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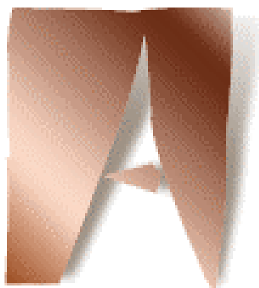

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SPIRULINA PLATENSIS AMMONIUM UPTAKE TESTS

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reference/*réference*	CCN5 to contract 13292/98/NL/MV
issue/*édition*	1
revision/*révision*	0
date of issue/*date d'édition*	February 2006
status/*état*	Final
Document type/*type dedocument*	Technical Note
Distribution/*distribution*	MELISSA all

CONFIDENTIAL DOCUMENT

APPROVAL

Title *titre*	*Spirulina platensis* ammonium uptake tests	issue *issue* 1	revision *revision* 0

author *auteur*	A. Masot , J. Albiol	date *date*	2006-02-10

approved by *approuvé by*	F. Gòdia	date *date*	2006-02-10

CHANGE LOG

reason for change / *raison du changement*	issue/ *issue*	revision/ *revision*	date/ *date*

CHANGE RECORD

Issue: Revision:

reason for change/ *raison du changement*	page(s)/ *page(s)*	paragraph(s)/ *paragraph(s)*

TABLE OF CONTENTS

1. Introduction *1*

2. Materials and methods *3*

2.1. Microorganism and culture medium **3**

2.1.1. Strains and inoculum 3

2.1.2. Culture medium 3

2.2. Bioreactor **5**

2.2.1. 7

2.2.2. Control and monitoring 8

2.2.3. Photobioreactor start-up 9

2.3. Analytic procedures **9**

2.3.1. Axenicity control 9

2.3.2. Total biomass analyses 9

2.3.3. Nitrogen analyses 10

2.3.4. Biomass elemental analyses 12

3. RESULTS AND DISCUSSION *13*

3.1. Test I: D=0.021 h-1 .... **13**

3.2. Test II: D=0.032 h-1 .... **16**

3.3. Test III: D=0.024 h-1 .... **20**

3.4. Test IV: D=0.026 h-1 .... **23**

3.5. Test V: D=0.028 h-1 .... **26**

3.6. Test VI: D=0.012 h-1 .... **31**

4. CONCLUSIONS *33*

5. References *35*

6. APPENDIX *36*

6.1. Light Calibration **36**

6.2. Equipment Sterilization **38**

6.3. Culture Axenicity Monitoring **39**

1. Introduction

The MELISSA project (Microbial Ecological Life Support System Alternative) is based on the connection between five compartments, colonised respectively by thermophilic anoxygenic bacteria, photohererotrophic bacteria, nitrifying bacteria, photosynthetic bacteria, higher plants and the crew.

Nitrate present in the outlet from the nitrifying compartment is the nitrogen source used by *S. platensis*. However, the ammonium obtained from the human urea could be used in this compartment as nitrogen source. Also a decrease in the O_2 supply to compartment III, would cause an increase in the ammonium income to compartment IVa. Thus, in order to evaluate the effects of ammonium consumption by *S. platensis* and to introduce some flexibility in the ammonium distribution inside the MELISSA loop, it is interesting to study to which extent *S. platensis* is able to assimilate ammonium.

In this framework, during this year several continuous culture tests at different dilution rates (in the range of 0.01-0.03 h^{-1}) and one step of light at each one were performed as described below.

Background: Nitrogen assimilation by *S. platensis*

Bibliographic studies on *S. platensis* nitrogen uptake show that although nitrate is the nitrogen source most often used in cultivation of this cianobacteria, ammonium can also be consumed (Ciferri, 1983; Richmond, 1986; Becker, 1994). Using nitrate as N source a decrease in cells growth rate is observed at high nitrate concentrations (16800 ppm $N\text{-}NO_3^-$; Ciferri, 1983). Alternatively, when using ammonium the apparent inhibition appears at lower concentrations (100 ppm $N\text{-}NH_4^+$; Richmond, 1988). However it seems that when both sources are present in the culture media at the same time, the cells have a higher affinity for ammonium and it is consumed first (Guerrero and Lara, 1987).

Previous studies about different nitrogen sources assimilation by *S. platensis* have already been carried out in the MELISSA group.

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Filali and Dubertret (1996) performed different tests to analyze the capability of *S. platensis* cells to grow on several nitrogen sources (nitrate, nitrite, ammonium, urea and aminoacids such as glutamine and adenine) supplied to the cultures individually or in combination as pairs or triads. The work was particularly focused on nitrate, urea and ammonium uptake at pH 9.5. They concluded that *S. platensis* is able to grow on classical mineral nitrogen compounds and also on organic compounds such as urea and aminoacids. In case of lack of usable nitrogen in the culture medium, cells consumed their phycocyanins and turned to a yellowish green colour. Concerning the growth on a single nitrogen source, the tolerance for concentration is high for nitrate (up to 1400 ppm $N\text{-}NO_3^-$) but low for urea and ammonium (42 ppm $N\text{-}NH_4^+$). Moreover, the inhibition due to an increase of nitrogen concentration appears sooner at low light intensities. Therefore, it is suggested that the nitrogen assimilation is related to the photosynthesis.

Although this first study was able to evaluate the *S. platensis* capability to grow on several nitrogen sources, the limitations of low light intensity (8 W/m^2) Erlenmeyer-flasks cultures suggest the necessity of further studies on nitrogen assimilation. As a consequence it was proposed to analyse the characteristics of nitrogen utilization under higher light intensities (up to 80-100 W/m^2) and in continuous cultures to mimic better the real situation of *S. platensis* in the MELISSA loop.

A second study (Lattenmayer C., 2001) was carried out at ESTEC laboratory to get some basic knowledge about growth kinetics of *S. platensis* using ammonium as nitrogen source in batch and continuous cultures.

They performed a continuous culture in a 3.2L Chemap Bioreactor with a stirrer speed of 200 rpm at pH 8.5, 35.5 ºC and a light intensity range of 50-80W/m^2. When light intensity was 80 W/m^2, they found a productivity decrease from 0.0106 $g.L^{-1}h^{-1}$ to 0.0086 $g.L^{-1}h^{-1}$ with an ammonium concentration inside the bioreactor of 38 ppm $N\text{-}NH_4^+$ and 80 ppm $N\text{-}NH_4^+$ respectively. In order to determine a critical value causing inhibitory effects between these two ammonium concentrations, they carried out

five experiments with Erlenmeyer-flasks at pH 8.5, 25ºC and a light intensity of 10 W/m^2 on one side using different ammonium concentrations.

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The results showed that at increasing ammonium concentrations the growth rate decreases. As a conclusion of this continuous and batch cultures, they assumed 70 ppm $N\text{-}NH_4^+$ as a critical value, which is close to the one observed by Richmond (100 ppm $N\text{-}NH_4^+$).

Nevertheless, this data is not sufficient enough to get a deep knowledge of the ammonium uptake and inhibition in *S. platensis* cultures, requiring more experimental data. Therefore in this work package several continuous cultures at different dilution rates and light intensities are performed.

The main objective of these cultures is to determine the inhibition effect at different reference conditions of light intensity (to be chosen between the 15-125 W/m^2 range) and dilution rate (0.012 h^{-1}- 0.032 h^{-1}), as well as the growth rates, biomass levels and its composition. The results will contribute to the formulation of mathematical models of the response observed. Those will allow foreseeing the behaviour of the strain when ammonium increases at the input of the compartment.

2. Materials and methods

2.1. Microorganism and culture medium

2.1.1. Strains and inoculum

The strain of *Spirulina platensis* used (*Arthrospira platensis* PCC 8005) was provided by the Pasteur Institute. It was revived and the subcultures were done using the Zarrouk-medium (Zarrouck, 1966) in Erlenmeyer-flasks with constant illumination and periodically agitation. The inoculum volume is fixed as the 10% of the working volume.

2.1.2. Culture medium

The medium used for pre-cultivation and batch phase is the one described by Zarrouk (1996) and consist of a macroelement solution (Table 1) and two microelement solutions (Table 2, 3) adjusted to pH 9.5.

Table 1.- *S. platensis* medium composition.

Compound	g/L
EDTA-Na·2 H_2O	0.08
NaCl	1
$FeSO_4$·7 H2O	0.01
Na_2CO_3	4.543
$NaHCO_3$	4.972
$MgSO_4$·7 H2O	0.20
$CaCl_2$	0.04
K_2SO_4	1
KH_2PO_4	0.5
$NaNO_3$	2.5
Solution	**mL/L**
A5	1.00
B6	1.00

Table 2.- Composition of A5 microelement solution

Compounds	g/L
H_3BO_3	2.860
$MnCl_2$·4 H_2O	1.810
$ZnSO_4$·7 H_2O	0.222
$CuSO_4$·5 H_2O	0.079
MoO_3	0.015

Table 3.- Composition of B6 microelement solution

Compounds	g/L
NH_4VO_3	0.023
$KCr(SO_4)_2$·12 H_2O	0.096
$NiSO_4$·7 H_2O	0.048
$(NO_3)_2Co$·6 H_2O	0.049
Na_2WO_4·2 H_2O	0.018
$Ti(SO_4)_2$+ $TiOSO_4$	0.048

Concerning the continuous cultures, as the goal of these tests is to evaluate the ammonium uptake of *S. platensis*, the nitrogen source was changed from sodium nitrate $NaNO_3$ 2.5 g/L (412 ppm N-NO_3^-) to ammonium sulphate $(NH_4)_2SO_4$ 1.10 g/L (233 ppm N-NH_4^+) and the pH solution was adjusted to 8.5 in order to avoid losing ammonium through the gas phase.

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The first tests results showed that with this concentration in the inlet medium, the ammonium concentrations inside the bioreactor were higher than the inhibition concentration observed in the bibliography (100 ppm $N\text{-}NH_4^+$; Richmond, 1986) and probably too high to allow the *S.platensis* growth. Therefore it was decided to reduce the ammonium concentration in the inlet medium to the half, which is 0.55 g/L ammonium sulphate (116 ppm $N\text{-}NH_4^+$). In this way the ammonium concentration in the inlet was high enough to supply the nitrogen requirement of the cells, but the remaining ammonium concentration inside the bioreactor could not reach high inhibitory levels.

A global quantity of 10L of culture medium is prepared and filtered to the inlet medium tank for several days. The microelement solution A5 and B6 added to the culture medium are from a 1 L stock conserved at 4ºC temperature.The evolution of the culture medium is followed on a daily basis by means of measuring the ammonium concentration and pH.

2.2.Bioreactor

A schematic representation of the experimental set-up is sketched in figure 1. All the equipment depicted is referenced and described in table 4.

