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MELiSSA

Growth capability of *Spirulina* on several nitrogen sources in the photosynthetic compartment of MELiSSA

Technical Note 33.2

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INTRODUCTION

The cyanobacteria Spirulina (*Arthrospira platensis*) are usually grown on nitrates because this nitrogen source do not set technical problems with culture mediums and is well accepted by the cells

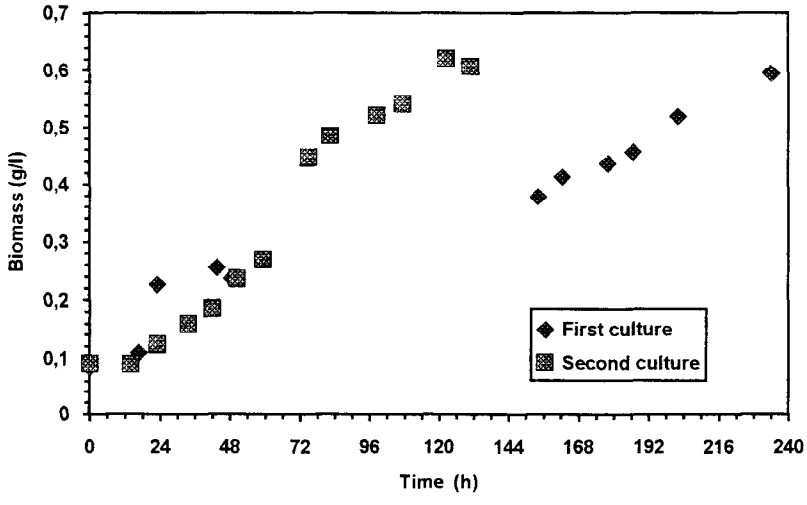
In a previous contract (ESTEC 8125/88/NL/FG), optimal culture conditions on nitrates were determined for modelling growth in the photosynthetic compartment, and since 1992, modelling of the whole closed ecosystem MELiSSA continues in the Laboratoire de Génie Chimique Biologique at Clermont-Ferrand.

In this ecosystem, the crew and the liquefying compartments produce urea and ammonium that must be oxidised in the nitrifying compartment to nitrite then nitrate before being used as nitrogen source by Spirulina in the photosynthetic compartment. It would therefore be interesting to determine to which extent Spirulina cells are able to directly use urea, ammonium or other nitrogen compounds produced by the preceding compartments in order to eventually introduce some flexibility in the MELiSSA loop or even to reduce the nitrifying compartment.

This work analyses the capability of Spirulina (*A. platensis*) cells to grow on several nitrogen sources, particularly on nitrate, urea and ammonium, supplied to the cultures individually or in combination by two or by three.

1 - CULTURE AND GROWTH OF *ARTHROSPIRA PLATENSIS* STRAIN PCC 8005

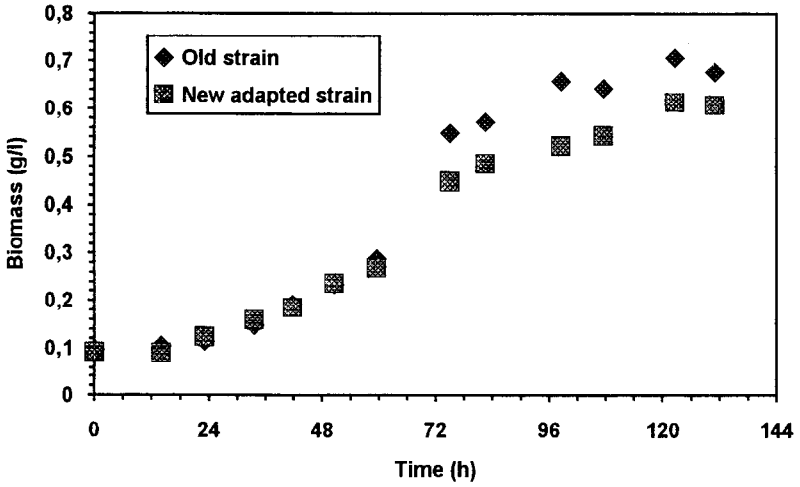
Our Spirulina strain being contaminated, we used the same axenic strain PCC 8005 provided by Institut Pasteur. Cultures were performed in 100 or 1000 ml erlenmeyer flasks in an orbital shaker (Gallenkamp) at 110 rpm, 35°C, and under 3% CO2 in air (~210 $l.h^{-1}$) and under a continuous light intensity of ~8 $W.m^{-2}$ (~2900 lux). Biomass production was calculated from turbidity measurements.



The first culture of this new strain on the Zarrouk (1966) culture medium provided an atypical growth curve: a very short initial exponential phase, during which biomass doubled, was followed by an unexpected 4 days lag, after what growth restarted again for a 2 - 3 weeks linear phase that stopped when nitrogen was exhausted in the culture medium. Moreover, the culture turned yellowish green and cells lysis occurred sooner than usual. This biphasic growth probably reflected the presence of two distinct *A. platensis* sub populations in the culture medium. The subsequent culture provided the classical growth characteristics of *A. platensis* on the Zarrouk culture medium, suggesting that one of these sub population was selected.

As shown below, the new strain selected after adaptation to the Zarrouk culture medium has acquired growth characteristics very similar to those of the old strain that was previously used for growth modelling. If significant, the slight difference in biomass estimations may only express

differences in the turbidity of the cultures that could result from differences in the shape of the trichomes, straight in the old strain and helicoidal in the new one.



This study was therefore performed on the new selected strain adapted to the Zarrouk culture medium.

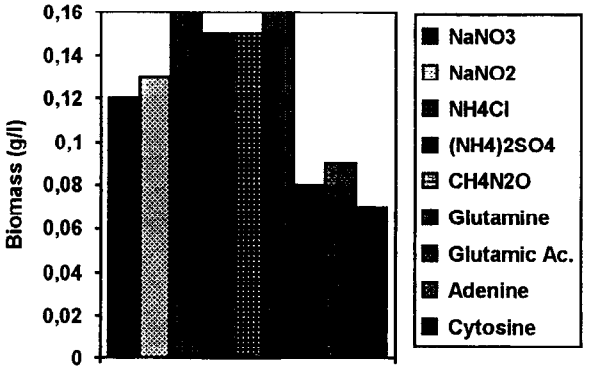
2 - GROWTH ON A SINGLE NITROGEN SOURCE

2 . 1 - Organic and inorganic nitrogen sources

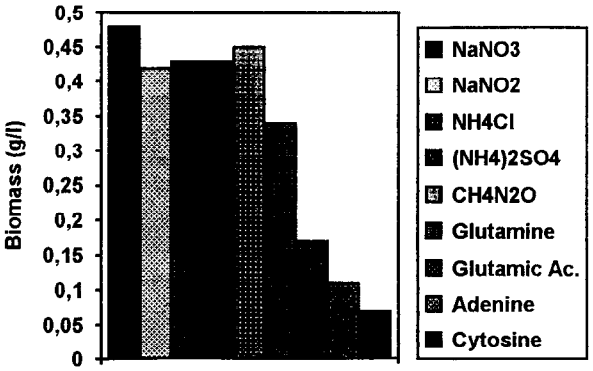
Cyanobacteria preferentially use inorganic nitrogen for growth, and particularly nitrates, nitrites and ammonium, but some of them are able to use organic nitrogen (Fogg *et al.*, 1973). Nitrate assimilation by cyanobacteria involves a transport system into the cells where it is sequentially reduced to ammonium by nitrate and nitrite reductases. Ammonium is then incorporated by the glutamine synthetase/glutamate synthase system.

These characteristics, that are very poorly documented for *A. platensis* (Soyer, 1992), were analysed by measuring the rate of biomass formation (Cornet, 1992) (biomass formed after two days of culture, before the appearance of a limitation) and the amount of biomass formed 3 days later. All cultures were inoculated with nitrate adapted precultures and contained different nitrogen compounds at the same concentration of atomic N-nitrogen, that was kept low (2 mM) because ammonium at least is known to be toxic at higher concentrations.

