Universitat Autònoma de Barcelona

# MELISSA collaboration agreement ECT/FG/CB/95.205

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### - TECHNICAL NOTE 25.120-

# Nitrate limitation tests

**Version :** 1

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# INTRODUCTION

During the previous research done for compartment IV of the MELISSA loop, a mathematical model describing the effect of light intensity on biomass growth and composition was developed. This model was validated for low and high dilution rate conditions of the airlift bioreactor of the MELISSA pilot plant. In the next step the effect of nitrogen limitation has to be incorporated. In a first approximation the model takes into account such effects on the growth rate via a Monod law. The parameters of the model were identified from preliminary batch roux flasks data (TN 19.1,TN 19.2) and they will have to be validated for the bioreactor continuous culture. In this technical note, the first continuous culture experiments done under nitrate limitation are reported. According to TN 24.2 the nitrate concentration values in the bioreactor must be lower than 4 or 5 times the Monod constant (Ks: $5.3x10^{-3}$ kg $NO^-_3/m^3$). To reach such conditions simulations were done using $100x10^{-3}$ kg $NO^-_3/m^3$ at the input flow and using dilution rates between 0.007-0.016 (Average light intensity at the bioreactor surface (Fr) between 50-200 $W/m^2$). In this conditions the simulated nitrate concentration in the bioreactor oscillates around $5x10^{-3}$ to $25x10^{-3}$ kg $NO_3^-/m^3$. According with such results it was decided with the MELISSA technical officer to test the behaviour of the *Spirulina platensis* culture around such conditions.

## Material and Methods

### Culture media

To perform this tests the Zarrouk salt mixture, as used by Cornet (1992), was used with a modification on its main nitrogen source content so as to obtain a nitrogen source limitation. To this purpose the concentration of Na $NO_3$ was decreased so as to obtain 0.1 $Kg/m^3$ of $NO_3^-$.

### Protein measurement.

Total protein content was determined using the modified Lowry method as described in appendix 2.

### Carbohydrate measurement.

Total carbohydrate content was determined using Herbert's phenol method as described in appendix 1.The EPS can be calculated from the total sugars present in the sample assuming that the saccharides contained in the active biomass are the 15% of the

total biomass free of the glycogen amount (according to TN 19.2). This approximation could not be applied because EPS could not be properly separated of the biomass in order to determine the glycogen present using the Palmsternia method. Therefore comparison with simulated values can be done by adding the simulated glycogen and exopolysaccharide content to the 15% of the simulated value of total biomass to obtain an approximation of the total carbohydrates.

**Chlorophyll and Phycocyanin measurement.**

Chlorophyll and phycocyanin content were determined using spectrophotometric determinations on extracts from freeze dried biomass as described in appendix 3.

**Nitrate concentration measurement.**

Nitrate concentration in the output culture media was determined in filtered samples using a Capillary Ion Analyser method, as described in appendix 4. It was also measured using UV measurement determinations either on-line (Dr. Lange Nitrax) or off line by means of the LCK 339 nitrate analysis kit. As it will be further discussed, due to the high affinity level for nitrate shown by the biomass in this culture conditions, the nitrate concentration in the bioreactor was always under the threshold level of the Nitrax on-line measurement and therefore those values are given as zero.

**Summary of the working conditions for each test.**

All the tests were performed in nitrate limitation conditions, corresponding to a nitrate concentration in the input medium of $100x10^{-3}$ kg $NO^{-}_{3}/m^{3}$. Dilution rate and light intensity were set as specified in table 1.

**Table 1** : Summary of operational conditions for the described tests.

| | Dil : 0.018 $H^{-1}$ | Dil : 0.012 $H^{-1}$ | Dil : 0.016 $H^{-1}$ | Dil : 0.05 $H^{-1}$ |
|---|---|---|---|---|
| Fr : 305 $W/m^2$ | A1 | B1 | C1 | - |
| Fr : 50.2 $W/m^2$ | A2 | B2 | C2 | D1 ;E1 |
| Fr : 305 $W/m^2$ | A3 | B3 | C3 | D2 |

## TEST A

The operational conditions, in the first nitrate limitation test, used a nitrate concentration in the feed medium of the bioreactor of $100x10^{-3}$ kg $NO^{-}_{3}/m^{3}$. Dilution rate was set to 0.018 $h^{-1}$ (residence time 55.55 h ; flow 0.126 l/h). Light intensity was set at 85 % of the controller action, which corresponds to a calculated Fr of 305 $W/m^{2}$. After setting up the conditions, dynamic evolution of the bioreactor culture performance to the steady-state was followed.

As it can be seen in figure 1, after changing the culture conditions biomass concentration was decreasing continuously, heading for the new steady state concentration. Biomass decreased until, presumably, the limitations have been released so as to allow biomass to grow again. After this point the biomass increased again towards a stabilisation point. Biomass seemed to stabilise around 0.49 $kg/m^{3}$ of total biomass. At this point culture variables were considered stable. This state will be referred as phase A1. Consequently three biomass samples were taken and freeze dried for the biomass analysis. Two samples were analysed at CNRS (France) for the determination of the main elements content, a third one was analysed at an analytical chemical service in UAB (Spain) for comparison purposes. The results of biomass elemental composition are given in table 2. A summary of the results obtained from the biomass macromolecular composition analysis is given in table 3.

Once the steady state was obtained, light intensity was changed in order to examine what was its effect on the biomass composition. The new level of light was 65.69 % of the light control action, which corresponds to a calculated Fr value of 50.2 $W/m^{2}$. Once the steady state was reached, culture samples were taken in order to measure the biomass macromolecular composition and the biomass elemental composition (tables 5 and 4). This steady state will be referred as A2.

At this point and in order to confirm the results obtained in the A1 steady state, the light intensity was set back to its previous value of the 85 % of the light intensity (Fr of 305 $W/m^{2}$ ). The results of this test, referred to as A3 can be seen in figure 4 and tables 6 and 7.

For each one of these cases the results calculated using the PHOTOSIM simulator for the steady states corresponding to the conditions of these tests can be found in tables 3,5 and 7.

## Phase A1 results

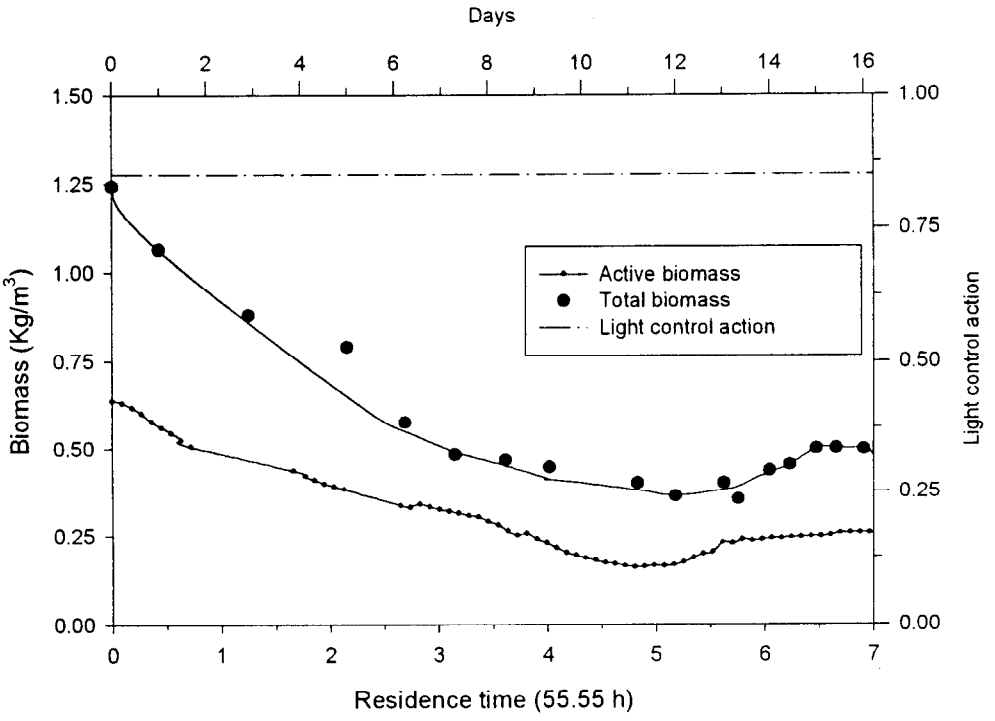



**Figure 1**: Evolution of biomass concentration at 0.018 $h^{-1}$ after setting up nitrate limitation (S0: 0.1 $kg/m^3$ $NO_3^-$). Fr: 305 $W/m^2$.

| A1 Element | Average (SdeM) +/-SD (%weight) | | Average (SAQ) SD (%weight) | Mol (SdeM) +/- SD | | Mol (SAQ) +/-SD | Simulation (Mol) | % Deviation from experimental |
|---|---|---|---|---|---|---|---|---|
| Sample | a | b | a | a | b | a | | |
| C | 41.4 +/- 0.05 | 35.9 +/- 0.1 | 42.05 +/-0.01 | 1 +/0.001 | 1 +/0.003 | 1 +/-3.4E-4 | 1 | |
| H | 6.1 +/- 0.05 | 4.91 +/- 0.1 | 6.23 +/-0.07 | 1.77 +/-0.01 | 1.66 +/-0.03 | 1.778 +/-2. E-2 | 1.619 | 6. |
| O | 39.9 +/-0.7 | 39.4 +/- 0.1 | - | 0.827 +/-0.01 | 0.821 +/-0.01 | - | 0.747 | 6.6 |
| N | 5.4 +/-0.1 | 4.84 +/- 0.03 | 5.59 +/-0.04 | 0.0976 +/- 0.002 | 0.115 +/-6.E-4 | 0.114 +/-7.5E-4 | 0.0703 (21%) | |
| P | 1.2 +/-0.2 | 1.1 +/- 0.5 | - | 0.0117 +/- 0.002 | 0.0119 +/- 0.005 | - | 0.0023 | 56 |
| S | 0.33 +/-7E-3 | 0.31 +/- 0.02 | 0.183 +/-0.006 | 0.003 +/-6.E-05 | 0.003 +/-2.E-4 | 0.0016 +/-5E-5 | 0.0112 | 242 |
| Ash % | 5.6 +/-0.8 | 13.5 +/- 0.3 | | | | | - | |

**Table 2** : Elemental composition obtained from samples of phase A1. Samples a and b. S. de M.: Service de Microanalyse C.N.R.S. (France). SAQ : Servei D'Analisi Química. U.A.B. (Spain).

| A1 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr $W/m^2$ | 305 | | 305 | |
| Light control action (%) | 85 | | 85 | |
| Total biomass (XT) $Kg/m^3$ | 0.49 | 0.06 | .482 | 1.1 |
| Active biomass (XA) $Kg/m^3$ | - | - | .163 | - |
| Chlorophyll (CH) $Kg/m^3$ | 0.0054 | 7E-04 | 0.00163 | 49 |
| Phycocyanins (PC) $Kg/m^3$ | 0.01 | 0.005 | 0.0175 | 53 |
| Proteins (P) $Kg/m^3$ | 0.16 | 0.01 | .111 | 21 |
| Vegetative biomass (XV) $Kg/m^3$ | - | - | .169 | - |
| Total carbohydrates $Kg/m^3$ | 0.20 | 0.03 | - | - |
| Exopolysaccharide (EPS) $Kg/m^3$ | - | - | .27 | - |
| Glycogen (G) $Kg/m^3$ | - | - | 0.006 | - |
| Spectrophotometer (XT) $Kg/m^3$ D.W. (Kontron) | 0.45 | | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Capill.Elec.) | 0.237 | - | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Dr. Lange) | 1.21 | 0.6 | 15.7 | 846 |

**Table 3:** Off-line results obtained at a dilution rate of 0.018 $h^{-1}$ and incident light flux of 305 $W/m^2$. Phase A1and Photosim simulation results for the same conditions.

