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Nitrogen and sulfur deprivation differentiate lipid accumulation targets of *Chlamydomonas reinhardtii*

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Nitrogen (N) and sulfur (S) have inter-related and distinct impacts on microalgal metabolism; with N starvation having previously been reported to induce elevated levels of the biodiesel feedstock material triacylglycerol (TAG), while S deprivation is extensively studied for its effects on biohydrogen production in microalgae.[1,2] We have previously demonstrated that N- and S-starved cells of *Chlamydomonas reinhardtii* display different metabolic trends, suggesting that different response mechanisms exist to compensate for the absence of those two elements.[3] We used *C. reinhardtii* CC-124 mt(-) and CC-125 mt(+) strains to test possible metabolic changes related to TAG accumulation in response to N and S deprivation, considering that gamete differentiation in this organism is mainly regulated by N.[4] Our findings contribute to the understanding of microalgal response to element deprivation and potential use of element deprivation for biodiesel feedstock production using microalgae, but much remains to be elucidated on the precise contribution of both N and S starvation on microalgal metabolism.

Due to high bioenergy outturn and the synthesis of high value added products associated with this group, microalgae are frequently investigated as a potential means for various biotechnological applications.[5] Biofuel production from microalgae has emerged as a promising way of partially remediating the dependency of global energy demand on fossil fuels. Compared with fossil fuels, biofuel production from microalgae is currently not cost-effective; however, continued increases in oil prices, together with a potential decrease in the cost of biodiesel from microalgae, are expected to make biofuels a viable alternative in the near future. A great variety of microalgae show distinct metabolic properties and are able to switch their metabolic output levels in response to different abiotic stress factors. Levels of TAG, a principal biodiesel feedstock, vary widely across microalgae, depending on their species-specific nature and environmental factors such as changes in element concentration or presence, light intensity and temperature.[6,7]

N vs. S Deprivation: Differential Survival Strategy of Microalgae

As a macroelement, N has a profound importance for microalgal metabolism and the limitation of this element is compensated by radical changes in several key metabolic pathways. In the process of acclimation to N deficiency, microalgae have been reported to degrade ribosomes and decrease enzyme activities involved in photosynthesis, glyoxylate cycle, gluconeogenesis and photosynthetic carbon fixation cycle while simultaneously inducing carotenoid production to protect against oxidative stress, increasing the expression levels of TAG synthesis related genes in significant quantities, and differentiating into gametes, considered a potential survival strategy since zygotes can withstand adverse conditions.[8,9] **Figure 1** shows that

