to the metabolism of alternative N sources [14], taking up urea, which is metabolized and the N is stored N in proteins and pigments.

The presence of NH_4^+ in the medium influences not only transport as also urea catabolism [53, 86]. NH_4^+ modifies urea transporters activity, decreasing their affinity for urea, and increases transporters turnover rate [86]. With similar regulation, the enzymes urea carboxylase and allophanate hydrolase, in the absence of NH_4^+, increase their basal activity of 10 to 100 times [53]. The presence of NH_4^+

ceases the accumulation of urea carboxylase enzyme and decreases allophanate hydrolase enzyme levels by 80% [53].

Moreover, the decrease of intracellular NH_4^+ stimulate the synthesis and enzymatic activity of glutamate dehydrogenase (GDH), GS and GOGAT [14, 42]; and increases the activity of pyruvate dehydrogenase (PDH) [87] increasing the levels of acetyl-CoA. The increase in acetyl-CoA lead to higher flux through TCA cycle and producing more C skeletons, which was redirected to a synthesis of macromolecules. In this study, an increment of isocitrate and malate in the treatments with urea was observed (Figure 6). These results suggest that under low levels of NH_4^+ and higher of urea, TCA cycle activity is increased to couple with increase in N assimilation. Similar responses were found at the protein level, when mitoproteome of *C. reinhardtii* growing in NH_4^+, NO_3^- as sources of N [88] and NH_4^+ starvation [42]. Isocitrate dehydrogenase (IDH) and 2-Oxoglutarate dehydrogenase (2-OGDH) were up-regulated, indicating increase in the production of C skeletons needed for amino acid biosynthesis [88].

The treatments with combined N sources, especially 25% urea, the one with the highest amount of NH_4^+, decreased the levels of some amino acids, organic acids and sugars compared to 100% NH_4^+ (Figure 7). This may have occurred because of the inhibition of urea metabolism by NH_4^+. In another study, combined NO_3^- and urea as N sources led to a greater accumulation of reserves [49].

During the STA phase the use of urea in the medium did not result in changes starch levels. However, in the LOG phase, starch, as well as the total carbohydrates contents increased when urea was applied as sole N source. Interestingly the total carbohydrates contents reached the highest in the 100% NH_4^+ treatment, followed by 100% urea. Combination of N sources lead to lower carbohydrates levels. It was also observed that the increase in the amount of urea increased the amount of some sugars- isomaltose, sucrose, galactinol and xylulose (Figure 7). This may be associated to C:N ratios, with increasing urea in the medium. The biosynthesis of starch in *C. reinhardtii* constitutes the first line of reserve accumulation [89]. The synthesis of starch and lipids does not occur simultaneously and there is still competition of the two metabolic pathways for carbon available for biosynthesis (glyceraldehyde-3-phosphate and 3-phosphoglycerate). The higher accumulation of TAGs occurs only in some mutant strains or when the carbon supply exceeds the capacity of starch synthesis [12, 90]. Wang et al. [43] characterized a strain of *C.*

reinhardtii mutant for starch production and demonstrated that although these cells do not require acetate to grow, lipid body production only occurs when acetate is added to the culture medium. This demonstrates that an exogenous C source is critical for lipid production.

In this study, we observed that urea lead to increase in total lipid contents in the STA phase (Figure 6E, 6F). In addition, it was verified that urea promotes changes in fatty acid profile (Figure 8). The changes in the fatty acid profile by urea supply is probably not related to the presence of alternative N sources, other than NH_4^+, but also the possibility of using urea as an extra C source. In the study of sequences corresponding to diacylglycerol acyltransferases (which catalyze the last step in TAG biosynthesis: the acylation of diacylglycerol to TAG), three of the six genes were up-regulated in the absence of NH_4^+ [40]. Studying the effects of CO_2 concentration on the FAMEs profile, Tsuzuki et. al. [91] found an increase of approximately 12% for palmitic acid when air enriched with 4% CO_2. The increase of palmitic acid observed in the present study achieved 29%. This reinforces our hypothesis that the C present in urea molecule is also being incorporated into biomass of *C. reinhardtii.*

5- CONCLUSIONS

Taken together our results suggest that urea promote several changes in C and N metabolism without drastic effects in growth. Moreover, the results indicate that urea provide additional C supply which alters lipids and total fatty acid production as well as the lipid profile. Nevertheless, further studies are still needed for better understanding the molecular basis of how urea metabolism actually lead to metabolite reprogramming and how it interferes with photosynthetic rates and production of C skeletons.

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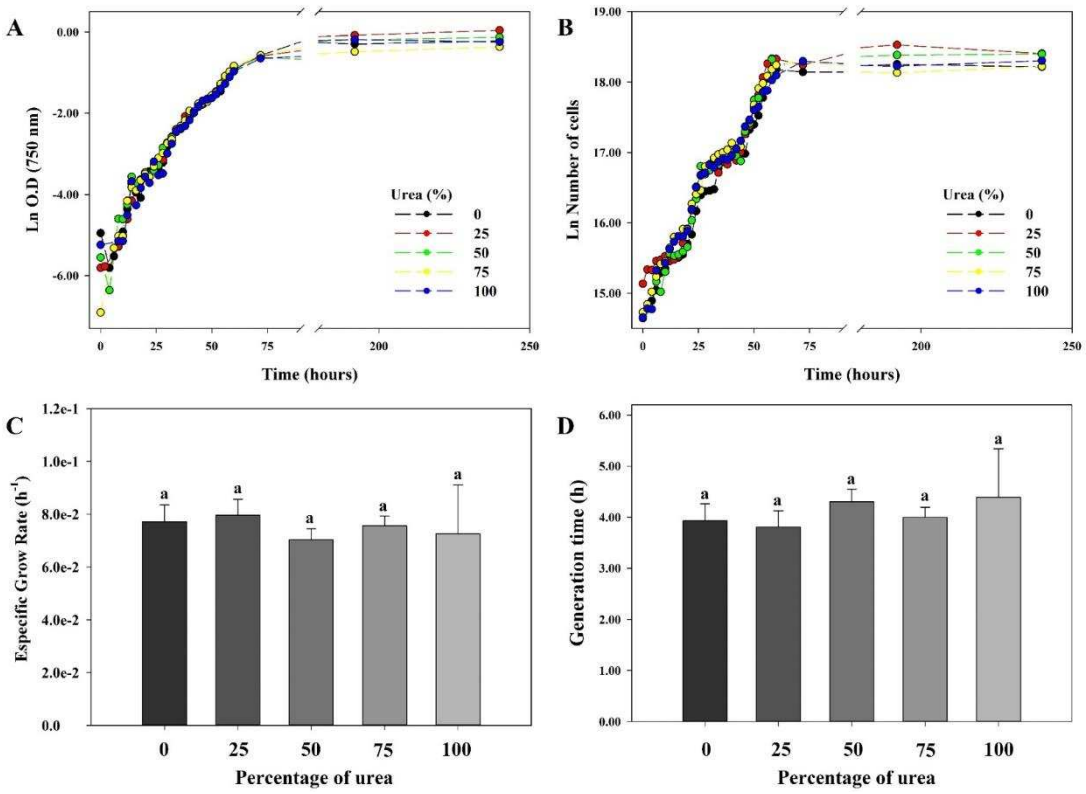