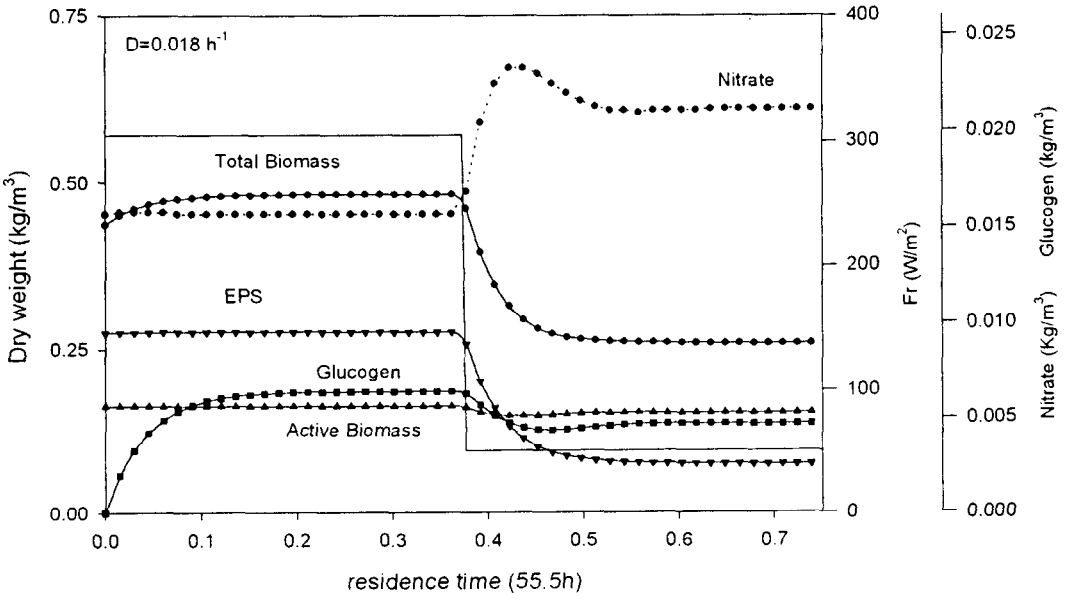


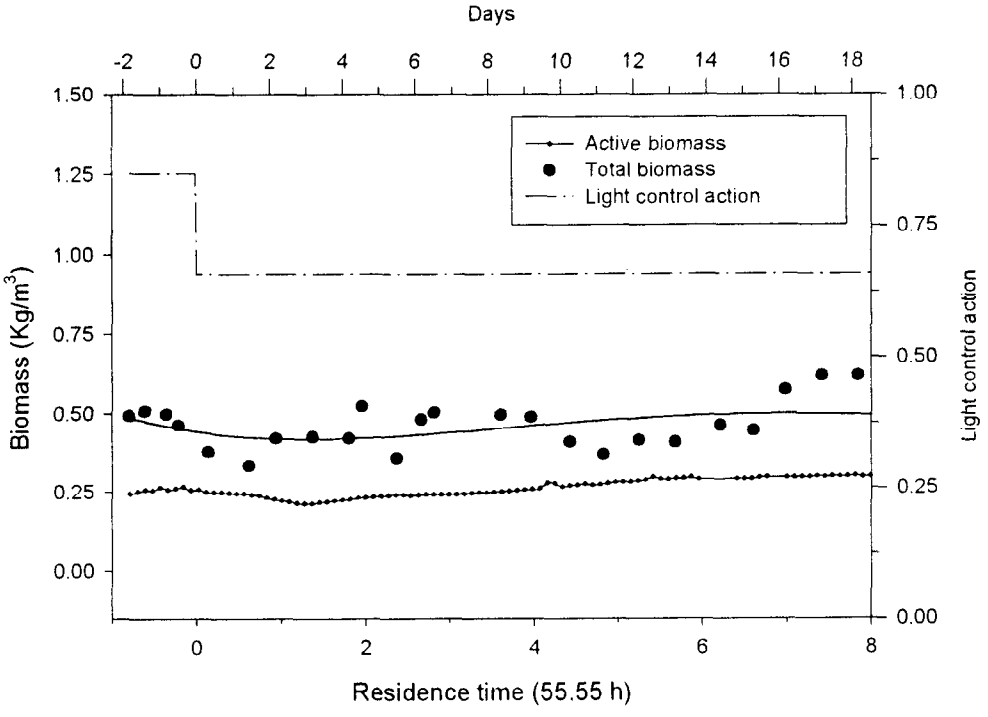

**Figure 2** Simulation of evolution of variables in nitrate limitation. Phase A1 to A2 (0.1 $kg/m^3$ $NO_3^-$). at 0.018 $h^{-1}$ dilution rate ( Fr: 305 →50.2 $W/m^2$).

## Phase A2 results

| A2 Element | Average (SdeM) +/-SD (%weight) | | Average (SAQ) +/-SD (%weight) | Mol (SdeM) +/-SD | | Mol (SAQ) +/-SD | Simulation (Mol) | % Deviation from experimental |
|---|---|---|---|---|---|---|---|---|
| sample | a | b | a | a | b | a | | |
| C | 40.3 +/-0.2 | 32.5 +/-0.1 | 40.9 +/-0.13 | 1 +/-0.004 | 1 +/-0.002 | 1 +/-0.003 | 1 | - |
| H | 5.9 +/-0.1 | 4.55 +/-0.1 | 6.1 +/-0.1 | 1.76 +/-0.02 | 1.68 +/-0.01 | 1.80 +/-0.03 | 1.594 | 6. |
| O | 38.5 +/-0.3 | 40.3 +/-0.12 | - | 0.82 +/-0.007 | 0.929 +/-0.003 | - | 0.585 | 24 |
| N | 5.3 +/-0.1 | 3.9 +/-0.03 | 5.6 +/-0.2 | 0.098 +/-0.002 | 0.103 +/-0006 | 0.118 +/-0.004 | 0.1068 | 0.5 |
| P | 1.14 +/-0.1 | 0.81 +/-0.2 | - | 0.011 +/-0.001 | 0.0097 +/-0.002 | - | 0.0043 | 41 |
| S | 0.32 - | 0.43 - | 0.18 +/-0.01 | 0.003 -. | 0.0049 - | 0.0016 +/-8.E-05 | 0.0082 | 76 |
| Ash % | 8.5 +/-0.5 | 17.4 +/-0.1 | | | | | - | - |

**Table 4 :** Elemental composition obtained from samples of phase A2. (0.1 kg/m$^3$ $NO_3^-$). at 0.018 h$^{-1}$ dilution rate and Fr: 50.2 W/m$^2$. Samples a and b. S. de M.: Service de Microanalyse C.N.R.S. (France). SAQ : Servei D'Analisi Quimica. U.A.B. (Spain).

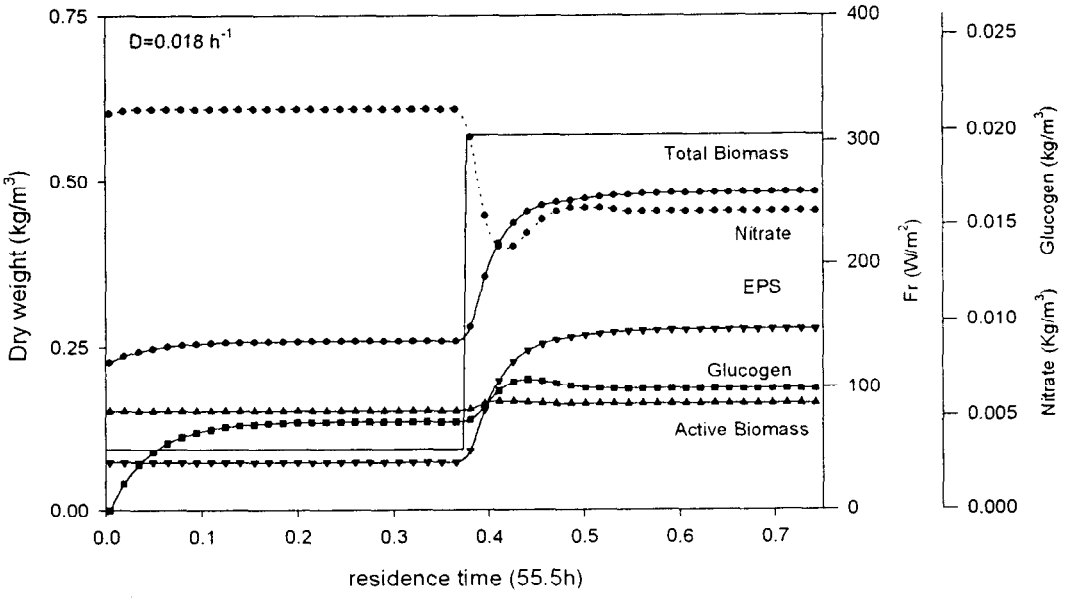
**Figure 3** Evolution of biomass concentration in nitrate limitation. Phase A1 to A2 (0.1 kg/m$^3$ $NO_3^-$). at 0.018 h$^{-1}$ dilution rate ( Fr: 305 →50.2 W/m$^2$).



| A2 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr $W/m^2$ | 50.2 | | 50. | - |
| Light control action (%) | 65.69 | | 65.69 | - |
| Total biomass (XT) $Kg/m^3$ | 0.49 | 0.06 | 0.258 | 33 |
| Active biomass (XA) $Kg/m^3$ | - | - | 0.153 | - |
| Chlorophyll (CH) $Kg/m^3$ | 0.006 | 7E-04 | 0.00153 | 62 |
| Phycocyanins (PC) $Kg/m^3$ | 0.018 | 0.005 | 0.0185 | 2 |
| Proteins (P) $Kg/m^3$ | 0.156 | 0.007 | 0.104 | 23 |
| Vegetative biomass (XV) $Kg/m^3$ | - | - | 0.157 | - |
| Total carbohydrates $Kg/m^3$ | 0.22 | 0.03 | - | - |
| Exopolysaccharide (EPS) $Kg/m^3$ | - | - | 0.074 | - |
| Glycogen (G) $Kg/m^3$ | - | - | 0.004 | - |
| Spectrophotometer (XT) Kg/m3 D.W (Kontron) | 0.45 | na | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Cap. Elec.) | 1.4 | 1 | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Dr. Lange) | 2.1 | 0.9 | 21 | 630 |

**Table 5 :** Off-line results obtained in nitrate limitation phase A2 (0.1 $kg/m^3$ $NO_3$) at a dilution rate of 0.018 $h^{-1}$ and incident light flux of 50.2 $W/m^2$. And PHOTOSIM simulated results for the same conditions.

**Figure 4** Simulation of evolution of variables during the return to the previous illumination conditions (Fr: 50.2 ->305 $W/m^2$).



## Phase A3 results

| A3 Element | Average (SdeM) SD (%weight) | | Average (SAQ) SD (%weight) | Mol (SdeM) SD | | Mol (SAQ) SD | Simulation | % Deviation from experimental |
|---|---|---|---|---|---|---|---|---|
| sample | a | b | a | a | b | a | | |
| C | 38.3 +/-0.1 | 38.9 +/-0.1 | 39.51 +/-0.01 | 1 +/-0.003 | 1 +/0.003 | 1 +/-0.0004 | 1 | - |
| H | 5.7 +/-0.03 | 5.6 +/-0.07 | 5.94 +/-0.08 | 1.771 +/-0.009 | 1.726 +/-0.021 | 1.80 +/-0.03 | 1.619 | 5.9 |
| O | 37.0 +/-0.2 | 36.4 +/-0.1 | - | 0.827 +/-0.005 | 0.701 +/-0.003 | - | 0.747 | 1.6 |
| N | 5.26 +/0.03 | 5.26 +/-0.04 | 5.59 +/-0.03 | 0.1029 +/-5.E-4 | 0.1158 +/-8.E-4 | 0.1214 +/-0.0006 | 0.0703 | 27 |
| P | 0.885 +/0.007 | 0.95 +/0.056 | - | 0.00893 +/-7.E-05 | 0.009 +/-6.E-4 | - | 0.0023 | 52 |
| S | 0.4 +/-0.03 | 0.39 +/-0.02 | 0.26 +/-0.03 | 0.0039 +/-3.E-4 | 0.0038 +/-2.E-4 | 0.0025 +/-0.003 | 0.0112 | 160 |
| Ash | 5.6 +/-0.8 | 12.5 +/-0.1 | | | | | | |

**Table 6 :** Elemental composition obtained from samples of test A3. (0.1 kg/m$^3$ $NO_3^-$). at 0.018 h$^{-1}$ dilution rate and Fr: 305 W/m$^2$. Samples a and b, S. de M.: Service de Microanalyse C.N.R.S. (France). SAQ : Servei D'Analisi Quimica. U.A.B. (Spain).