All cultures of *S. platensis* were grown in a Biostat L BRAUN 2L working volume cylindrical photobioreactor (Ref.001).

Illumination of the bioreactor is obtained using 15 halogen lamps (Ref.007), distributed radially around the external wall, in 5 columns (Ref.006) containing, each one, 3 lamps. The light spectrum emitted by halogen lamps matches with the photosynthetic absorption range of the micro algae *S. platensis.* Light intensity supply control is described in the control section (2.2.1.4).

The culture is stirred mechanically by an agitation axis with two 6-blade Rushton turbines (Ref.003). The agitation speed is set at the same value as in the cultures performed by Lattenmayer (2001), 200 rpm.

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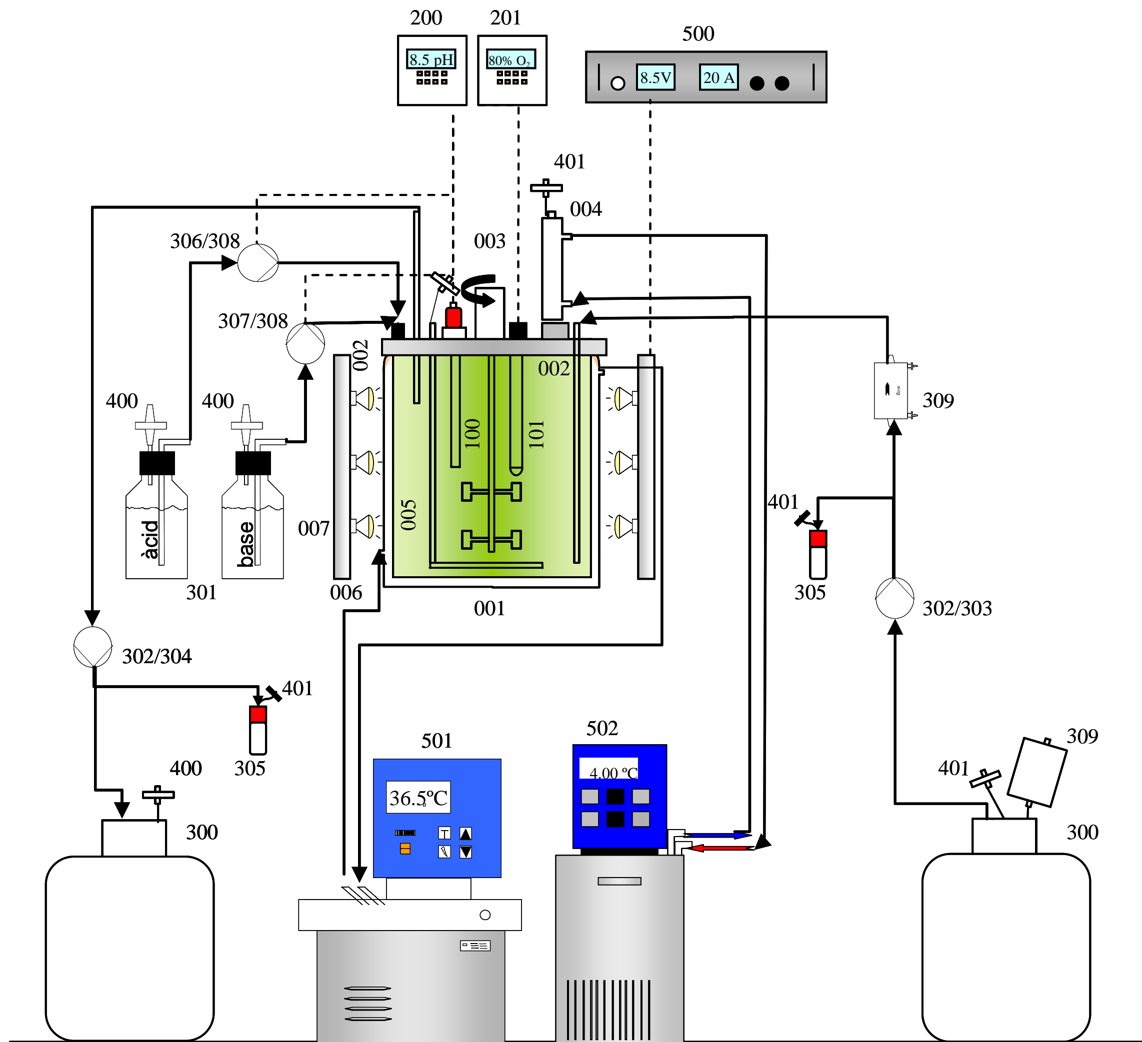

Figure 1.- General set-up of Biostat Braun 2L used for the *S. platensis* cultures. More detailed information about the referenced equipment is reported in table 4.

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Table 4.- List and description of the experimental equipment used for the culture of *S. platensis* at laboratory scale. References can be identified in figure 1.

	ELEMENT	REF.	DESCRIPTION	MODEL	TRADE	ORIGIN
REACTOR	Tank	001	glass, 2L	Biostat	Braun	Germany
	Lid	002	stainless steel	Biostat	Braun	Germany
	Mechanically stirring	003	35-250/280-2200 rpm	RZR	Heidolph	Germany
	Condenser	004	stainless steel	Biostat	Braun	Germany
	Air difusor	005	stainless steel	Biostat	Braun	Germany
	Lamps support	006	stainless steel	Custom manufactered	Custom manufactered	Spain
	Lamps	007	Halogen lamp,12V,20W	type 215	Sylvania	Belgium
SENSORS	pH sensor	100	autoclavable	InPro3000/120	Mettler Toledo	Zürich,Switzerland
	O_2 dissolved sensor	101	autoclavable	73052	Mettler Toledo-Ingold	Zürich,Switzerland
CONTROL	pH controller	200	20-253V,AC/DC,2VA	pH 2100	Mettler Toledo	Zürich,Switzerland
	O_2 controller	201	20-235V,AC/DC,2VA	O2 4100	Mettler Toledo	Zürich,Switzerland
LIQUID LOOP	I/O medium tank	300	polypropylene, 10L	2136	Nalgene	Hereford,UK
	Acid/base bottle	301	500 ml	Pyrex	Bibby	Staffordshire, UK
	I/O medium peristaltic pump	302	4 channels, 8 rollers	Reglo MS4/8-100	Ismatec	Zürich,Switzerland
	Inlet medium pump tube	303	ID=2.06mm, Wall=0.8mm	070540-151/SC0316	Pharmed/Ismatec	Zürich,Switzerland
	Outlet medium pump tube	304	ID=2.79mm, Wall=0.9mm	070540-181/SC0318	Pharmed/Ismatec	Zürich,Switzerland
	Sample tube	305	50 ml	G125	Alco	Barcelona,Spain
	Acid peristaltic pump	306	peristaltic	Ismatec	Ismatec	Zürich,Switzerland
	Base peristaltic pump	307	peristaltic	Ismatec	Ismatec	Zürich,Switzerland
	Pump tube	308	norprene	06402-14	Masterflex	Illinois,USA
	Liquid filter	309	0.22 mm	Opticap	Millipore	Bedford,MA,USA
GAS LOOP	Air filter	400	50 mm, 0.22 mm	Millex	Millipore	Bedford,MA,USA
	Air filter	401	25 mm, 0.22 mm	Millex	Millipore	Bedford,MA,USA
AUXILIARY EQUIPMENT	Power supply	500	0-15V/0-40A	SM1540-D	Delta Elektronika	Zirikzee, Holland
	Thermostatic bath	501	Jacket temperature regulator	D8	Haake GH	Germany
	Refrigeration bath	502	Condenser temperature regulator	Frigiterm	J.P Selecta	Barcelona, Spain

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2.2.1. Control and monitoring

All the monitored and controlled parameters of *S. platensis* culture are listed in table 5 and described in the following paragraphs. Parameters measured off-line are basically biomass analyses, which are described more widely in section 2.3.

Table 5. – Monitored and controlled parameters of *S. platensis* culture.

Monitored parameters	**On-line**	Dissolved oxygen (DO) Temperature pH
	Off-line	Biomass concentration (dry weight, optical density) Ammonium and nitrate medium concentration Elemental biomass analyses [CNHS]
Controlled parameters		Temperature pH Light intensity

2.2.1.1. Oxygen monitoring

The oxygen is monitored by an O_2 amplifier (Ref.201), which receives the value of dissolved oxygen (DO) measured with a polarographic oxygen probe (Ref.101). The controller shows also the culture temperature.

2.2.1.2. Temperature control

The temperature is controlled at 36.5ºC by means of a thermostatic bath (Ref.501), which circulates water through the external glass jacket of the photobioreactor.

2.2.1.3. pH control

An autonomous controller (Ref.200) regulates the pH of the culture media by addition of acid (HCl 1.5M) or base (NaOH 1M). The controller receives the value measured by the pH sensor (Ref.100) and actuates accordingly on the acid pump (Ref.306) or the base pump (Ref.307).

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2.2.1.4. *Light intensity control*

Light intensity is modified by means of changing the voltage supplied to the lamps by a 15V-40A-power supply (Ref.500). The calibration of the light intensity at the surface of the reactor, expressed in W/m^2, as a function of the voltage is given in Appendix 6.1.

2.2.2. Photobioreactor start-up

In order to start-up a culture the following steps have to be performed:

1. Light intensity calibration.
2. pH sensor calibration. (Ph probe is calibrated before autoclaving and the calibration corrected after autoclaving if necessary based on the initial pH reading of the bioreactor medium and the external value taken from an initial sample.)
3. Equipment sterilisation: Equipment sterilization is performed as described in appendix 6.2
4. O_2 dissolved sensor calibration (Oxygen probe is calibrated to 100 % reading in equilibrium with bubbled air after autoclaving. 0% reading is previously adjusted to the electrical zero).
5. Photobioreactor inoculation ($V_{inoculation}$=10% $V_{bioreactor}$).

2.3. Analytic procedures

2.3.1. Axenicity monitoring

Maintenance of axenic conditions of the culture is monitored according to the procedure described in appendix 6.3.

2.3.2. Total biomass analyses

2.3.2.1. *Dry weight*

Dry weight of *S. platensis* is determined as follows: first culture broth is filtered through a 0.47 μm filters (previously dried and weighted), then filters are dried until constant weight in a microwave oven (20 min., 150 W) and cooled to room temperature in a desiccator. Afterwards, filters are weighted again to determine the biomass dry weight by means of the difference

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between the weight of the filter and its weight before filtering the sample. Dry weight value is calculated as the arithmetical average of three samples.

