Universitat Autònoma de Barcelona
Dep. Enginyeria Química
08193 Bellaterra, Barcelona, Spain

MELISSA

Memorandum of Understanding
ECT/FG/MMM/97.012

Contract Number: ESTEC/CONTRACT13292/98/NL/MV

TECHNICAL NOTE: 47.5

Tests with the 3 linked bench compartments

Version: 1
Issue: 0

CREUS N.; ALBIOL, J.; GÒDIA, F.

JULY 2001

Document Change Log

Version	Issue	Date	Observations
Draft	0	20/3/01	Preliminary Version
1	0	26/07/01	Final Version

TABLE OF CONTENTS

1.- INTRODUCTION

The MELISSA project (Microbiological Ecological Life Support System Alternative) of the European Space Agency (ESA) is a tool for the development of a biological life support system to be used during Manned Space Missions. In order to achieve this purpose the project proposes the connection between five compartments, four of which contain microbial organisms and one higher plants.

The liquefying compartment, or compartment I, is responsible for the biodegradation of human faecal material and other wastes generated by the crew. The volatile acids, ammonium, gases and soluble components produced during the fermentation are fed into the second compartment.

The anoxygenic phototrophic compartment II metabolizes some of the compounds produced in the anaerobic liquefying compartment, with edible biomass generation. To achieve this goal *Rhodospirillum rubrum* and *Rhodobacter capsulata* are cultured in an anaerobic environment, either in photoheterotrophic or in photoautotrophic conditions.

The objective of compartment III (nitrifying compartment) is to transform the ammonium ions present in the exit stream from compartment II into nitrate, the most appropriate nitrogen source assimilated by the cells cultured in compartment IV. It consists in a packed-bed reactor with cells of two bacterial strains (*Nitrosomonas europaea* and *Nitrobacter winogradskyi*) immobilized onto polystyrene beads (Biostyr).

Compartment IV has as its main task the carbon dioxide removal and supply of oxygen for the crew respiration generating at the same time edible biomass as food supply. This compartment is currently implemented in airlift reactors where *Spirulina platensis* is cultivated. This cyanobacteria presents a high nutritional value and contains all the essential aminoacids, besides cysteine, in the adequate concentrations according to the FAO proposed standards.

The higher plants compartment is the basic food supplier for the crew.

To assure the satisfactory operation of the system, it is important to study the connection between these bioreactors not only at optimal conditions but also taking into account possible deviations in the behavior of any of them.

Dynamic operation of a loop of bioreactors will result in transitory states, especially when the system is driven from one steady state to another one or due to an eventual malfunction. In these cases, incomplete consumption of substrates may result and the effect of those on the subsequent bioreactors must be well characterized in order to foresee their behavior and ensure the proper operation of the loop.

Once the optimal operation of the connection of compartments II, III and IV has been successfully tested (Creus *et al.*, 1999), two of the major possible deviations from the optimal conditions have been performed and analyzed, such as the income of acetic acid in compartment III and nitrite and ammonium in compartment IV. These experiments have been done first, with the bioreactors disconnected and after during the connection of the three compartments.

2.- SET-UP AND MATERIALS AND METHODS

2.1.- SET-UP

As it is shown in figure 1 the experimental set up consists in three different bioreactors (compartments II, III and IV) and the necessary separation units to connect them. This same set up was used in TN 43.8 (Creus *et al.*, 1999). A brief description of each compartment and the different steps required in their connection are given in this section.

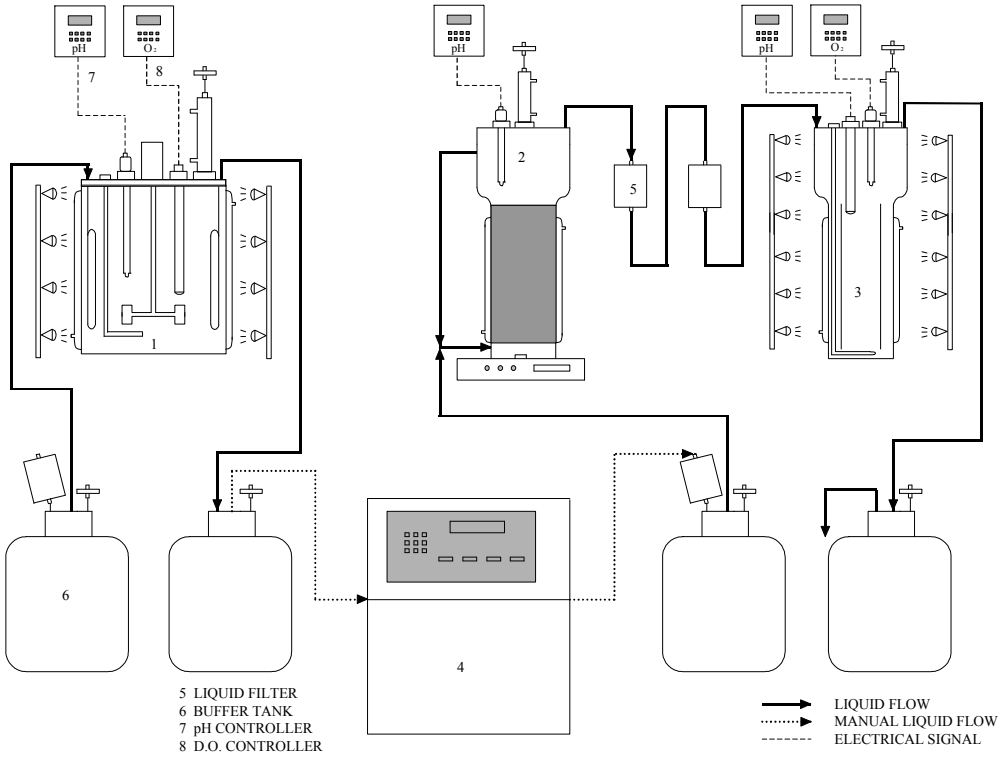

Figure 1. - Diagram of the experimental set up for the connection of compartments II, III and IV of MELISSA loop at bench scale.