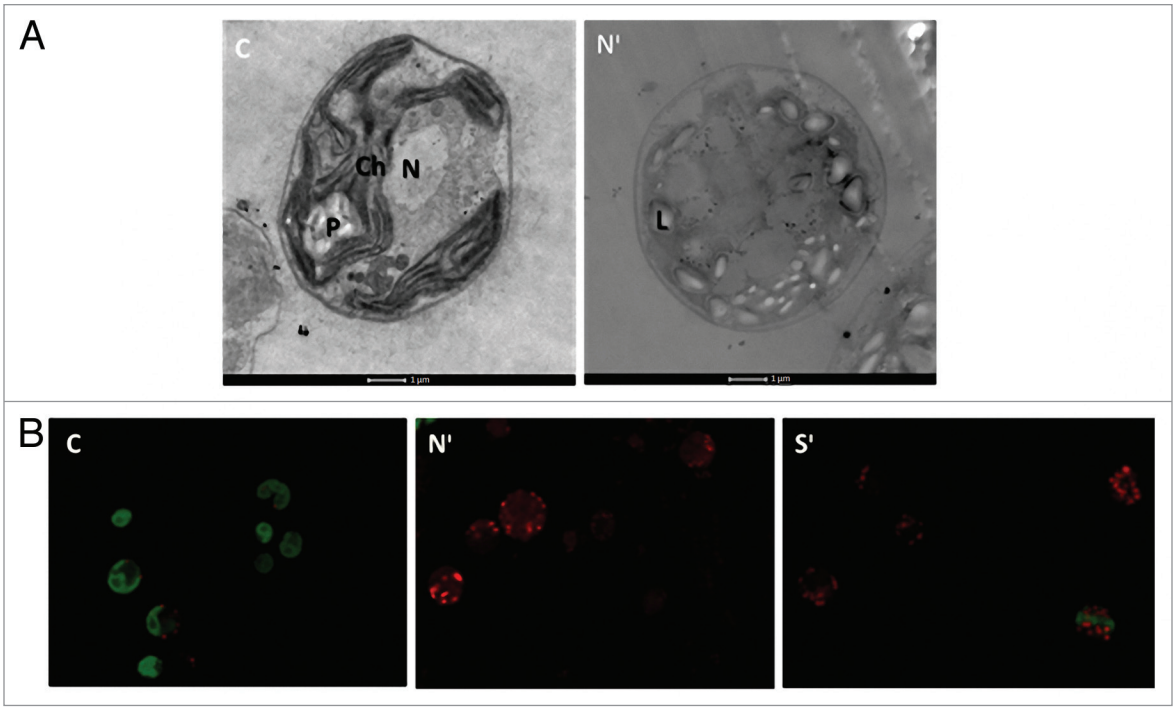

Figure 1. Transmission electron microscopy (A) and confocal fluorescence microscopy (B) images of control, N-starved and S-starved *C. reinhardtii* cells sampled on fifth day of incubation. In the fluorescence images green represents chlorophyll autofluorescence and light red represents Nile red fluorescence. Abbreviations: C, control; N′, N-deprived cells; S′, S-deprived cells; Ch, chloroplast; P, pyrenoid; N, nucleus; L, lipid bodies.

nitrogen deprivation causes dramatic anatomical changes in *C. reinhardtii*. Most notably the chloroplast is degraded into smaller sphere-like sub-compartments and cytoplasmic lipid droplets are formed (**Fig. 1A**). Besides these, chloroplast degradation is not as fast as N deprivation in S-deprived cells (**Fig. 1B**) supporting the data reported previously.[3] Microalgae have been reported to synthesize arylsulphatase to recover SO_4^{-2} from SO_4^{-2} esters, upregulate acyltransferases and ATP sulfurylase expression, downregulate proteins involved in translation and folding, and decrease chloroplast ribosomal polypeptides greatly.[10,11] Furthermore, cessation of cell cycle, upregulation of the enzymes of the oxidative pentose phosphate pathway, N- or S- scavenging proteins, activation of various mechanisms for reactive oxygen species removal, repression of Calvin cycle enzymes and decrease of photosynthetic activity are observed during both types of deprivations.[12,13] Such changes represent attempts to survive the increased oxidative stress associated with nutrient deprivation and to recover N or S from the environment by highly selective uptake processes for those elements.

Work in our lab recently demonstrated that S-deprived samples of *C. reinhardtii* mt(+) and mt(-) strains display increased growth rates, cell volumes, neutral lipid and TAG accumulation compared with their N-deprived equivalents, while a more rapid decrease in chlorophyll content was observed in *C. reinhardtii* cultures under N deprivation (**Table 1**). Furthermore, the metabolic changes associated with nutrient starvation occurred in a time-dependent manner, generally reaching a maximum on the fourth and fifth days of starvation and decreasing or remaining stable afterwards.[3] This trend may indicate that vegetative cells of *C. reinhardtii* can mitigate the effects of N and S starvation for four to five days before the stress associated with long-term nutrient deprivation leads to autophagy to recycle part of the cytoplasm including organelles. N deficiency was reported to induce autophagy, which is a self-degrading process common in eukaryotes that provides needed energy and raw materials for cellular repair, in many organisms[14] including *C. reinhardtii*.[15] Longer starvation period that autophagy response is insufficient would lead cell death. Dead cells may then be scavenged by their conspecifics for their N or S content, allowing limited growth and a stable cell count.

Our studies showed that N starvation generally yielded similar effects as S starvation, but the negative impacts on cell count, total protein and chlorophyll levels were much more severe (**Table 1**). This result is likely caused by the relative importance and abundance of N compared with S, such that while S can be salvaged from dead cells or obtained from intracellular stores, N must be supplied constantly for adequate growth. N content of dry *C. reinhardtii* biomass is known to be over 10-fold greater than the S content.[2] As such, a much greater mass of N is necessary for *C. reinhardtii*, while a comparatively lesser amount of S, such as that found in the initial cells inoculated into the S-free medium, may be enough to partially facilitate growth. Compared with S-starved cells, N-starved *C. reinhardtii* cells also displayed a lower amount of enlargement, which may also be correlated with the greater metabolic stress N-starved samples undergo (**Table 1**). We have observed that cellular functions are affected more rapidly in N-starved

