Nitrate and nitrite as mixed source of nitrogen for *Chlorella vulgaris*: growth, nitrogen uptake and pigment contents

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Chlorella vulgaris **was grown using mixed sources of nitrogen (nitrate and nitrite). Starting from B3N as basal medium, nitrate was substituted by nitrite keeping total nitrogen constant over 7 conditions: 0, 20, 40, 50, 60, 80 and 100 % NO_2^-. Growth rate, nitrogen uptake, photosynthetic apparatus status and pigment contents were monitored. Nitrite addition triggered a growth rate inhibition from early introduction (20 % NO_2^-, 81 $mg_{NO_2^-}$/l). Nitrate uptake rate increased with nitrate content in the culture medium (maximum at 5.87 mg_N/l/d, 100 % NO_3^-), while nitrite uptake remained constant around 2.93 mg_N/l/d. Photosynthetic apparatus was not impacted by the nitrogen source substitution. Pigments profiles (chlorophyll *a*, *b* and total carotenoids) were not statistically different for all the tested conditions. From a biotechnological perspective, this finding rules out the use of nitrite substitution as a pigment manipulating stress strategy.**

Nitrogen | Nitrite | Nitrate | Growth | Pigment

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1. Introduction

Shifting from the sole biofuel production, biotechnologists are nowadays regarding microalgae as a promising source of food, feed and added value molecules with applications in nutraceutical and pharmaceutical sectors (1). Still, microalgae use is not restricted to molecules production. From an environmental perspective, microalgae cultivation offers several benefits: carbon dioxide sequestration and some water pollutions remediation (mainly nitrogen and phosphorous). This last point is of note as it can be aligned with two bottlenecks hindering large scale microalgae farming: water requirement and culture costs.

From an industrial perspective, wastewater is associated to a non negligible treatment cost. Using it to grow microalgae would turn a waste into a co-product and a possible source of profit. This approach has long been focused low added value usages for the produced biomass, such as anaerobic digestion (2) or biofuel production (3). Nowadays new applications arise such as nutrient recovery (nitrogen, phosphorous , ...)(4). Sadly, most of the time, industrial streams have an incomplete composition with respect to microalgal growth requirements. As a consequence, they require the addition of targeted elements to allow cells cultivation. Hence, it is possible to take advantage of this formulation stage to optimize medium composition towards targeted molecules production (5). In this regard, nitrogen is a powerful means of manipulating cells response as it takes part in amino, proteins, enzymes and nucleic acids syntheses.

Nitrogen can be delivered in various forms to the culture. The most common one being nitrate (NO_3^-), still other options such as ammonium (NH_4^+), urea ($CO(NH_2)_2$) or nitrite (NO_2^-) can be used. From a metabolic perspective, green microalgae favour ammonium as they can readily use it. Indeed, other forms have to be transformed by a succession of enzymatic pathways. Put simply, these pathways can be illustrated with nitrate assimilation as it engages most of them sequentially. First, nitrate is transported into the cell. In the cytoplasm, nitrate is reduced into nitrite by the nitrate reductase. Then, nitrite is transported into the chloroplast where nitrite reductase catalyzes its reduction into ammonium. Finally, ammonium is integrated via the GS/GOGAT cycle (6). Over the course of this cycle, ammonium binds with glutamate to form glutamine before being split into two glutamate molecules again. While this pathway clearly spotlights how nitrate, nitrite and ammonium are assimilated, urea is not mentioned. In order to use urea as nitrogen source, microalgae use another enzyme, urea amidolyase, which splits urea, after transportation, into two ammonia molecules, latter entering the GS/GOGAT cycle. For the sake of completion, one has to mention organic sources of nitrogen other than urea, namely amino acids, purine and pyrimidine. While little is known about the two lasts, amino acids assimilation mechanisms are widely diversified even within the Chlorophyta phylum (7). For example, *Tetraselmis* species transport and deaminate amino acids intracellularly while *Chlorella* species do the same extracellularly.