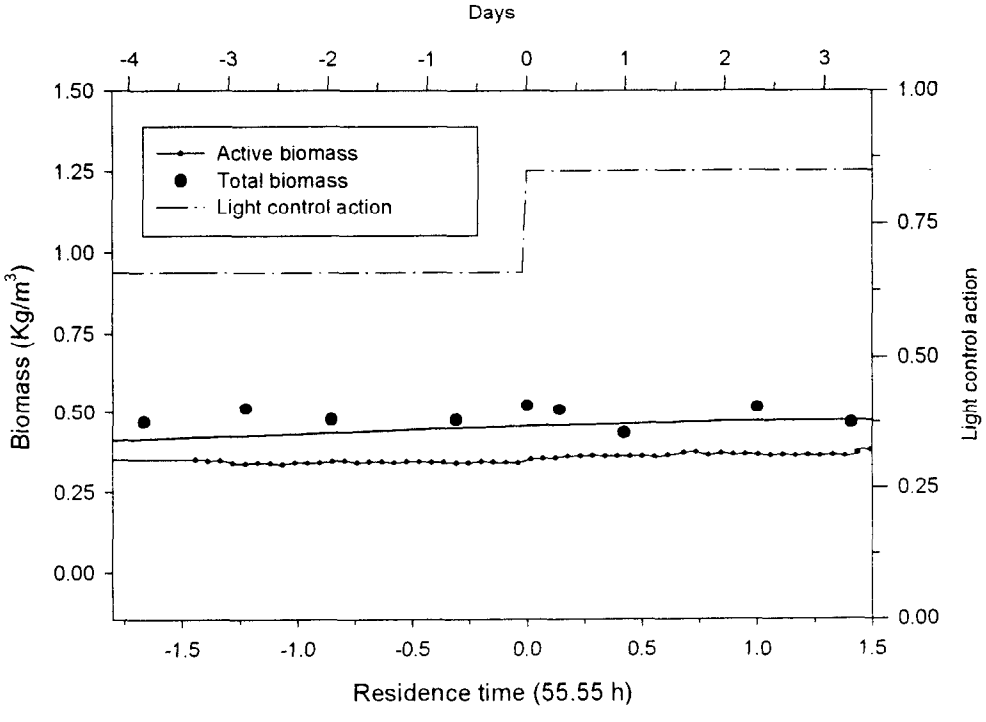



**Figure 5** Evolution of the biomass during the return to the previous illumination conditions (Fr: 50.2 ->305 W/m$^2$).

| A3 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr $W/m^2$ | 305 | | 305 | - |
| Light control action (%) | 85 | | 85 | - |
| Total biomass (XT) $Kg/m^3$ | 0.45 | 0.06 | .482 | 5 |
| Active biomass (XA) $Kg/m^3$ | - | - | .163 | - |
| Chlorophyll (CH) $Kg/m^3$ | 0.01 | 0.006 | 0.00163 | 28 |
| Phycocyanins (PC) $Kg/m^3$ | 0.018 | 0.007 | 0.0175 | 2 |
| Proteins (P) $Kg/m^3$ | 0.14 | 0.02 | .111 | 14 |
| Vegetative biomass (XV) $Kg/m^3$ | - | - | .169 | - |
| Total carbohydrates $Kg/m^3$ | 0.20 | 0.02 | - | - |
| Exopolysaccharide (EPS) $Kg/m^3$ | - | - | .27 | - |
| Glycogen (G) $Kg/m^3$ | - | - | 0.006 | - |
| Spectrophotometer (XT) $Kg/m^3$ D.W (Kontron) | 0.49 | na | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Cap. Elec.) | 0 | na | - | |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Dr. Lange) | 1.9 | na | 15.7 | 500 |

**Table 7** Off-line results obtained in nitrate limitation phase A3 (0.1 $kg/m^3$ $NO_3^-$) at a dilution rate of 0.018 $h^{-1}$ and incident light flux of 305 $W/m^2$. And PHOTOSIM simulated results for the same conditions.

## TEST B

For the second part of the test the dilution rate was set to $0.012h^{-1}$ (residence time 83.33 $h^{-1}$; flow 0.084 l/h). The nitrate concentration in the input of the bioreactor was maintained as in A test at $100x10^{-3}$ kg $NO^{-}_{3}/m^{3}$. At the beginning of the test the light intensity was set at 85 % of the controller action, which corresponds to a calculated Fr of 305 $W/m^2$. After setting up the conditions, dynamic evolution of the bioreactor culture performance to the steady-state was followed. The steady state reached is referred as B1. A summary of the results obtained of biomass macromolecular composition analysis are given in table 8. The biomass level reached can be seen figure 7. The results of biomass elemental composition are presented in table 9.

Once the steady state was obtained, light intensity was changed in order to observe what was its effect on the biomass composition. The new level of light was of 65.69 % of the light control action, which corresponds to a calculated Fr value of 50.2 $W/m^2$. Once the steady state was reached, culture samples were taken in order to measure the biomass macromolecular and elemental composition. This steady state will be referred as B2. Biomass levels and results of macromolecular analysis are given in table 10. Results of the elemental composition are shown in table 11.

At this point and in order to confirm the results obtained in the B1 steady state, the light intensity was set back to its previous value of the 85 % of the light intensity (Fr of 305 $W/m^2$ ). The results of this test, referred to as B3, can be seen in figure 8 and biomass levels and macromolecular analysis results in table 12. Elemental analysis results are given in table 13.

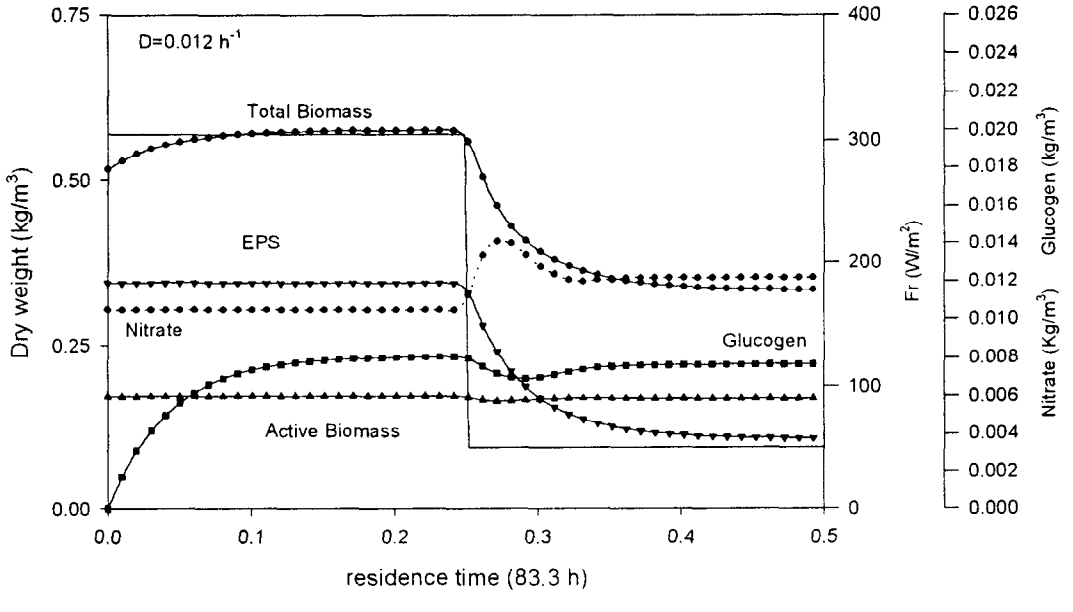
For each case the results offered by the PHOTOSIM simulator for the steady states corresponding to the conditions of this tests can be found in tables 8, 10 and 12 and figures 6 and 9.

## Phase B1 results

| B1 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr $W/m^2$ | 305 | | 305 | - |
| Light control action (%) | 85 | | 85 | - |
| Total biomass (XT) $Kg/m^3$ | 0.41 | 0.05 | 0.578 | 29 |
| Active biomass (XA) $Kg/m^3$ | - | - | 0.173 | - |
| Chlorophylls (CH) $Kg/m^3$ | 0.005 | 0.001 | 0.00173 | 46 |
| Phycocyanins (PC) $Kg/m^3$ | 0.018 | 0.002 | 0.0139 | 16 |
| Proteins (P) $Kg/m^3$ | 0.136 | na | 0.118 | 9 |
| Vegetative biomass (XV) $Kg/m^3$ | - | - | 0.1815 | - |
| Total carbohydrates $Kg/m^3$ | 0.236 | 0.04 | - | - |
| Exopolysaccharide (EPS) $Kg/m^3$ | - | - | 0.344 | - |
| Glycogen (G) $Kg/m^3$ | - | - | 0.0085 | - |
| Spectrophotometer (XT) $Kg/m^3$ D.W (Kontron) | 0.56 | 0.01 | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Cap. Elec.) | 1.8 | 1. | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Dr. Lange) | 1.7 | 0.6 | 10.5 | 360 |

**Table 8 :** Off-line results obtained in nitrate limitation phase B1 (0.1 $kg/m^3$ $NO_3^-$) at a dilution rate of 0.012 $h^{-1}$ and incident light flux of $305W/m^2$. And PHOTOSIM simulated results for the same conditions.

**Figure 6**: Simulation of evolution of variables in nitrate limitation. From phase B1 to B2 (0.1 $kg/m^3$ $NO_3^-$). at 0.012 $h^{-1}$ dilution rate ( Fr: 305→50.2 $W/m^2$).



| B1 Element | Average (SdeM) | SD | Mol (SdeM) | SD | Simulation | % Deviation from experimental |
|---|---|---|---|---|---|---|
| C | 41.82 | 0.05 | 1 | 0.001 | 1 | - |
| H | 6 | 0.06 | 1.721 | 0.016 | 1.623 | 4 |
| O | 39.17 | 0.05 | 0.70 | 0.001 | 0.766 | 7 |
| N | 5.6 | 0.001 | 0.115 | 0.0005 | 0.0634 | 31 |
| P | 0.885 | 0.007 | 0.007 | 7.E-04 | 0.0021 | 63 |
| S | 0.79 | 0.08 | 0.003 | 0.0002 | 0.0115 | 200 |
| Ash | 6.25 | 0.06 | | | | |

**Table 9 :** Elemental composition obtained from samples of test B1. (0.1 kg/m$^3$ $NO_3^-$). at 0.012 h$^{-1}$ dilution rate and Fr: 305 W/m$^2$. (S.deM.) : Service de Microanalyse C.N.R.S. (France).

## Phase B2 results

| B2 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr W/m$^2$ | 50.2 | | 50.2 | - |
| Light control action (%) | 65.69 | | 65.69 | - |
| Total biomass (XT) Kg/m$^3$ | 0.49 | 0.03 | 0.332 | 23 |
| Active biomass (XA) Kg/m$^3$ | - | - | 0.170 | - |
| Chlorophyll (CH) Kg/m$^3$ | 0.005 | na | 0.00170 | 46 |
| Phycocyanins (PC) Kg/m$^3$ | 0.029 | 0.01 | 0.0156 | 31.7 |
| Proteins (P) Kg/m$^3$ | 0.210 | 0.01 | 0.1163 | 31.6 |
| Vegetative biomass (XV) Kg/m$^3$ | - | - | 0.1772 | - |
| Total carbohydrates Kg/m$^3$ | 0.273 | 0.03 | - | - |
| Exopolysaccharide (EPS) Kg/m$^3$ | - | - | 0.108 | - |
| Glycogen (G) Kg/m$^3$ | - | - | 0.0077 | - |
| Spectrophotometer (XT) Kg/m3 D.W (Kontron) | 0.61 | 0.03 | - | - |
| $NO_3^-$ ($10^{-3}$ Kg/m$^3$) (Cap. Elec.) | 2.6 | na | - | - |
| $NO_3^-$ ($10^{-3}$ Kg/m$^3$) (Dr. Lange) | 1.3 | 0.9 | 12.2 | 580 |

**Table 10 :** Off-line results obtained in nitrate limitation phase B2 (0.1 kg/m$^3$ $NO_3^-$) at a dilution rate of 0.012 h$^{-1}$ and incident light flux of 50.2 W/m$^2$. And PHOTOSIM simulated results for the same conditions.