45 h culture



113 h culture



After two days of culture (45 h), these nitrogen sources may be classified into 3 groups on the basis of their capability to promote biomass formation, and therefore, to be rapidly assimilated:
- a group containing ammonium salts, urea and glutamine on which growth is rapid;

- a group containing nitrate and nitrite that are assimilated slower;
- a group containing glutamic acid, adenine and cytosine on which growth is very slow.

A. platensis is therefore able to use for growth, not only the classical mineral sources of nitrogen , nitrites nitrates and ammonium, but also and with different efficiencies, several organic sources of nitrogen, urea, aminoacids (glutamine, casein hydrolysate - not shown), puric and pyrimidic bases. Glutamine is the best used aminoacid and it allows, at the concentration of 1 mM the same growth rate than that obtained with NH4Cl. Moreover, concentrations of glutamine in the range 2 - 30 mM N-nitrogen do not inhibit growth (not shown) of *A. platensis*, contrary to *Phormidium laminosum* (Tapia et al., 1995). Glutamic acid is used at concentrations up to 16 mM (not shown), but with less efficiency than glutamine

Nitrogen of puric or pyrimidic bases is quite not assimilated when provided to the cultures under that form. The cultures do not form significant biomass and rapidly change to a yellowish - green colour that expresses that cells are using nitrogen of their phycocyanins.

After 5 days of culture (113 h), nitrogen, which is exhausted in the culture medium, has allowed the formation of ~ 0.45 g/l biomass in the two first groups. This deficiency in nitrogen is compensated by the degradation of phycocyanins, responsible for most of the cultures having lost their blue-green colour (except those grown on nitrite or nitrate) and becoming green or even orange. This effect is reversible and cells reconstitute normal levels of phycocyanins in 48 h following the addition of nitrogen (except in the form of puric or pyrimidic bases).

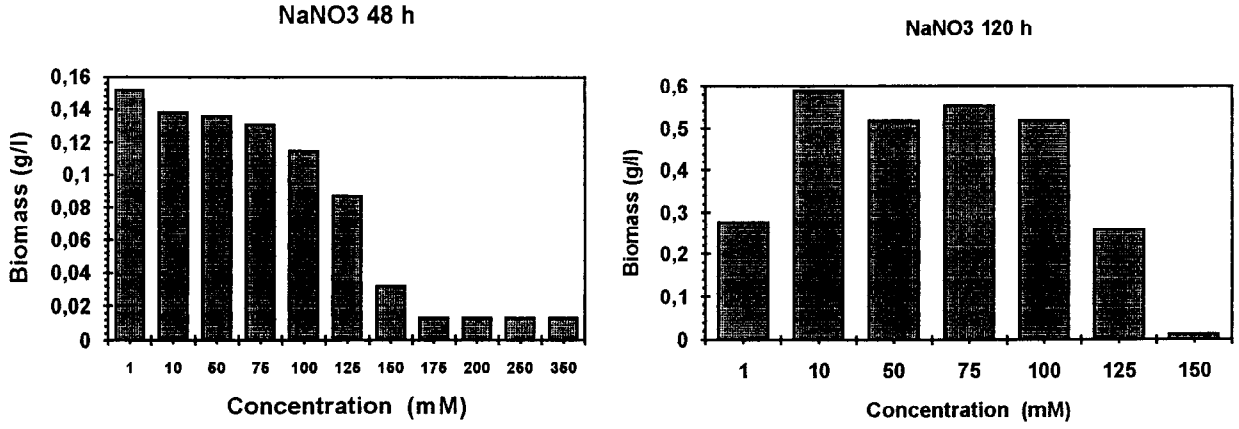
We analysed in more details the growth of *A. platensis* in the presence of the 3 main nitrogen sources, nitrate, urea and ammonium produced by MELiSSA.

2 . 2 - Effects of several parameters on growth of *A. platensis*

2 . 2 . 1 - Optimal concentrations for growth rate

Growth rate was measured by the biomass formed 2 days after inoculation, before

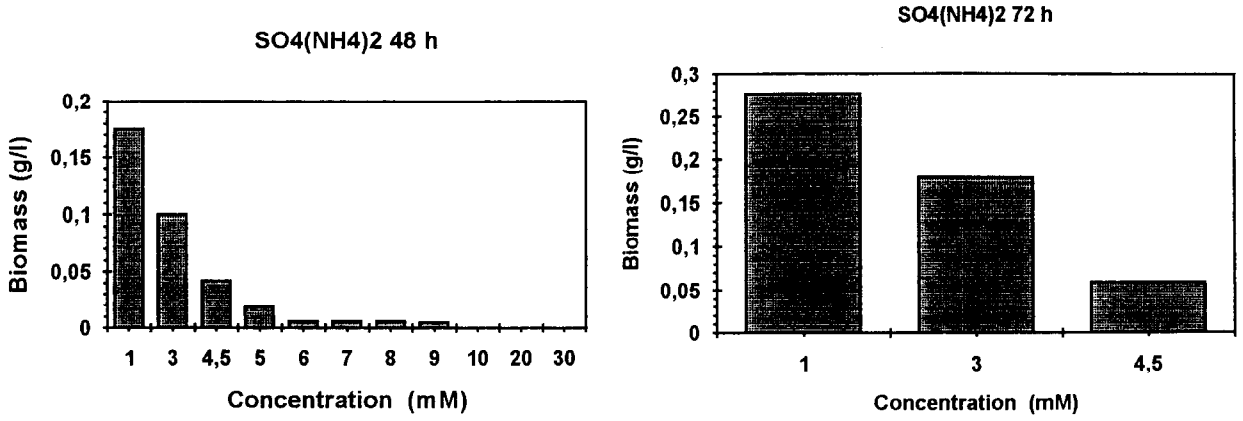
Sodium Nitrate - Growth is faster at low nitrate concentrations, but is only significantly slowed down at concentrations higher than about 100 mM. However, low concentrations entail a rapid appearance of nitrogen limitation and the subsequent cessation of growth (in 3 - 4 days at 1 mM) while growth continues for more than a week for nitrate concentrations higher than 10 mM



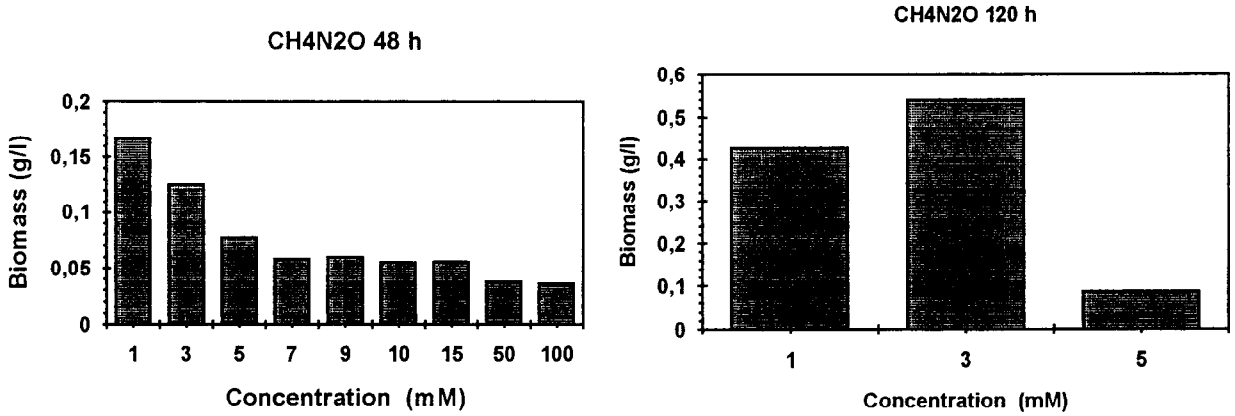
A. platensis therefore may be grown on a large range of nitrate concentration, between 1 and 50 mM, with an optimum at 1 mM for growth rate.

Ammonium sulphate - The lower the ammonium concentration, the higher the growth rate; the tolerance to high concentrations is very limited since growth rate is already reduced by 3 mM

ammonium. Growth continues for at least 5 days (120 h) at 1 - 3 mM ammonium (2 6 mM nitrogen) and higher concentrations result in a rapid (about 48 h) cell lysis.