From a biotechnological perspective, ammonium is prone to stripping (up to 80 % (8)) and can inhibit microalgae growth (9). Therefore, urea, nitrate and nitrite remain first choice sources of nitrogen. The two first have been widely studied in the context of wastewater treatment with various aims (among other, biofuels (10, 11) and pigments production (12)). On the contrary, studies on nitrite remain few. Available researches have shown notable effects of nitrite as nitrogen source: smaller cells (13), macronutrients composition alteration (lower protein content) (14), polysaccharides production (15). Singularly, to date, no authors have studied the potential impact of nitrate substitution by nitrite on microalgae pigment profile despite the fact that high nitrite

content perturbs cell photosynthetic apparatus. Nitrite can disturb the photosynthetic electron transport chain at the PSII level in two ways. On the acceptor side, nitrite slows down electron transfer from QA to QB inducing PSII jamming. On the donor side, ammonia (originating from nitrite) disrupts the Oxygen Evolving Complex (16). These damages affect cell growth on the short term and may activate a long term adaptation strategy. That is why the absence of study of the pigment profile response is all the more surprising. In addition, nitrogen is mandatory for chlorophyll synthesis. Regarding carotenoids, their content could also be modified as these molecules have two roles: antioxidants and broadening light absorption spectrum. Finally, from a wider perspective, protein synthesis depending upon nitrogen, its alteration may also have an impact on cell pigment profile and photosynthetic apparatus.

As media formulated from wastewater will contain a proportion of nitrite as nitrogen source, it is important to study its potential beneficial or detrimental effects. In this work, a special focus was set on pigment profiles as those molecules can represent a potential valorization for the produced biomass. Indeed, owing to their coloring and antioxidant properties, microalgae are regarded as a feed supplement of choice for aquaculture (17). To lead this work, *Chlorella vulgaris* was cultivated using modified B3N medium with nitrogen source replacement from 100 % NO_3^- to 100 % NO_2^-. The monitored outcomes were growth rate, nitrogen uptake, pigment contents and photosynthetic apparatus status.

2. Materials and methods

2 1. Strain & culture medium

The strain used for this study was *Chlorella vulgaris* (CV 211-11b) obtained from SAG Culture Collection, Germany. Cells were subcultured using B3N medium, 75 µmolPhotonPAR/m^2/s, 20 °C, 100 rpm agitation, duplicate. The passaging procedure was characterized by 1/100 sampling, 250 ml flasks, 50 ml culture medium. The culture medium was chosen among three candidates with increasing nitrogen content: BBM (2.94 mM), B3N (8.82 mM) and BG-11 (17.6 mM). The aim was to select the one that offered the highest amount of nitrogen, without inducing inhibition (18). This would allow nitrogen levels to be roughly constant throughout the culture, or at least avoid early depletion, thus allowing to measure its intrinsic effect. In addition, it prevents nitrogen starvation during subculturing which is known to induce higher nitrogen uptake later on (7). The three media gave similar results in term of generation time (± SD, n=3): BBM - 9h49 ± 17 min, B3N - 10h55 ± 43 min, BG-11 - 9h31 ± 10 min. Still, as BG-11 induced minor foaming, B3N was chosen as reference medium in spite of its lower nitrogen content compared to BG-11.

2 2. Culture protocol

Seven media were formulated following B3N recipe. For all those media, the total molar nitrogen content was constant, only the nitrogen source was varied, either nitrate (from $NaNO_3$) or nitrite (from $NaNO_2$). The tested nitrites fractions were: 0, 20, 40, 50, 60, 80 and 100 % NO_2^-. Cultures were conducted in 250 ml flask with 50 ml culture medium, placed in the same conditions as the subculturing. Inoculations were performed weekly. Cultures were stopped after 7 days, which were sufficient to enter stationary phase. The study was carried out in biological triplicates in axenic condition. After stopping the culture, cells were processed for pigment content analysis.

2 3. Cell concentration monitoring

Samples were drawn out of the flasks twice daily (1 ml). Cell growth was monitored using optical density (Shimadzu UV-1800 spectrophotometer) as proxy of cells dry weight. Samples were diluted to an optical density of 0.4 or less before recording the value. The optical density was acquired at 750 nm, as it allows to almost only account for cell walls and not pigments, hence measuring only for biomass. A calibration curve linking optical density and dry weight was produced using late exponential phase cells (7 points, OD range from 0.02 to 0.5, $R^2 = 0.997$). With this curve it is possible to compute biomass density from optical density readings. Finally, frequent microscope observations were done to check the cell condition and potential contamination.