2.3.2.2. Optical density

The optical density measured at 750 nm is a direct measurement of the S. platensis concentration according to the Beer-Lambert law, which is the linear relationship between absorbance and concentration of an absorbing species. When working in concentration units of molarity, the Beer-Lambert law is written as:

$$A = e\ b\ c$$

where:

A = absorbance (no units, $A = \log_{10} P_0 / P$)
e = molar absorbtivity or extinction coefficient ($L\ mol^{-1}\ cm^{-1}$)
b = path length of the sample, (cm)
c = concentration of the compound in solution ($mol\ L^{-1}$)

Neither exopolysaccharides nor cyanobacteria pigments absorb at these wavelength. Thus, these measurements reflect only the scattering of light produced by the presence of the microorganisms, fact that is directly related with biomass concentration. The optical density is measured by a spectrophotometer (Kontron Instrument, Uvikon 941 Plus, Italy).

2.3.3. *Nitrogen analyses*

2.3.3.1. Ammonium concentration (ppm N-NH4+)

Ammonium was measured using UV measurement determinations by means of LCK 305 ammonium analysis kits (Dr. Lange Nitrax, analyse range 1-12 ppm $N\text{-}NH_4^+$).

<u>Basis:</u> Ammonium ions react with the hypochloride and salicylate ions in presence of nitroferrocyanide. Nitroferrocyanide acts as a catalyser (pH=12.6) forming iodophenol blue. Iodophenol blue is quantified measuring the absorption at 694 nm (Dr. Lange Xion 500 spectrophotometer).

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Procedure: Add 0.5 ml of filtered sample (diluted if necessary to be inside the measuring range) to ammonium analysis kit, mix it by manual agitation and wait 15 minutes. The absorbance of the kit is measured with the spectrophotometer (Dr. Lange Xion 500) at 694 nm. Figure 2 shows the correlation between Dr. Lange kit units given by the spectrophotometer and the ammonium concentration.

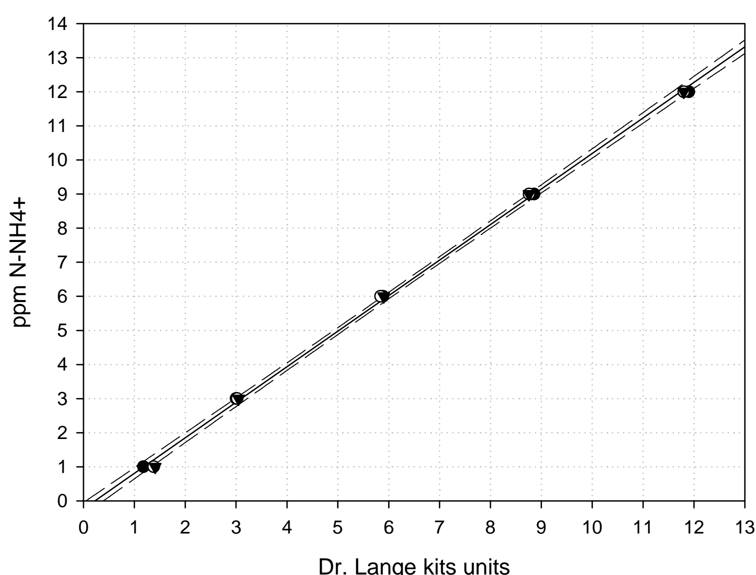

Figure 2.- Standard curve for ammonium analyses.
Linear regression values (y=a+b*x): a= -0.2318 ± 0.0633; b=1.0424±0.0087; r^2=0.9990

2.3.3.2. *Nitrate concentration (ppm N-NO3-)*

Nitrate is measured using UV measurement determinations by means of LCK 339 nitrate analysis kits (Dr. Lange Nitrax, analyse range 0.23-13.5 ppm $N\text{-}NO_3^-$).

Basis: Nitrate ions, in presence of sulphuric or phosphoric acid, react with 2,6-dimethylphenol forming 4-nitro-2,6-dimethylphenol which is quantified measuring the absorption at 370 nm (Dr. Lange Xion 500 spectrophotometer).

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Procedure: Add 1 ml of filtered sample (diluted if necessary to be inside the measuring range) into the test cuvette. After adding 0.2 ml of LCK 339 solution, the cuvette is mixed strongly. The absorbance is measured after 15 minutes with the spectrophotometer (Dr. Lange Xion 500). Nitrate concentrations in ppm $N\text{-}NO_3^-$ are obtained using the following standard curve (Figure 3).

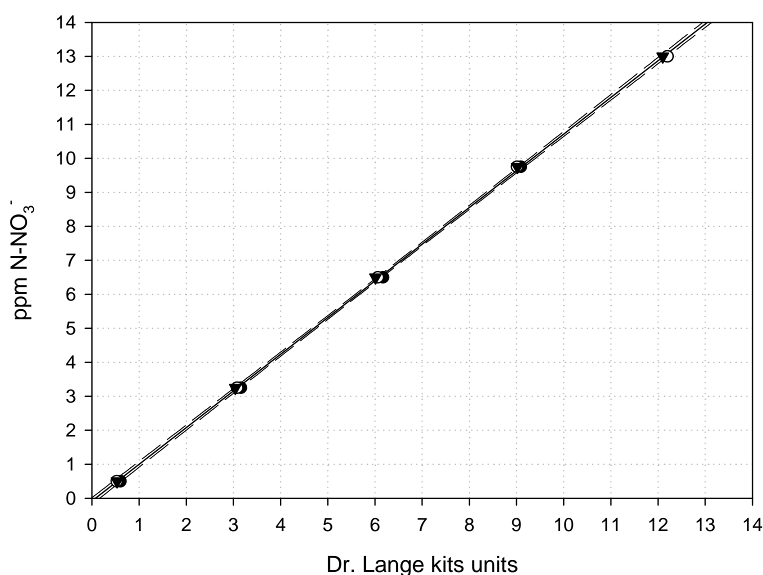

Figure 3.- Standard curve for nitrate analyses.
Linear regression values (y=a+b*x): a= -0.0069 ± 0.0329; b=1.0782±0.0044; r^2=0.9998

2.3.4. Biomass elemental analyses

2.3.4.1. Elemental composition (C, N, H, S)

Elemental analyses were performed by the analytical service of UAB. The determination of C, N, H and S composition is done with the sample combustion inside a Sn capsule with O_2 atmosphere in a furnace at 1800ºC.

Basis: The combustion transforms organic sample components to the corresponding oxides, obtaining a gas mixture of CO_2, N_2, N_xO_y, H_2O, SO_2 and SO_3. The exothermic reaction, which turns Sn into SnO_2(s), releases heat that increases the capsule temperature till 1800 ºC. SnO_2 remains as a solid in the combustion zone. Inorganic sample components also remain as solid oxides in the combustion zone.

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The gases formed and the O_2 excess flow with He as a carrier gas to a reactor with WO_3, which produce the transformation of the gas mixture into the unique species for each element, obtaining CO_2, N_2, H_2O, SO_2 and the O_2 and He in excess. This mixture is transported to a reactor at 500 ºC containing CuO, which reacts with the O_2 in excess to give CuO(s).

The remaining gases CO_2, N_2, H_2O and SO_2 are carried with He to a gas chromatograph (Porapak column, Waters Associates Inc.) where they are separated and measured using a thermal conductivity detector, which detects a signal proportional to the amount of component.

Sample preparation: The biomass sample is in freeze-dried form.

Results: The quantification is performed by interpolation on an appropriate reference curve.

3. RESULTS AND DISCUSSION

In order to evaluate the behaviour of *S. platensis* using ammonium as a nitrogen source, different continuous cultures at various dilution rates have been performed. These are presented in the following sections where the dilution rate (D) used for the continuous cultures is already included in the section title.

Once the steady state is reached, a step of light is done, so that the influence of light in ammonium uptake and cell growth can be analyzed. Experimental conditions are described with detail for each case and the results obtained are commented after depicted.

The first ammonium continuous cultures were performed in an air-lift reactor, but after detecting the ammonium loss through the gas phase by means of an acid trap, the reactor was changed into a mechanically stirred reactor. These first cultures are not presented due to the lack of a complete measurement of the ammonium loss, which results in the impossibility to close nitrogen balance.

3.1. Test I: D=0.021 h^{-1}

First it was decided to perform a culture in an intermediary dilution rate value. Conditions and evolution of compartment IVa in the first culture are presented in table 6 and figure 4 respectively.

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Table 6.- Operational conditions of test I shown in figure 4.

Parameter	Phase I	Phase II
Light (W/m^2)	33	125
Q_L (L/day)	1.05	1.05
ι (h)	46	46
D (h^{-1})	0.021	0.021

During this test culture conditions were set as described in Table 6. Light intensity at the surface of the bioreactor (Fr) was initially fixed at 33 W/m^2. When the experiment reached 210 hours of operation and a steady state was reached (more than 4 residence times with established values), light intensity was increased from 33 W/m^2 to 125 W/m^2.

During the evolution of the culture, an increase in the biomass concentration is observed after an increase in the available light intensity followed by a new steady state. This response confirms that, in these conditions, the biomass growth is limited by light intensity and as a result non consumed ammonium remains in the bioreactor.

Consequently, as the ammonium concentration in the inlet flow remains constant, it can be seen that when the biomass concentration increases, the ammonium concentration in the outlet flow diminishes.

The levels of ammonium remaining in the bioreactor, of around 63 ppm $N\text{-}NH_4^+$ at phase I and around 26 ppm $N\text{-}NH_4^+$ after light increase (Phase II), do not cause a measurable inhibition effect on the biomass growth in these conditions (Table 6).