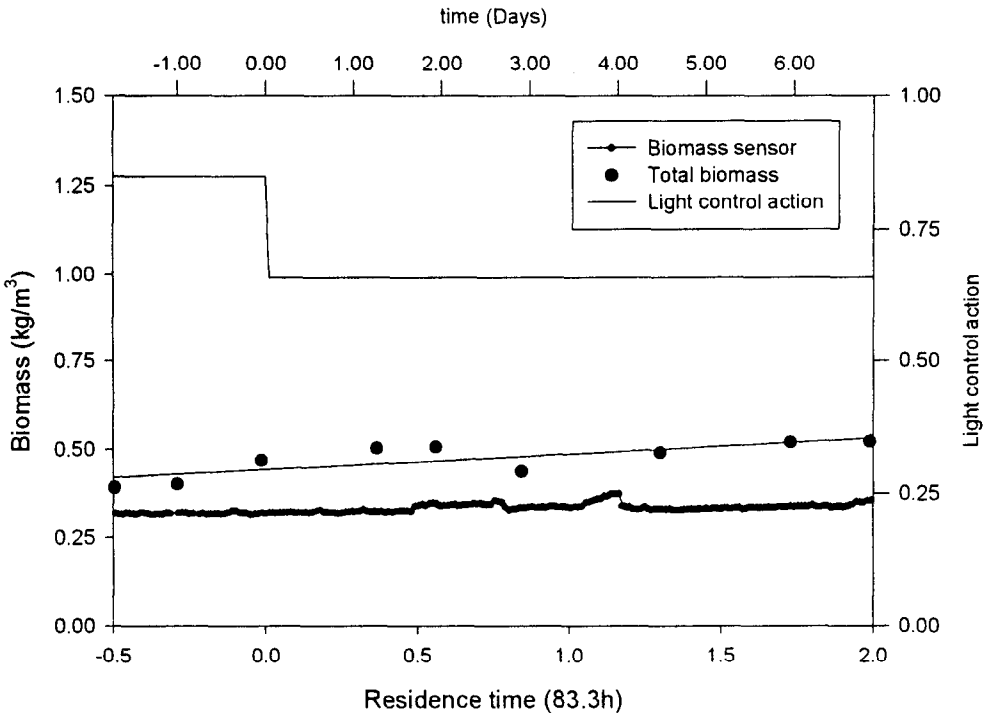



**Figure 7**: Evolution of biomass concentration in nitrate limitation. From phase B1 to B2 (0.1 kg/m$^3$ $NO_3^-$). at 0.012 h$^{-1}$ dilution rate ( Fr: 305→50.2 W/m$^2$).

**Table 11 :** Elemental composition obtained from samples of test B2. (0.1 kg/m$^3$ $NO_3^-$). at 0.012 h$^{-1}$ dilution rate and Fr: 50.2 W/m$^2$. (S.deM.) : Service de Microanalyse C.N.R.S. (France).

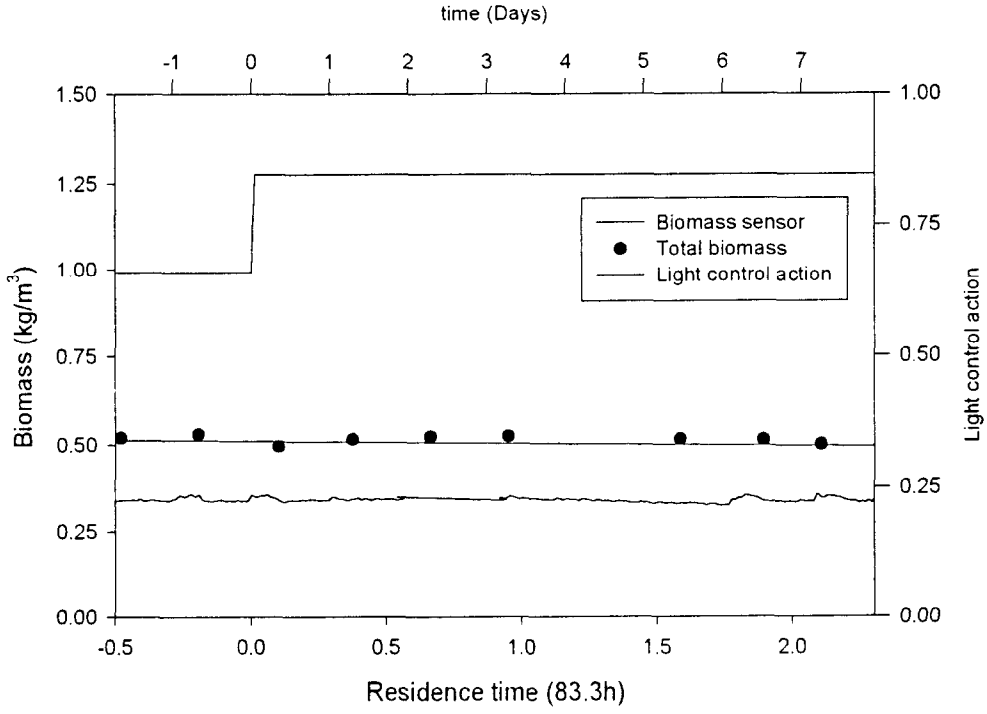
| B2 Element | Average (SdeM) | SD | Mol (SdeM) | SD | Simulation | % Deviation from experimental |
|---|---|---|---|---|---|---|
| C | 42.35 | 0.04 | 1 | 0.001 | 1 | - |
| H | 6.24 | 0.01 | 1.769 | 0.002 | 1.60 | 6.7 |
| O | 37.52 | 0.001 | 0.66 | - | 0.619 | 4 |
| N | 4.7 | 0.006 | 0.095 | 0.001 | 0.1143 | 14 |
| P | 0.70 | 0.2 | 0.0064 | 0.002 | 0.0037 | 30 |
| S | 0.26 | 0.05 | 0.0023 | 0.0002 | 0.0088 | 200 |
| Ash | 8.2 | 0.2 | - | - | - | - |

## Phase B3 results

| B3 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr $W/m^2$ | 305 | | 305 | - |
| Light control action (%) | 85 | | 85 | - |
| Total biomass (XT) $Kg/m^3$ | 0.5 | 0.03 | 0.578 | 11 |
| Active biomass (XA) $Kg/m^3$ | 0.29 | | 0.173 | 28 |
| Chlorophylls (CH) $Kg/m^3$ | 0.005 | 0.0007 | 0.00173 | 46 |
| Phycocyanins (PC) $Kg/m^3$ | 0.013 | 0.003 | 0.0139 | 5 |
| Proteins (P) $Kg/m^3$ | 0.155 | 0.02 | 0.118 | 17 |
| Vegetative biomass (XV) $Kg/m^3$ | - | - | 0.1815 | - |
| Total carbohydrates $Kg/m^3$ | 0.265 | 0.03 | - | - |
| Exopolysaccharide (EPS) $Kg/m^3$ | - | - | 0.344 | - |
| Glycogen (G) $Kg/m^3$ | - | - | 0.0085 | - |
| Spectrophotometer (XT) $Kg/m^3$ D.W (Kontron) | 0.68 | 0.02 | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Cap. Elec.) | 0.3 | na | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Dr. Lange) | 2.4 | 0.7 | 10.5 | 240 |

**Table 12 :** Off-line results obtained in nitrate limitation phase B3 (0.1 $kg/m^3$ $NO_3^-$) at a dilution rate of 0.012 $h^{-1}$ and incident light flux of 305 $W/m^2$. Also shown PHOTOSIM simulated results for the same conditions.

**Figure 8 :** Evolution of biomass concentration in nitrate limitation. From phase B2 to B3 (0.1 $kg/m^3$ $NO_3^-$). at 0.012 $h^{-1}$ dilution rate ( Fr: 50.2→305 $W/m^2$).



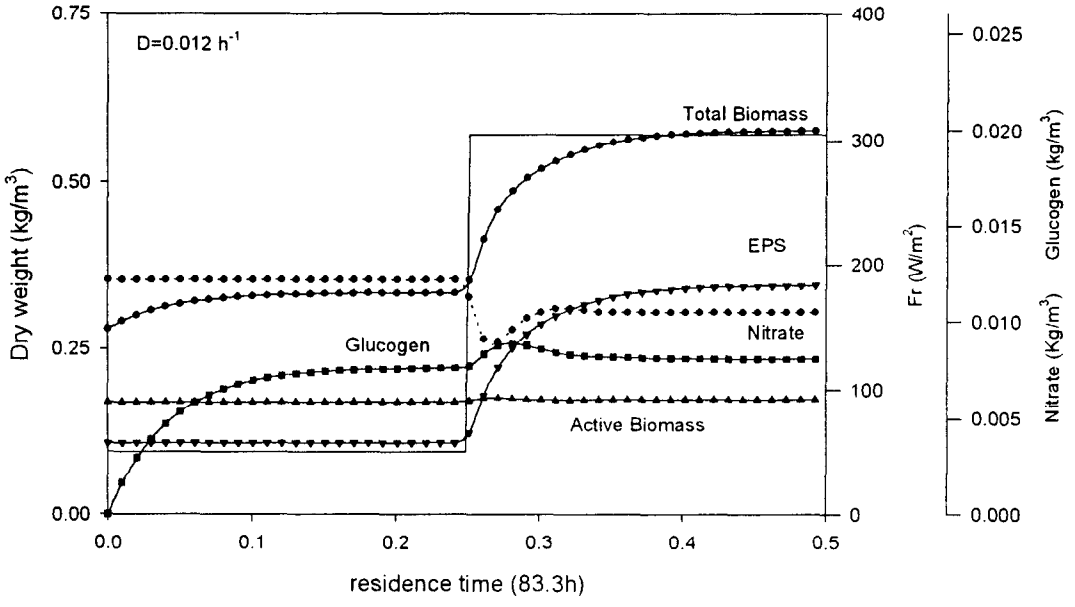



**Figure 9 :** Simulation of evolution of biomass concentration in nitrate limitation. From phase B2 to B3 (0.1 kg/m$^3$ $NO_3^-$). at 0.012 h$^{-1}$ dilution rate ( Fr: 50.2→305 W/m$^2$).

**Table 13** : Elemental composition obtained from samples of test B3. (0.1 kg/m$^3$ $NO_3^-$). at 0.012 h$^{-1}$ dilution rate and Fr: 305 W/m$^2$. (S.deM.): Service de Microanalyse C.N.R.S. (France).

| B3 Element | Average (SdeM) | SD | Mol (SdeM) | SD | Simulation | % Deviation from experimental |
|---|---|---|---|---|---|---|
| C | 42.72 | 0.06 | 1 | 0.001 | 1 | - |
| H | 6.25 | 0.01 | 1.757 | 0.002 | 1.623 | 5 |
| O | 39.6 | 0.1 | 0.695 | 0.002 | 0.766 | 7 |
| N | 5.2 | 0.02 | 0.105 | 0.0003 | 0.0634 | 28 |
| P | 0.71 | 0.3 | 0.0064 | 0.002 | 0.0021 | 47 |
| S | 0.28 | 0.01 | 0.0024 | 0.0001 | 0.0115 | 270 |
| Ash | 5.2 | 0.3 | - | - | - | - |

## TEST C

For the third part of the test the dilution rate was set to $0.016h^{-1}$ (residence time 62.5 $h^{-1}$; flow 0.112 l/h). The nitrate concentration in the input of the bioreactor was maintained as in previous tests at $100x10^{-3}$ kg $NO^{-}_{3}/m^{3}$. At the beginning of the test the light intensity was set at 85 % of the controller action, which corresponds to a calculated Fr of 305 $W/m^2$. After setting up the conditions, dynamic evolution of the bioreactor culture performance to the steady-state was followed. The steady state thus reached is referred to as C1. A summary of the results obtained from the biomass macromolecular composition analysis can be seen in table 14. The biomass level reached are presented in figure 11. The results of biomass elemental composition are given in table 15.

Once the steady state was obtained, light intensity was changed in order to see what was its effect on the biomass composition. The new level of light was 65.69 % of the light control action, which corresponds to a calculated Fr value of 50.2 $W/m^2$. Once the steady state was reached, culture samples were taken in order to measure the biomass macromolecular and elemental composition. This steady state will be referred as C2. Biomass levels and macromolecular, analysis are given in table 16. Results of the elemental composition are given in table 17.

At this point and in order to confirm the results obtained in the C1 steady state, the light intensity was set back to its previous value of the 85 % of the light intensity (Fr of 305 $W/m^2$). The results of this test, referred to as C3, can be seen in figure 12 and table 18 for a summary of biomass levels, protein and carbohydrate measurements. Elemental composition is given in table 19. For each case the results offered by the PHOTOSIM simulator for the steady states corresponding to the conditions of this tests are given in tables 14, 16 and 18 and figures 10 and 13.