Figure 1: Growth Parameters: Growth curve of *C. reinhardtii* CC125 cultivated under different proportions of urea and ammonium (NH_4^+) based in Ln of optical density (**A**) and based in Ln of number of cells (**B**). **C** – Especific Grow rate ($\mu_{máx}$) in h^{-1}, **D**- Generation time in hours. Values represent the mean ± SD of five replications. Means followed by the same lowercase letters do not differ by Tukey test ($p < 0.05$).

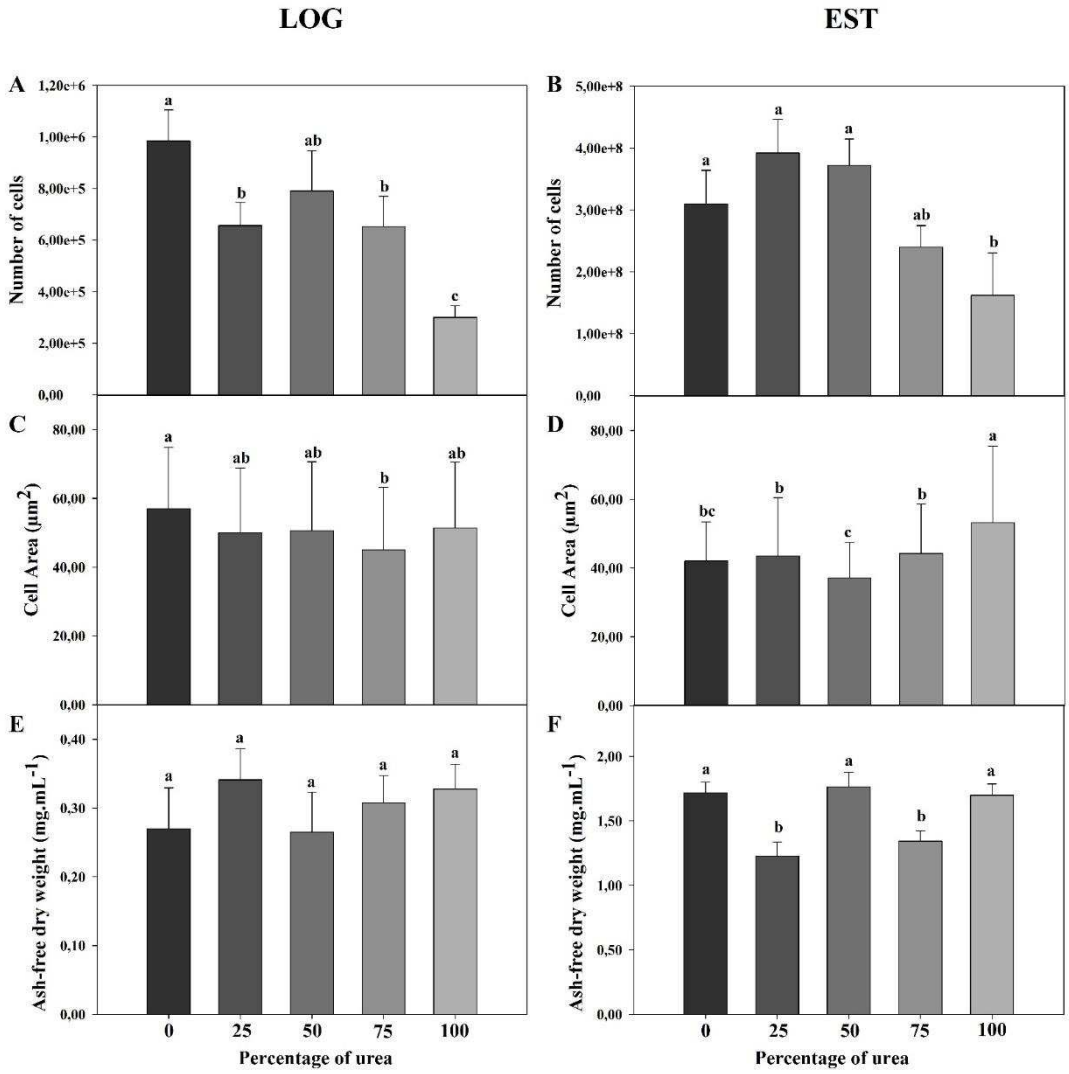


Figure 2: Growth and biomass production of *C. reinhardtii* CC 125 cultivated under different proportions of urea and ammonium (NH_4^+). The graphs to the left refer to the logarithmic phase (LOG) and the right to stationary phase (STA). **A** and **B**, number of cells after 50 h and 240 h of cultivation, respectively; **C** and **D**, cell area of 100 cells (μm^2); **E** and **F**, Ash-free dry weight (mg mL^{-1}). Values represent the mean ± SD of four replications. Means followed by the same lowercase letters do not differ by Tukey test ($p < 0.05$).