Urea - Results are similar to those obtained with ammonium, with however a better tolerance for high concentrations. Growth continues for at least 5 days (120 h) at 1 - 5 mM ammonium (2 10 mM nitrogen) and higher concentrations also result in a rapid (about 48 h) cell lysis.

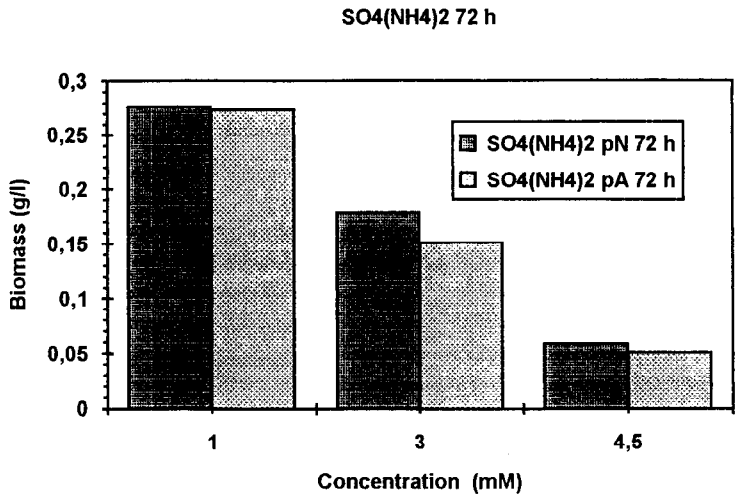


It therefore appears that, for the 3 analysed nitrogen sources, the optimal nitrogen concentration for rapid growth rate is around 2 mM. Higher concentrations delay the appearance of a limitation in the case of nitrate but result in a rapid cell lysis in the case of urea and ammonium. If not optimal for growth rate, the high concentration of nitrate in the Zarrouk culture medium (29.4 mM) is acceptable by cells and delays the appearance of nitrogen limitation.

2 . 2 . 2 -Effect of pre adaptation of the inoculum to the used nitrogen source

The low tolerance of *A. platensis* to high nitrogen concentrations, particularly of ammonium and urea may result from a lack of adaptation of the inoculums that was previously grown on nitrate. In that case, this tolerance could be increased by pre adaptation of cells to the different used nitrogen sources.

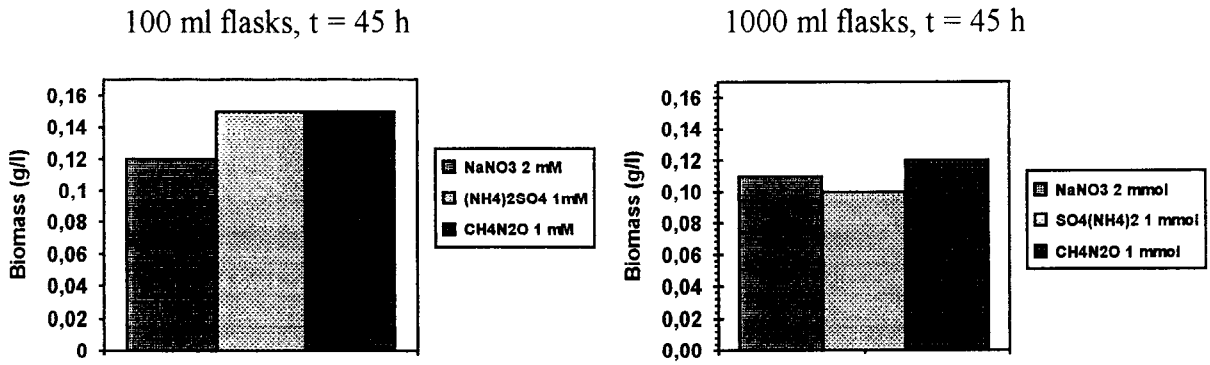
Culture mediums containing different concentrations of ammonium were inoculated with non adapted precultures grown on nitrate (pN) or preadapted during a 12 days culture on ammonium (pA). The similarity of the obtained results for each culture condition show that the susceptibility of *A. platensis* to high concentrations of ammonium is constitutive and does not result from a lack of adaptation.



2 . 2 . 3 - Effects of light intensity and toxicity of nitrogen sources

The early growth cessation and cell lysis with urea and ammonium suggest that these nitrogen sources are toxic even at low concentrations. This toxicity appeared to be higher when Spirulina cells were grown in larger flasks, in which cell shadowing increases the dark volume and therefore decreases the amount of light energy available for growing cells. This observation suggested that light energy may be involved in nitrogen metabolism.

Effect of light energy at low nitrogen concentrations



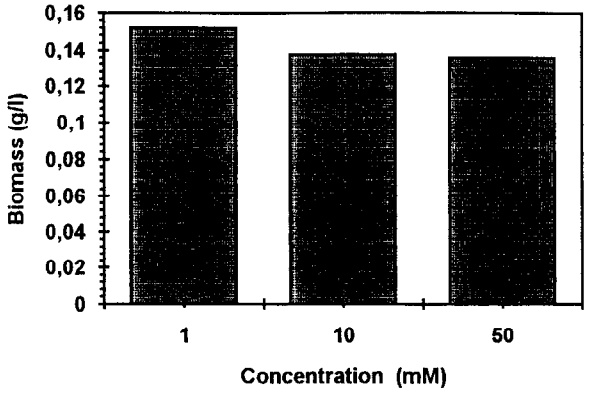
At low, non toxic nitrogen concentrations, the relative decrease in available light energy in 1000 ml flasks, compared to 100 ml flasks resulted, as expected in a slight decrease in the rate of biomass production, particularly in the presence of ammonium and urea (20% to 30%). This difference persisted during growth.

Effect of light energy at high nitrogen concentrations

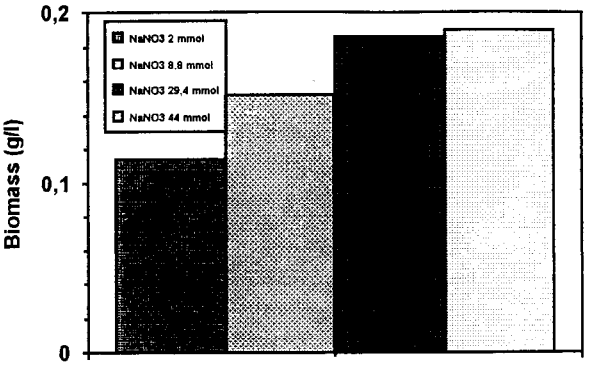
Sodium nitrate

At high light intensity (100 ml flasks), one find again the best growth on low nitrogen concentration. The lower light intensity in the 1000 ml flasks leads, as expected, to a decrease in the formed biomass, but for low nitrogen concentrations only since under these low light conditions, the utilisation of nitrate become proportional to its concentration.

100 ml flasks, t = 48 h



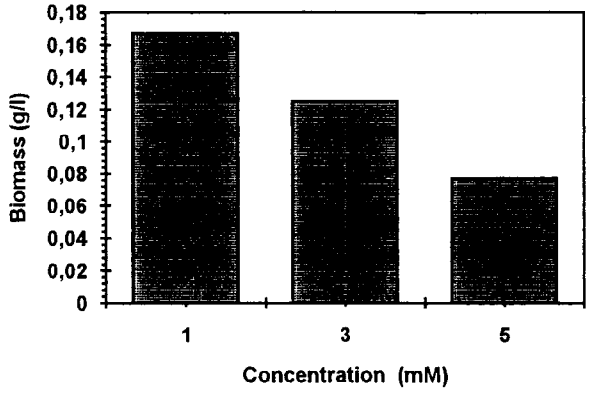
1000 ml flasks, t = 45h



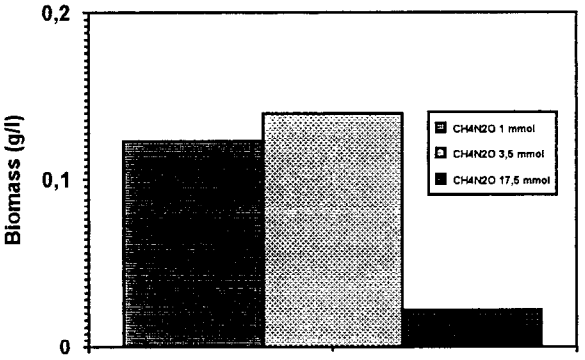
Urea

The toxicity of increasing concentrations of urea is confirmed at high light intensity (100 ml flasks). The reduced available light energy in the 1000 ml flasks results in a decrease of biomass formation, which becomes, as for nitrate, proportional to the concentration of urea. However, these conditions of limited light energy increase the susceptibility of cells to the toxic effects of urea since cell lysis appears sooner than at higher light intensity (about 2 - 3 days).