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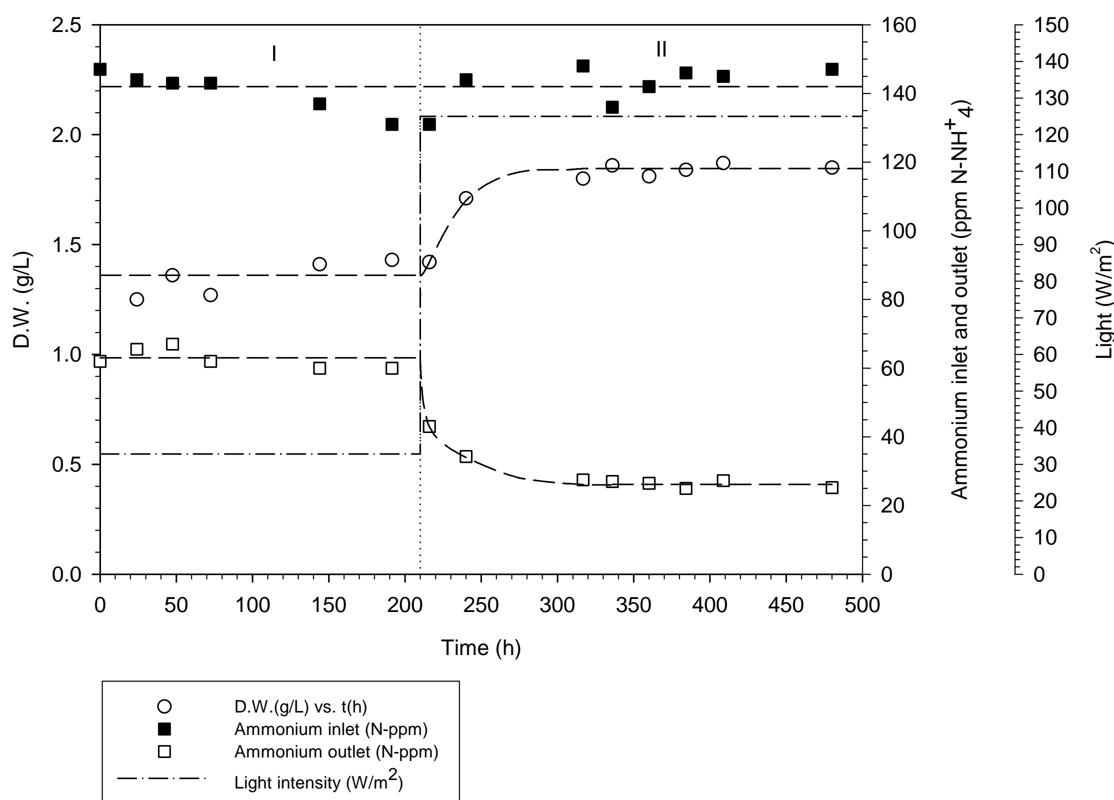

Figure 4.- Evolution of compartment IVa using ammonium as nitrogen source. The operational conditions of this experiment are summarized in table 6. Phase I: The light intensity was set at 33 W/m^2 during the initial 210 hours of the test. Phase II: After the first steady-state light intensity was increased at 125 W/m^2.

In order to evaluate with more detail the behaviour of this culture, a summary of the main average parameter values (such as biomass levels, productivity, ammonium uptake per unit biomass) reached at each steady state are reported in Table 7.

Table 7.- Main parameters values of test I shown in figure 4.

Parameter	Phase I	Phase II
Biomass concentration (g/L)	1.36 ± 0.09	1.84 ± 0.02
Productivity (mg/L h)	29 ± 2	40.4 ± 0.5
NH_4^+ input (ppm N- NH_4^+)	141±6	143 ± 4
NH_4^+ output (ppm N- NH_4^+)	63±3	26 ± 1
$Y_{X,N}$ (mg N/g *S. platensis*)	57.4± 5.2	63.4± 3

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Regarding to the values shown in Table 7, both steady states are successfully reached, and the values of culture parameters present a low standard deviation.

These results confirm that a culture, performed at stable and fixed conditions, has a higher productivity when the light available is higher. Although apparently the nitrogen consumption per biomass unit seems to be higher at higher light intensity, the difference is not statistically significant (α=0.05).

In order to study whether the light intensity affects the biomass composition, the elemental composition analysis of these two steady states has been carried out. Table 8 reports the average value of 5 measurements for C, N, H and S, as well as their dispersion.

Table 8.- Results of the elemental composition analysis described as the average value of 5 measurements ± standard deviation. Percentages do not add to 100% due to the lack of determination of oxygen, phosphorous and ashes. Last column shown the corresponding elemental formula.

Phase	C (%)	H (%)	N (%)	S (%)	Elemental formula
I	47.28±0.30	6.82±0.12	11.31±0.21	0.58±0.04	$CH_{1.7302}N_{0.2050}S_{0.0045}$
II	48.03±0.59	7.08±0.17	10.13±0.34	0.52±0.07	$CH_{1.8568}N_{0.1848}S_{0.0037}$

No significant difference between C, H, N and S composition of *S. platensis* biomass from phase I and II is detected. It can be therefore concluded that under the experimental conditions tested, light intensity has no change on measured elements.

From this first culture it can be confirmed the capability of *S. platensis* to grow using ammonium as a nitrogen source. Furthermore an ammonium concentration of 63 ppm $N\text{-}NH_4^+$ do not cause any apparent inhibitory effect. A light step up produces an increase of productivity, but no remarkable change in elemental composition.

3.2. Test II: D=0.032 h^{-1}

As the culture at intermediary dilution rate value (0.021 h^{-1}) was successfully performed, a second culture with a higher dilution rate was proposed. Table 9 summarizes operational conditions of this culture and Figure 5 shows its evolution.

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In order to allow a progressive adaptation of the cells to the culture conditions a gradual increase in flow rate was performed (phase I, figure 5). Phase II starts when the desired input flow rate is reached.

Table 9.- Operational conditions of test II shown in figure 5.

Parameter	Phase I	Phase II
Light (W/m^2)	125	125
Q_L (L/day)	0.51-1.35	1.55
ι (h)	93-35	30.8
D (h^{-1})	0.011-0.028	0.0325

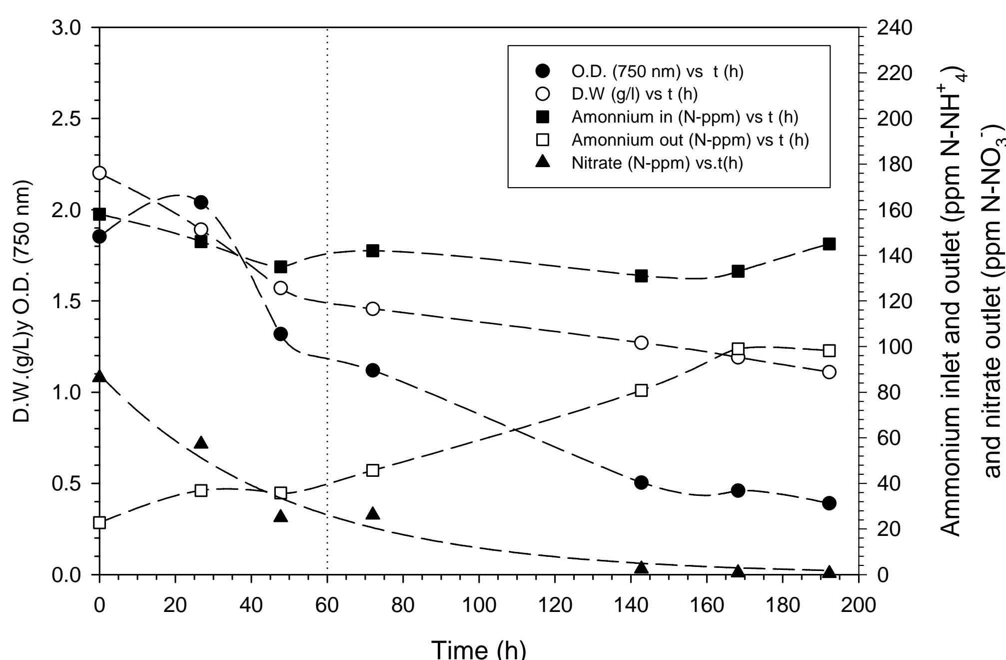

Figure 5.- Evolution of compartment IVa using ammonium as nitrogen source at 125 W/m^2. Phase I: During the initial 60 h the input flow rate was gradually increased. Phase II: After 60 hours the desired dilution rate was set at 1.55 L/d.

The nitrate is present in the outlet during phase I. This is due to the fact that batch culture is done with nitrate instead of ammonium, as explained in culture media section (see 2.12). However, it can be seen that the evolution of nitrate concentration in compartment IVa is

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usually in agreement with the nitrate wash-out curve confirming that ammonium cells have a higher affinity for ammonium and it is consumed first in front of other nitrogen sources (Guerrero and Lara, 1987, Creus, 2003).

It is known that an increase in dilution rate will result in a lower biomass concentration at steady state in comparison with the previous experiment. As ammonium concentration in the input flow is the same the result will be a higher ammonium concentration at the steady state.

Once the desired dilution rate is reached biomass levels tend to decrease and ammonium values to increase and seem to stabilize after 5 residence times. However cells started to aggregate and turn into a yellowish colour. Consequently, as the pH and the temperature values had any significant deviation from the set point ones, it was considered that an inhibition was taking place and the culture was discontinued. Taking into account the average values of main parameters at the two last sampling points (Table 10), it can be calculated that the productivity obtained in this culture (40.8 mg L^{-1} h^{-1}) is almost the same as the one obtained in the first one (40.4 mg L^{-1} h^{-1}), both at a light intensity of 125 W/m^2.

Table 10.- Main parameters values of test II shown in figure 5.

Parameter	Phase II
Biomass concentration (g/L)	1.25±0.15
Productivity (mg/L h)	40.8±4.8
NH_4^+ input (ppm N- NH_4^+)	138±7
NH_4^+ output (ppm N- NH_4^+)	81±25
$Y_{X,N}$ (mg N/g *S. platensis*)	44±15

The presence of aggregates surely implies that the dry weight measured in the output flow no longer reflects the situation inside the bioreactor. This would explain why the dry weight value does not decrease as the optical density.

Cultures with higher dilution rates, such as 0.042 h^{-1} (Creus *et al*. 2002) have already been reached in previous experiments with nitrate. On the other hand the biomass aggregation and the change in pigment content effects observed in the culture can be the result of the action of an inhibiting factor such as for example a high light intensity or an excess of ammonium. The

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low biomass level reached produces an increase in the ammonium concentration, leading to have a concentration peak of 100 ppm N-NH_4^+ inside the bioreactor. Such a high ammonium concentration can produce a inhibitory effect to the *S. platensis* growth and can have an influence on protein synthesis and the capacity of the biomass to recover.

Previous experiments under conditions of nitrate limitation result in similar modifications of biomass aggregation and colour (Vernerey 2000). It is possible that the metabolic alterations resulting from deficient nitrogen incorporation, either due to a limitation or due to an inhibition, have an effect on the photosynthetic system due to the limited nitrogen availability for synthesis of either the proteins or the pigments, like the phycocyanins, involved in the process. As a result a different sensitivity to light intensity might result. In this case light intensity could be an inhibiting factor at different nitrogen source levels for different nitrogen sources.

Therefore, it seems that when ammonium is the nitrogen source used, the bioreactor cannot reach a steady state under these conditions. The low biomass composition present in the bioreactor didn't allow to obtain enough freeze-dried biomass to carry out the elemental composition analysis.

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3.3. Test III: D=0.024 h^{-1}

Another example of the tests performed to delimit the operating conditions driving the *S. platensis* cultures to inhibiting conditions, can be seen in the following experiment of compartment IVa.

Table 11.- Operational conditions of test III.