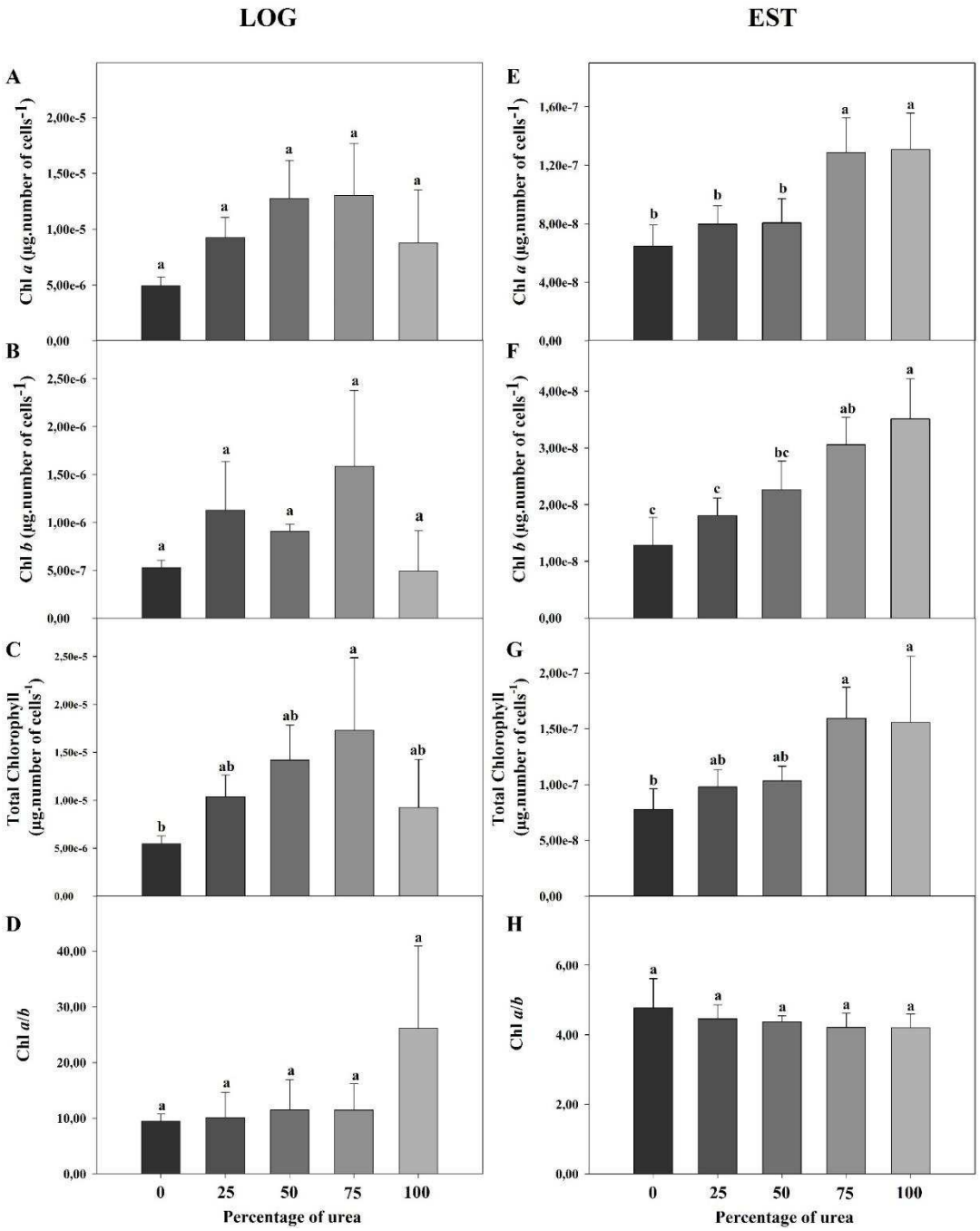


Figure 3: Chlorophyll content of *C. reinhardtii* CC 125 cultivated under different proportions of urea and ammonium (NH_4^+). The graphs to the left refer to the logarithmic phase (LOG) and the right to stationary phase (STA). **A** and **E**, chlorophyll *a* (µg number of cells^{-1}); **B** and **F**, chlorophyll *b* (µg number of cells^{-1}); **C** e **G** total chlorophyll content (µg number of cells^{-1}); **D** and **H**, chlorophyll *a*/*b* ratio. Values represent the mean ± SD four replications. Means followed by the same lowercase letters do not differ by Tukey test ($p < 0.05$).

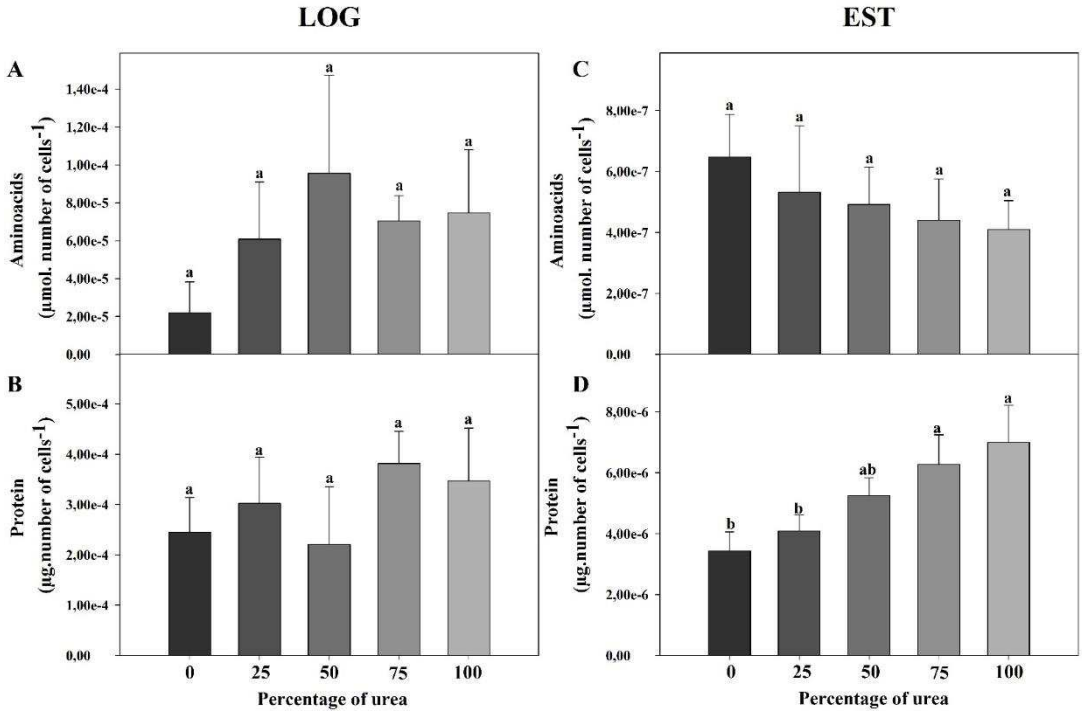


Figure 4: Nitrogen containing metabolites of *C. reinhardtii* CC 125 cultivated under different proportions of urea and ammonium (NH_4^+). The graphs to the left refer to the logarithmic phase (LOG) and the right to stationary phase (STA). **A** and **C**, total free amino acids content (µmol number of cells^{-1}); **B** and **D**, total protein content (µg number of cells^{-1}). Values represent the mean ± SD four replications. Means followed by the same lowercase letters do not differ by Tukey test ($p < 0.05$).