Compartment II

Cultures corresponding to the second compartment are done in a 3 L stirred tank bioreactor (APPLIKON). A control unit (APPLIKON ADI 1012) regulates the pH of the culture media at 6.9 by addition of acid (HCl 1M) or base (NaOH 1.5M), stirs the culture mechanically to assure a perfect mixing (300rpm) and monitorizes the absence of oxygen. The anaerobic conditions are obtained bubbling He inside the fermentor.

The temperature, set at 30ºC, is controlled by an external water jacket.

Illumination of the bioreactor is obtained using a radial disposition of a set of 15 halogen lamps (*SYLVANIA* Halogen –Professional de luxe-, 12V, 20W, ∅ 50mm.) distributed in 10 columns containing one or two lamps successively. Two 35V –18A power supplies provide the necessary electrical power. Changing the voltage supplied to the lamps, the light intensity is modified. A calibration of the light intensity in W/m^2 at the surface of the reactor as a function of the voltage is given in Appendix I.

Connection between compartment II and III

Due to the fact that the outlet of compartment II contains biomass, a removal step is necessary. To this purpose a centrifugation step is introduced in the liquid effluent of compartment II previous to the transfer operation to compartment III. The volumes managed during the continuous runs at bench scale, are too small to use a continuous centrifuge. Thus, a batch centrifuge (BECKMAN J2-21 M/E) (10000rpm, 4ºC, 20min) is used. As the centrifugation operation is a discontinuous process, two buffer tanks, one for the outlet of compartment II and the other for the inlet of compartment III are required. Then the collection of the liquid effluent for centrifugation and the storage of the biomass free medium after the centrifugation step are allowed. This centrifugation step is done daily as the quality of *Rhodospirillum rubrum* decreases significantly when it is stored during a longer period.

To avoid contamination of the centrifuged media, sterilization before introducing it to the input storage tank of the third compartment is required. To this purpose two filtering steps using liquid filters (MILLIPORE OPTICAP^{TM} 4") of 0.22μm are introduced.

Compartment III

Implementation of compartment III is done using a packed-bed column, as described previously in TN 37.510 (Pérez *et al.*, 1997).

The pH is controlled by an autonomous controller (CRISON pH/mV 252). Regulation of the pH at 8.4, is achieved by means of acid (CO_2) or base (Na_2CO_3 40g/L) addition. Temperature is controlled at 30ºC by an external water jacket. Aeration is done through a gas sparger. Part of the effluent of the bioreactor is recirculated in order to achieve a higher conversion. A magnetic stirrer is used to ensure the perfect mixing between the inlet and the recirculation.

Connection between compartments III and IV

These two compartments can be directly connected. However, due to the fact that the outlet of compartment III contains some biomass, two filtering steps through liquid filters (MILLIPORE OPTICAP^{TM} 4") of 0.22μm are included. One in the output of compartment III and another one at the input of compartment IV. In this way, the two compartments are isolated allowing a disconnection in case of a malfunction of one of them.

Compartment IV

The bioreactor used is a 3 L airlift with an internal glass made cylindrical part (for separating riser and downcomer sections). The introduction of a gas phase in the riser causes a difference in the medium density with respect to the downcomer, providing the internal liquid mixing of the reactor.

An autonomous controller (METTLER TOLEDO PH2100) regulates the acid (HCl 0.5M) or base (NaOH 0.75M) addition to control the pH at 9.5. The oxygen is monitored by measuring the DO (dissolved oxygen) with a polarographic oxygen probe (METTLER TOLEDO O_2 4100). Temperature is controlled at 36.5ºC by means of an external water jacket.

Illumination of the bioreactor is obtained using a radial disposition of a set of 25 halogen lamps (*SYLVANIA* Halogen –Professional de luxe-, 12V, 20W, ∅ 50mm.) distributed in 5 columns containing 5 lamps. One 15V–40A power supply provides the necessary electrical power. Changing the voltage supplied to the lamps, the light intensity is modified. A calibration of the light intensity in W/m^2 at the surface of the reactor as a function of the voltage is given in Appendix II.

2.2.- MATERIALS AND METHODS

2.2.1.- Strains and inoculum

The strain of *Rhodospirillum rubrum* used (ATCC 25903) is obtained from the American Type Culture Collection. It is revived and the subcultures are done using their recommended medium. The inoculum volume is fixed as the 10% of the working volume (2.4 L).

The strains of *Nitrosomonas europaea* and *Nitrobacter winogradsky*i used are obtained from the American Type Culture Collection: *Nitrosomonas europaea* (ATCC 19718) and *Nitrobacter winogradsky*i (ATCC 25391). A co-culture (*Nitrosomonas europaea* and *Nitrobacter winogradsky*i) obtained from the operation of a Biostat B reactor (Pérez *et al*., 1997) is used to inoculate the reactor.