Parameter	Phase I	Phase II	Phase III
Light (W/m^2)	125	125	90
Q_L (L/day)	0.43-0.92	1.14	1.14
ι (h)	112-52	42	42
D (h^{-1})	0.009-0.019	0.024	0.024

Since in the previous culture (test II) a stable *S. platensis* culture could not be obtained with a high dilution rate (0.032 h^{-1}), it was decided to perform a new test with a dilution rate slightly lower than in test II, but higher than in test I (0.021 h^{-1}). Table 11 shows the culture conditions used for each of the different phases. As it was done in test II, phase I corresponds to a transitory phase with an increasing input flow rate between batch and continuous phases. Starting in phase I from the biomass levels obtained after a batch culture, the continuous culture was started in phase II at slightly lower dilution rate (0.024 h^{-1}) than in the previous experiment The results of the evolution of this culture are depicted in Figure 6 and 7.

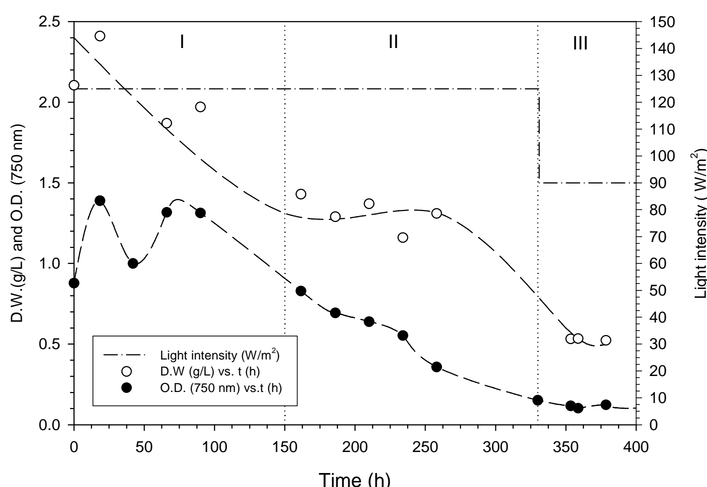

Figure 6.- Evolution of cell concentration in compartment IVa under test conditions summarized in Table 11. Phase I: Intermediary phase between batch-continuous phases with increasing input flow rate at 125 W/m^2. Phase II: Dilution rate set at 0.024 h^{-1} and light intensity at 125 W/m^2. Phase III: A light step down (Fr=90 W/m^2) is performed.

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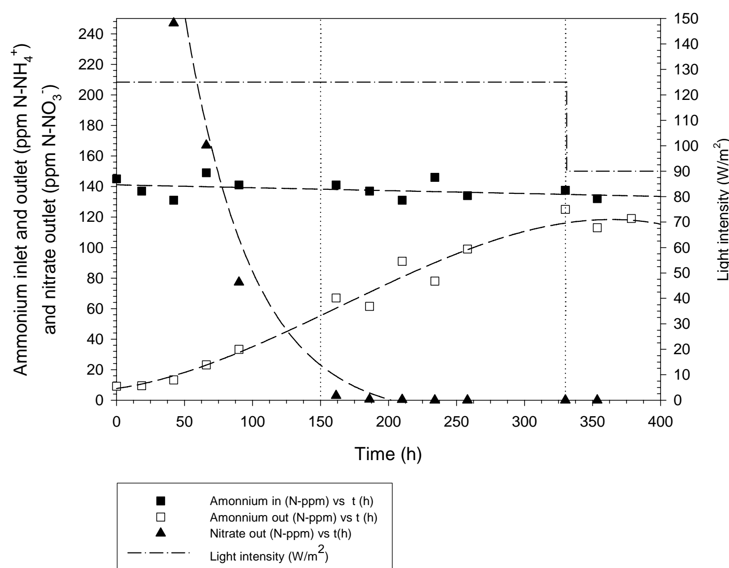

Figure 7.- Evolution of ammonium concentration in the inlet and the outlet flow and outlet nitrate concentration in IVa under test conditions summarized in Table 11. Phase I: Intermediary phase between batch-continuous phases with increasing input flow rate at 125 W/m^2. Phase II: Dilution rate set at 0.024 h^{-1} and light intensity at 125 W/m^2. Phase III: A light step down (Fr=90 W/m^2) is performed.

As mentioned in test II nitrate present in the outlet is because of the batch culture media. Again the evolution of nitrate concentration in compartment IVa is in agreement with the nitrate wash-out curve confirming that cells have a higher affinity for ammonium and it is consumed first in front of other nitrogen sources. When phase II begins, there is a negligible nitrate concentration inside the bioreactor.

The biomass levels in phase II were expected to be between the ones reached in the previous two experiments described (1.84 and 1.25 g/L), as light intensity is the same (125 W/m^2) and dilution rate is between the two previously tested values (0.021 and 0.032 h^{-1}).

Consequently the ammonium levels have to be lower than in the previous test, but probably higher than in the initial one. Both parameters (biomass and ammonium concentration) had values between the range fixed by the two previous tests, as it can be seen in table 12.

Table 12.- Main parameters values of test III shown in figure 6 and 7.

Parameter	Phase II	Phase III
Biomass concentration (g/L)	1.31±0.10	0.53
Productivity (mg/L h)	31.1±2.4	12.6
NH_4^+ input (ppm N- NH_4^+)	137±6	134±4
NH_4^+ output (ppm N- NH_4^+)	79±15	119±8
$Y_{X,N}$ (mg N/g *S. platensis*)	44.9±15.7	35.7

Although the dry weight initially appeared to have a tendency to level around a concentration of 1.3 g/L (see Table 12), the optical density showed a continuous decrease in its value reflecting a reduction in cell concentration. This lower biomass concentration, caused by some inhibiting factor, leads to a gradual increase of the outlet NH_4^+ concentration (79 ppm N-NH_4^+). Once again it could be argued that, since the dry weight is lower than test I, local light intensity reaches higher values that could be inhibitory.

To verify that light intensity is not the inhibiting factor light was decreased to 90 W/m^2. However the biomass washout did not decrease. It was therefore accepted, as the most probable hypothesis, that the incapacity to recover was due to the ammonium accumulation, which reached a concentration of 119 ppm N-NH_4^+ in these conditions. The elementary composition of the biomass obtained in phase II is shown in Table 13.

Table 13.- Results of the elemental composition analysis described as the average value of 2 measurements ± standard deviation. Percentages do not add to 100% due to the lack of determination of oxygen, phosphorous and ashes. Last column shown the corresponding elemental formula.

Phase	C (%)	H (%)	N (%)	S (%)	Elemental formula
II	40.05±0.10	6.41±0.10	9.05±0.01	1.37±0.14	$CH_{1.9204}N_{0.1937}S_{0.0128}$

The nitrogen percentage in test III is lower than in the first one, where the ammonium concentration has not reached inhibitory levels.

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3.4. Test IV: D=0.026 h^{-1}

As in the previous tests a high local light intensity is one of the possible factors for the *S. platensis* growth inhibition, a test with the same conditions as test III is carried out, but starting the culture at a lower light intensity. This culture operates under conditions described in Table 14.

Table 14.- Operational conditions of test IV shown in Figures 8 and 9.

Parameter	Phase I	Phase II	Phase III
Light (W/m^2)	33	33	125
Q_L (L/day)	0.5-1.16	1.23	1.23
ι (h)	96-41	39	39
D (h^{-1})	0.01-0.024	0.026	0.026

The culture evolution is depicted in Figures 8 and 9.

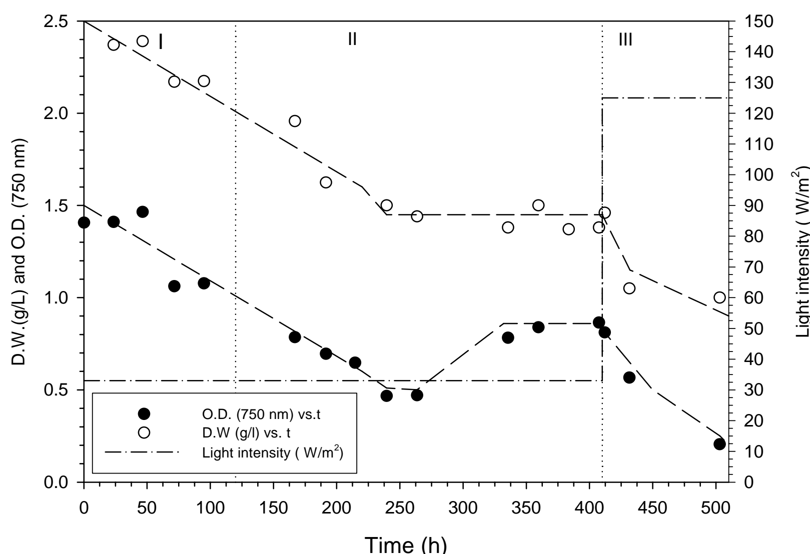

Figure 8.- Evolution of dry weight and O.D in compartment IVa in test conditions summarized in Table 14. Phase I: Intermediary phase batch-continuous with increasing input flow rate at 33 W/m^2. Phase II: Dilution rate set at 0.026 h^{-1} and light intensity at 33 W/m^2. Phase III: A light step up (Fr=125 W/m^2) is performed.

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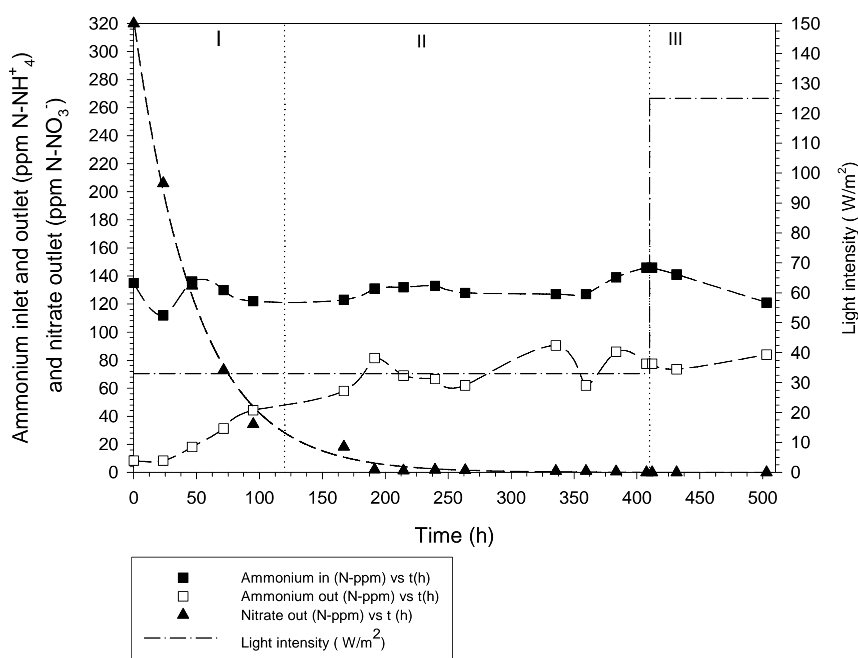

Figure 9.- Evolution of ammonium in the input and in the output and nitrate in the output in compartment IVa in test conditions summarized in Table 14. Phase I: Intermediary phase batch-continuous with increasing input flow rate at 33 W/m^2. Phase II: Dilution rate set at 0.026 h^{-1} and light intensity at 33 W/m^2. Phase III: A light step up (Fr=125 W/m^2) is performed.