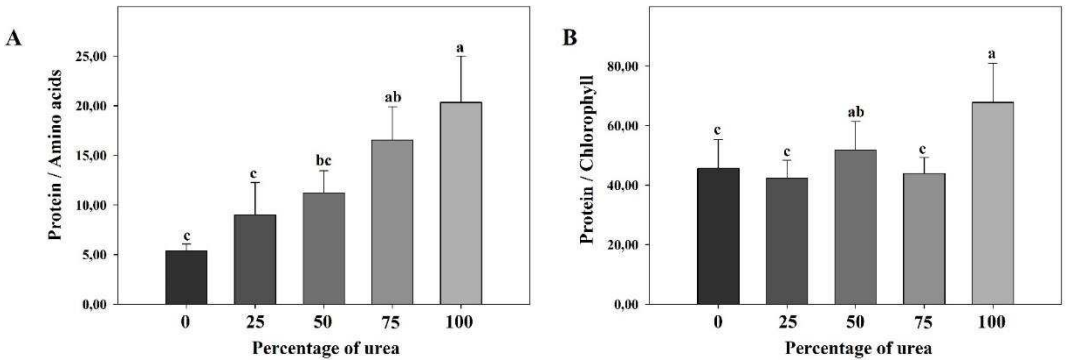


Figure 5: Nitrogen containing metabolites ratio of *C. reinhardtii* CC 125 cultivated under different proportions of urea and ammonium (NH_4^+). The graphs refers to stationary phase (STA). **A** protein/amino acids ratio and **B**, protein/chlorophyll ratio. Values represent the mean ± SD four replications. Means followed by the same lowercase letters do not differ by Tukey test ($p < 0.05$).

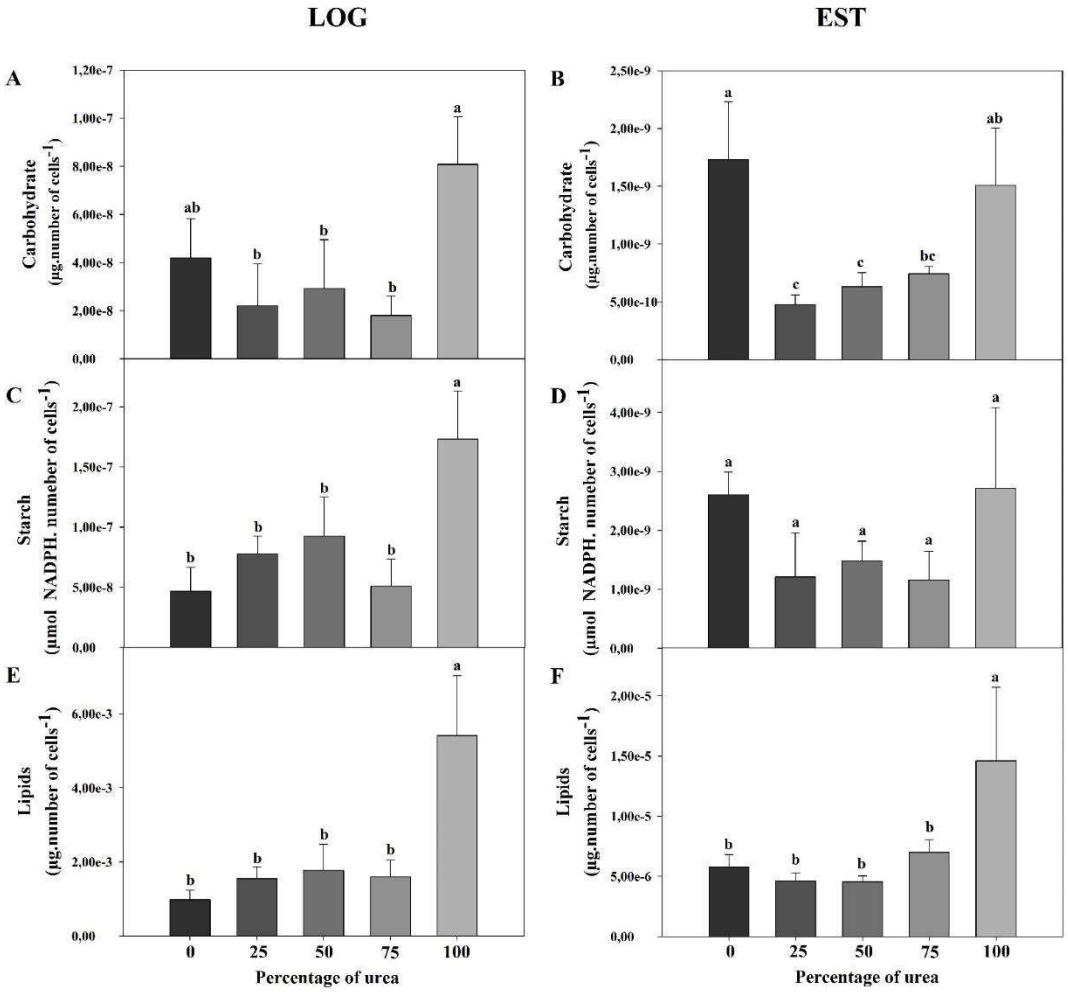


Figure 6: Carbon containing metabolites ratio of *C. reinhardtii* CC 125 cultivated under different proportions of urea and ammonium (NH_4^+). The graphs to the left refer to the logarithmic phase (LOG) and the right to stationary phase (STA). **A** and **B**, total carbohydrate content (µg. Number of cells^{-1}); **C** and **D**, Starch content (µM NADPH number of cells^{-1}); **E** and **F** total lipids content (µg. Number of cells^{-1}). Values represent the mean ± SD four replications. Means followed by the same lowercase letters do not differ by Tukey test ($p < 0.05$).