The *Spirulina platensis* strain used is obtained from the Pasteur Institute*: Arthospira platensis* (PCC 8005). *S. platensis* is revived and the subcultures are done using their recommended medium. The inoculum's volume is fixed as the 10% of our working volume (2.5 L).

2.2.2.- Culture media

Connection of compartments II, III and IV

To be able to connect the three compartments a culture media containing all the necessary compounds for the growth of *Rhodospirillum rubrum*, *Nitrosomonas europaea, Nitrobacter winogradsky*i and *Spirulina platensis*, is designed (Creus *et al.*, 1999). The media used is based in a combination of the used media for each individual compartment and uses the micronutrient solutions of each individual compartment. It is described in table 2.1.

Product	**1 L**	
CH_3COONa	3.42	g
EDTA-Na·2 H_2O	0.10	g
$MnCl_2$·2 H_2O	0.01	g
$FeSO_4$·7 H_2O	0.03	g
KH_2PO_4	0.40	g
$NaHCO_3$	0.25	g
$MgSO_4$·7 H_2O	1.20	g
$CaCl_2$·2 H_2O	0.09	g
Trace element	1.00	mL
Biotin	1.00	mL
$(NH_4)_2SO_4$	2.73	g
$CuSO_4.5H_2O$	4.00E-06	g
Na_2HPO_4	0.49	g
$ZnSO_4.7H_2O$	4.30E-06	g
$(NH_4)_6Mo_7O_{27}.4H_2O$	0.18	g
K_2SO_4	0.55	g
A5	1.00	mL
B6	1.00	mL

Table 2.1. - Media composition for the connection tests of compartments II, III and IV. Where the trace element solution, biotin solution, A5 and B6 solutions can be found in tables 2.2, 2.3, 2.4 and 2.5

A5	
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 2.2.- Composition of A5 solution

B6	
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

Table 2.3.- Composition of B6 solution

Trace elements	
Compounds	*g/100mL*
Iron citrate	0.300
$MnSO_4 \cdot H_2O$	0.002
H_3BO_3	0.001
$CuSO_4 \cdot 5\ H_2O$	0.001
$(NH_4)_6MoO_{27} \cdot 4\ H_2O$	0.002
$ZnSO_4$	0.001
EDTA-Na	0.050
$CaCl_2 \cdot 2\ H_2O$	0.020

Table 2.4.- Trace elemnts solution composition

Biotin	
Compound	*g/L*
Biotin	0.015

Table 2.5.- Biotin solution composition

The most important characteristics of this media are the ammonium concentration which is set to give a 400 N-ppm ammonium concentration in the outlet of compartment II and is expected to be transformed to NO_3^- in compartment III, and the acetic concentration which is varied along the experiments. Carbon source in compartment IV is given by CO_2 bubbling.

Compartment III

The culture medium used in compartment III when disconnected from compartment II and IV is based in a mixture of an adapted medium for *Nitrosomonas europaea* (Wijffels, 1994) and an adapted medium for *Nitrobacter winogradskyi* (Hendrikus *et al.*, 1992) and (Wijffels, 1994). This medium is described in (Pérez, 1997) and is given in table 2.6.

***N. europaea* and *N.winogradskyi* medium**	
Compounds	*g/L*
$FeSO_4 \cdot 7\ H_2O$	2.5E-3
KH_2PO_4	0.68
$NaHCO_3$	0.8
$MgSO_4 \cdot 7\ H_2O$	0.052
$CaCl_2 \cdot 2\ H_2O$	7.4E-4
$(NH_4)_2SO_4$	1.32
$CuSO_4 \cdot 5\ H_2O$	4.0E-6
Na_2HPO_4	0.71

$ZnSO_4 \cdot 7\ H_2O$	4.3E-6
$(NH_4)_6Mo_7O_{27} \cdot 4H_2O$	0.177

Table 2.6.- *N. europaea* and *N. winogradskyi* medium composition

Connection between compartments III and IV

To be able to connect compartments III and IV, a media containing all the necessary compounds for the growth of *Nitrosomonas europaea, Nitrosomonas winogradsky*i and *Spirulina platensis* has to be defined. A medium based on the combination of the above-described media for each compartment has been designed and it is presented in table 2.7.

Connection III and IV medium	
Compound	*g/L medium*
EDTA-Na$\cdot 2\ H_2O$	0.08
$FeSO_4 \cdot 7\ H_2O$	0.01
KH_2PO_4	0.68
$NaHCO_3$	0.80
$MgSO_4 \cdot 7\ H_2O$	0.20
$CaCl_2 \cdot 2\ H_2O$	0.04
$(NH_4)_2SO_4$	1.32
Na_2HPO_4	0.71
$(NH_4)_6Mo_7O_{27} \cdot 4\ H_2O$	0.18
Dissolution	*mL/L medium*
A5	1.00
B6	1.00

Table 2.7.- Composition of the medium used for the connection of compartments III and IV (A5 and B6 compositions are presented in tables 2.2 and 2.3)

2.2.3.- Analytic procedures

Cell concentration

- Dry weight

S. platensis and *R. rubrum* dry weight are determined by filtering through a 0.45µm and 0.22µm respectively pre-weighted filters, dried until constant weight in a microwave (20 min., 150 W) and cooled down in a desiccator.

