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Understanding microalgae behavior under alternating energy starvation and N-starvation conditions to develop “feast-and-famine” processes

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Feeding organic substrates to microalgae cultures in photo-heterotrophic, mixotrophic, or fully heterotrophic processes could provide effective solutions for enhancing the economic and environmental sustainability of industrial microalgae cultivation, particularly when wastewaters or agro-industrial by-products are used as organic substrate sources. However, controlling bacterial contamination remains a major challenge that hinders the scalability of such processes. The application of feast and famine strategies has been shown to mitigate contamination issues. Nevertheless, comprehensive kinetic models that can predict microalgal behavior under variable nutrient stress conditions are lacking. This study aims to provide a preliminary understanding of microalgae behavior under different combinations of energy and nitrogen starvation conditions. The objective is not only to understand the responses to specific starvation conditions but also to examine how behavior changes depending on the previous environmental conditions of the inoculum. In this paper, various combinations of nutrient availability conditions were tested on a strain of Chlorella sorokiniana. Under heterotrophic nitrogen starvation, microalgae growth followed a logistic model, with a fattening factor of 2.8 and a maximum specific growth rate (µmax) of 0.032 h⁻¹. Under energy starvation conditions, with or without nitrogen, on average 16% biomass reduction was consistently observed within 48 hours, with no significant effect from prior nutrient conditions. Cell concentration exhibited different behaviors, with cell numbers increasing under most energy starvation conditions, except when microalgae were transferred from nitrogen starvation to simultaneous nitrogen and energy starvation. Conventional first-order models were inadequate to describe biomass decay under energy starvation conditions. Therefore, kinetic models that account for the consumption of intracellular components, such as carbohydrates, should be developed.

The results of this study provide insights that can be used to develop innovative models to predict microalgae behavior under variable nutrient availability conditions. These insights allow the development of new cultivation processes more environmentally sustainable than the conventional ones.

* 1. Introduction

[Microalgae](https://www.sciencedirect.com/topics/earth-and-planetary-sciences/microalga) are photosynthetic [microorganisms](https://www.sciencedirect.com/topics/earth-and-planetary-sciences/micro-organism) that have emerged as promising systems for the production of food, feed and other bio-based products. However, their widespread application in many economic sectors is currently limited by the high production cost of conventional phototrophic processes (Ruiz et al., 2016). In addition to the conventional phototrophic metabolism, by which microalgae use light as energy source and CO2 as carbon source; they can also use an heterotrophic metabolism, by which organic substrates are employed as both carbon and energy source. In the mixotrophic metabolism, phototrophy and heterotrophy are performed in parallel. Supplementing organic substrate to photobioreactors, by implementing mixotrophic or photo-heterotrophic processes, can reduce production costs and energy demand due to the improved biomass productivity achieved with the heterotrophic metabolism. Such kinds of strategies are particularly attractive when wastewaters or by-products are used as sources of organic substrate.

However, the main limitation hindering the scale-up of these processes is the excessive bacteria contamination arising when organic substrates is provided to non-axenic photobioreactors (Dragone, 2022). The application of properly designed “feast-and-famine” processes has been shown in recent years to control bacterial contamination while maintaining improvements in biomass productivity given by the heterotrophic metabolism (Di Caprio, 2021). However, these processes require cultivation under varying nitrogen and energy starvation conditions. The behavior of microalgae under these variable environmental conditions has been poorly studied, hindering the development of appropriate kinetic models that could be used for a better process design (Di Caprio, 2021).

In this study, for the first time, microalgae were systematically cultivated under different combinations of variable environmental conditions including optimal growth, N-starvation, energy starvation and combined N and energy starvation. The changes in biomass concentration, cell concentration and elemental composition of algae were monitored.

* 1. Materials and methods
		1. Microalgae maintenance

*Chlorella sorokiniana* SAG 211/8k was maintained in 300-mL Erlenmeyer flasks, in non-axenic conditions, 150 rpm orbital shaking, in BG-11 medium, 24/24 h irradiation at 80 μmol s–1 m–2 and at 25 ± 3 °C. From flaks, microalgae were inoculated into 500 mL (h = 35 cm, d = 5 cm) column glass photobioreactors (PBRs) at 0.1 g L–1, in modified M-8 medium, with the following nutrient concentration (mM): KNO3 29.7, KH2PO4 5.4, Na2HPO4∙2H2O 1.5, MgSO4∙7H2O 1.6, CaCl2∙2H2O 0.09, EDTA ferric sodium salt 0.3, Na2EDTA∙2H2O 0.1, H3BO3 0.001, MnCl2∙4H2O 0.07, ZnSO4∙7H2O 0.01,CuSO4∙5H2O 0.0073. Chemicals were purchased from Sigma Aldrich.