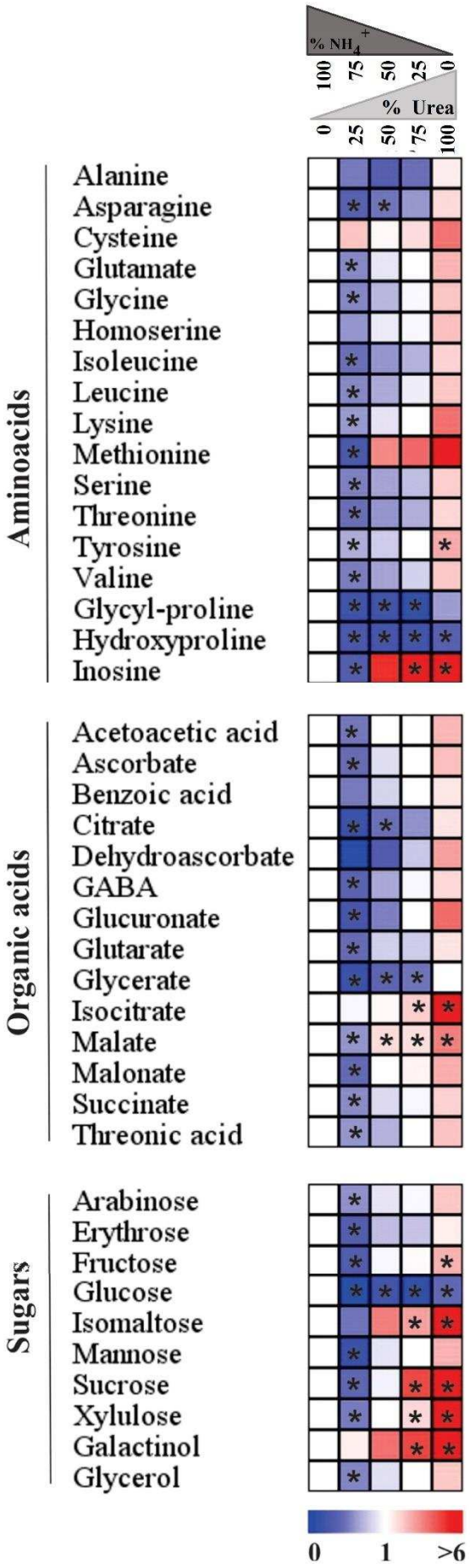

% NH4+
100 75 50 25 0
% Urea
0 25 50 75 100
Aminoacids
Alanine
Asparagine
Cysteine
Glutamate
Glycine
Homoserine
Isoleucine
Leucine
Lysine
Methionine
Serine
Threonine
Tyrosine
Valine
Glycyl-proline
Hydroxyproline
Inosine
Organic acids
Acetoacetic acid
Ascorbate
Benzoic acid
Citrate
Dehydroascorbate
GABA
Glucuronate
Glutarate
Glycerate
Isocitrate
Malate
Malonate
Succinate
Threonic acid
Sugars
Arabinose
Erythrose
Fructose
Glucose
Isomaltose
Mannose
Sucrose
Xylulose
Galactinol
Glycerol
0 1 >6

Figure 7: Metabolite profiling of *C. reinhardtii* CC 125 cells cultivated under different proportions of urea and ammonium (NH_4^+) in the stationary phase. Heat map representing the changes in relative metabolite contents determined as described in secction 2.4 of Materials and Methods. Data are normalized to the internal standard and cell number. Asterisk demarcates values that were judged to be significantly different from the treatment with 100% NH_4^+ ($p < 0.05$) at the same time point following the performance of Student's t tests.