2 4. Nitrate and nitrite concentrations monitoring

Nitrate and nitrite concentrations were determined over the growth course. Samples were filtered and diluted before being presented to an ICS-5000+ Ion Chromatography system (Thermo Fisher Scientific) coupled with a conductivity detector. All ions were identified by comparison to their retention time with standard solutions. Quantification was achieved using the height of the peak in external calibration, the range of concentrations was from 0.2 to 10 mg/l. All standards were purchased from Sigma-Aldrich with a TraceCert quality.

2 5. Photosynthetic apparatus status

At the end of the culture, AquaPen AP 110-C PAM fluorometer was used to qualify photosynthetic apparatus status using Fv/Fm ratio measurement. Before starting these analyses, cells were adapted to a dark environment for 15 minutes.

2 6. Pigment profiles

Finally, the cells were washed twice with milliQ water (4 °C, 11000 rpm, 10 minutes). Biomass pellet was frozen before being freeze-dried (1 day primary drying, 2 days secondary drying, Christ alpha 1-2 LD +). 3 to 4 mg of dried microalgae powder was homogenized in 10 ml pure methanol using MP Biomedicals FastPrep42 bead miller. Preliminary tests showed that *Chlorella vulgaris* was recalcitrant to pigment extraction. Thus, for production runs, homogenized cells were left in 60 °C water bath for 20 minutes (19). Liquid was then filtered (0.22 µm) and absorbance spectrum was recorded. Finally, Wellburn's equations for pure methanol, high precision spectrophometer (Eq. 1, 2 and 3) were used to quantify chlorophyll *a*, chlorophyll *b* and total carotenoids (20):

$$Chl_a = 16.72\ A_{665.2nm} - 9.16\ A_{652.4nm} \quad \textbf{(1)}$$

$$Chl_b = 34.09\ A_{652.4nm} - 15.28\ A_{665.2nm} \quad \textbf{(2)}$$

$$Car_{x+c} = (1000\ A_{470nm} - 1.63\ Chl_a - 104.96\ Chl_b)/221 \quad \textbf{(3)}$$

2 7. Mathematical treatment

Cell growth rate (μ) was calculated in the exponential growth phase using classical equation (Eq. 4):

$$\mu = \frac{ln(C_{t_2}) - ln(C_{t_1})}{t_2 - t_1} \quad \textbf{(4)}$$

Nitrogen uptake rates, either total nitrogen, NO_2^--N (nitrogen in nitrite) or NO_3^--N (nitrogen in nitrate) were computed thanks to ion chromatography measurements. They were taken as the variation of the species of interest over the culture (Eq. 5). This is of course a simplification as the cultures exhibited lag phases and stationary phases, still this is similar to what others authors did (21, 22) and makes comparison possible.

$$R_N = \frac{C_{N,t_2} - C_{N,t_1}}{t_2 - t_1} \quad \textbf{(5)}$$

Finally, statistical significance was assessed using non-parametric Kruskal-Wallis test, as it is better suited for small datasets such as triplicated measurement points. When the null hypothesis was rejected ($p < 0.05$), data were further analyzed using pairwise Mann-Withney U test.

3. Results

3 1. Growth and nitrogen concentrations over time

The growth rates of *Chlorella vulgaris* under different nitrogen sources mixtures are reported in Figure 1. As one can see, the cell growth rate is constant, around 0.82 1/d, from 100 to 40 % nitrite fraction. Lower fractions of nitrite induce better cells performances: 0.97 and 1.30 1/d for 20 and 0 % nitrite, respectively.