## Phase C1 results

| C1 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr $W/m^2$ | 305 | | 305 | - |
| Light control action (%) | 85 | | 85 | - |
| Total biomass (XT) $Kg/m^3$ | 0.48 | 0.06 | 0.51 | 4 |
| Active biomass (XA) $Kg/m^3$ | - | - | 0.167 | - |
| Chlorophylls (CH) $Kg/m^3$ | 0.006 | na | 0.00167 | 51 |
| Phycocyanins (PC) $Kg/m^3$ | 0.021 | 0.01 | 0.0165 | 15 |
| Proteins (P) $Kg/m^3$ | 0.155 | 0.002 | 0.1144 | 18 |
| Vegetative biomass (XV) $Kg/m^3$ | - | - | 0.1745 | - |
| Total carbohydrates $Kg/m^3$ | 0.23 | 0.02 | | - |
| Exopolysaccharide (EPS) $Kg/m^3$ | - | - | 0.2971 | - |
| Glycogen (G) $Kg/m^3$ | - | - | 0.0072 | - |
| Spectrophotometer (XT) $Kg/m^3$ D.W (Kontron) | 0.61 | 0.05 | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Cap. Elec.) | 0 | - | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (calculated) | 2.1 | 0.3 | 13.6 | 380 |

**Table 14 :** Off-line results obtained in nitrate limitation phase C1 (0.1 $kg/m^3$ $NO_3$) at a dilution rate of 0.016 $h^{-1}$ and incident light flux of 305 $W/m^2$. Also shown the PHOTOSIM simulated results for the same conditions.

**Figure 10 :** Simulation of evolution of variables in nitrate limitation. From phase C1 to C2 (0.1 $kg/m^3$ $NO_3^-$). at 0.016 $h^{-1}$ dilution rate ( Fr: 305→50.2 $W/m^2$).

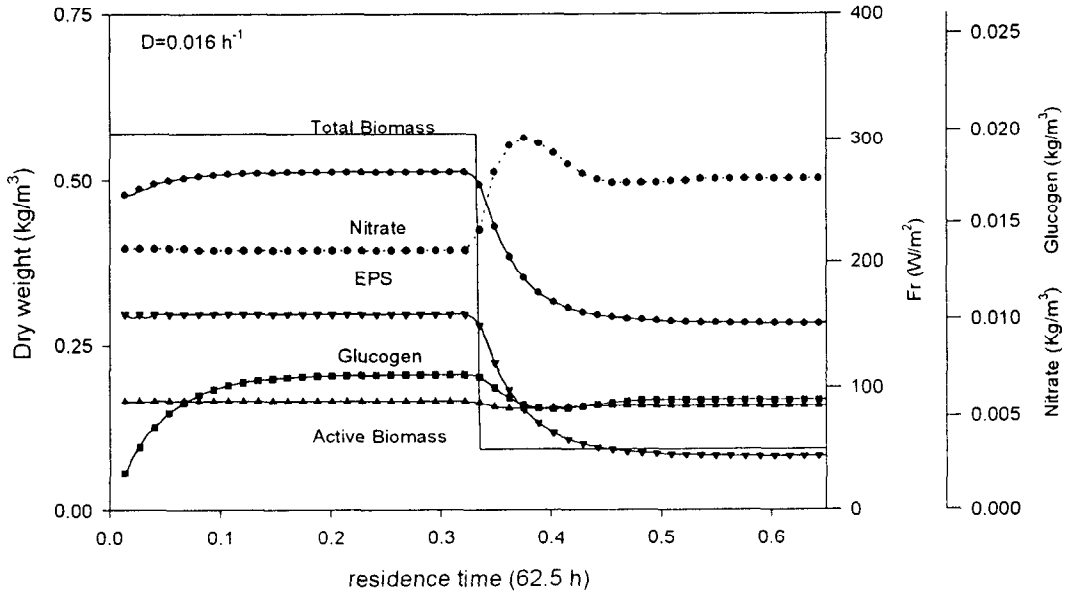


**Table 15** : Elemental composition obtained from samples of test C1. (0.1 kg/m$^3$ $NO_3^-$). at 0.016 h$^{-1}$ dilution rate and Fr: 305 W/m$^2$. (S.deM.) : Service de Microanalyse C.N.R.S. (France).

| C1 Element | Average (SdeM) | SD | Mol (SdeM) | SD | Simulation | % Deviation from experimental |
|---|---|---|---|---|---|---|
| C | 36.04 | 0.05 | 1 | 0.001 | 1 | - |
| H | 5.3 | 0.05 | 1.756 | 0.014 | 1.62 | 5 |
| O | 38.65 | 0.17 | 0.8 | 0.003 | 0.753 | 4 |
| N | 4.1 | 0.01 | 0.097 | 0.003 | 0.0681 | 21 |
| P | 0.1 | 0.12 | 0.001 | 0.0012 | 0.0022 | 85 |
| S | 0.39 | 0.03 | 0.004 | 0.00025 | 0.0113 | 130 |
| Ash | 15.4 | 0.23 | - | - | - | - |

## Phase C2 results

| C2 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr W/m$^2$ | 50.2 | | 50.2 | - |
| Light control action (%) | 65.69 | | 65.69 | - |
| Total biomass (XT) Kg/m$^3$ | 0.54 | 0.03 | 0.283 | 34 |
| Active biomass (XA) Kg/m$^3$ | - | - | 0.160 | - |
| Chlorophyll (CH) Kg/m$^3$ | 0.006 | 0.0008 | 0.00160 | 52 |
| Phycocyanins (PC) Kg/m$^3$ | 0.025 | 0.004 | 0.018 | 20 |
| Proteins (P) Kg/m3 | 0.14 | 0.03 | 0.109 | 16 |
| Vegetative biomass (XV) Kg/m$^3$ | - | - | 0.166 | - |
| Total carbohydrates Kg/m$^3$ | 0.24 | 0.03 | - | - |
| Exopolysaccharide (EPS) Kg/m$^3$ | - | - | 0.084 | - |
| Glycogen (G) Kg/m$^3$ | - | - | 0.006 | - |
| Spectrophotometer (XT) Kg/m$^3$ D.W (Kontron) | 0.67 | 0.02 | - | - |
| $NO_3^-$ ($10^{-3}$ Kg/m$^3$) (Cap. Elec.) | 0 | - | - | - |
| $NO_3^-$ ($10^{-3}$ Kg/m$^3$) (Dr. Lange) | 1.1 | 0.1 | 17.3 | - |

**Table 16:** Off-line results obtained in nitrate limitation phase C2 (0.1 kg/m$^3$ $NO_3^-$) at a dilution rate of 0.016 h$^{-1}$ and incident light flux of 50.2 W/m$^2$. PHOTOSIM simulated results for the same conditions are also shown.

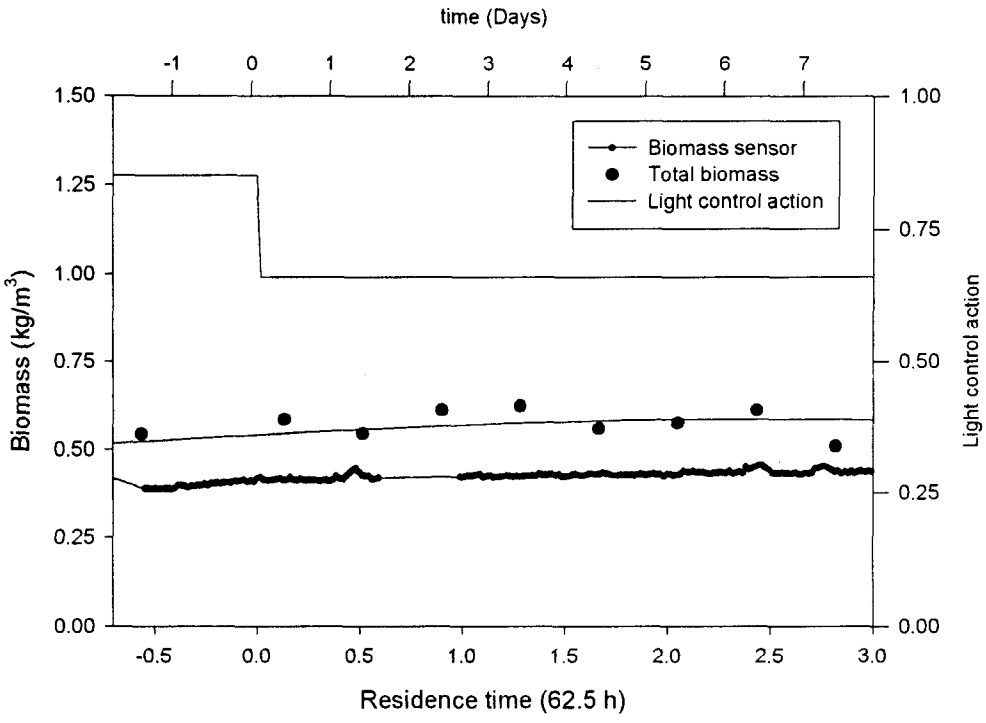



**Figure 11 :** Evolution of biomass concentration in nitrate limitation. From phase C1 to C2 (0.1 kg/m$^3$ $NO_3^-$). at 0.016 h$^{-1}$ dilution rate ( Fr: 305→50.2 W/m$^2$).

**Table 17 :** Elemental composition obtained from samples of test C2. (0.1 kg/m$^3$ $NO_3^-$). at 0.016 h$^{-1}$ dilution rate and Fr: 50.2 W/m$^2$.
(S.deM.) : Service de Microanalyse C.N.R.S. (France).

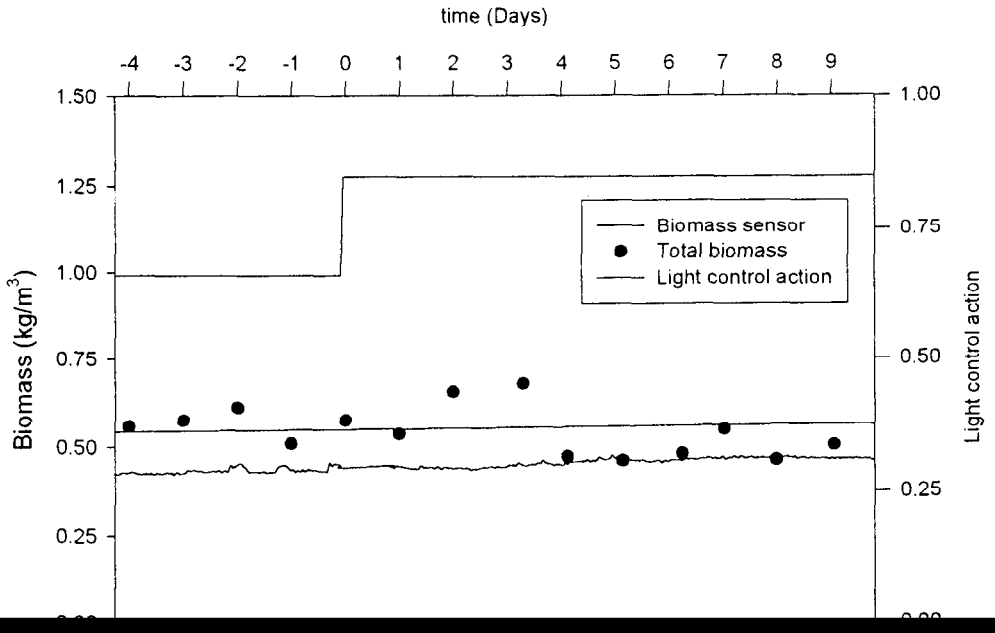
| C2 Element | Average (SdeM) | SD | Mol (SdeM) | SD | Simulation | % Deviation from experimental |
|---|---|---|---|---|---|---|
| C | 38.4 | 0.07 | 1 | 0.001 | 1 | - |
| H | 5.6 | 0.01 | 1.742 | 0.014 | 1.596 | 6 |
| O | 40.8 | 0.2 | 0.8 | 0.003 | 0.5948 | 18 |
| N | 4.24 | - | 0.094 | - | 0.1231 | 22 |
| P | 1.24 | 0.05 | 0.012 | 0.0005 | 0.004 | 50 |
| S | 0.38 | - | 0.004 | 0.00047 | 0.0084 | 77 |
| Ash | 9.3 | 0.15 | - | - | - | - |

## Phase C3 results

| C3 | EXPERIMENTAL | SD | SIMULATION | % Deviation from experimental |
|---|---|---|---|---|
| Fr $W/m^2$ | 305 | | 305 | - |
| Light control action (%) | 85 | | 85 | - |
| Total biomass (XT) $Kg/m^3$ | 0.52 | 0.07 | 0.51 | 1 |
| Active biomass (XA) $Kg/m^3$ | - | - | 0.167 | - |
| Chlorophyll (CH) $Kg/m^3$ | 0.006 | 9E-04 | 0.00167 | 51 |
| Phycocyanins (PC) $Kg/m^3$ | 0.019 | 2E-04 | 0.0165 | 19 |
| Proteins (P) $Kg/m^3$ | 0.20 | 0.01 | 0.1144 | 32 |
| Vegetative biomass (XV) $Kg/m^3$ | - | - | 0.1745 | - |
| Total carbohydrates $Kg/m^3$ | 0.28 | 0.02 | - | - |
| Exopolysaccharide (EPS) $Kg/m^3$ | - | - | 0.2971 | - |
| Glycogen (G) $Kg/m^3$ | - | - | 0.0072 | - |
| Spectrophotometer (XT) $Kg/m^3$ D.W (Kontron) | 0.74 | 0.05 | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (Off-line) | 0.3 | na | - | - |
| $NO_3^-$ ($10^{-3}$ $Kg/m^3$) (calculated) | 1.3 | 0.4 | 13.6 | 670 |

**Table 18:** Off-line results obtained in nitrate limitation phase C3 (0.1 $kg/m^3$ $NO_3$) at a dilution rate of 0.016 $h^{-1}$ and incident light flux of 305 $W/m^2$. PHOTOSIM simulated results for the same conditions are also shown.