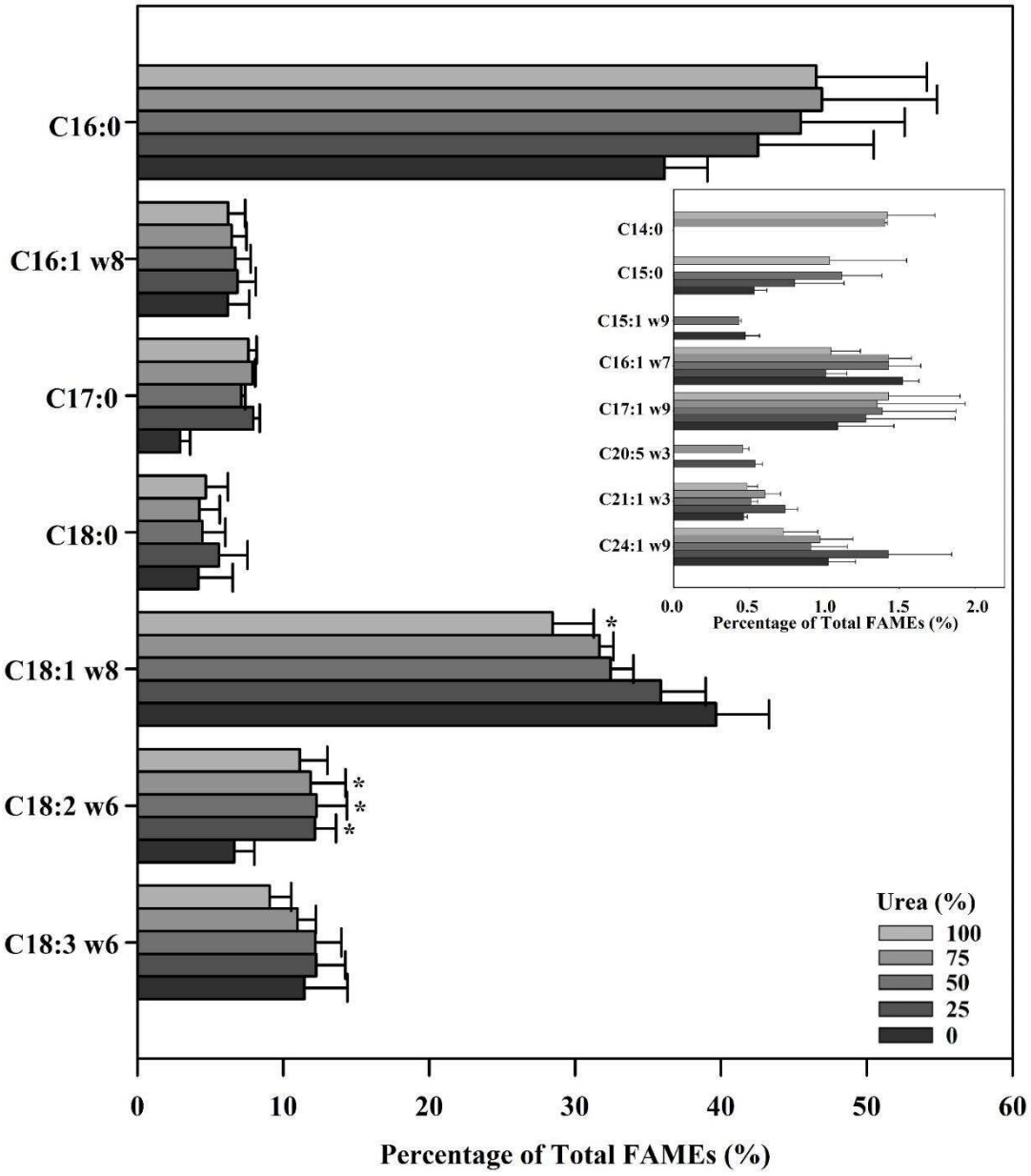
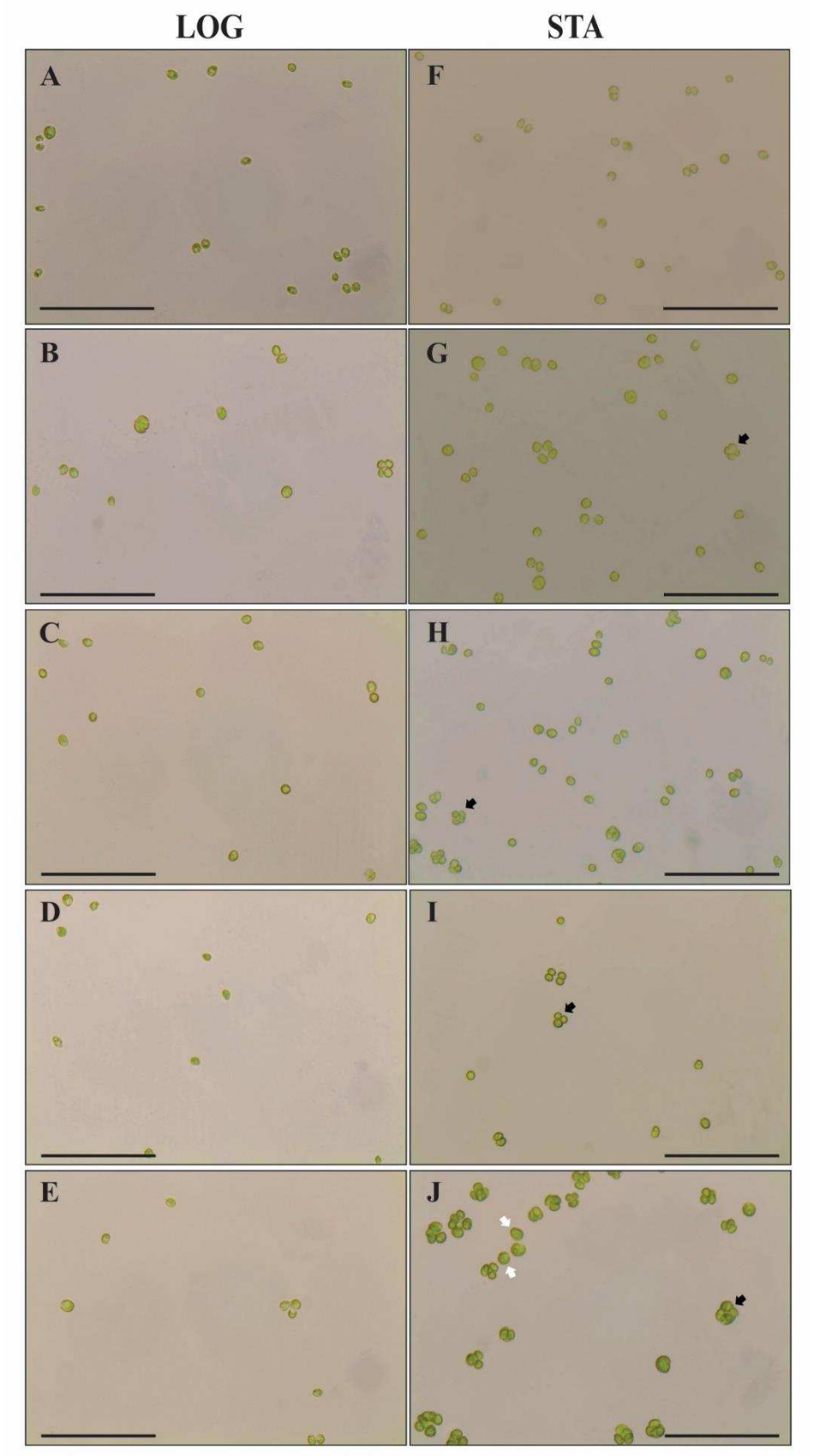


Figure 8: Fatty acid profile (FAMEs) of *C. reinhardtii* CC 125 cells cultivated under different proportions of urea and ammonium (NH_4^+) in the stationary phase. Values represent the mean ± SD of four replicates. Asterisk demarcates values that were judged to be significantly different from the treatment with 100% NH_4^+ ($p < 0.05$) at the same time point following the performance of Student's t tests.



Supplemental Figure 1: Images of *C. reinhardtii* CC125 cells cultivated under different proportions of urea and ammonium (NH_4^+). The graphs to the left refer to

the logarithmic phase (LOG) and the right to stationary phase (STA). **A**-0% urea, 100% NH_4^+; **B**-25% urea, 75% NH_4^+; **C**- 50% urea, 50% NH_4^+; **D**- 75% urea, 25% NH_4^+; **E**- 100% urea, 0% NH_4^+; **F**-0% urea, 100% NH_4^+; **G**-25% urea, 75% NH_4^+; **H**- 50% urea, 50% NH_4^+; **I**- 75% urea, 25% NH_4^+; **J**- 100% urea, 0% NH_4^+. The black arrows indicate cell clusters formed during cell division and the white arrows indicate some cells of the 100% urea treatment with a visually larger diameter than other treatments. The bar corresponds to 100 μm. Images were photographed under Olympus CKX 41 light microscope in 40X objective.