Another important parameter to monitor is the concentration of each of the nitrogen source over time. Indeed, in order to claim assessment of the nitrogen sources intrinsic effects, the associated concentrations have to remain high enough during the whole run. Figure 2 reports the nitrite and nitrate concentrations for the case that would be the most prone to the exhaustion of one of the nitrogen source (80 % NO_2^- 20 % NO_3^-). In this configuration, nitrite depletion over the run is 11.5 ± 3.6 % and nitrate depletion is 36.2 ± 10.1 %. Regarding the sources ratio (N-NO_2^-/N-NO_3^-), it increases from 4.05 ± 0.02 at the beginning of the culture to 5.41 ± 0.90 at the end of the run. Absolute concentrations and nitrogen species ratio remained reasonably stable. Thus, the protocol was deemed acceptable to claim the investigation of the intrinsic effect of the blend of the nitrogen sources.

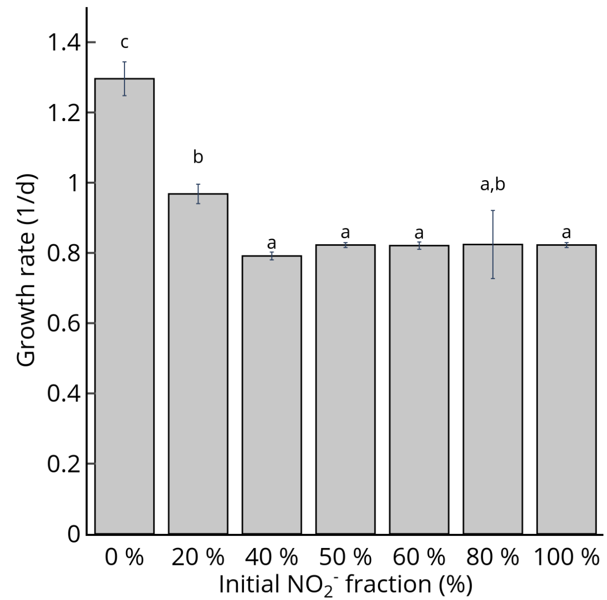

Fig. 1. Growth rate versus NO_2^- fraction. Error bar: standard deviation (n=3). Letters: groups with the same statistical significance (p>0.05)

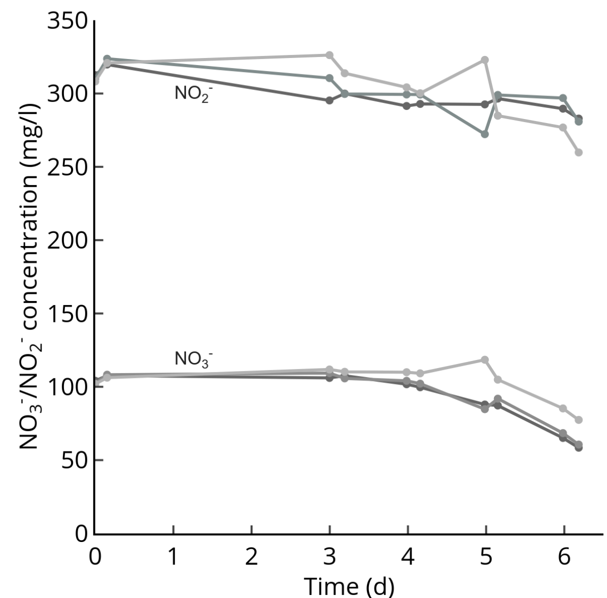

Fig. 2. Nitrogen sources concentration over the course of a culture (80 % NO_2^- / 20 % NO_3^-). Color: each run within the triplicate

3 2. Nitrogen uptake

Figure 3 presents the nitrogen uptake rate for the different conditions. As one can see, increasing the nitrate fraction increased the total nitrogen uptake rate. Still, total nitrogen uptake is the sum of the contributions of N-NO_3^- and N-NO_2^- uptake rates. Looking at those two rates individually, an almost linear increase of N-NO_3^- uptake rate with NO_3^- fraction is observed ($R_{N-NO_3^-} = 0.064 f_{NO_3^-}$, R^2 = 0.99). On the contrary, N-NO_2^- uptake rate plateaus from 20 to 60 % initial NO_3^- fraction; around 2 mg_N/l/d, before decreasing.