After nearly 3 residence times of continuous operation with an increasing flowrate at 33 W/m^2 (Phase I), dilution rate was set at 0.026 h^{-1} (Phase II). The nitrate initially present in the outlet is the result of the fact that the batch culture was done with nitrate instead of ammonium as nitrogen source as already mentioned.

After about 5 residence times (Phase II) the entire nitrate remaining from the batch culture has been washed out and the ammonium concentration reached levels over 76 ppm $N\text{-}NH_4^+$. Previous results indicate that these ammonium concentrations are around the inhibitory levels. However, biomass concentration reaches a stable level around a biomass concentration of 1.4 g/L (see table 15) during around 5 residence times (from 200 to 400 hours).

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Table 15.- Main parameters values of test IV shown in figure 8 and 9.

Parameter	Phase II	Phase III
Biomass concentration (g/L)	1.41±0.05	1.20±0.22
Productivity (mg/L h)	36.2±1.4	30.8±5.7
NH_4^+ input (ppm N- NH_4^+)	133±9	136±13
NH_4^+ output (ppm N- NH_4^+)	76±13	78±5
$Y_{X,N}$ (mg N/g *S. platensis*)	40.8±8.9	48.3±15.4

The biomass elemental composition from the first steady state (phase II) has a lower carbon and nitrogen content (see Table 16) than in the composition already presented. Microscopic observation of the cells (Figure 10) was performed in order to evaluate if the difference in biomass composition was also reflected as a difference in the biomass morphology.

Table 16.- Results of the elemental composition analysis described as the average value of 2 measurements ± standard deviation. Percentages do not add to 100% due to the lack of determination of oxygen, phosphorous and ashes. Last column shown the corresponding elemental formula.

Phase	C (%)	H (%)	N (%)	S (%)	Elemental formula
II	34.48±0.21	5.28±0.16	7.94±0.01	1.51±0.07	$CH_{1.8379}N_{0.1973}S_{0.0164}$

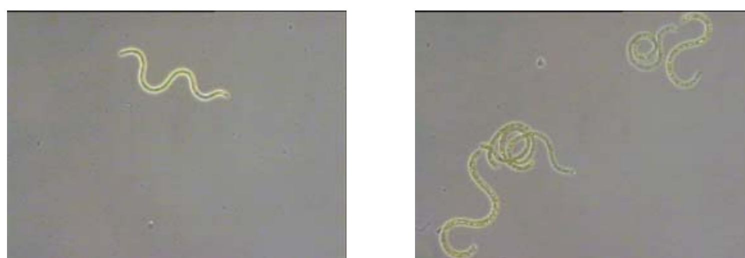

Figure 10.- Morphology of *S. platensis*

When the light intensity is relatively low (33 W/m^2), the culture reaches a steady state at this dilution rate. At this point starts phase III, where the increase light intensity would allow for an increase in biomass and consequently in ammonium consumption. However, it seems that which such a high light intensity value and with an ammonium concentration around the inhibitory levels (76 ppm N-NH_4^+), the culture becomes unstable.

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3.5. Test V: D=0.028 h^{-1}

In the previous culture one observes that since the dilution rate is quite high (0.026 h^{-1}), the biomass concentration and, consequently, its ammonium uptake are quite low. As a result, the outlet ammonium concentration is quite high and oscillates around the inhibitory concentrations which are located around 70 ppm $N\text{-}NH_4^+$. In this situation any potential deviation or change in the conditions, such as a light step, can lead to an unstable situation of the culture, which can be unable to be recovered. Increasing the dilution rate will result in a decreased level of biomass concentration and therefore an increased level of ammonium. It is desired to have an ammonium concentration inside the bioreactor low enough to avoid operating around the inhibitory concentrations, but without being a limiting factor for the biomass growth. For this reason it is decided to decrease the inlet ammonium concentration, which will allow to operate at an increased growth rate at similar ammonium levels to the previous ones and therefore to define with more accuracy the inhibitory range of ammonium in the *S. platensis*. During this culture, operational conditions were set as described in Table 17.

Table 17.- Operational conditions of compartment IVa for test V shown in Figures 11 and 12.

Parameter	Phase I	Phase II	Phase III	Phase IV	Phase V	Phase VI
Light (W/m^2)	33	63	125	15	33	52
Q_L (L/day)	1.37	1.37	1.37	-	1.37	1.37
ι (h)	35	35	35	-	35	35
D (h^{-1})	0.028	0.028	0.028	batch	0.028	0.028

Figures 11 and 12 show the evolution of the culture during these tests.

Light intensity at the surface of the bioreactor was initially 33 W/m^2. In these conditions the culture stabilizes around a biomass concentration of 1.16 g/L. This result confirms that at low light intensity a steady state can be obtained similarly to the previous ones. The other average values for some parameters such as productivity and nitrogen consumption are summarized in Table 18.

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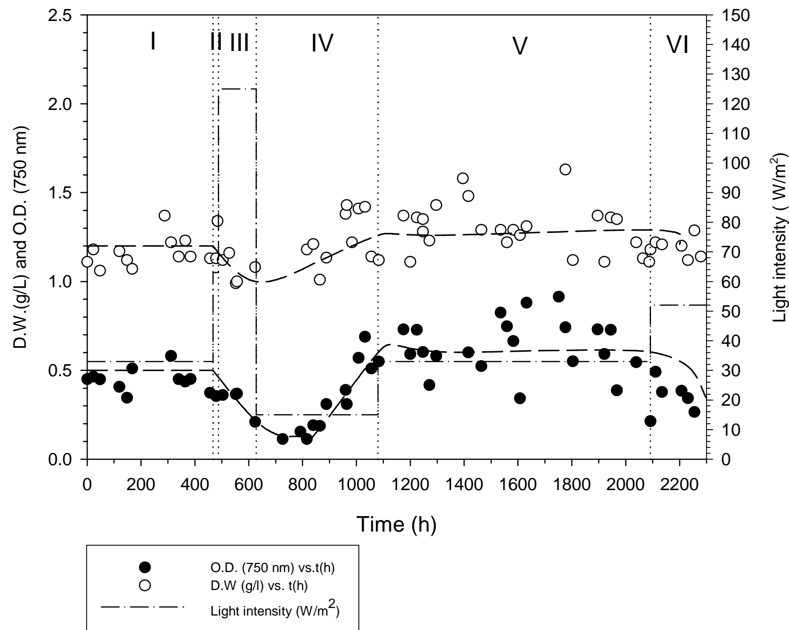

Figure 11.- Evolution of cell concentration in compartment IVa under test conditions summarized in Table 17. Phase I: Fr=33 W/m^2; Phase II: Fr=63 W/m^2; Phase III Fr=125 W/m^2; Phase IV Fr=15 W/m^2; Phase V Fr=33 W/m^2; Phase VI Fr=52 W/m^2

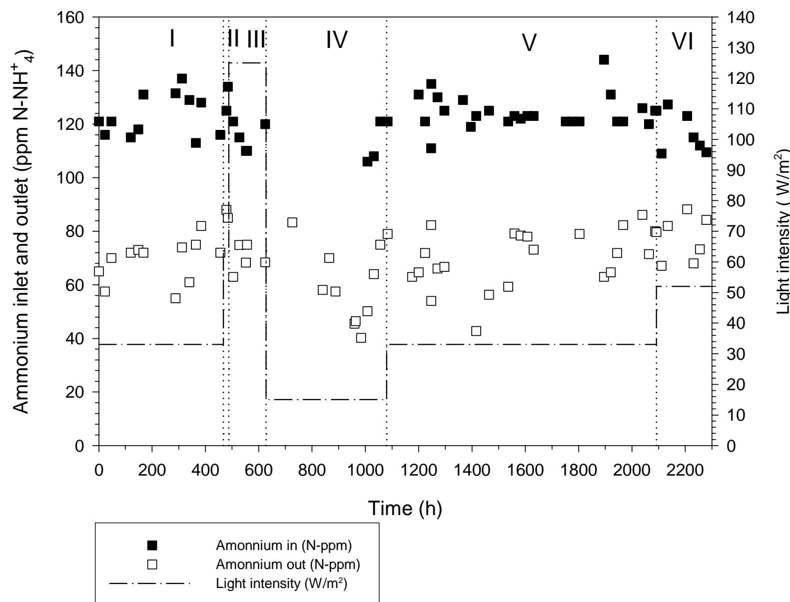

Figure 12.- Evolution of ammonium in the input and in the output and nitrate in the output in compartment IVa in test conditions summarized in Table 17. Phase I: Fr=33 W/m^2; Phase II: Fr=63 W/m^2; Phase III Fr=125 W/m^2; Phase IV Fr=15 W/m^2; Phase V Fr=33 W/m^2; Phase VI Fr=52 W/m^2.

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Table 18.- Main parameters values of test V shown in figure 11 and 12.

Parameter	Phase I	Phase V	Phase VI
Biomass concentration (g/L)	1.16±0.08	1.29±0.14	1.21±0.06
Productivity (mg/L h)	32.5±2.3	36.1±3.9	33.9±1.8
NH_4^+ input (ppm N- NH_4^+)	123±8	124±6	113±10
NH_4^+ output (ppm N- NH_4^+)	69±8	71±11	77±8
$Y_{X,N}$ (mg N/g *S. platensis*)	46.5±8.9	42.8±11.2	30.2±8.8

Once the first steady state was reached, a light intensity increase was gradually performed, from 33 W/m^2 to 63 W/m^2 and a few hours later to 125 W/m^2. In the same way as it was observed in test II, where the dilution rate (0.032 h^{-1}) and the light intensity (125 W/m^2) were also high, the biomass turns into a yellowish colour and forms thick clusters. A sample of this biomass was observed by microscope (Figure 13).