Supplemental Table 1: Composition of the culture medium Tris Acetate Phosphate (TAP medium)

Solution	**Composition (for 1L)**
Phosphate Buffer	108 g de K_2HPO_4; 56g de KH_2PO_4 e H_20 q.s.p 1L
Nutrient Stock Solution I *	40g de NH_4Cl; 10g de $MgSO_4.7H_20$; 5g de $CaCl_2.2H_20$ e H_20 q.s.p 1L
Nutrient Stock Solution II**	55.7g de KCl; 10g de $MgSO_4.7H_20$; 5g de $CaCl_2.2H_20$ e H_20 q.s.p 1L
Hutner's trace metal solution	22g de $ZnSO_4.7H_2O$; 11,4g de H_3BO_3; 5,06g de $MnCl_2$; 1.61g de $CoCl_2.6H_2O$; 1.57g de $CuSO_4.5H_2O$; 1,1g de $(NH_4)_6Mo_7O_{24}.4H_2O$; 4,99g de $FeSO_4.7H_2O$ e 50g de EDTA.

*To make a TAP medium, Add 20 ml 1 M Tris, pH 7.0, 1 ml phosphate buffer, 1 ml Hutner's trace metal solution, and 10 ml nutrient stock solution I to approximately 0.5 L of bidistilled water, adjust pH to 7.0 with 1.0 mL glacial acetic acid, and add water to a final volume of 1 L.
** To make a TAP-N (nitrogen-deficient medium), prepare TAP as described above but replace nutrient stock solution I with nutrient stock solution II.

Supplemental Table 2: Fold change ±SER in relative levels of primary metabolites of *Chlamydomonas reinhardtii* grown under different proportions of urea and ammonium. Values presented are means of four biological replicates. The bold numbers that were judged to be significantly different (Students't test, $p \leq 0.05$).

	Percentage of Urea (%)				
	0	**25**	**50**	**75**	**100**
Amino acids					
Alanine	1,00 ± 0.37	0,51 ± 0.27	0,36 ± 0.31	0,46 ± 0.21	1,45 ± 0.89
Asparagine	1,00 ± 0.25	**0,37 ± 0.08**	**0,49 ± 0.16**	0,61 ± 0.21	1,82 ± 1.21
Cysteine	1,00 ± 0.36	2,25 ± 1.20	1,22 ± 0.74	1,76 ± 0.73	3,68 ± 3.61
Glutamate	1,00 ± 0.15	**0,56 ± 0.10**	0,89 ± 0.07	1,03 ± 0.23	2,48 ± 1.13
Glycine	1,00 ± 0.17	**0,55 ± 0.09**	0,72 ± 0.22	0,96 ± 0.16	2,21 ± 0.94
Homoserine	1,00 ± 0.27	0,60 ± 0.16	0,90 ± 0.07	0,95 ± 0.11	2,28 ± 0.96
Isoleucine	1,00 ± 0.15	**0,47 ± 0.12**	0,60 ± 0.29	0,70 ± 0.17	1,97 ± 1.13
Leucine	1,00 ± 0.18	**0,56 ± 0.09**	0,67 ± 0.23	0,91 ± 0.15	2,16 ± 0.95
Lysine	1,00 ± 0.23	**0,62 ± 0.14**	0,88 ± 0.18	1,00 ± 0.36	3,73 ± 3.29
Methionine	1,00 ± 0.10	**0,29 ± 0.09**	3,35 ± 3.32	3,92 ± 1.66	6,35 ± 2.92
Serine	1,00 ± 0.17	**0,53 ± 0.11**	0,65 ± 0.30	0,74 ± 0.15	2,06 ± 1.08
Threonine	1,00 ± 0.18	**0,46 ± 0.13**	0,60 ± 0.34	0,69 ± 0.17	1,88 ± 1.16
Tyrosine	1,00 ± 0.14	**0,68 ± 0.10**	0,79 ± 0.25	1,05 ± 0.22	**2,69 ± 1.12**
Valine	1,00 ± 0.16	**0,52 ± 0.11**	0,65 ± 0.26	0,80 ± 0.17	2,14 ± 1.02
Glycyl-proline	1,00 ± 0.08	**0,21 ± 0.08**	**0,23 ± 0.20**	**0,14 ± 0.07**	0,63 ± 0.56
Hydroxyproline	1,00 ± 0.24	**0,29 ± 0.08**	**0,25 ± 0.15**	**0,24 ± 0.10**	**0,37 ± 0.19**
Inosine	1,00 ± 0.12	**0,35 ± 0.10**	4,89 ± 4.49	**5,61 ± 2.28**	**18,55 ± 6.18**
Pyroglutamic acid	1,00 ± 0.15	**0,53 ± 0.09**	0,83 ± 0.07	0,95 ± 0.18	2,29 ± 0.99
Organic Acids					
Acetoacetic acid	1,00 ± 0.25	**0,50 ± 0.04**	1,07 ± 0.12	1,10 ± 0.22	2,47 ± 1.21
Ascorbate	1,00 ± 0.17	**0,45 ± 0.15**	0,86 ± 0.07	1,00 ± 0.11	2,31 ± 1.00
Benzoic acid	1,00 ± 0.41	0,51 ± 0.31	0,82 ± 0.14	0,98 ± 0.38	1,58 ± 1.07