**Figure 12 :** Evolution of biomass concentration in nitrate limitation. From phase C2 to C3 (0.1 $kg/m^3$ $NO_3^-$). at 0.016 $h^{-1}$ dilution rate ( Fr: 50.2→305 $W/m^2$.).



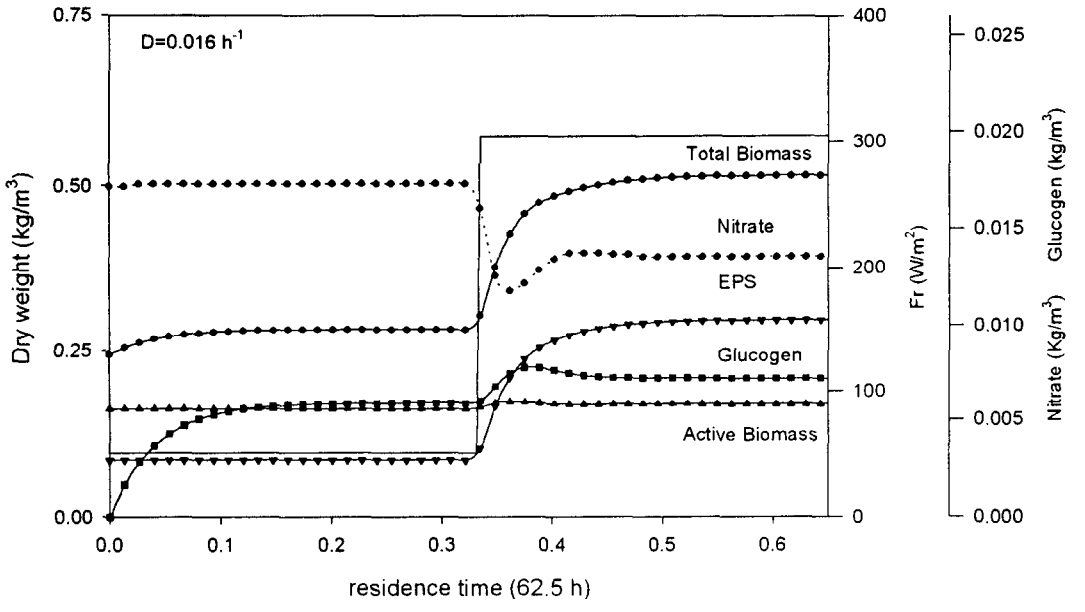



**Figure 13 :** Simulation of evolution of variables in nitrate limitation. From phase C2 to C3 (0.1 kg/m$^3$ $NO_3^-$). at 0.016 h$^{-1}$ dilution rate ( Fr: 50.2→305 W/m$^2$.)

**Table 19 :** Elemental composition obtained from samples of test C3. (0.1 kg/m$^3$ $NO_3^-$). at 0.016 h$^{-1}$ dilution rate and Fr: 305 W/m$^2$. (S.deM.) : Service de Microanalyse C.N.R.S. (France).

| C3 Element | Average (SdeM) | SD | Mol (SdeM) | SD | Simulation | % Deviation from experimental |
|---|---|---|---|---|---|---|
| C | 42.9 | 0.11 | 1 | 0.001 | 1 | - |
| H | 6.2 | - | 1.735 | - | 1.62 | 5 |
| O | 39.2 | 0.1 | 0.685 | 0.001 | 0.753 | 7 |
| N | 5.04 | 0.02 | 0.1 | 0.0003 | 0.0681 | 23 |
| P | 0.8 | 0.2 | 0.007 | 0.002 | 0.0022 | 48 |
| S | 0.31 | 0.007 | 0.004 | 7.E-5 | 0.0113 | 130 |
| Ash | 5.46 | 0.01 | - | - | - | - |

## Complementary Tests

### TEST D

Due to the cells high affinity for the nitrate observed during the previous tests it was decided to perform a new test at a higher dilution rate. For this test the dilution rate was set to 0.05 $h^{-1}$ (residence time 20 h, flow 0.350 l/h). The nitrate concentration in the input of the bioreactor was maintained as in previous tests at $100x10^{-3}$ kg $NO^-_3/m^3$ . At the beginning of the test the light intensity was set at 65.69 % of the controller action, which corresponds to a calculated Fr of 50.2 $W/m^2$ . After setting up the conditions, dynamic evolution of the bioreactor culture performance to the steady-state was followed. The steady state thus reached is referred to as D1.

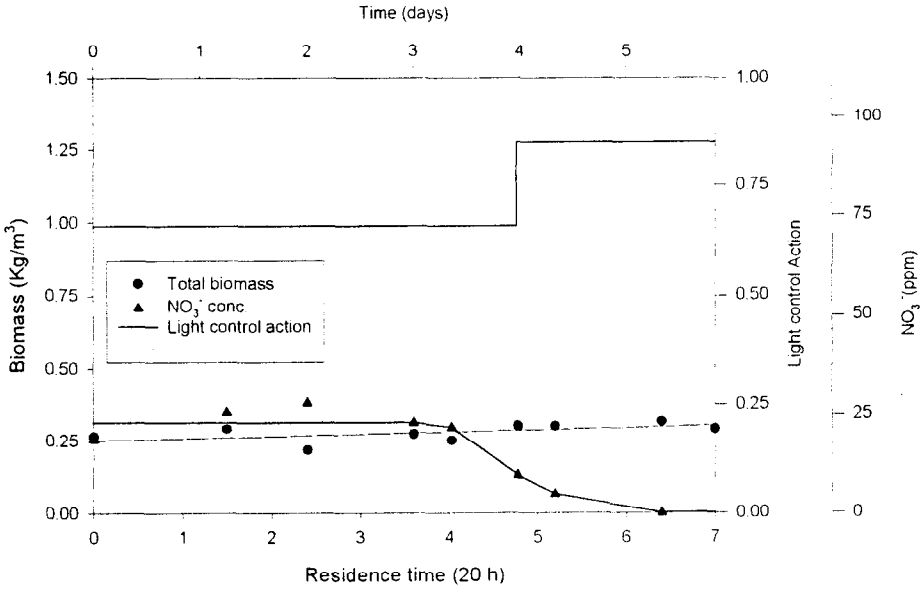



**Figure 14 :** Evolution of biomass concentration in nitrate limitation. Test D1→D2. (0.1 kg/$m^3$ $NO_3^-$). at 0.05 $h^{-1}$ dilution rate ( Fr: 50.2→305 $W/m^2$.)

After 3 residence times the conditions were considered stable and light intensity was increased setting the controller at 85% of its range. This corresponds to a calculated Fr of 305 $W/m^2$ . The results obtained in both states are given in figure 14. The second phase is referred as D2. During this tests the biomass level was lower that in previous tests. This suggested that the nitrate levels remaining in the bioreactor could be higher.

Indeed when the nitrate levels were measured off line they were found to be higher than the ones found before. However the nitrate concentration decreased when light was increased, but no measurable increase in total biomass could be observed.

**Table 20 :** Elemental composition obtained from samples of test D1. (0.1 kg/m$^3$ $NO_3^-$). at 0.05 h$^{-1}$ dilution rate and Fr: 50.2 W/m$^2$. (SAQ.) : Servei D'Analisi Química. U.A.B. (Spain).

| D1 Element | Average (SAQ) | SD | Mol (SdcM) | SD |
|---|---|---|---|---|
| C | 41.6 | 0.02 | 1 | 0.001 |
| H | 5.6 | 0.14 | 1.61 | 0.04 |
| O | - | - | - | - |
| N | 5.62 | 0.04 | 0.116 | 0.0008 |
| P | - | - | - | - |
| S | 0.3 | 0.035 | 0.0027 | 0.0003 |

**Table 21 :** Elemental composition obtained from samples of test D2. (0.1 kg/m$^3$ $NO_3^-$). at 0.05 h$^{-1}$ dilution rate and Fr: 305 W/m$^2$. (SAQ.) : Servei D'Anàlisi Química. U.A.B. (Spain).

| D2 Element | Average (SAQ) | SD | Mol (SdcM) | SD |
|---|---|---|---|---|
| C | 38.7 | 0.2 | 1 | 0.006 |
| H | 5.65 | 0.001 | 1.752 | - |
| O | - | - | - | - |
| N | 6.68 | 0.001 | 0.148 | - |
| P | - | - | - | - |
| S | 0.245 | 0.035 | 0.0024 | 0.0003 |

## TEST E

To ascertain the previous unexpected behaviour, the D culture was stopped, the bioreactor cleaned and started with and inoculum comming from the previous culture. The dilution rate was set at 0.05 $h^{-1}$ and at an Fr of 50.2 $W/m^2$. This time the illumination conditions were not changed in order to evaluate the long term behaviour of the biomass. After setting up the conditions, dynamic evolution of the bioreactor culture performance to the steady-state was followed. The steady state thus reached is referred to as E. During this test two biomass samples (E1(res time : 17.7) and E2(res time : 28.4)) were lyophilised to allow for the biomass elemental analysis. Those analyses were done only in UAB due to time constraints (tables 22 and 23 ). Nitrate in the culture media was completely consumed.

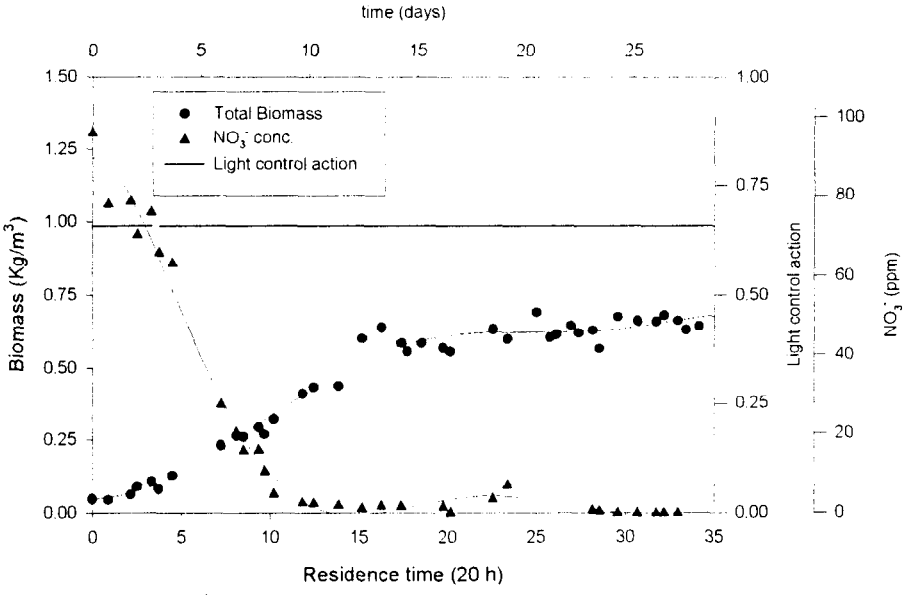



**Figure 15 :** Evolution of biomass concentration in nitrate limitation. Test E (0.1 $kg/m^3$ $NO_3^-$). at 0.05 $h^{-1}$ dilution rate ( Fr: 50.2 $W/m^2$.).