PBRs were maintained at 27 ± 1 °C, pH at 6.7 ± 0.5 and fed with 1 L min–1 air and 35 mL min–1 pure CO2, under continuous light supply at 500 ± 50 μmol s–1 m–2 photons with led light lamps (GROWSTAR L-QB1). The photon flux was measured at different heights of the PBR and the mean value is reported.

* + 1. Cultivation under different environmental conditions

Experiments were conducted in duplicate in 500 mL PBRs in the conditions described in the following:

* Optimal Growth (OG): microalgae were cultivated as described for maintenance in 2.1 at initial concentration of 0.1 g L-1.
* N-starvation (N-): microalgae from OG condition were harvested and inoculated at 0.5 g L-1 into PBRs with fresh modified M-8 medium without KNO3 and with 10 g L-1 glucose. PBRs were fed with only air and maintained under dark by covering them with aluminum foils.
* Energy starvation after N-starvation (E-af\_N-): after cultivation in N-, cells were harvested and inoculated at 1.5 g L-1 into PBRs with fresh M-8 medium. PBRs were fed with only air and maintained under dark by covering them with aluminum foils.
* Energy and N-starvation after optimal growth (E-N-af\_OG): after cultivation in OG, cells were harvested and inoculated at 1.5 g L-1 into PBRs with fresh M-8 medium without KNO3. PBRs were fed with only air and maintained under dark by covering them with aluminum foils.
* Energy starvation after optimal growth (E-af\_OG): after cultivation in OG, cells were harvested and inoculated at 0.85 g L-1 into PBRs with fresh M-8 medium. PBRs were fed with only air and maintained under dark by covering them with aluminum foils.
* Energy and N-starvation after N-starvation (E-N-af\_N-): after cultivation in N-, cells were harvested and inoculated at 1 g L-1 into PBRs with fresh M-8 medium without KNO3. PBRs were fed with only air and maintained under dark by covering them with aluminum foils.
	+ 1. Parameter estimation

Biomass to glucose yield was estimated by Eq. 1, with S0 and S(t) the initial glucose concentration (g L-1 ) and the glucose concentration measured at time t, respectively.

$Y\_{{X}/{S}}=\frac{X-X\_{0}}{S\_{0}-S(t)}$ (1)

The accumulation ability under N-starvation was estimated with the maximum fattening factor (ηmax), determined from non-linear fitting of data with the logistic model (Eq. 2) revised in the form of Eq. 3, by considering that Xmax can be expressed as Xmax = ηmax X0.

$X\left(t\right)=\frac{X\_{max}X\_{0}e^{μ\_{max}t}}{X\_{max}+X\_{0}\left(e^{μ\_{max}t}-1\right)} $ (2)

$η=\frac{X(t)}{X\_{0}}=\frac{η\_{max}e^{μ\_{max}t}}{η\_{max}+e^{μ\_{max}t}-1} $ (3)

Where X0 and Xmax are the initial and maximum biomass concentration (g L-1), while µmax is the maximum specific growth rate (h-1).

* + 1. Determination of biomass and cell concentration

Biomass concentration was determined by filtering a known volume of microalgae suspension through 0.2 µm cellulose acetate filters, which were then dried at 105 °C. Microalgae cell concentration was measured by optical counting in a Thoma chamber by using an optical microscope (Leitz Laborlux 12).

* + 1. Chemical characterizations

Biomass elemental composition was determined by CHNS analysis carried out by an elemental analyzer (EA 1110 CHNS/O). Glucose in the medium was determined by using the phenol-sulfuric acid method (Dubois method): 1 mL of 5% phenol solution and 5 mL of 96% sulfuric acid were added to 2 mL sample, mixed, cooled 10 min at environmental temperature and 10 min in a water bath at 27 °C, then absorbance was read at 490 nm and quantification made using a calibration line.