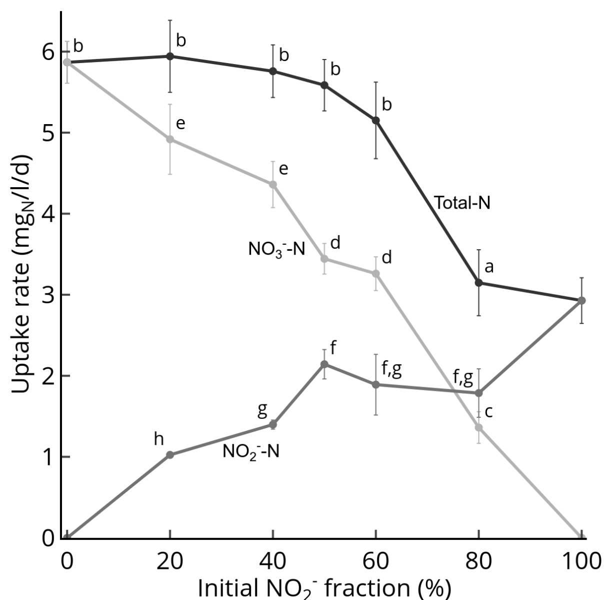

Fig. 3. Total-N, NO_3^--N and NO_2^--N uptake rates versus NO_2^- fraction. Error bar: standard deviation (n=3). Color: light grey N-NO_3^-, grey - N-NO_2^-, dark grey - total nitrogen. Letters: groups with the same statistical significance (p>0.05)

3 3. Photosynthetic apparatus

Fv/Fm ratios ranged from 0.79 to 0.81, exhibiting no statistically significant differences between samples.

3 4. Pigment contents

Pigment contents of the different cultures are presented in Figure 4. Despite visual trends, statistical analysis shows that none of the pigment exhibits a statistically significant different trend (p-value of 0.23, 0.45 and 0.52 for chlorophyll *a*, *b* and total carotenoids respectively).

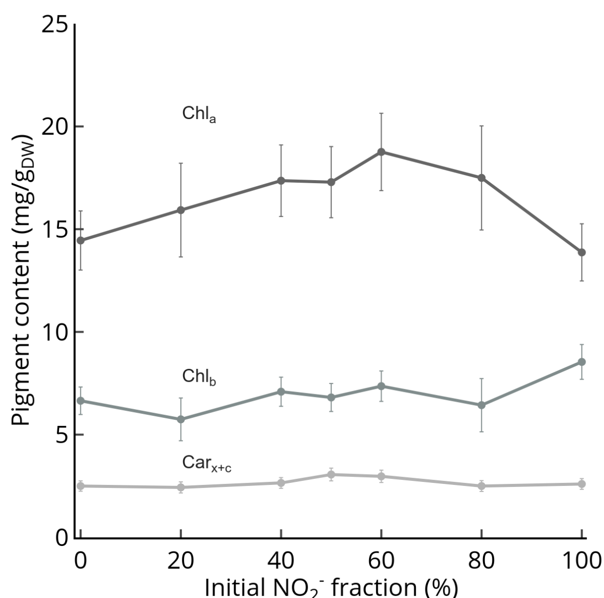

Fig. 4. Pigment contents versus NO_2^- fraction. Error bar: standard deviation (n=3). No statistically significant difference could be found

4. Discussion

Only few authors studied the impact of nitrite addition alone to the culture medium of *Chlorella vulgaris*, which limits the number of studies we can refer to. Furthermore, direct comparison is hindered, in some cases, by the fact that some authors supplemented their culture with high CO_2 content (23), or used very high nitrogen concentration (order of magnitude of g/l). Nevertheless, the inhibition observed by nitrite addition to the medium has also been reported for value of 20 $mg_{N-NO_2^-}$/l (or 66 $mg_{NO_2^-}$/l) (24), which corresponds to the 80 % NO_3^- / 20 % NO_2^- in our study (or 81 $mg_{NO_2^-}$/l).