- Optical density

The optical density measured at 750nm and 700nm is a direct measurement respectively of the *S. platensis* and *R.rubrum* concentration. Polysaccharides do not absorb at these wavelengths. Thus, these measurements reflect only the diffusion of the

light produced by the presence of the microorganisms, fact that is directly related with biomass concentration. The spectrophotometer used is a Kontron Instrument, Uvikon 941, Italy.

Ammonium, nitrite and nitrate concentrations

- Ammonium

Ammonium was measured using UV measurement determinations by means of LCK 305 ammonium analysis kits (Dr. Lange Nitrax).

BASIS: 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 694nm.

- Nitrate

Nitrate is measured using UV measurement determinations by means of LCK 339 nitrate analysis kits (Dr. Lange Nitrax).

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.

- Nitrite

Nitrite is measured using UV measurement determinations by means of LCK 341 nitrite analysis kits (Dr. Lange Nitrax).

BASIS: Nitrite ions, in acid solutions, react with primary aromatic amines to form diazone salts which are quantified measuring the absorption at 524 nm.

Acetic concentration

Acetic acid concentration is determined either by:

- high resolution liquid chromatography (HPLC) (Hewlett Packard 1050 with an Aminex HPX-87H ionic exchange column and an HP 1047 index refraction detector.
- or gas chromatography (Hewlett Packard 5890).

PHB concentration

PHB concentration is measured following the procedure described by Comeau et al. (Comeau *et al.,* 1988) by means of gas chromatography (Hewlett Packard 5890) with an automatic capillar injector 7376A

Axenicity control

The fermentors broth and the media are checked for bacterial by microscopy (ZEISS AXIOSKOP).

3.- RESULTS AND DISCUSSION

3.1.- Effect of an acetic acid inlet in compartment III

A potential deviation from the steady state in compartment II would cause an acetic acid increase in the income of compartment III. In order to study this effect two different experiments are performed.

3.1.1.- Effect of an inlet of acetic acid in compartment III

First, different acetic acid concentrations are added in the used media of compartment III (described in section 2.2.2) and its effect in the evolution of this compartment is studied. This experiment is done with compartment III disconnected. Thus, before testing the effect of an outlet of acetic acid from compartment II in the whole connection loop, the effect of an income of acetic acid in compartment III is tested.

During this experiment compartment III is operating under the conditions described in table 3.1.

	Compartment III
T (ºC)	30
pH	8.2
V_{util} *(L)*	0.475
Q_L *(mL/min)*	0.556
[acetic acid]	0-20 mM

Table 3.1.- Summary of the operation conditions of compartment III during this experiment

The evolution of the effect of different acetic acid concentrations in the inlet of compartment III is presented in figure 3.1 a and 3.1 b.