The nitrogen content of the biomass is slightly lower than in previous experiments and its steady state concentration, slightly higher. Microscopic examination of the biomass (figure 16), showed a morphological change compared to the cells maintained in culture flasks under non limiting conditions (figure 17). This suggested that either a long term adaptation of biomass has taken place or that a variant, with a higher affinity for the nitrate, and therefore more adapted to the culture conditions used, has been selected

during the continuous culture. To test this a sample of biomass from the bioreactor was withdrawn and cultured under non limiting conditions, at Clermont Ferand University laboratories. After a period of time of around two months the strain has reverted to its usual morphological appearance. This fact suggests that a long term adaptation of the biomass to the nitrate limiting conditions has taken place.

**Table 22 :** Elemental composition obtained from samples of test E1. (0.1 $kg/m^3$ $NO_3^-$). at 0.05 $h^{-1}$ dilution rate and Fr: 50.2 $W/m^2$. (SAQ.) : Servei D'Analisi Química. U.A.B. (Spain).

| E1 Element | Average (SAQ) | SD | Mol (SdeM) | SD |
|---|---|---|---|---|
| C | 26.6 | 0.2 | 1 | 0.007 |
| H | 3.1 | 0.007 | 1.384 | 0.003 |
| O | - | - | - | - |
| N | 3.9 | 0.03 | 0.124 | 0.0009 |
| P | - | - | - | - |
| S | 0.13 | 0.02 | 0.0019 | 0.0003 |

**Table 23** : Elemental composition obtained from samples of test E2. (0.1 $kg/m^3$ $NO_3^-$). at 0.05 $h^{-1}$ dilution rate and Fr: 50.2 $W/m^2$. (SAQ.) : Servei D'Analisi Química. U.A.B. (Spain).

| E2 Element | Average (SAQ) | SD | Mol (SdeM) | SD |
|---|---|---|---|---|
| C | 27.3 | 1. | 1 | 0.038 |
| H | 3.3 | 0.19 | 1.438 | 0.085 |
| O | - | - | - | - |
| N | 4.15 | 0.32 | 0.130 | 0.01 |
| P | - | - | - | - |
| S | 0.02 | 0.006 | 0.0003 | 8.E-5 |

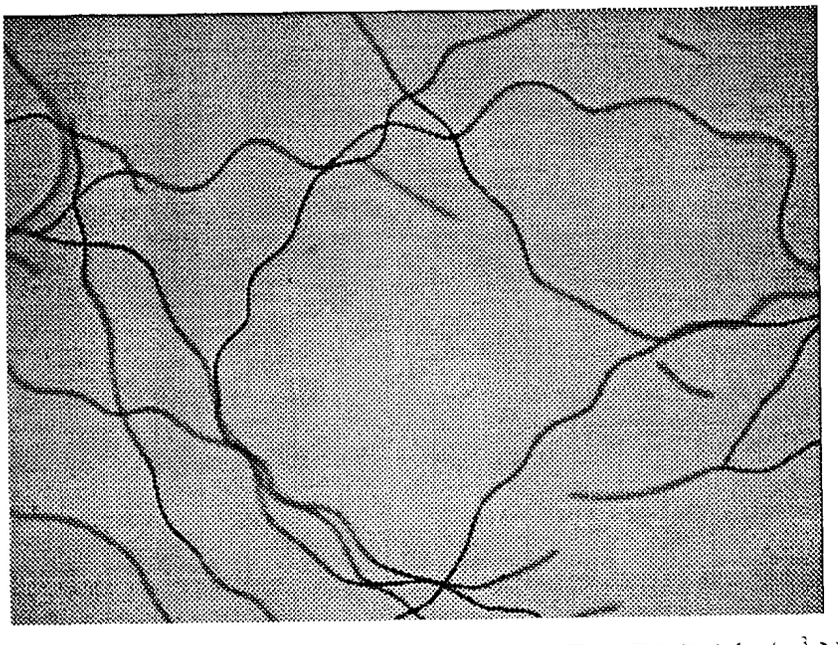
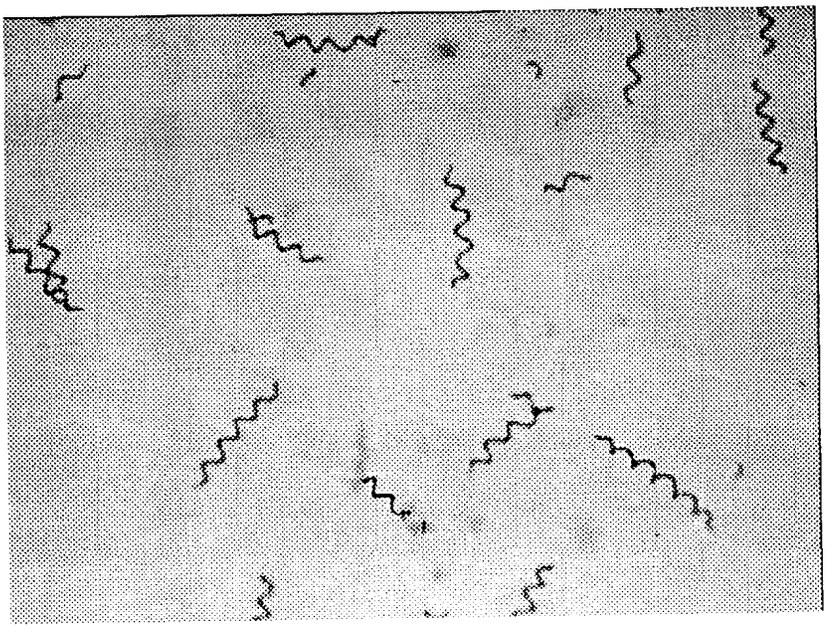


**Figure 16 :** Picture of biomass in nitrate limitation. Test E2 (0.1 kg/m$^3$ $NO_3^-$). at 0.05 h$^{-1}$ dilution rate ( Fr: 50.2 W/m$^2$.) Microscopic examination x5.

**Figure 17 :** Picture of biomass batch stock culture. Microscopic examination x5.



## Discussion of the results

The tests performed had the purpose to evaluate the behaviour of *Spirulina* cells and the evolution of macromolecular and elemental composition under nitrate limitation conditions. In table 24 an averaged summary of the biomass levels, protein and total carbohydrate measured for the different tests, done under the different culture conditions of tests A, B and C are given. In all the cases the biomass has been maintained at values around 0.5 g/l DW, which according to the biomass averaged elemental compositions determined (table 25), would correspond to the nearly complete exhaustion of the nitrogen source.

If a nearly complete consumption of the nitrogen source is accepted, and biomass elemental composition obtained is taken into account, it is possible to calculate the total biomass that would be obtained. In table 24 the calculated biomass concentrations, assuming a complete consumption of the nitrogen source, can be compared with the measured values. In all cases the calculated values are inside the experimental deviations of the measured values and therefore there is a good agreement among the biomass measured, the elemental analysis and the very low nitrate concentrations found in the culture medium.

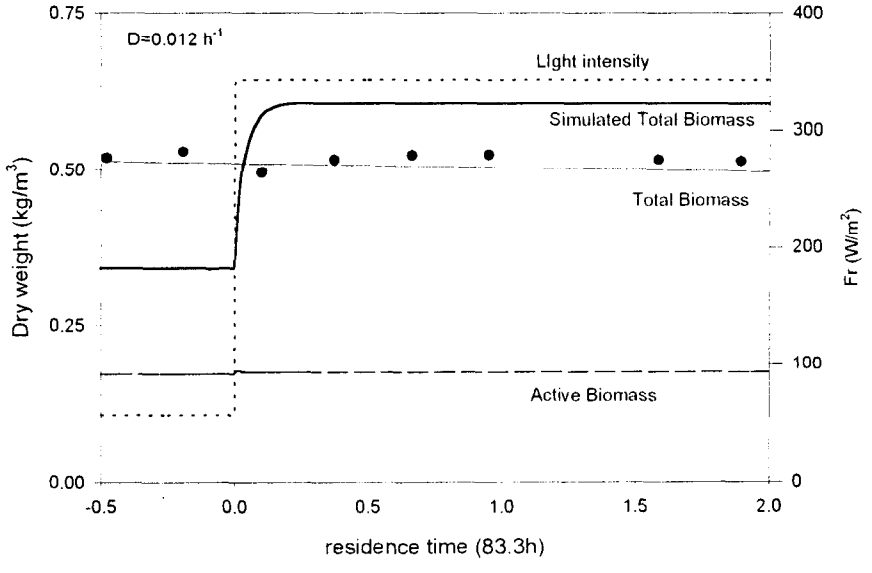



**Figure 18 :** Comparison among experimental and simulated biomass concentrations for $0.012h^{-1}$ (test B2-B3).

Lack of biomass concentration changes with light intensity indicate that in the conditions employed, nitrate was the limiting factor for the cells growth, as changes in light intensity did not change biomass levels in the bioreactor. Measured protein and carbohydrate content do not show a significant variation within the usual precision of their corresponding analysis methods (table 24).

As con be seen in figure 18 real and simulated values differ mainly in that the values measured indicate a constant concentration of the total biomass, while the model

indicates an increase in the total biomass. This increase in the values predicted by the model is mainly the result of the predicted increase in EPS and glucogen. In the experiments done, total biomass concentration do not change with light intensity. In the same way the simulated active biomass is maintained constant in the simulation. This may indicate that the EPS and glycogen contents are more constant than they were under under light limitation.

The real nitrate values in the bioreactor vessel were generally too low to be measured with confidence by the current available methods, as they were below of their threshold of measurement. This indicates that the affinity for the nitrate is higher than it is considered in the model. A modification of the value of the affinity parameter in the model will allow to obtain a nitrate value nearer to the measured one. This change will also increase the value of the simulated total biomass concentration at the steady state, approaching the values of the measured total biomass to the simulated values with low light intensity.

All this data indicate the modifications that should be pursued in the model so as to use it under nitrate limitation conditions until more data is obtained.

Averaged biomass elemental composition, table 25, shows only a slight variation among the different tests. This deviation is believed to be only due to the experimental variability. In conclusion no significant biomass composition changes were measured, taking into account the experimental error, although minor changes might have occurred. In any case, they are too small to be considered significant.

Comparison of those data with tests D and E suggest that a more complex adaptation takes place during a long term maintenance of the cells under nitrate limiting conditions. This can be seen either in the morphological change (figure 16) as well as in the preliminary elemental analysis (tables 22 and 23). Therefore more complete tests are needed to properly evaluate the importance and consequences of such changes.

Evaluation of the deviation obtained in measuring the dry weight suggested that an improvement should be pursued. Therefore future dry weight determinations will be done using a different kind of filters allowing for a higher volume of the biomass to be filtered and used for the biomass dry weight determinations.

| T | DILUTION RATE ($h^{-1}$) | LIGHT INTENSITY ($W/m^2$) | TOTAL BIOMASS ($Kg/m^3$) +/- SD | CALCULATED BIOMASS ($Kg/m^3$) +/-SD | TOTAL CARBOHYDR. ($Kg/m^3$) +/-SD | PROTEINS ($Kg/m^3$) +/-SD |
|---|---|---|---|---|---|---|
| A | 0,018 | 305 | 0,47 +/-0,04 | 0,43 | 0,200 +/-0,01 | 0,15 +/-0,01 |
| A | 0,018 | 50.2 | 0,49 +/-0,03 | 0,5 | 0,22 +/-0,03 | 0,16 +/-0,01 |
| C | 0,016 | 305 | 0,50 +/-0,03 | 0,5 | 0,25 +/-0,04 | 0,19 +/-0,03 |
| C | 0,016 | 50.2 | 0,54 +/-0,03 | 0,53 | 0,24 +/-0,03 | 0,14 +/-0,03 |
| B | 0,012 | 305 | 0,44 +/-0,04 | 0,42 | 0,25 +/-0,02 | 0,15 +/-0,01 |
| B | 0,012 | 50.2 | 0,49 +/-0,03 | 0,48 | 0,27 +/-0,03 | 0,21 +/-0,03 |

**Table 24 :** Averaged macromolecular composition of the different samples for the different test conditions assayed in this TN.