* + 1. **Data analysis**

All experiments were replicated at least twice. Significant difference among samples were tested by analysis of variance (ANOVA) in Microsoft Office Excel, considering α = 0.05. Errors are indicated as ± standard deviation

* 1. Results and discussion
		1. From optimal growth to N-starvation

A significant portion of our understanding of microalgae behavior regards optimal growth conditions, where all nutrients are available and cells grow in a balanced mode. Among various stress conditions, N-starvation is the most extensively studied in microalgae, as it is commonly used to enhance the accumulation of lipids and starch. These compounds accumulate when nitrogen depletion restricts the synthesis of proteins and nucleic acids. Under N-starvation microalgae increase their mass through the accumulation of organic compounds, up to a maximum accumulation ability, referred to as the maximum fattening factor: ηmax (Di Caprio, 2022). This behavior can be effectively described by a logistic model. Since the experiments in this study were conducted under heterotrophic conditions with N-starvation, the logistic model predicts well the kinetic behavior, independent of the initial biomass X0, when normalized as η = f (µmax, ηmax, t) (Figure 1a).



Figure 1: Microalgae biomass accumulation under N-starvation and result of data fitting with logistic model (a). Different symbols indicate independent repetitions of the experiment. Biomass to substrate yield (YX/S) at different batch sampling times (b).

The estimated ηmax = 2.8 ± 0.6 indicates that under N-starvation, *C. sorokiniana* can increase its biomass by up to 2.8 its initial value X0.This ηmax value is consistent with typical values previously reported for microalgae (Di Caprio, 2022). Similarly, the µmax = 0.032 ± 0.06 h-1 aligns well with previous findings for *C. sorokiniana* grown on glucose at the same temperature (Karimian et al., 2022). Some heterogeneity was observed among the repeated N-starvation experiments, likely due to differences in the physiological properties of the different inocula used. Indeed, parallel replicates using the same inoculum showed much higher reproducibility (Figure 1a). After 4 days of cultivation, biomass accumulation attained η = 0.9 ηmax, which can be considered a sufficient completion of the accumulation phase in a production process. Achieving η = 0.9 ηmax could be considered a good compromise among biomass production and productivity, as achieving η closer to ηmax would significantly reduce the productivity of the batch. During the N-starvation experiment, there was not any problem of contamination by other microorganisms as N depletion hinder cell duplication of any other potential contaminant. The N-starvation experiment also allowed for the determination of the biomass-to-glucose yield factor YX/S (Figure 1b). YX/S was 0.28 ± 0.03 after 24h of cultivation, with no significant variation observed at longer time points. The YX/S valueafter 5 h was higher, but with higher associatederror, likely due to a transient phase in which glucose uptake occurred before being converted into storage compounds such as starch and lipids (Patel et al., 2022). A previous study reported a higher YX/S between 0.36-0.38 (Patel et al., 2022).

* + 1. Microalgae behavior under energy starvation

The behavior of microalgae under energy starvation conditions is poorly studied, as this is an unproductive phase and typically considered irrelevant for industrial applications. However, energy starvation accounts for approximately for half of the time microalgae spend in photobioreactors in outdoor plants. This is especially true in regions, such as Europe, where night typically comprises half of the day. This means that microalgae are currently typically cultivated under feast (daytime) and famine (nighttime) conditions. Furthermore, various innovative heterotrophic and photo-heterotrophic processes under feast and famine conditions have been described for microalgae cultivation with wastewaters, aimed at controlling bacteria contamination (Di Caprio, 2021). Previous studies have shown that during the night phase, microalgae can lose between 1-25 % of their biomass (Edmundson & Huesemann, 2015). Additional data are required to understand how the conditions experienced prior to entering energy starvation influence microalgae behavior. When microalgae were moved from N-starvation to energy starvation (in the presence of nitrogen), biomass concentration decreased over a 4 day period. Comparable maximum biomass losses were observed across all energy starvation conditions tested (p = 0.08), with an average loss of −16 ± 6%, which is in consistent with previous measures (Edmundson & Huesemann, 2015). This finding suggests that the environmental conditions in which microalgae were cultivated before entering energy starvation phase do not affect the extent of biomass loss (Figure 2). First-order kinetic models are commonly used to describe cell decay, particularly for predicting bacteria decay under energy starvation (Wang & Witarsa, 2016). However such models are inapplicable to microalgae, as their decay is limited to a fraction of the initial biomass. Two alternative approaches could be considered: i) splitting the initial biomass X0 into two sub-fractions, a fixed percentage of which undergoes decay, or ii) using structured models that account for intracellular components (e.g. carbohydrates), and include decay reactions specific to these components.