100 ml flasks, t = 48 h



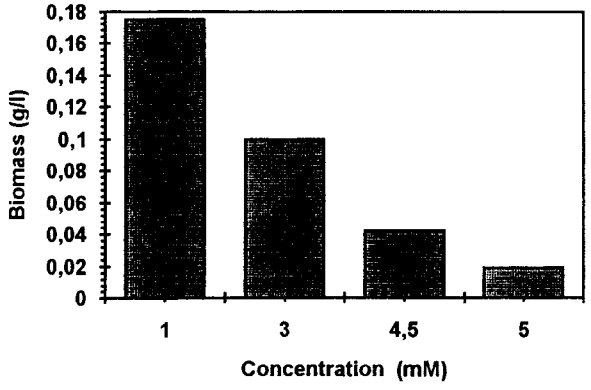
1000 ml flasks, t = 45h



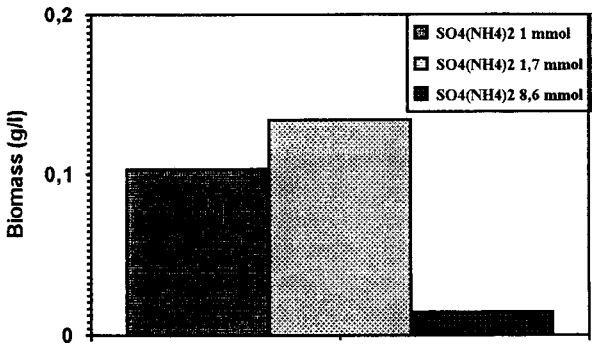
Ammonium sulphate

Results are similar to those obtained with urea, with an even more marked susceptibility of cells to increased ammonium concentrations under reduced light intensity, resulting in an earlier cell lysis. (1 - 3 days).

100 ml flasks, t = 48 h



1000 ml flasks, t = 45h



These measurements thus show that the available light energy affects the utilisation of nitrate, ammonium and urea by *A. platensis*.

- Under conditions of limitation of biomass production by low light energy, nitrogen utilisation is proportional to its concentration.
- The toxicity of increasing concentrations of nitrate, urea and ammonium appears earlier under conditions of reduced light energy.

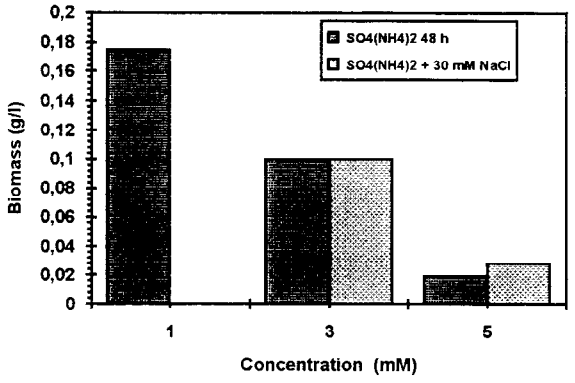
These results suggest that the efficiency of transmembrane transport systems or of regulatory mechanisms of intracellular nitrogen concentration or even of detoxification systems could be dependent on light energy. One may then consider that these processes would be controlled by ferredoxin, which is the final acceptor of the photosynthetic electron transport chain, and which is known to play a key role in several photoregulated processes.

2 . 2 . 4 - Effects of Na^+ ions on growth and on nitrogen utilisation

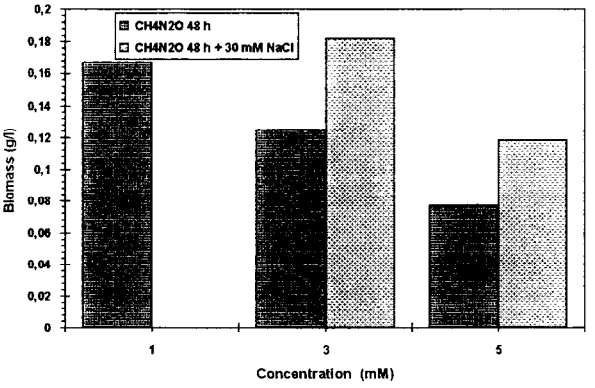
Several studies have shown that Na^+ ions may facilitate the transport of nitrogen compounds, nitrate particularly (Rodriguez *et al.*, 1994), through the plasma membrane of cyanobacteria. However, Boussiba (Boussiba, 1990) showed that they are not involved in the transport of ammonium into *Synechococcus* cells.

The effects of 30 mM NaCl on the utilisation of ammonium and urea at different concentrations have been analysed. The results show that Na^+ ions do not exert any effect on ammonium utilisation by *A. platensis*, maybe because at the pH of the culture, the great majority of ammonium ions are converted to ammonia, a neutral little molecule able to freely diffuse through the cell wall and the plasma membrane, or because Na^+ ions are not involved in the used transport system.

Ammonium



Urea



On the contrary, Na^+ ions allow a better utilisation of urea and they could even participate to a detoxification system since they exert a protective effect against increasing urea concentrations

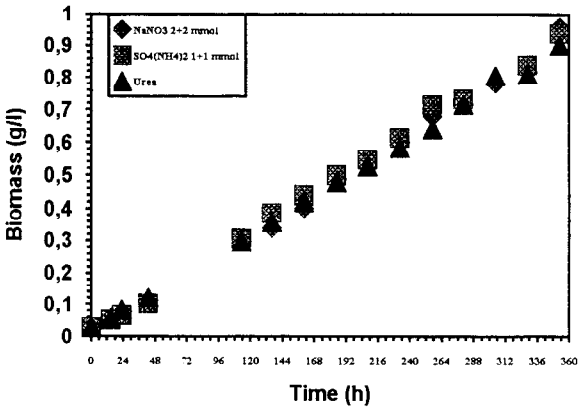
These differential effects of Na^+ ions suggest the existence of several distinct processes for the entry in the cell of the different nitrogen compounds.

2 . 3 - Optimal culture of *A. platensis* on nitrate, ammonium or urea

For cultivation of *A. platensis* under optimal conditions, toxic effects of high nitrogen concentrations should be reduced, sufficient light energy should be provided, growth rate and biomass production should be maximised. In that perspective, cultures were performed in which the nitrogen concentration was maintained at a low level ($\leq$2 mM in order to limit its toxicity and to allow a maximal growth rate) by successive additions of limited amounts of the used nitrogen source, each 5 days, before its exhaustion in the culture medium (in order to delay the appearance of a

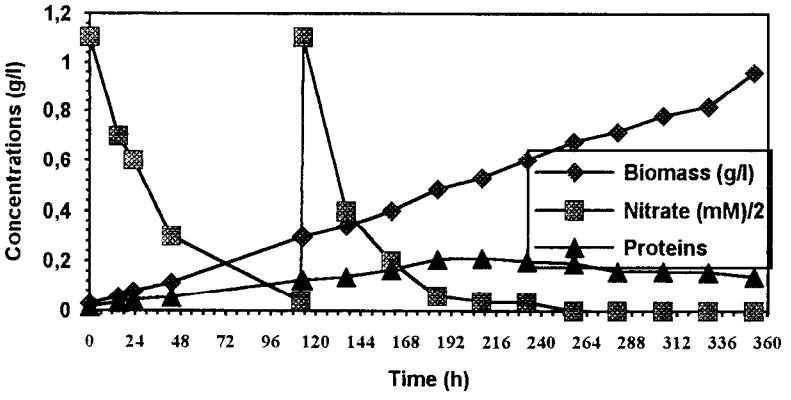
nitrogen limitation and to allow maximal biomass production). The light energy delivered to the cultures was increased during growth by reducing the distance between the cultures and the light sources (2.8 to 5 kLux)

The growth curves obtained with nitrate, urea and ammonium are very similar, with a very short exponential phase followed by a long linear phase during which nitrogen does not become limiting and during which high amount of biomass is formed at high rate.