**Table 25 :** Averaged elemental composition of the different samples for the different test conditions essayed in this TN. Averages have been done using the results of the analyses done at : Service de Microanalyse C.N.R.S. (France).

| TEST | A | | C | | B | |
|---|---|---|---|---|---|---|
| Dilution rate ($h^{-1}$) | 0.018 | | 0.016 | | 0.012 | |
| Light intensity | 305 | 50.2 | 305 | 50.2 | 305 | 50.2 |
| %C | 38.6 +/-2.2 | 36.4 +/-5.5 | 39.5 +/-4.9 | 38.4 +/-0.1 | 42.3 +/-1 | 42.3 +/-0.1 |
| %H | 5.6 +/-0.5 | 5.2 +/-0.9 | 5.74 +/-0.6 | 5.6 +/-0.01 | 6.13 +/-0.2 | 6.25 +/-0.01 |
| %O | 38.18 +/-1.74 | 39.44 +/-1.26 | 38.95 +/-0.42 | 40.8 +/-0.2 | 39.38 +/-0.29 | 37.52 +/-0.01 |
| %N | 5.2 +/-0.2 | 4.6 +/-0.96 | 4.6 +/-0.7 | 4.2 - | 5.4 +/-0.3 | 4.7 +/-0.1 |
| %P | 1.0 +/-0.2 | 0.98 +/-0.2 | 0.45 +/-0.5 | 1.2 +/-0.05 | 0.75 +/-0.06 | 0.71 +/-0.2 |
| %S | 0.36 +/-0.05 | 0.38 +/-0.08 | 0.35 +/-0.05 | 0.38 - | 0.31 +/-0.04 | 0.26 - |
| Ash | 10.9 +/-3.6 | 12.96 +/-6.3 | 10.46 +/-7.0 | 9.355 - | 5.73 +/-0.7 | 8.20 - |

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# Appendix 1

## Total carbohydrates measurement

### Phenol method (Herbert 1971)

**A - Reactants:**

1.- Concentrated sulphuric acid (96-97%).

2. -Phenol 5% (w/v)

3.- Glucose

**B - Sample treatment:**

- Centrifuge 10 ml of culture suspension. Discard supernatant.
- Wash with DDW (Double Distilled Water).
- Centrifuge in the same conditions. Discard the supernatant.
- dissolve in DDW.

(alternatively dissolve a sample of freeze dried biomass in DDW).

**C- Analysis**

-Add to 1 ml sample

1ml Phenol and mix carefully

5ml of sulphuric acid. Mix carefully.

- Wait 10 min.
- Cool the tubes (15 min. in water 25 °C).

-Read absorbance ,of the sample and the blank, at 488 nm, against DDW.

**D-Results**

-Prepare a standard calibration curve using glucose samples (0-100 mg/l).

-Use the standard curve to calculate the concentration of the 1 ml sample by interpolation of the absolute absorbance.

# Appendix 2

## Total Proteins Measurement

**Lowry modified method**.

**A- Material and Reactants**

-Spectrophotometer. Fixed wavelength 750 nm

-Reactants:

1.-Modified Lowry Protein Reagent (Pierce)

2.-Folin-Ciocalteau reagent 2N (fresh each day).

3.- BSA standard (2 mg/l) (in NaOH 1N)

4.- NaOH 1N

**B- Sample treatment.**

Cell Suspension:

-Centrifuge 15 ml of cell suspension. (15 min. 12000 g. 8 °C). Discard the supernatant.

- Wash the pellet with distilled water (or MgCl 95 mg/ml). Discard the supernatant.

- Complete the pellet with 10 ml NaOH 1N.

- Cap the tube and heat it (100 °C, 5 min.).

Freeze dried sample:

- Prepare a sample containing 0.25 mg/ml of freeze dried biomass in NaOH 1N.

- Cap the tube and heat it (100 °C, 5 min.).

**C- Standard solution preparation**

Prepare a set of standard solutions, in the same diluent as the unknown samples, within the range of 50 to 500 mg/l. Using a BSA stock solution of 2 mg/ml, the standards can be prepared adding to the test tubes the following volumes:

| Protein (mg/l) | 0 | 40 | 100 | 200 | 300 | 400 | 500 |
|---|---|---|---|---|---|---|---|
| BSA Stock (ml) | 0 | 0.02 | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 |
| Diluent (ml) | 1 | 0.98 | 0.95 | 0.9 | 0.85 | 0.8 | 0.75 |

Once the standard solutions are prepared, cap the tube and heat it at 100 °C during 5 min. in the same way as you do with the sample

**D- Procedure.**

To complete the test, follow the instructions in the following table:

| | |
|---|---|
| Sample: | 0.4 ml |
| Modified Lowry reagent | 2. ml |
| Vortex. Incubate | 10 min. |
| Folin Ciocalteau 1 N Solution | 0.2 ml |
| Vortex incubate | 30 min. (exactly) |
| Read Absorbance at 750 nm | |

**E- Results.**

Calculate the net absorbance, subtracting the absorbance of the blank from the sample and standard solutions. Trace a curve of protein concentration v.s. absorbance with the results obtained with the standard solutions. Calculate the protein concentration of the sample by interpolation of its net absorbance value in the standard curve.

Note: The absorbance obtained is pH and time dependent.

# Appendix 3

## Chlorophyll and phycocyanin measurement.

**Chlorophyll Measurement.**

**A- Material and Reactants**

-Acetone 80%.
-0.45 µm Millipore filters (acetone resistant).
-Sonifyer.
-Dual cell spectrophotometer.

**B- Sample treatment and procedure.**

-Filter 10 ml of a culture suspension to separate the culture media.
-Add the filter to a 5 ml 80% acetone in a test tube.
-Submit sample to sonification 30' to disperse the sample.
-Wait 2 min.
-Filter the sample with the Millipore filter.
-Measure absorbance in the spectrophotometer at 663 nm against an acetone blank..
-Calculate concentration using 8200 $m^{-2}$/Kg at 663 nm as absorption coefficient. Or alternatively as (Sestak 1971):
Chlorophyll a ($x10^{-3}$ Kg/$m^3$)=11.78*A(664)-2.29*A(647) ;

**Phycocyanin Measurement.**

**A- Material and Reactants**

-0.45 µm Millipore filters.
-Sonifyer.
-0.05M K-Phosphate buffer pH 7, 25 mM EDTA.
-Liquid nitrogen.
-Dual cell spectrophotometer.

**B- Sample treatment and procedure.**

-Filter 10 ml of a culture suspension to separate the culture media.
-Add 5 ml of phosphate buffer.
-Submit sample to sonification 30' to disperse the sample. And break tricomes.
-Freeze the sample in liquid nitrogen.
-Let the sample equilibrate overnight at 20 °C.
-Measure the absorbance of the sample at 652 and 615 nm against a phosphate blank .
-Calculate phycocyanin concentration as (Siegelman 1980):

Phycocyanin (PC)(Kg/m3)=(A(615)-0.474*A(652))/5.34 ;

Allophycocyanin (APC) (Kg/m3)= A(652)-0.208*A(615))/5.09 ;

# Appendix 4

## Nitrate concentration measurement

**Capillary Ion Analysis.**

**A- Material.**

-Waters Capillary Ion Analyzer (CIA) .

-Ultra pure water.

**B-Procedure.**

Dilute two volumes of a filtered sample with one volume of a deionized water.

Take 0.5 ml of the diluted sample and analyse the ion content using the following conditions (Method N-701a):

Electrolite : Sulphate/OFM-OH

Capillar : Fused silica 75 µm.

Power source : Negative

Potential for analysis : 15kV

Injection time : 30 seconds (hidrostatic)

Detection : 185 nm. (Mercury lamp)

Temperature : 25 °C.

**C-Results.**

Interpolate the area of the peak given by the analyser in a calibration curve to obtain the nitrate concentration.

**D Calibration curve.**

To obtain the calibration curve, different samples of Zarrouk culture media were prepared with different nitrate concentrations. This samples were analysed as described above for the bioreactor samples.

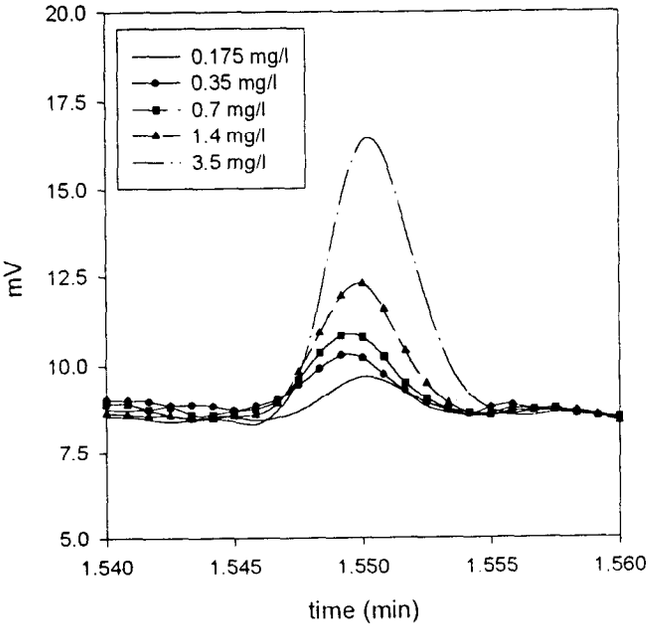
In figure 18 it can be seen and example of the electroforogram obtained using a 3.5 ppm sample of nitrate concentration.

As it can be seen there is a big peak previous the nitrate peak, which forces a dilution of the sample to be able to separate both peaks. However the peak separation is good enough to measure sample concentrations as low as 0.260 ppm (x $10^{-3}$ $Kg/m^3$) which once diluted correspond to a 0.175 ppm. An example

of the peaks obtained with those low concentrations of nitrate can be seen in figure 18. The figure has been amplified to show only the zone of the electroforogram showing the nitrate peak.

Following the above described procedure a calibration curve can be obtained. An example of a calibration curve can be seen in figure 20.

**Figure 19 :** Picture of the peaks obtained for different nitrate concentrations.



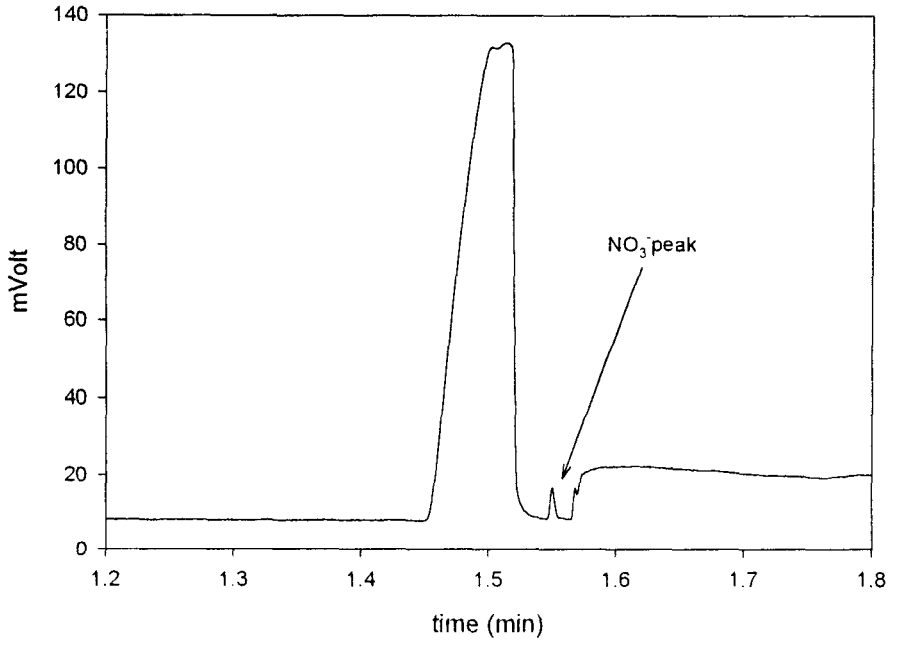



**Figure 20 :** Picture of the nitrate peak obtained at 3.5 mg/l compared with the rest of the non separated culture components..

**Figure 21 :** Example of a calibration curve that can be obtained using the peaks at different $NO_3^-$ concentrations shown in figure 18.