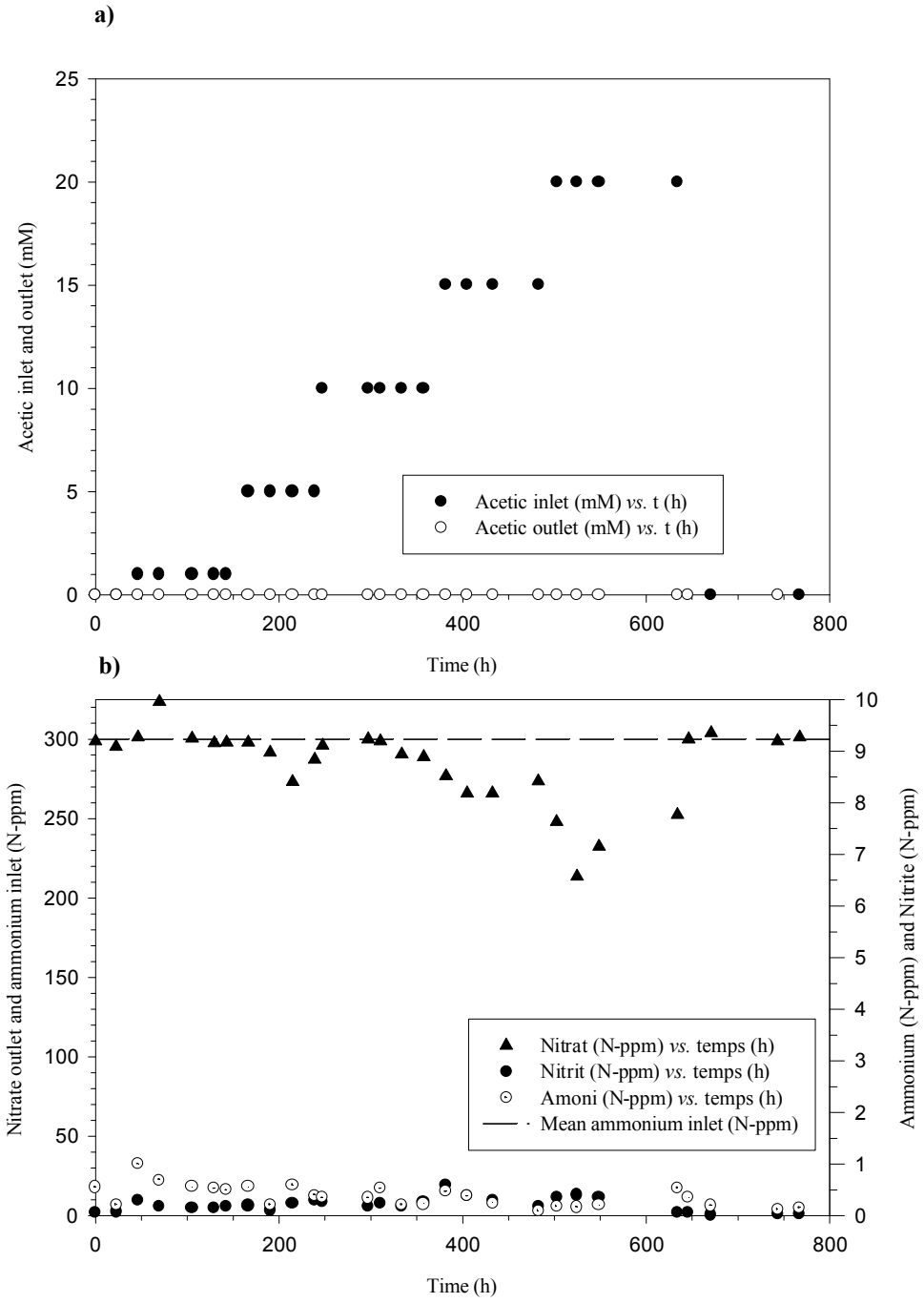

Figure 3.1.- a) Evolution of the acetic acid concentration in compartment III. **b)** Evolution of ammonium, nitrate, nitrite in the outlet of compartment III. Ammonium inlet concentration: 300N-ppm

As can be observed in figure 3.1 the satisfactory performance of compartment III is not altered by the presence of acetic acid. The ammonium conversion into nitrate is complete during this experiment, which is the objective of the *Nitrosomonas europaea* and *Nitrobacter winogradskyi* co-culture.

Non acetic acid is found in the outlet of compartment III. Thus, all the acetic acid entering in this compartment is completely consumed. It has been previously reported that *Nitrosomonas europaea* and *Nitrobacter winogradskyi* can partially use acetic acid as carbon source (Bock, 1976, Steinmüller and Bock, 1976, Smith and Hoare, 1977,

Matin, 1978, Krümmel and Harms, 1982). However, the reported amount of carbon from acetic acid that they can incorporate is quite low, being around 275 nmoles/mg increase in dry weight of the culture. Nevertheless, the tests mentioned before were performed in batch cultures and a higher incorporation of acetic acid by lithotrophic microorganisms in chemostats has been also described (Matin, 1978).

Along this experiment, 13.38 g of acetic acid (0.22 moles) have entered in compartment III. If it is assumed that all the acetic acid has been assimilated by microorganisms having a yield $Y_{X/S}$ of 0.36 g biomass/g substrate, equal to the mean biomass/substrate yield of different bacteria which use acetic acid as substrate (Abbot and Clamen, 1973), 4.82 g of biomass would be formed. Fact that would mean a production of 0.198 g of biomass per day or 0.8 g/L. This biomass production seems to be quite significant if compared to previous data. After a year and a half of functioning of a column exactly the same as the one used in this experiment, the column had 44 g biomass / L_{bed} (Pérez, 2000) which supposes a biomass production of 0.7 g/month, being the bed volume 0.286 L. This production is only one seventh of the production obtained in this experiment. However, the fact that in this experiment acetic acid was present and, thus, could be use as carbon source, has to be taken into account.

The decrease of the nitrate outlet concentration when high acetic acid concentrations are consumed, a gap of 50 N-ppm is found in the N balance as can be seen in figure 3.1b, can be also due to the biomass assimilation of this compound.

3.1.2.- Effect of an outlet of acetic acid in compartment II in the connection of the three compartments.

Once known that the normal operation of compartment III is not disturbed by the presence of acetic acid, the effects of an outlet of acetic acid in compartment II are tested in the whole connection. To obtain this behavior, changes in the dilution rate or decreases in the incident light of compartment II, are performed.

Table 3.2 presents the operational conditions used in the first test.

	Compartment II					***Comp. III***	***Comp. IV***
	I	**II**	**III**	**IV**	**V**		
Light (W/m^2)	136	92	59	34	18.1	0	20
D (h^{-1})	0.08	0.08	0.08	0.08	0.08	0.07	0.017
Q_L (mL/h)	200	200	200	200	200	33.33	41.6 (*)
i (days)	0.52	0.52	0.52	0.52	0.52	0.59	2.5
C_0 (gC/L)	0.5	0.5	0.5	0.5	0.5		

Table 3.2.- Operational conditions of experiment I. (*) This flow rate is bigger than the flow rate in compartment III due to base addition

Figures 3.2, 3.3 and 3.4 present the evolution of the three compartments along these changes.