Citrate	1,00	±	0.13	**0,20**	**±**	**0.16**	**0,47**	**±**	**0.13**	0,59	±	0.21	1,66	±	1.23
Dehydroascorbate	1,00	±	1.26	0,06	±	0.03	0,33	±	0.26	0,78	±	0.33	2,88	±	1.73
GABA	1,00	±	0.18	**0,39**	**±**	**0.09**	0,66	±	0.31	0,95	±	0.51	1,85	±	0.68
Glucuronate	1,00	±	0.41	**0,26**	**±**	**0.07**	0,53	±	0.24	1,10	±	0.26	3,82	±	1.88
Glutarate	1,00	±	0.00	**0,45**	**±**	**0.04**	0,80	±	0.01	0,79	±	0.11	1,63	±	0.77
Glycerate	1,00	±	0.27	**0,20**	**±**	**0.03**	**0,41**	**±**	**0.08**	**0,49**	**±**	**0.09**	1,05	±	0.73
Isocitrate	1,00	±	0.21	0,95	±	0.26	1,23	±	0.34	**2,04**	**±**	**0.45**	**9,07**	**±**	**4.87**
Malate	1,00	±	0.10	**0,60**	**±**	**0.19**	**1,79**	**±**	**0.33**	**1,70**	**±**	**0.26**	**3,47**	**±**	**1.86**
Malonic acid	1,00	±	0.19	**0,44**	**±**	**0.14**	1,03	±	0.20	1,34	±	0.31	2,61	±	1.60
Succinate	1,00	±	0.17	**0,57**	**±**	**0.07**	0,84	±	0.13	0,95	±	0.16	1,97	±	1.00
Threonic acid	1,00	±	0.14	**0,61**	**±**	**0.07**	0,70	±	0.37	1,05	±	0.23	2,24	±	1.12
Sugars															
Arabinose	1,00	±	0.16	**0,59**	**±**	**0.09**	0,88	±	0.08	0,96	±	0.17	2,17	±	1.07
Erythrose	1,00	±	0.24	**0,35**	**±**	**0.08**	0,74	±	0.14	0,76	±	0.15	1,48	±	0.89
Fructose	1,00	±	0.12	**0,34**	**±**	**0.21**	0,95	±	0.15	1,18	±	0.25	**2,58**	**±**	**1.07**
Fructose-1-P	1,00	±	0.11	**0,24**	**±**	**0.13**	**0,54**	**±**	**0.03**	**0,65**	**±**	**0.03**	1,38	±	0.67
Fructose-6-P	1,00	±	0.03	**0,12**	**±**	**0.05**	0,97	±	0.14	2,08	±	0.66	4,16	±	3.25
Glucose	1,00	±	0.24	**0,04**	**±**	**0.05**	**0,23**	**±**	**0.22**	**0,11**	**±**	**0.02**	**0,42**	**±**	**0.28**
Isomaltose	1,00	±	0.34	0,49	±	0.21	3,49	±	1.95	**2,88**	**±**	**0.88**	**12,17**	**±**	**4.83**
Mannose	1,00	±	0.14	**0,18**	**±**	**0.09**	0,88	±	0.07	1,01	±	0.20	2,57	±	1.17
Sucrose	1,00	±	0.35	**0,41**	**±**	**0.19**	0,93	±	0.42	**4,47**	**±**	**1.67**	**16,13**	**±**	**11.92**
Xylulose	1,00	±	0.11	**0,52**	**±**	**0.13**	1,11	±	0.18	**1,88**	**±**	**0.43**	**6,16**	**±**	**3.78**
Galactinol	1,00	±	0.39	1,44	±	0.68	3,74	±	2.49	**4,53**	**±**	**2.35**	**12,11**	**±**	**5.88**
Glycerol	1,00	±	0.26	**0,55**	**±**	**0.10**	0,87	±	0.06	1,02	±	0.15	2,16	±	0.90

Supplemental Table 3: Fatty Acid Profile (as percentage of FA with respect to Total Fatty Acid Fraction detected on the chromatography) in *C. reinhardtii* CC 125 mt+ growing in urea. Values are expressed as mean ± SD. The bold numbers that were judged to be significantly different (Students't test, $p \leq 0.05$).

	Percentage of Urea (%)														
	0			**25**			**50**			**75**			**100**		
C14:0	ND[a]		-	ND		-	ND		-	1.41	±	0.02	1.42	±	0.31
C15:0	0.54	±	0.08	0.81	±	0.33	1.12	±	0.27	ND		-	1.04	±	0.51
C15:1 w9	0.48	±	0.09	ND		-	0.44	±	0.02	ND		-	ND		-
C16:0	36.13	±	2.94	42.54	±	7.94	45.47	±	7.12	46.92	±	7.90	46.53	±	7.60
C16:1 w7	1.52	±	0.11	1.01	±	0.14	1.43	±	0.21	1.43	±	0.15	1.05	±	0.20
C16:1 w8	6.21	±	1.47	6.85	±	1.25	6.70	±	1.04	6.46	±	1.02	6.22	±	1.18
C17:0	1.52	±	0.67	1.69	±	0.44	1.67	±	0.27	1.78	±	0.19	0.84	±	0.57
C17:1 w9	1.09	±	0.37	1.28	±	0.60	1.39	±	0.49	1.35	±	0.59	1.43	±	0.48
C18:0	4.18	±	2.35	5.58	±	1.96	4.45	±	1.58	4.26	±	1.39	4.70	±	1.51
C18:1 w8	39.66	±	3.63	35.89	±	3.08	32.45	±	1.56	31.67	±	0.96	**28.47**	**±**	**2.81**
C18:2 w6	6.64	±	1.37	**12.17**	**±**	**1.44**	**12.28**	**±**	**2.09**	**11.88**	**±**	**2.39**	11.13	±	1.88
C18:3 w6	11.44	±	2.96	12.26	±	2.00	12.20	±	1.77	10.98	±	1.26	9.07	±	1.46
C20:5 w3	ND		-	0.55	±	0.05	ND		-	0.46	±	0.04	ND		-
C21:1 w3	0.47	±	0.03	**0.74**	**±**	**0.09**	0.52	±	0.05	0.61	±	0.11	0.49	±	0.07
C24:1 w9	1.03	±	0.18	1.43	±	0.42	0.91	±	0.24	0.97	±	0.22	0.73	±	0.23
ΣSFA	42.37	±	6.04	50.62	±	10.67	52.71	±	9.24	54.37	±	9.50	54.53	±	10.50
ΣMUFA	50.46	±	5.88	47.20	±	5.58	43.84	±	3.61	42.49	±	3.05	38.39	±	4.97
ΣPUFA	18.08	±	4.33	24.98	±	3.49	24.48	±	3.86	23.32	±	3.69	20.20	±	3.34

[a] ND = not detected.

* **w** refers to the double bond positions given from the methyl end of the carbon chain.