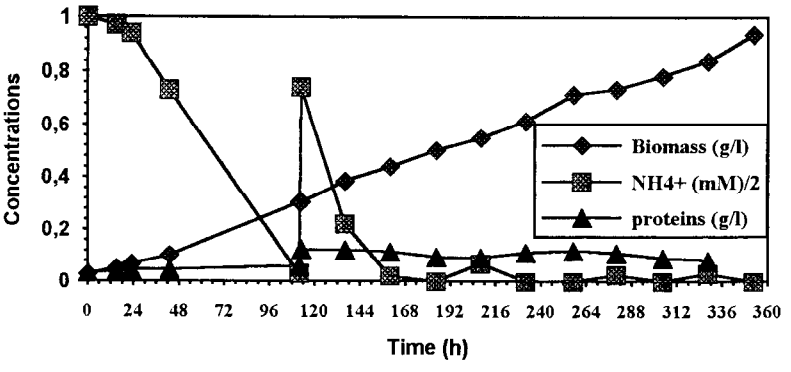


The 3 following figures show the details these growth curves, with in parallel the evolution of the concentration of proteins and of the used nitrogen sources in the culture medium

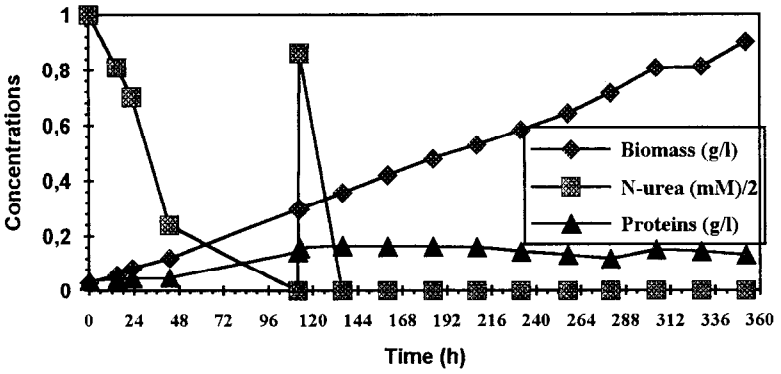
Sodium nitrate



Ammonium sulphate



Urea



3 - GROWTH ON TWO MIXED NITROGEN SOURCES

Cyanobacteria usually use nitrate as preferential nitrogen source. However, several nitrogen compounds may be simultaneously available in the environment, and in the particular case of nitrate and ammonium, cells first use ammonium then nitrate. Moreover, it has been shown that the presence of ammonium decreases or even inhibits the utilisation of nitrate by several cyanobacteria (Guerrero and Lara, 1987).

These characteristics are poorly documented for *A. platensis*. They were specified by analyses of growth on several binary combinations of nitrogen sources, nitrate + urea, nitrate + ammonium and urea + ammonium. In each case, several experiments were performed in which the concentration of total nitrogen and the proportion of the two used nitrogen sources varied. Growth was measured by the amount of biomass formed after 2 days culture (43 h) and during the subsequent 3 days (115 - 43 h).

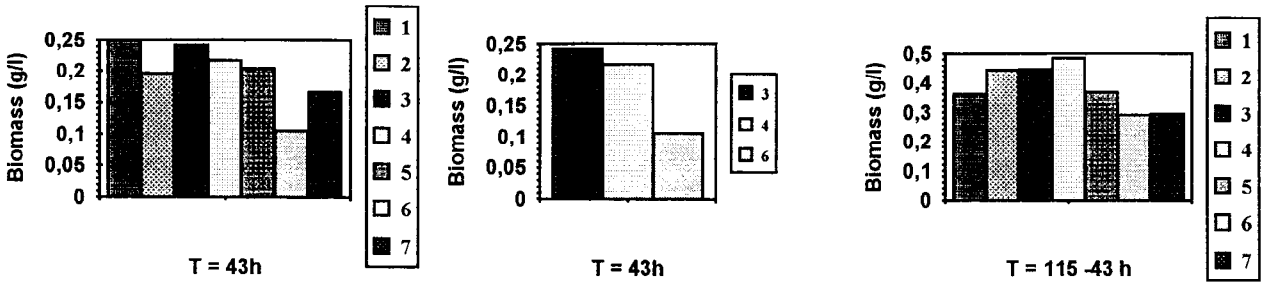
3 . 1 - Nitrate and Urea

Experimental conditions : Experiments are classified on the basis of increasing amounts of nitrogen.

Experiment	Σ N-nitrogen mM	$N\text{-}NO_3^-$ mM	%	$N\text{-}CH_4N_2O$ mM	%
1	3,8	1,4	*36,8*	2,4	*63,2*
2	4,2	1,4	*33,3*	2,8	*66,7*
3	5,3	4,5	*84,9*	0,8	*15,1*
4	8,5	4,5	*52,9*	4,0	*47,1*
5	10,1	7,7	*76,2*	2,4	*23,8*
6	11,7	4,5	*38,5*	7,2	*61,5*
7	13,3	7,7	*57,9*	5,6	*42,1*

Growth at 43 h and between 43 and 115 h.

After 43 h culture, and for concentrations of total nitrogen lower than 10 mM, growth on these mixed nitrogen sources is more important (0.22 - 0.25 g biomass/l) than that observed with nitrate or urea alone (0.17 - 0.18 g/l). Increasing concentrations of nitrogen in the culture medium still appear to inhibit growth, but nitrogen seems to exert a protective effect against the toxicity of urea. This is clearly manifest in the central graph which shows that the inhibitory effect of urea is less marked in the presence of 4.5 mM nitrate than alone.



Between 43 and 115 h, the increase in the biomass formed in experiments 1 - 4, in which both the concentrations of total nitrogen and of nitrate increase, may be correlated to nitrate exhaustion, and then would express a preferential utilisation of nitrate for growth. The reduced biomass formation in experiments 3 - 5 confirms the toxicity of high nitrogen concentrations

In conclusion, in the combination nitrate - urea, nitrate seems to be first assimilated; the concentration of total nitrogen should range between 4 and 10 mM and urea, even if less toxic in the presence of nitrate, should not exceed 30% of total nitrogen.

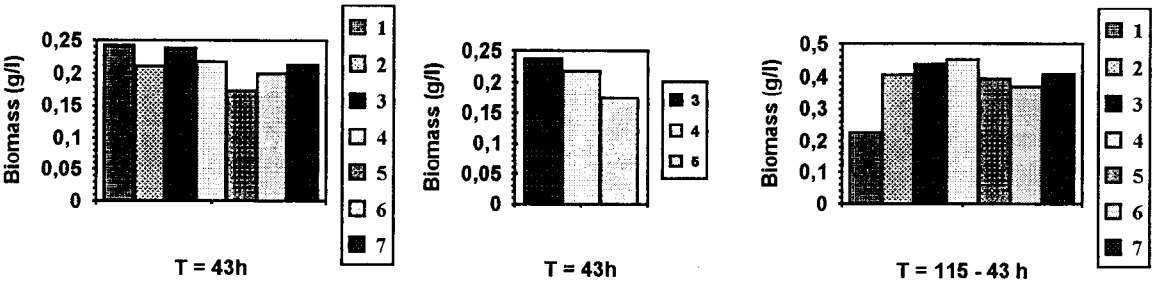
3 . 2 - Nitrate and Ammonium

Experimental conditions : Experiments are classified on the basis of increasing amounts of nitrogen.

Experiment	Σ N-nitrogen mM	$N\text{-}NO_3^-$ mM	%	$N\text{-}NH_4^+$ mM	%
1	2,6	1,4	*53,8*	1,2	*46,2*
2	4,2	1,4	*33,3*	2,8	*66,7*
3	4,9	4,5	*91,8*	0,4	*8,2*
4	6,5	4,5	*69,2*	2,0	*30,8*
5	8,1	4,5	*55,6*	3,6	*44,4*
6	8,9	7,7	*86,5*	1,2	*13,5*
7	10,5	7,7	*73,3*	2,8	*26,7*

Growth at 43 h and between 43 et 115 h

Again, the amount of biomass formed in 2 days on these 2 combined nitrogen sources is higher than on each of them. The toxicity of ammonium, that is still manifest (central graph) is attenuated by nitrate, and to a larger extent than for urea ; in consequence, growth on this combination of nitrogen sources seems to be less susceptible to increasing nitrogen concentration. This inhibition of nitrate assimilation by ammonium, which likely results from a competition between the transport systems for these two compounds, is less marked than in other cyanobacteria (Guerrero and Lara, 1987).