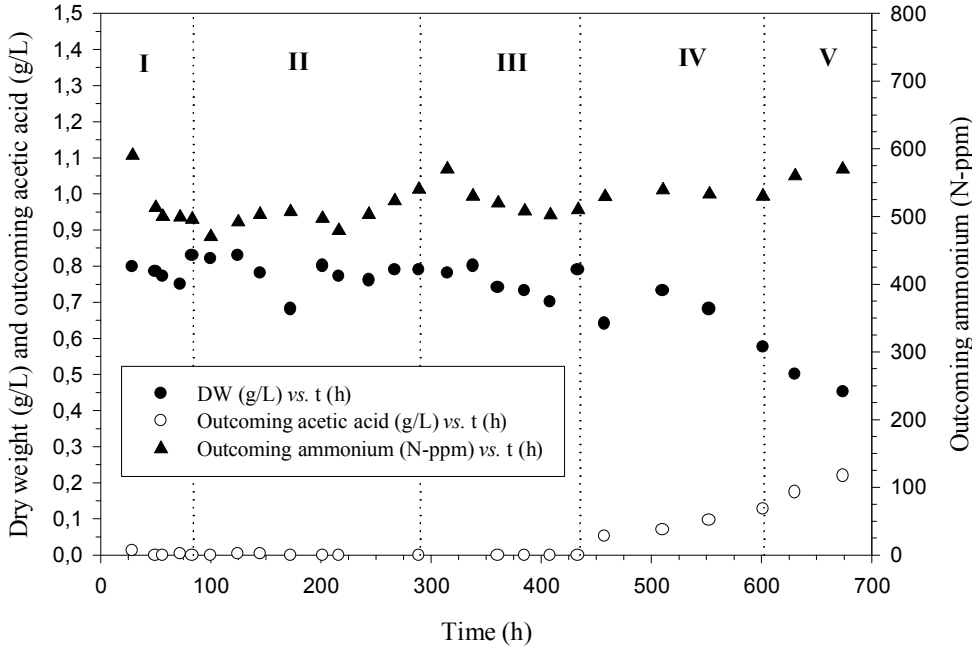

Figure 3.2.- Evolution of compartment II

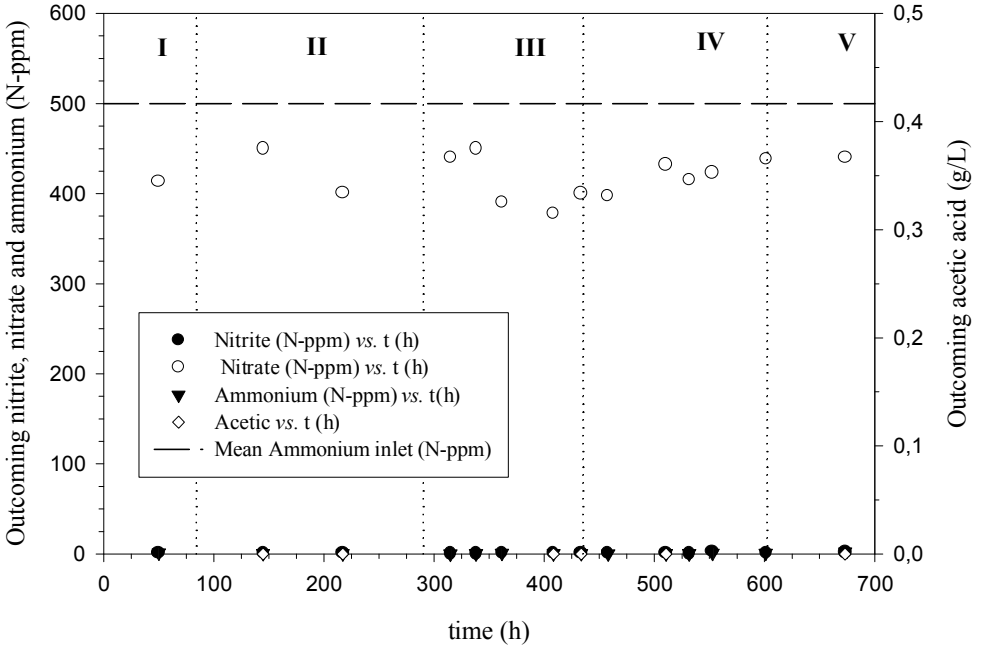

Figure 3.3.- Evolution of compartment III

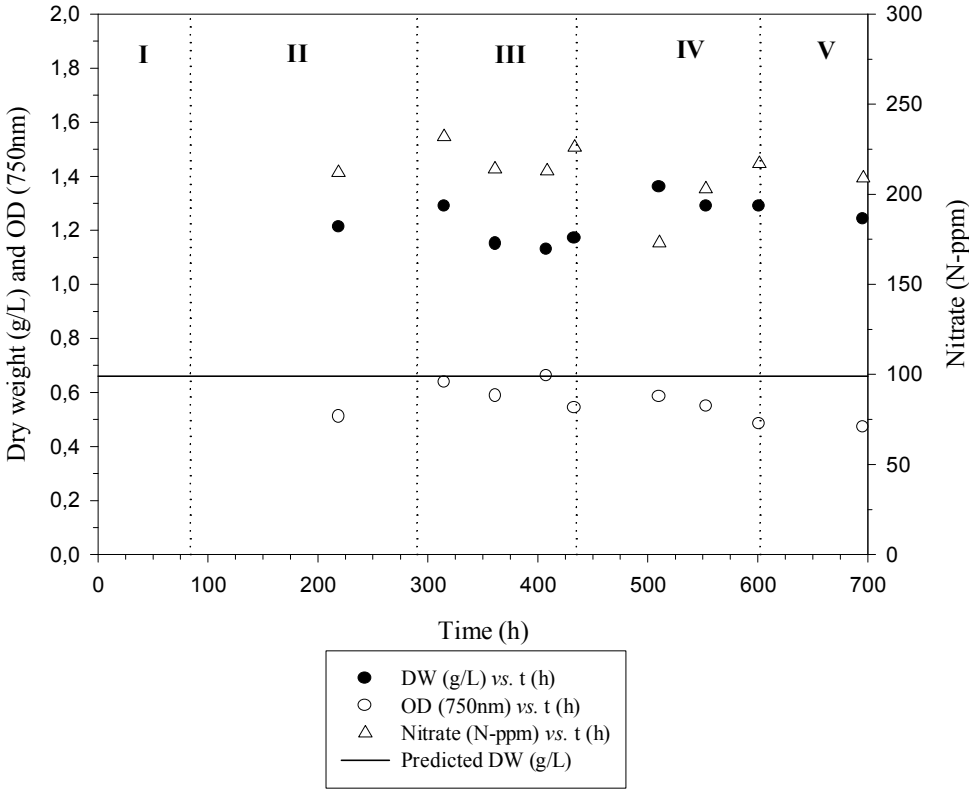

Figure 3.4.- Evolution of compartment IV

After having observed the evolution of compartment II at D=0.08 h^{-1} , the D was increased in the following test. Table 3.3 presents the operational conditions used in the second experiment.