Regarding uptake rates the observed trends have also been reported. *Chlorella vulgaris* faster growth with nitrate compared to nitrite was also observed by other scholars (15). The early absorption of nitrite (day 3 for NO_2^- and day 5 for NO_3^-, Fig. 2) can be explained by the fact that this source of nitrogen requires one less enzymatic transformation and is therefore easier to mobilize, before it triggers its inhibitory effect. The linear trend of nitrate absorption with its amount within the medium was also described (18). The fact that we also observe it in the case of a mixture would suggest that nitrate and nitrite uptakes are independent to some extend for the tested concentrations. Biomass composition analysis would be of help in confirming this assumption, for example by showing a lower protein, hence nitrogen, content of cells grown with nitrite only. In the absence of our own analysis, we can refer to other authors findings. Indeed, they confirmed that cultures led with nitrite exhibited lower protein content (41 % instead of 51 %) than their nitrate counterpart (14).

Regarding absolute values uptake rates, Table 1 summarized the nitrogen uptake rates reported by other authors with *Chlorella vulgaris*. In our work, under pure nitrate, *Chlorella vulgaris* was capable of removing 5.87 mg_N/l/d, which compares well with other studies. Regarding pure nitrite, the strain removed 2.93 mg_N/l/d. Here again, the performances are in agreement with other authors works.

All these findings being in agreement with other authors reporting, they build confidence in the quality of the performed runs. This allows to discuss with serenity original results on photosynthetic apparatus status and pigments profiles.

Regarding photosynthetic apparatus status, Fv/Fm ratio around 0.8 indicates full functionality and the lack of stress impacting this part of the cells. This finding has to be considered in the light of other authors work on short term response (few hours). They reported a negative effect on PSII with a Light Harvesting Complex state transition toward higher PSI affinity (16). Hence, in our case, microalgae adapted to the stress imposed over their photosynthetic apparatuses. After, one week cultivation, they are able to restore an healthy photosynthetic apparatus, which is nevertheless less productive, as growth is still negatively impacted. Further analysis would be required to precisely link this performance decrease to actual perturbation of Oxygen Evolving Complex or QA/QB electron transfer.

Finally, pigments profiles were not reported by other au-

Nitrogen origin	Gross N uptake rate (mg_N/l/d)	Corrected N uptake rate (mg_N/l/d)	Correction	Reference
NO_3^-	5.87	-	-	Present work
	5.6	-	-	(25)
	18.7	4.68	Normalized by illumination	(18)
	5.42	-	-	(22)
NO_2^-	2.93	-	-	Present work
	2.5	-	-	(24)
	0.8	-	-	(15)
	10.2	2.06	Interpolation & Normalized by illumination	(21)

Table 1. Nitrogen uptake rates obtained from literature. Selection criteria: species *Chlorella vulgaris*, grown under air. Normalized by illumination: the reported work was led under 75 µmolPhotonPAR/m^2/s, other authors used other configurations, e.g. 600 µmolPhotonPAR/m^2/s on a 12h/12h period in (18). In order to compare likes to likes, their results were normalized by the ratio of the illuminations

thors, thus comparison is not possible. Still, the fact that chlorophyll *a* and *b* contents are not statistically different for all the tested conditions suggests that the photosynthetic apparatus of the cell is not severely impaired by the addition of nitrite. In addition, the stability of the total carotenoid content would show that switching from nitrate to nitrite as nitrogen source did not stress high enough to induce antioxidant response. From a biotechnological perspective, this finding has two implications. On the one hand, it is possible to grow *Chlorella vulgaris* using nitrite and expect the same amount of pigments as if using nitrate, though not the same productivity as growth rate is lower. On the other hand, it advocates against the use of nitrite in order to implement a pigment accumulating stress strategy.

5. Conclusion

Chlorella vulgaris was grown under mixed sources of nitrogen made of nitrate and nitrite. Nitrite addition triggered a growth rate inhibition from early introduction (81 $mg_{NO_2^-}$/l). Nitrate uptake rate showed a linear trend following nitrate content in the culture medium, while nitrite uptake remained constant around 2.0 mg_N/l/d. Pigments profiles (chlorophyll *a*, *b* and total carotenoids) were not statistically different for all the tested conditions. From a biotechnological perspective, this finding rules out the use of nitrite substitution, in the tested amounts, as a pigment manipulating stress strategy.

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AUTHOR CONTRIBUTIONS

WL and VP initiated and designed the study. AM and WL led the experimental work with the help of NC, EM and VP. All the authors critically interpreted the results. VP drafted the manuscript, the other authors corrected it. All authors approve the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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