The amount of biomass formed during the 3 subsequent days confirms that, if nitrogen is not exhausted (experiments 2 to 7), growth is not affected by nitrogen concentrations up to ~ 10 mM in

this system. The amount of biomass formed during this period (~ 0.4 g/l) is similar to that formed in the system nitrate - urea.

Growth of *A. platensis* on the combination nitrate - ammonium is maximal and independent of nitrogen concentration between 3 and 11 mM; nitrate seems to be preferentially used and ammonium slightly inhibits growth and should not exceed 30% of total nitrogen

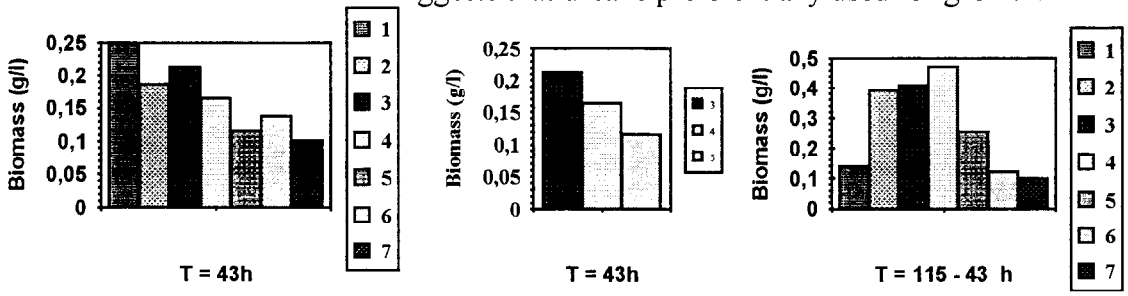
3 . 3 - Ammonium and Urea

Experimental conditions : Experiments are classified on the basis of increasing amounts of nitrogen.

Experiment	Σ N-nitrogen mM	$N\text{-}CH_4N_2O$ mM	%	$N\text{-}NH_4$ mM	%
1	2,4	1,2	*50,0*	1,2	*50,0*
2	4,0	1,2	*30,0*	2,8	*70,0*
3	4,4	4,0	*90,9*	0,4	*9,1*
4	6,0	4,0	*66,7*	2,0	*33,3*
5	7,6	4,0	*52,6*	3,6	*47,4*
6	8,0	6,8	*85,0*	1,2	*15,0*
7	9,6	6,8	*70,8*	2,8	*29,2*

Growth at 43 h and between 43 et 115 h

In this combination of urea and ammonium, increasing concentrations of nitrogen exert a very significant inhibition on the of biomass formed during the first 2 days, that is not surprising since both these compounds appeared toxic at quite low concentrations. However the higher biomass formation when they are furnished at similar concentrations, but in combination (experiments 5, 6 and 7) rather than individually shows that these compounds mutually reduce their toxicity. The reduction of biomass formation by increasing concentrations of ammonium in the presence of constant concentration of urea suggests that urea is preferentially used for growth.



During the subsequent 3 days, the reduced formation of biomass expresses the exhaustion of nitrogen in the culture medium in experiments 1 - 3, and the toxicity of high concentrations of ammonium + urea in experiments 4 - 7.

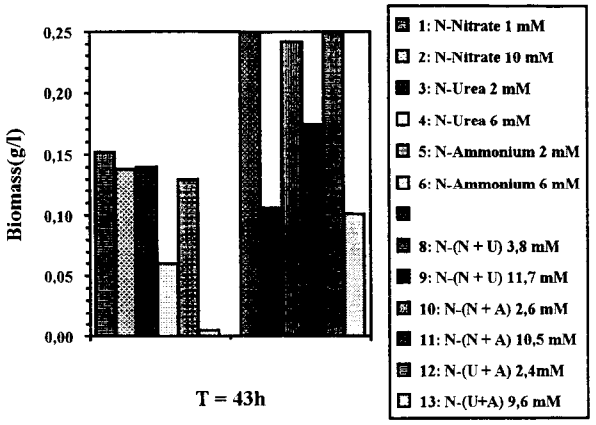
In this combination ammonium - urea, urea seems to be first and preferentially used for growth while ammonium exerts a negative effect. Best growth conditions are obtained with a total nitrogen concentration ranging between 4 - 7 mM and a proportion of ammonium not exceeding 50%.

3.4 - Conclusions

From this analysis of growth of *A. platensis* on two combined nitrogen sources, it principally appears that :

- *A. platensis* can grow on all the analysed binary combinations of nitrate, urea and ammonium.

- At low concentration, growth is even better on these combinations than on each of these individual nitrogen sources.
- Increasing nitrogen concentrations are less toxic than with individual compounds



4 - GROWTH ON THREE MIXED NITROGEN SOURCES

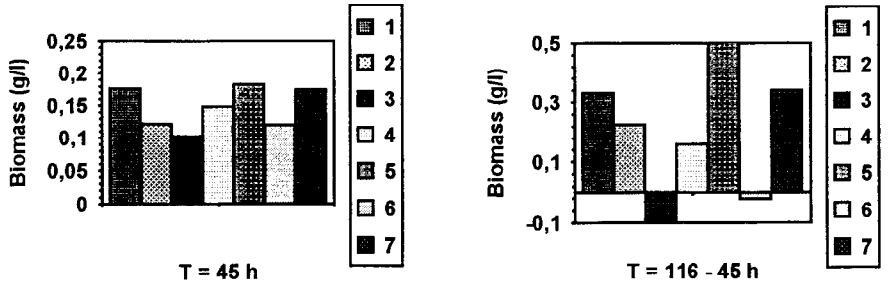
4 . 1 - Effects of nitrogen concentration

Experimental conditions : Experiments are classified on the basis of nitrogen concentration.

	Σ N-nitrogen mM	N-Urea mM	%	N-Ammonium mM	%	N-Nitrate mM	%
1	12,4	4,8	*38,7*	4,0	*32,3*	3,6	*29,0*
2	12,8	1,6	*12,5*	4,0	*31,3*	7,2	*56,2*
3	14,0	8,0	*57,1*	2,4	*17,2*	3,6	*25,7*
4	14,4	4,8	*33,3*	2,4	*16,7*	7,2	*50,0*
5	14,8	1,6	*10,8*	2,4	*16,2*	10,8	*73,0*
6	16,0	8,0	*50,0*	0,8	*5,0*	7,2	*45,0*
7	16,4	4,8	*29,3*	0,8	*4,8*	10,8	*65,9*

Growth at 43 h and between 43 et 115 h

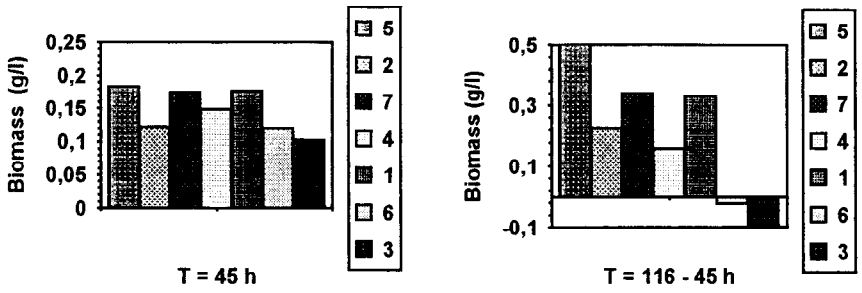
- classification by increasing total nitrogen concentration



Cells grow on this ternary combination of nitrogen sources, but the amount of biomass produced after 2 days of culture (0.10-0.18) is lower than that obtained with the binary combinations, and even in some cases with the individual compounds. Nitrogen concentration does not seem to be responsible for the observed differences in biomass production, particularly during the 3 subsequent days. The only classification criterion that allowed to point out a simple correlation was

the urea concentration, confirming its toxicity in this system. The biomass production decreases with increasing concentrations of urea, that may even entail a cell lysis at 8 mM.