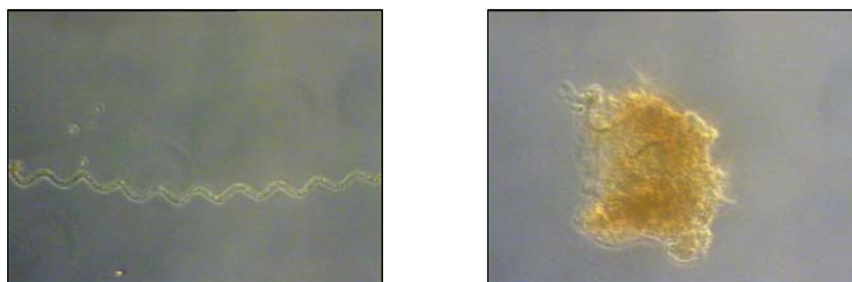

Figure 13.- Morphology S .platensis at the end of Phase III, before starting the batch phase (IV). The right photo shows a S .platensis cell trapped inside a thick cluster.

As it can be seen in the picture, there are some *S. platensis* cells alive, but trapped inside heterogeneous aggregates formed by cells and presumably exopolysaccharides. At this point it was decided to change the culture operation into a batch mode and to decrease the light intensity to 15 W/m^2. The aim of these changes was to evaluate the capacity of cells to recover. Phase IV correspond to this batch phase where the culture was able to recover and in phase V it returned successfully to a steady state similar to the initial one, confirming the complete recovery of the culture.

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However, the morphology of *S. platensis* cells from this culture is still different than the one of the cells from a culture using nitrate as nitrogen source in non limiting conditions but similar to the ones previously found in nitrate limiting conditions (Vernerey 2000).

Therefore it is possible that full recovery requires a longer period. For comparison, a sample of the pilot plant bioreactor, where the *S. platensis* grow with nitrate as the only N source, is taken and observed by microscope. Figure 14 evidences the *S. platensis* morphological changes depending the nitrogen source used (NH_4^+ or NO_3^-).

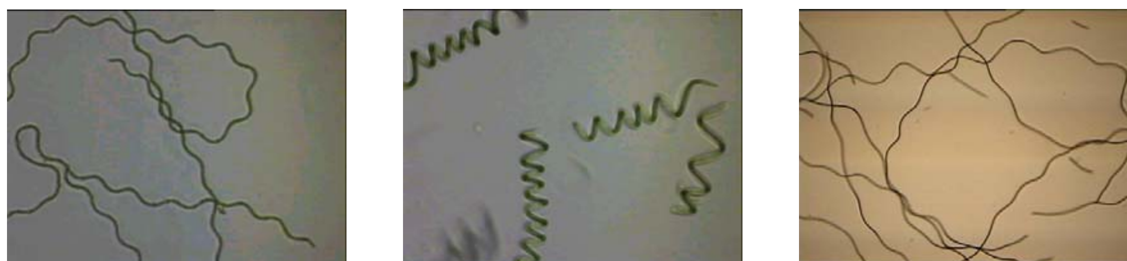

Figure 14.- Morphology *S. platensis* using different nitrogen source. Left photo: *S. platensis* culture in the laboratory bioreactor using ammonium as nitrogen source. It corresponds to the Phase IV shown in Figure 9 and 10 (magnification x10). Middle photo: *S. platensis* culture in a pilot plant bioreactor (V=77L) using nitrate as a nitrogen source (magnification x10). Right photo: Picture of biomass in nitrate limitation. (0.1 kg/m3 NO_3^-) at 0.05 h^{-1} dilution rate (Fr: 50 W/m^2, 7L bioreactor, magnification x5, from Vernerey et al. 1996).

Once the biomass had recovered during the batch phase, phase V starts setting the light at 33 W/m^2 and the dilution rate as in the other previous continuous phases. The culture is maintained in operation during 1000 hours with stable conditions evolution and with a biomass concentration level quite similar to the one reached in the first phase (see Table 18), confirming again the stability of the steady state.

After this second steady state, a step up of light to 52 W/m^2 is done. Although once again (as in the transition from phase I to III), when the increase of light intensity is performed, the biomass forms thick clusters (Figure 15). Contrary to phase III, it seems that the culture is able to reach a new steady state (Table 18).

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Table 19 shows the elementary biomass composition not only of the biomass of the clot from the different steady states, but also of the clusters formed in the phases where the light was set high (phases III and VI).

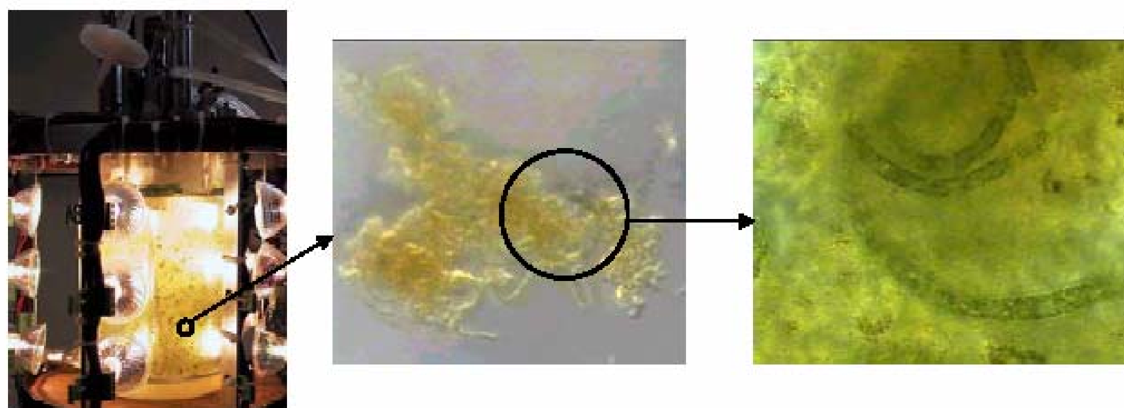

Figure 15.- Culture and morphology of *S. platensis* at Phase VI where Fr=52 W/m^2. The clots formed after the light increase are observed by microscope. Photo magnification: middle 20x, right 100x. In the right photo *S. platensis* is trapped in the clot.

Table 19.- Results of the elemental composition analysis described as the average value of 2 measurements ± standard deviation except for clots which is a determination of 3 different samples because its irregular composition. Percentages do not add to 100% due to the lack of determination of oxygen, phosphorous and ashes. Last column shown the corresponding elemental formula.

Phase	C (%)	H (%)	N (%)	S (%)	Elemental formula
I	41.79±0.18	6.28±0.06	9.46±0.05	0.76±0.23	$CH_{1.8019}N_{0.1939}S_{0.0068}$
I	45.21±0.11	6.64±0.15	11.10±0.13	0.39±0.08	$CH_{1.7611}N_{0.2104}S_{0.0032}$
III (Clots)	18.36±0.30	3.55±0.08	2.00±0.04	0.70±0.08	$CH_{2.3214}N_{0.0934}S_{0.0142}$
V	37.47±0.04	5.46±0.08	8.64±0.03	0.54±0.02	$CH_{1.7488}N_{0.1977}S_{0.0053}$
V	35.14±0.69	5.30±0.15	8.02±0.08	0.94±0.06	$CH_{1.8065}N_{0.1975}S_{0.0100}$
VI	37.10±0.21	5.58±0.04	9.01±0.01	0.45	$CH_{1.8035}N_{0.2082}S_{0.0045}$
VI (Clots)	18.69	3.25	4.03	1.21	$CH_{2.0867}N_{01848}S_{0.0042}$

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3.6. Test VI: D=0.012 h^{-1}

To end with these series of experiments, which have lead to a better understanding of the *S. platensis* culture using ammonium as nitrogen source, a last test with a low dilution rate (0.012 h^{-1}) was carried out.

The other operational conditions of *S. platensis* compartment during test VI are shown in table 20.

Table 20.- Operational conditions of test VI.

Parameter	Phase I	Phase II
Light (W/m^2)	33	76
Q_L (L/day)	0.60	0.60
ι (h)	80	80
D (h^{-1})	0.012	0.012

The evolution of this test at a low dilution rate (0.012 h^{-1}) is depicted in figure 16.

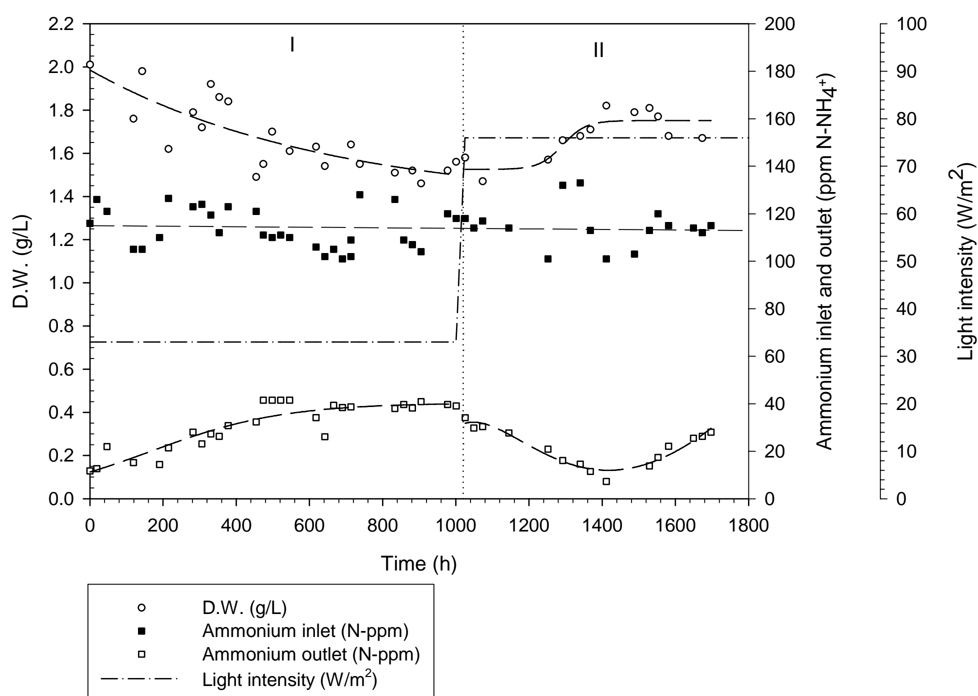

Figure 16.- Evolution of compartment IVa using ammonium as nitrogen source with a dilution rate of 0.001 h-[1]. Phase I: Light intensity set at 33 W/m^2. Phase II: After 1000 hours a step up of light to 76 W/m^2 is performed.

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The culture started with a high biomass value (2 g/L) formed in the previous batch phase. As the dilution rate desired has a low value (0.012 h^{-1}), it was not necessary to have an intermediary phase between batch and phase I with an increasing input flow rate. As a consequence, when the continuous operation starts, biomass begins to decrease until reaches a stable value around 1.56 g/L.

This value is in agreement with other biomass levels obtained also at a light intensity of 33 W/m^2, but at different dilution rates. The biomass density is higher when the dilution rate is lower at a fixed light intensity, since the biomass is 1.56 g/l when D=0.012 h^{-1}; 1.36 when D=0.021 h^{-1} and 1.16 g/L when D =0.028 h^{-1}.