Table 1. Changes in growth and biochemical parameters in wild type *C. reinhardtii* CC-124 and CC-125 strains after four days of N or S deprivation

Parameters tested	*C. reinhardtii* CC-124 (mt -)		*C. reinhardtii* CC-125 (mt +)	
	N deprivation	S deprivation	N deprivation	S deprivation
Cell Growth	83% decrease	65% decrease	66% decrease	49% decrease
Total biovolume	62.6% decrease	220% increase	54.6% decrease	310% increase
Relative dry weight	32% decrease	27% decrease	23% decrease	20% decrease
Protein level	88% decrease	89% decrease	87% decrease	89% decrease
Chlorophyll content	61% decrease	26% decrease	89% decrease	74% decrease
Carotenoid content	3.6-fold increase	2.8-fold increase	1.9-fold increase	2.3-fold increase
Cell biovolume	2.9-fold increase	6.1-fold increase	1.7-fold increase	5.8-fold increase
Starch level	2.3-fold increase	3.4-fold increase	4.3-fold increase	4.7-fold increase
Relative polisaccharide level	8.1-fold increase	9.9-fold increase	13.1-fold increase	8.6-fold increase
Total neutral lipid level	2.4-fold increase	2.6-fold increase	1.7-fold increase	3-fold increase
Relative TAG level	6.9-fold increase	15.3-fold increase	29.1-fold increase	16.5-fold increase

Detailed information can be found in our recent paper.[3]

microalgal cells. N starvation leads to an almost instantaneous cell growth arrest due to the obligatory presence of N in every protein and most metabolites cannot be compensated by autophagy or other recycling pathways. On the other hand, S deprivation leads to less sudden but again severe responses in overall metabolism and cellular functions. This temporal delay in response probably corresponds to a period of cellular recycling by autophagy and better accumulation of stress marker molecules (carotenoid, TAG, etc.).

S Deprivation May Be Used as a Potential Means for TAG Production from Microalgae

As previously reported,[3] chlorophyll content decreased rapidly upon both S and N starvation, while a corresponding increase in carotenoid content was also observed. *C. reinhardtii* is known to restructure its photosynthetic machinery upon S deprivation, resulting in a decrease in the expression of many of the proteins making up the photosystem complexes I and II within 24 h.[1] Such adjustments occur to minimize oxidative stress, as reactive oxygen species (ROS) are generated during photosynthesis and the shutdown of the latter may afford a measure of control over their levels.[16] As such, the decrease in chlorophyll synthesis is interpreted to be a part of the alteration, deactivation and disassembly of photosynthetic complexes as a response to oxidative stress resulting from S starvation. Likewise, N deprivation is closely associated with the degradation of ribulose-1,5-bisphosphate carboxylase oxygenase to recycle the latter's N content[17] and the depletion of this protein may necessitate alterations in the mechanism of photosynthesis, leading to the decrease in chlorophyll content observed in N-deficient *C. reinhardtii*.[18,19] An increase in carotenoid content, observed in both N- and S-starved samples, is a response to the stress conditions brought about by nutrient deficiency and is consistent with previous studies.[20] As such, their accumulation may be a stress response intended to prevent oxidative damage. Our recent investigation showed that both starch and neutral lipids greatly accumulate in S-deprived *C. reinhardtii* and that those increases correspond to the rapid decrease in protein levels observed during the first day of starvation. Production of starch took priority over lipid synthesis, suggesting that the two metabolites may compete and that the disturption of starch metabolism may increase lipid production capacity, as has been suggested previously.[2,21] While both metabolites were found to increase greatly upon starvation with no apparent antagonistic effects, this is likely the result of cell enlargement caused by S deprivation instead of a true lack of competition between lipid and starch synthesis. Our results suggest that a global shutdown in energetic functions may occur upon S deprivation. Flagella are almost always lost after third day of S deprivation and chlorophyll levels drop considerably, leading to the conclusion that anabolic reactions are severely reduced in that particular cell. Herein, we propose that upon reduction of energy consumption, the trend of metabolism favors storage of energetic denser molecules. Lipids are highly energetic molecules having a higher energy yield per gram than sugars, while starch is known to be the densest form of sugars that is usually used for storage in plants.

Records detailing the use of N starvation to increase lipid production for biodiesel production exist in literature,[2] our study suggests that S starvation is the preferable approach due to the lack of adequate cell growth and biovolume attainment upon N exposure.

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