- classification by increasing urea concentration



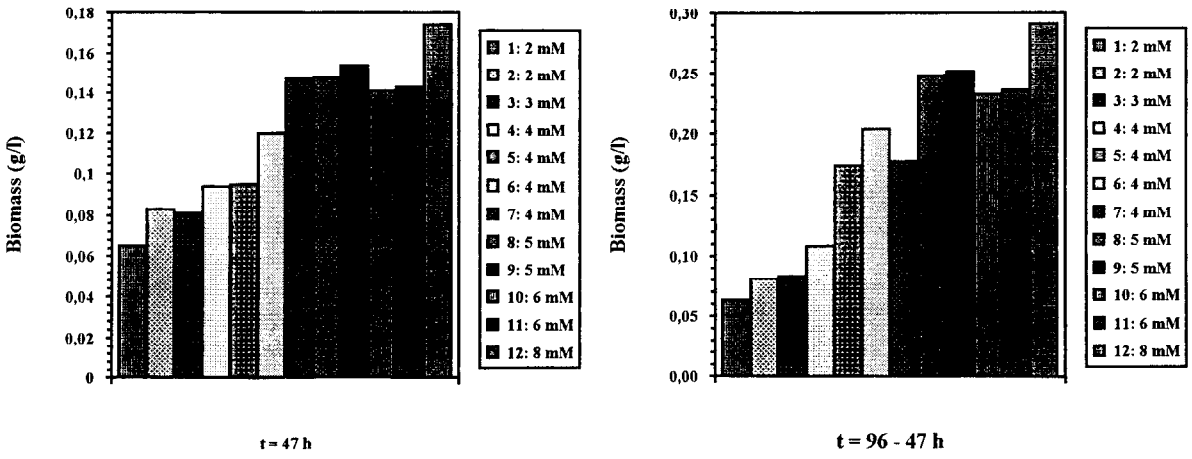
4 . 2 - Effects of the proportion of the three nitrogen sources

In each of these experiments, nitrate, ammonium and urea were supplied to the growing cells in different, non lethal proportions, but at a constant final nitrogen concentration of 10 mM.

Experimental conditions.

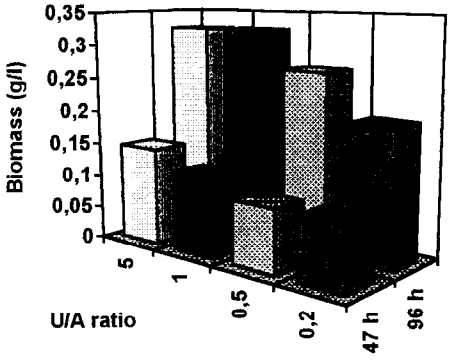
	N-Nitrate mM	N-Urea mM	N-Ammonium mM
1	2	3	5
2	2	5	3
3	3	2	5
4	4	1	5
5	4	2	4
6	4	3	3
7	4	5	1
8	5	2	3
9	5	4	1
10	6	3	1
11	6	1	3
12	8	1	1

Growth at 47 h and between 47 h and 96 h : classification on the basis of nitrate concentration.



This classification shows that for 80% nitrate in this ternary system, the amount of formed biomass is very similar to that formed with same concentration of nitrate alone. The formation of biomass decreases for lower nitrate concentrations, showing that increasing proportions of the other partners, urea and ammonium exert a very significant inhibition of growth, particularly above 50%.

Among these partners, ammonium seems to be the more toxic since this inhibition of growth increases with decreasing urea/ammonium ratios in experiments 4 - 7 in which the concentration of nitrate is constant



4 . 3 - Effects of pH and of sodium hydrogen carbonate concentration on growth

Experimental conditions

Effects of pH and of sodium hydrogen phosphate on nitrogen utilisation was analysed with the ternary system described paragraph 4 . 1, in which total nitrogen concentration and nitrate, urea and ammonium proportions varied as recall in this table.

	Σ N-nitrogen mM	N-Nitrate mM	%	N-Urea mM	%	N-Ammonium mM	%
1	14	3,6	*25,7*	8,0	*57,1*	2,4	*17,2*
2	12,4	3,6	*29,0*	4,8	*38,7*	4,0	*32,3*
3	16	7,2	*45,0*	8,0	*50,0*	0,8	*5,0*
4	14,4	7,2	*50,0*	4,8	*33,3*	2,4	*16,7*
5	12,8	7,2	*56,2*	1,6	*12,5*	4,0	*31,3*
6	16,4	10,8	*65,9*	4,8	*29,3*	0,8	*4,8*
7	14,8	10,8	*73,0*	1,6	*10,8*	2,4	*16,2*

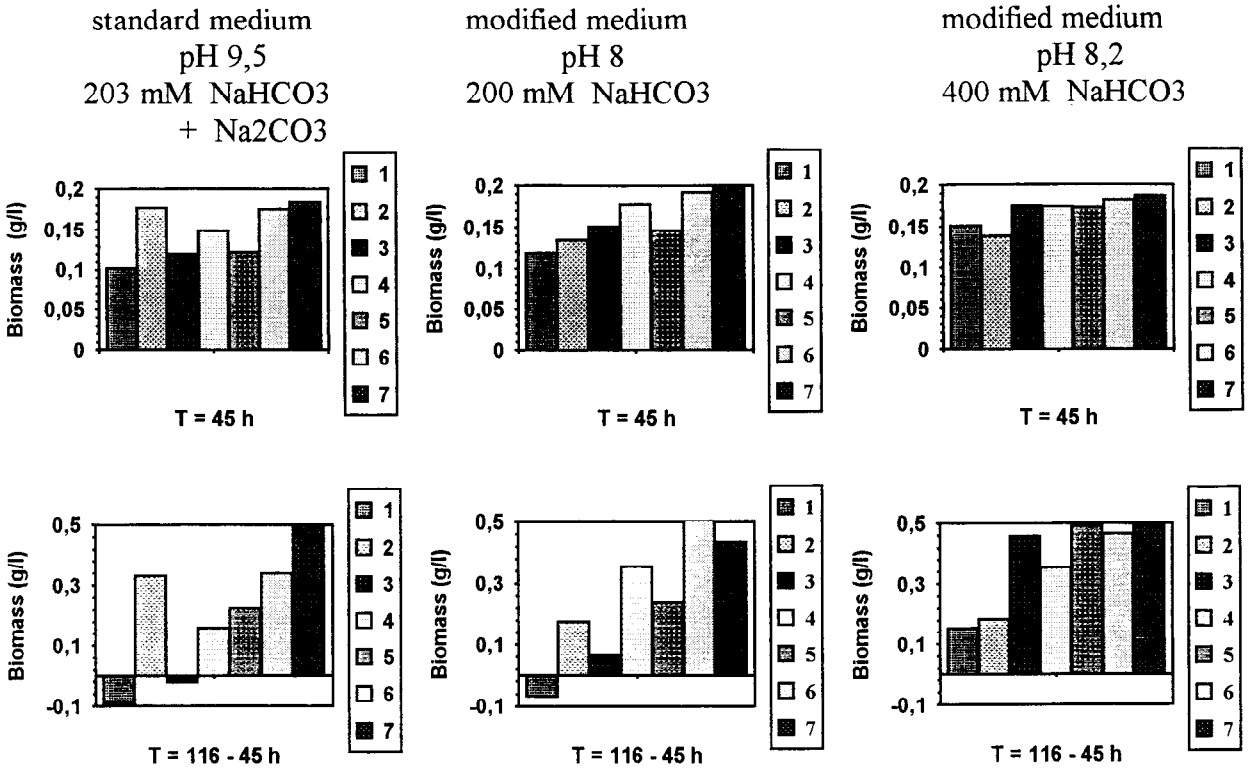
Each of these conditions was produced in the Zarrouk culture medium in which pH and sodium hydrogen carbonate concentration were modified as follow compared to the standard medium:

- pH 9.5, 203 mM Na2CO3 + NaHCO3
- pH 8, 200 mM NaHCO3
- pH 8,2, 400 mM NaHCO3

Growth at 45 h and between 45 and 116 h ; classification on the basis of nitrate concentration.