The other main parameters values hardly oscillate, which mean that a first steady state is reached with success. In table 21 are summarized average main parameters values obtained in test VI.

Table 21.- Main parameters values of test VI shown in figure 16.

Parameter	Phase I	Phase II
Biomass concentration (g/L)	1.56±0.07	1.74±0.06
Productivity (mg/L h)	18.7±0.8	20.8±0.7
NH_4^+ input (ppm N- NH_4^+)	111±8	113±9
NH_4^+ output (ppm N- NH_4^+)	38±4	18±7
$Y_{X,N}$ (mg N/g *S. platensis*)	48.2±6.4	57.2±6.6

After the first steady state at 33 W/m^2, a light step up is performed. In the same way as it was observed in test I, the biomass level is higher when more light is supplied to the culture. Furthermore although the ammonium concentration in the inlet remains constant, the ammonium concentration in the outlet is lower.

Table 22 show the elemental composition of the biomass obtained in each steady state of this test.

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Table 22.- Results of the elemental composition analysis described as the average value of 2 measurements ± standard deviation . Percentages do not add to 100% due to the lack of determination of oxygen, phosphorous and ashes. Last column shown the corresponding elemental formula.

Phase	C (%)	H (%)	N (%)	S (%)	Elemental formula
I	39.49±4.22	5.85±0.99	9.31±1.12	0.82±0.14	$CH_{1.7779}N_{0.2020}S_{0.0078}$
II	25.68±0.56	3.83±0.08	5.49±0.06	0.81±0.17	$CH_{1.7901}N_{0.1831}S_{0.0118}$

4. CONCLUSIONS

In this technical note several *S. platensis* continuous cultures at a different dilution rates have been performed using ammonium as a nitrogen source. In order to evaluate the influence of the light intensity in these tests, at least one step of light is performed in each culture.

As it can be seen from the results, it can be confirmed the capability of *S. platensis* to grow using ammonium as a nitrogen source. Moreover, when nitrate is present in the outlet due to that batch culture is done with nitrate instead of ammonium, the evolution of nitrate concentration in compartment IVa is usually in agreement with the nitrate wash-out curve. Therefore it can also be confirmed that the biomass shows a higher affinity for ammonium in front of other nitrogen sources, as already mentioned in previous studies Guerrero and Lara (1987) and Creus (2003).

More specifically performed experiments allow to reach the following conclusions:

- At the culture conditions tested the continuous cultures reach a stable steady state at N-NH_4^+ values in the bioreactor below 65 ppm at low light intensities. In those conditions increasing light intensity allows to reach a new stable steady state.
- At N- NH_4^+ values in the bioreactor higher than 65 ppm, light intensity appears to have deleterious effects at lower light intensities that with nitrate cultures. In those conditions, light intensity Fr values higher than 33 W/m^2 resulted in cell aggregation, culture decoloration, increased synthesis polymeric material and cell elongation. Those conditions force to stop the continuous cultures.

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- The effects produced by the increase in light intensity described in the previous paragraph can be reversed maintaining the culture in batch conditions at low light intensities (15 W/m^2).

The effects on the biomass observed at high ammonium concentrations and increased light intensities are identical to those observed in cultures of nitrate limitation (Vernerey et al. 1996). This fact may indicate that the effect of ammonium inhibition acts limiting the rate of ammonium incorporation as a nitrogen source. As a result, when light intensity is increased carbon fixation is increased but all processes depending on the nitrogen source incorporation, like protein synthesis, can not increase. In those conditions cells would cease division an produce exopolymers. The process might affect protein incorporation in the photosynthesis system and impair its synthesis and repair resulting in the yellowish colour. Those possibilities recommend to further study the effects of nitrogen source supply in this compartment.

The obtained results provide an increased knowledge of *Arthrospira platensis* behaviour in front of increased ammonium concentrations which can result as an unstable operation of compartment III. As a result it can be proposed to design a control strategy maintaining ammonium levels below 65 ppm N- NH_4^+ in the bioreactor. In case the combination of ammonium levels reached and light intensity result in the modification of the cells behaviour as described, the effect is reversible and can be easily reversed by introducing a recovery period at low light intensity and batch culture.

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6. APPENDIX

6.1. Light Calibration

The light availability determination is of key importance for the operation of photobioreactors. Once light intensity at the bioreactor's surface is known, the light availability at any point of the bioreactor can be determined.

The light intensity at the bioreactor's surface is obtained by measuring the light intensity at the axis of the bioreactor, using a spherical light sensor (Li-Cor, LI-193SA, Lincoln, USA) that integrates the light reaching its radial illuminated surface. Conversion of the light intensity measured by the spherical sensor to the light intensity at the surface of the bioreactor is done using the following equation:

$$Fr = \frac{Eb \cdot rb}{\pi \cdot Rb}$$

Fr: Light flux at the bioreactor's surface (W/m^2)

Eb: Light intensity measured by the sensor ($\mu mol/m^2s$)

rb: Sensor's radius (30 mm)

Rb: Bioreactor's radius (65 mm)

Fc: Conversion coefficient (0.291 W s/µmol)

Photosynthetically active radiation (PAR), expressed in W/m^2, describes the radiation in wavelengths useful for photosynthesis of plants (400-700 nm) and is the term used in the light transfer mathematical models developed. Generally radiation is measured and reported in terms of photons and when a spherical sensor is used, the radiation is reported specifically as the photosynthetic photon flux rate (PPFR) for the 400-700 nm waveband expressed in $\mu mols/m^2s^2$.

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For the application of the previously developed light transfer mathematical models, it is necessary to convert the units of the spherical sensor ($\mu mols/m^2s^2$) to W/m^2. The conversion coefficient used is 0.291, which has been previously calculated by J.F.Cornet by integration of the used lamps spectra in the range 350–750 nm used by *Spirulina platensis* cells.

The Eb values are measured in the empty bioreactor with water circulating through the external jacket. Light intensity measurements are done at different vertical positions (assuming 0 at the bioreactor's base) and at different voltages supplied to the lamps. The results of these measurements are plotted in Figure 17.

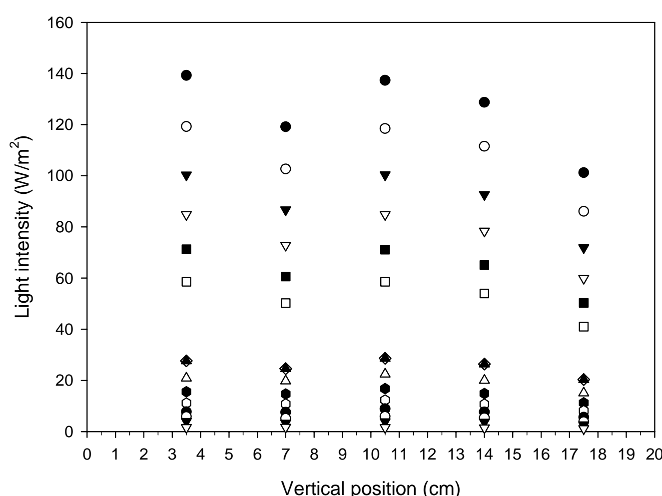

Figure 17.- Light intensity measured by the sensor, which is located in bioreactor's centre, at different vertical positions supplying different voltages (assuming 0 cm the bottom part of the reactor).

Averaging the measurements obtained at different vertical positions, the average light intensity value for each voltage supplied to the lamps is obtained. The light intensity values measured by the sensor in $\mu mols/m^2s^2$, are converted to Fr values using the above mentioned formula and conversion factor. Figure 18 shows the relationship between the voltage supplied to the lamps and the Fr of the bioreactor.

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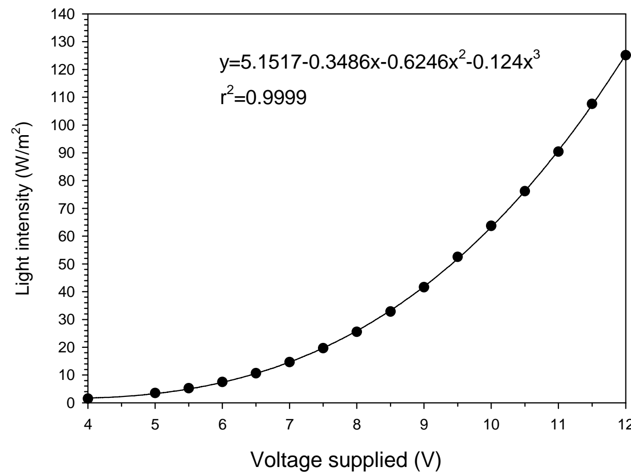

Figure 18 .- Relation between average light intensity at bioreactor's surface and voltage supplied to lamps.

6.2. Equipment Sterilization

Elimination of all transmissible agents, such as bacteria and viruses, from a surface, a piece of equipment or microorganism culture media is performed by heat sterilization.

Culture equipment is distributed in 3 groups.

Input bottle, including input liquid filter, gas filter, and pump tubes.

Output bottle, gas filter and pump tubes.

Bioreactor including, precalibrated pH and oxygen probes, ph bottles and connecting tubes and culture medium for the batch period.

Each group of equipment is sterilized in an autoclave (Varioklav 400) at 121°C (250°F), at 103 kPa (15 psi) above atmospheric pressure, for 30 minutes. The steam and pressure transfer sufficient heat to the equipment to assure reaching a safe condition.

Once autoclaved input and output bottles are connected to the bioreactor by means of the corresponding quick connectors in the neighbourhood of a Bunsen burner flame and following the corresponding sterility safety procedures.

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6.3. Culture Axenicity Monitoring

The media and culture broth are checked weekly for bacterial contamination by microscopic examination.

The samples are obtained from the input and output sampling points, following the same sterility safety procedures as the ones used for the input and output daily sampling.

One drop of each sample is deposited in a microscopic counting slide and covered with a proper cover slide attempting to break as few *Spirulina* as possible.

Magnification of the microscope is progressively increased from 100x, 200x, 400x, 1000x. focusing the surroundings of the cyanobacteria.

Finally observation is repeated using phase contrast.

For each magnification at 400x and 1000x, 10 different counting fields are observed and searched for any signs of microscopic life such as clear bacteria, yeast or fungi forms with or without movement. Taking into account that the observation is not performed under sterile conditions, a culture is considered axenic if not more than 10 suspected contaminants are found in total in all the fields.

If the culture shows clear signs of contamination (*v.g.* a significant amount of microorganisms is found of the order of the cultured microorganism) the culture is terminated.

Should the observation result in observation of microorganisms but in a low number, the sampling is repeated daily and microscopic observation monitored for an increase in the microscopic counts until a definitive diagnostic of contamination/not contamination is issued. In such event the culture is terminated.

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