Different behaviors were observed with regards to cell division. When microalgae were shifted from N-starvation to energy starvation, substantial cell division occurred (Figure 2b). This was because microalgae utilized the accumulated organic carbon as an energy source and the available nitrate in the medium as a nitrogen source for the synthesis of proteins and nucleic acids (functional biomass). This description is supported by previous findings (Di Caprio et al., 2019) and by the observed decrease in carbon content from 51% to 43.8% and increase in nitrogen content from 2.5% to 5.9% during the energy starvation phase (Table 1). Cell concentration increased 3-4 times. Under these conditions, cell concentration can only increase to a limited extent due to the finite amount of energy stored inside cells during the previous N-starvation phase.

A relevant increase in cell concentration was observed even when cells shifted from optimal growth conditions to energy starvation (with or without nitrogen) (Figure 3d,f). This increase was due to a fraction of cells that had already reached their “decision point” during optimal growth. The decision point is a regulatory checkpoint in the cell cycle, that, once attained, induce cells to proceed on completing their cell cycle, regardless of the new environmental conditions (Borowitzka et al., 2016). To sustain this cell division, organic compounds, such as carbohydrates, were likely consumed. When cells were shifted from optimal growth to energy starvation (with or without nitrogen), there was a relevant increase in nitrogen and sulfur in the elemental composition of the biomass, indicating an increase in protein content.

Table 1: Elemental composition of microalgae biomass after different cultivation conditions.

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|  | C (%) | H (%) | N (%) | S (%) |
| N-starvation (end) | 51 ± 1 | 8.1 ± 0.2 | 2.5 ± 0.6 | 0.1 ± 0.1 |
| Energy starvation after N-starvation (end) | 43.8 ± 0.2 | 7.0 ± 0.1 | 5.9 ± 0.1 | 0.23 ± 0.02 |
| Energy and N-starvation after N-starvation (end) | 51.6 ± 0.2 | 8.35 ± 0.05 | 3.1 ± 0.1 | n.d. |
| OG (end) | 46 ± 2 | 7.14 ± 0.01 | 8 ± 1 | 0.3 ± 0.1 |
| Energy and N-starvation after OG (end) | 48.0 ± 0.1 | 7.1 ± 0.1 | 9.11 ± 0.01 | 0.46 ± 0.01 |
| Energy starvation after OG (end) | 46.8 ± 0.3 | 7.4 ± 0.2 | 8.9 ± 0.2 | 0.39 ± 0.01 |

Only when microalgae were transferred from N-starvation to simultaneous energy and N starvation condition, no significant cell division occurred. In this case, cells under N-starvation had exited the cell cycle and entered the G0 phase. When these cells were exposed to both energy and N starvation, they could not divide because, despite having stored organic compounds, they lacked nitrogen to synthetize functional biomass. In this scenario, there were not significant changes in the elemental composition of the cells. These insights allow to understand how to choose the harvesting phase for the biomass in a process with variable nutrient availability. A biomass richer in nitrogen (N) would have a higher protein content, while biomass with a lower N percentage would be richer in carbohydrates and lipids.



Figure 2: Microalgae biomass decay during the different energy starvation phases (with X0 the initial biomass of the energy starvation phase) (a, c, e, g). Variation in cell concentration as compared to the initial concentration of each energy starvation phase (b, d, f, h).Data from two biological replicates are reported.

* 1. Conclusions

The insights presented in this study provide a preliminary understanding of microalgal behavior under varying nutrient availability conditions, including fluctuations in nutrient levels. Until now, there has been limited knowledge about these aspects, particularly regarding the effects of energy starvation conditions, and ho the physiological property of the inoculum affects its behavior under energy starvation condition. Nitrogen starvation can be effectively described using a logistic model that incorporates the maximum fattening factor (ηmax) and specific growth rate as key parameters. In contrast, the behavior under energy starvation conditions is more complex. Conventional first-order kinetic models are not applicable, as only 16% of the initial biomass decays on average, after which the biomass stabilizes. Cell concentration exhibits a different pattern compared to biomass, with significant increases in cell concentration observed under all stress conditions tested, except when cells were transferred from nitrogen starvation to both nitrogen and energy starvation. Variations in the elemental composition of the biomass are driven by nitrogen uptake and the synthesis or degradation of organic storage molecules. Future studies should incorporate structured models that account for the synthesis and degradation of intracellular compounds to more accurately describe microalgal behavior under varying nitrogen and energy starvation conditions.

These models are expected to enable the development of new processes that can use resources (water, energy, nutrients) more efficiently.

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