*** Effects of pH on growth**. Decreasing from 9.5 to 8 the pH of the standard culture medium has few effects on growth; however,

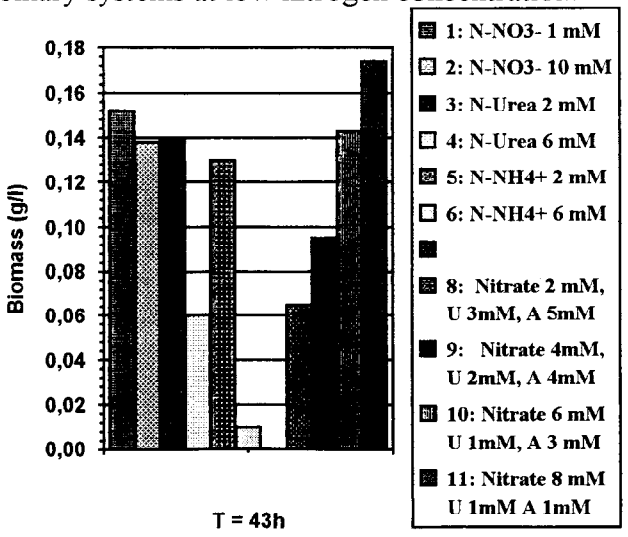
- at pH 8, growth seems to be more directly related to nitrate concentration, suggesting that nitrate is preferentially used as nitrogen source by *A. platensis* at this pH;
- this pH slightly reduces the toxicity of urea.



* **Effects of sodium hydrogen carbonate**. The comparison of growths at pH 8 - 8.2 on simple or double concentrations of NaHCO3 shows that increased concentrations of sodium hydrogen carbonate:
- allow a better use of nitrogen at low nitrate concentration,
- decrease the toxicity of nitrogen, particularly that of urea, (experiments 1 and 3 at 116 - 45 h),
- and therefore allows a better utilisation of urea, since less toxic (exp. 3 at 116 - 45 h).

4 . 4 - Conclusions

It therefore appears that *A. platensis* is able to grow on mixed nitrate, ammonium and urea. However, this growth is less efficient than on nitrate alone because the toxicity of ammonium and urea is not reduced by nitrate as in the case of the binary combinations, and no stimulation of growth was observed as in the binary systems at low nitrogen concentration.



These experiments also show that pH and NaHCO3 concentration participate in the utilisation of nitrogen. Some differences in growth characteristics on standard Zarrouk culture medium or during our experiments may result from differences in the relative amounts of nitrogen and sodium hydrogen carbonate, with a C/N ratio equal to 7 in the standard medium and ranging between 12 and 100 in our experiments.

GENERAL CONCLUSIONS AND PERSPECTIVES

Usable Nitrogen sources:

• *A. platensis* cells are able to use for growth the classical mineral nitrogen compound, nitrate, nitrite and ammonium, but also organic compounds such as urea and aminoacids. They do not use nitrogen of puric or pyrimidic bases.

• Cells compensate the absence of usable nitrogen in the culture medium by the degrading their phycocyanins. The resulting altered state does not significantly affect the transport and the metabolism of nitrogen and cells reconstitute in 2 days normal levels of phycocyanins when nitrogen becomes available in the culture medium.

Growth on a single nitrogen source

• Growth rate increases when nitrate, urea or ammonium concentrations in the culture medium decrease. Optimal concentrations for high growth rates are about 2 mM of atomic nitrogen, and if tolerance for higher concentrations is relatively high for nitrate (up to about 100 mM), it is very low for urea and particularly for ammonium that both slow down growth already at 3 mM and even cause a rapid lysis of cells at higher concentrations.

• This low tolerance of *A. platensis* to increasing concentrations of ammonium and urea is constitutive since not resulting from a lack of previous adaptation of inoculums.

• The utilisation of nitrate, ammonium and urea by *A. platensis*, and the toxicity of these compounds are modified by the available light energy:
- Under conditions of limitation of biomass production by low light intensity, nitrogen utilisation becomes proportional to its concentration;
- The toxicity of increasing nitrogen concentrations appears sooner at low light intensities.

These results suggest that the efficiency of transport systems or of regulatory mechanisms of intracellular nitrogen or even of detoxification systems are related to the available light energy.

• Na^+ ions do not modify the utilisation of ammonium by *A. platensis*, either because not used by the ammonium transport system or because at the pH of the culture, ammonium ions are converted to ammonia which can freely diffuse through the plasma membrane. On the contrary, Na^+ ions allow a better use of urea and could even participate to a detoxification system. These results suggest the existence of distinct transport systems for the different nitrogen sources.

• Fast growing cultures producing high amounts of biomass can be obtained by maintaining during long periods the nitrogen concentration at low levels (~2 mM) by successive additions of the used nitrogen source, and by increasing the light intensity in order to compensate cell-shadowing

Growth on 2 combined nitrogen sources

• *A. platensis* cells grow on all binary combinations of nitrate, ammonium and urea, with a maximal rate for low total nitrogen concentration, in a narrow range for the ammonium - urea combination, but in a much larger range when nitrate is present.

• When present, nitrate seems to be used first and seems to be responsible for growth characteristics very similar to those observed when it is the only nitrogen source. In consequence, nitrate in sufficient proportion (~60%) reduces the toxicity of urea and of ammonium, which appears at concentrations higher than when these compounds are individually supplied to the cultures.

• In the ammonium - urea combination, urea seems to be initially and principally used. The toxic effects of each of these compounds already appear at concentrations that are still low, but significantly higher however than when individually supplied to the cultures. Mixed ammonium and urea mutually reduce their toxicity and therefore allow better growth.

• Some concentration ratios of the combined nitrogen sources allow, by some synergy effect, a growth rate 30% higher than that obtained in the best conditions with a single nitrogen source

Growth on 3 combined nitrogen source

• In ternary combination, nitrate seems to be the nitrogen source used first ; in proportion higher than 80%, it allows a biomass production similar to that obtained when it is supplied alone to the cultures. For lower proportions, i.e. when the proportion of the urea - ammonium couple becomes higher than 20%, these two compounds become toxic, particularly for high ammonium/urea ratios

• Contrary to some binary combinations, none ternary combination induces by some synergy any stimulation of growth.

• Preliminary measurements suggest that a decrease of pH or an increase of the sodium hydrogen carbonate concentration in the culture medium reduce the toxicity of urea and, in consequence, may allow a better use of nitrogen at low nitrate concentrations.

Perspectives

This study shows that it is possible to introduce some flexibility in MELiSSA since growth of *A. platensis* in the photosynthetic compartment appears to be not dependant on nitrate or nitrite produced in the nitrifying compartment, and is able to use, at low concentrations, other nitrogen sources directly produced by other upper compartments of the closed ecosystem, and particularly, urea and ammonium produced by the crew and the liquefying compartments respectively. However, it appeared during this study that several parameters, such as pH, concentration of Na^+ ions or sodium hydrogen carbonate, available light energy, adaptation of cells to high light intensity or efficiency of gas transfer in the culture participate to nitrogen utilisation for growth, and therefore should be analysed in more details.

Particularly, a point that remains to be elucidated is the eventual interrelationships between photosynthesis and nitrogen assimilation. The light intensity used during this study (~8 $W.m^{-2}$) was relatively low for culture of *A. platensis*, and gas (particularly CO2) transfer in flasks under the used orbital stirring was limiting ; it therefore would be of the highest interest to analyse the characteristics of nitrogen utilisation under higher light intensities, up to 80 - 100 $W.m^{-2}$ and under conditions of increased gas transfer, by direct air - CO2 bubbling in the stirred culture.

The photosynthetic compartment of MELiSSA may be used for secondary applications, particularly in the fields of environment ant water treatment. The results obtained in this study show that one may consider the possibility to use cyanobacteria (and particularly *A. platensis*) for the treatment of waters contaminated by nitrogen from single or mixed sources, on condition that growth of *A. platensis* on the different nitrogen compound that are susceptible to contaminate aqueous effluents has been analysed.

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