	Compartment II		***Comp. III***	***Comp. IV***
	I	**II**		
Light (W/m^2)	260	260	0	20
D (h^{-1})	0.08	0.12	0.07	0.017
Q_L (mL/h)	200	288	33.3	41.6 (*)
i (days)	0.52	0.35	0.59	2.5
C_0 (gC/L)	0.5	0.5		

Table 3.3.- Operational conditions of experiment I. (*) This flow rate is bigger than the flow rate in compartment III due to base addition

Figures 3.5, 3.6 and 3.7 present the evolution of the three compartments along these changes.

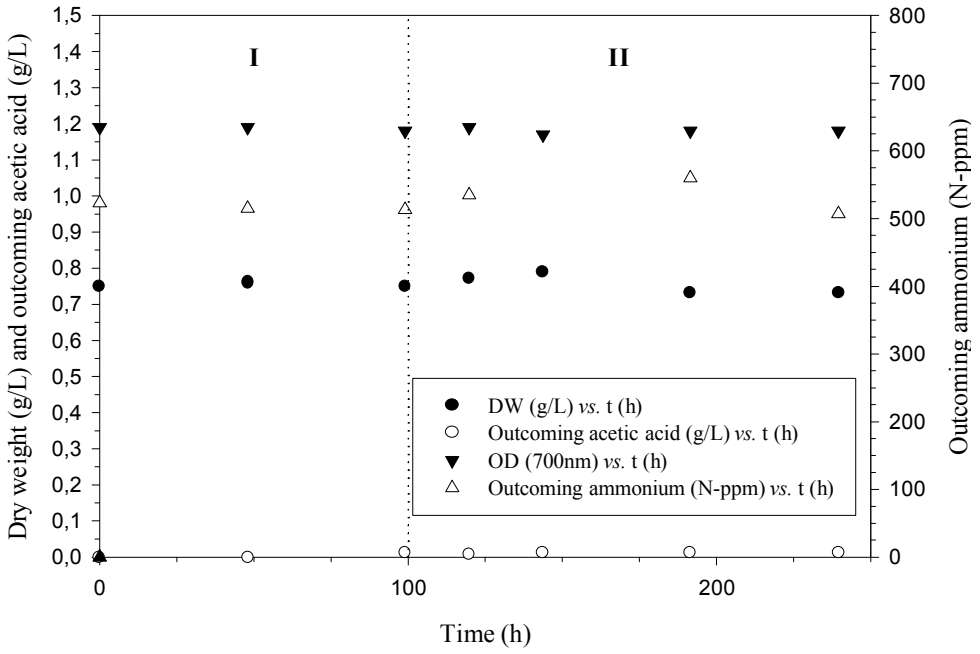

Figure 3.5.- Evolution of compartment II

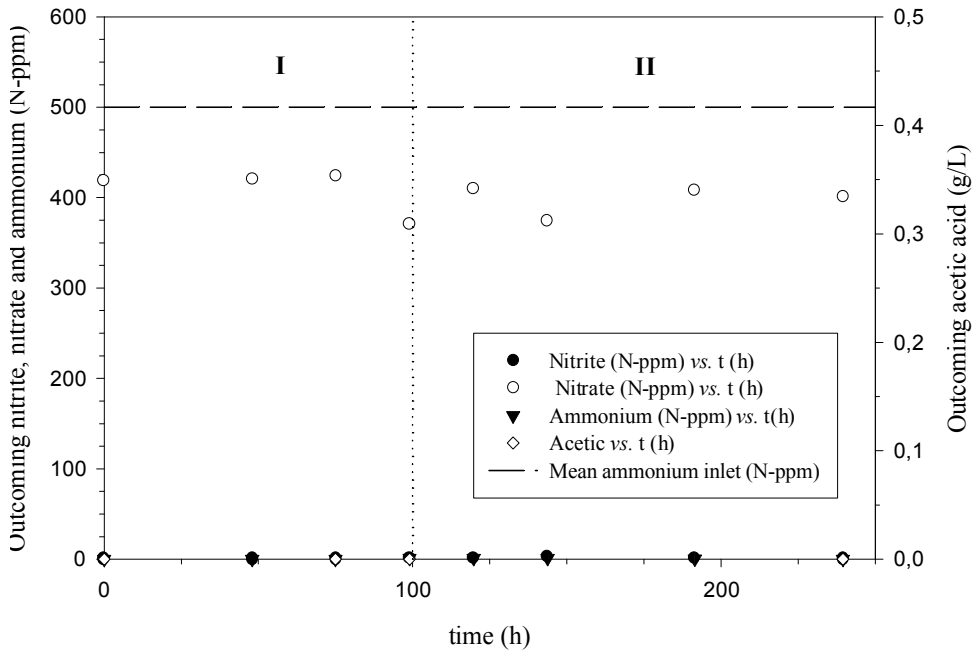

Figure 3.6.- Evolution of compartment III

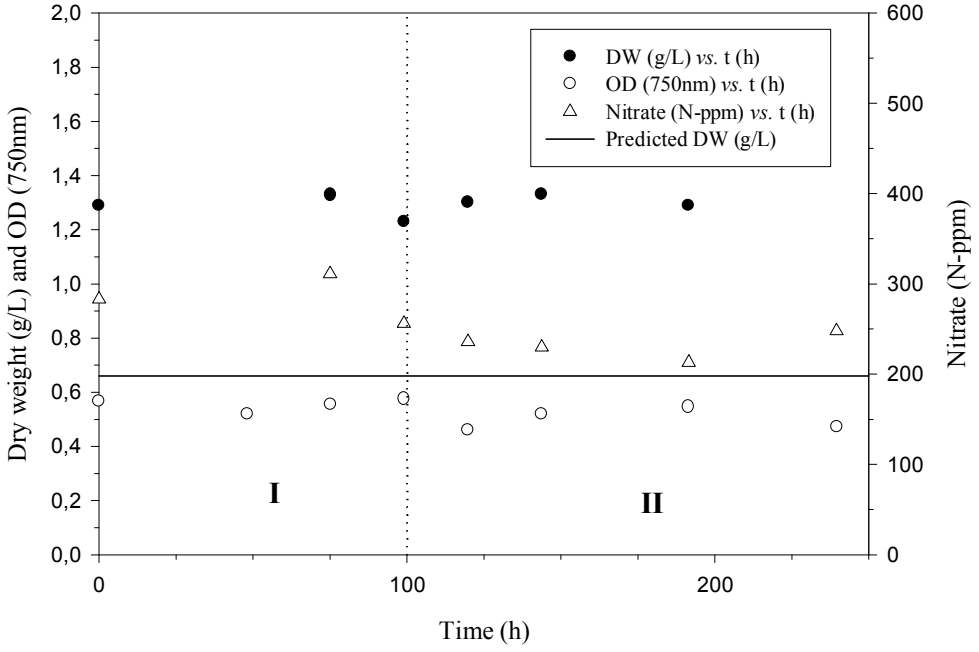

Figure 3.7.- Evolution of compartment IV

At D=0.12 h^{-1}, almost no acetic acid is found in the outlet but some cell attachment problem occurred. Thus the initial carbon concentration is decreased in the following test. Table 3.4 presents the operational conditions used in the third experiment.

	Compartment II							*Comp. III*	*Comp. IV*
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**		
Light (W/m^2)	260	224	192	138	92	58	14.7	0	20
D (h^{-1})	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.07	0.017
Q_L (mL/h)	288	288	288	288	288	288	288	33.33	41.6 (*)
i (days)	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.59	2.5
C_0 (gC/L)	0.4	0.4	0.4	0.4	0.4	0.4	0.4		

Table 3.4.- Operational conditions of experiment I. (*) This flow rate is bigger than the flow rate in compartment III due to base addition

Figures 3.8, 3.9 and 3.10 present the evolution of the three compartments along these changes.

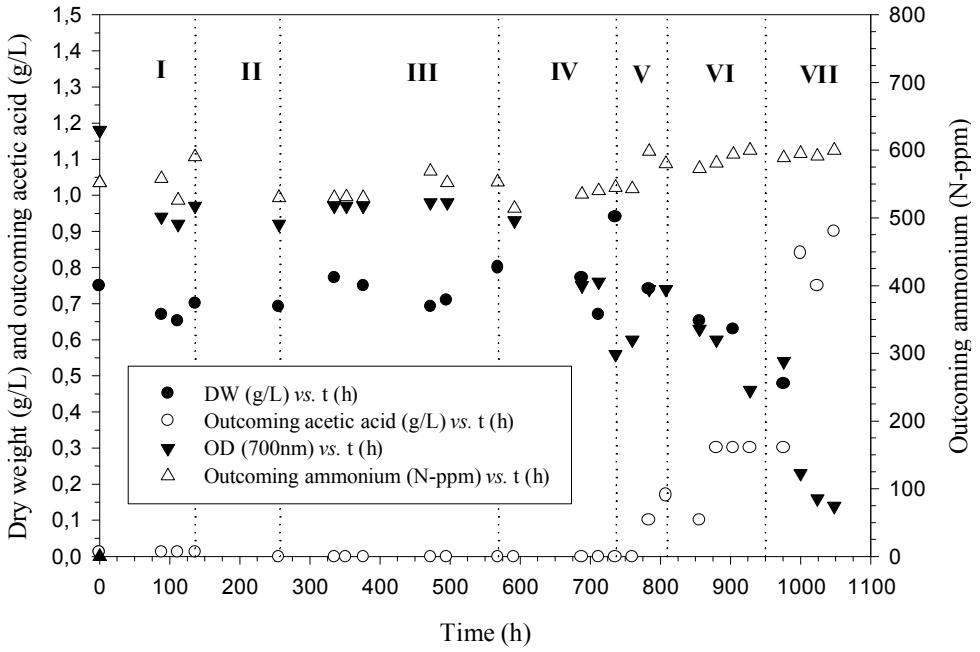

Figure 3.8.- Evolution of compartment II

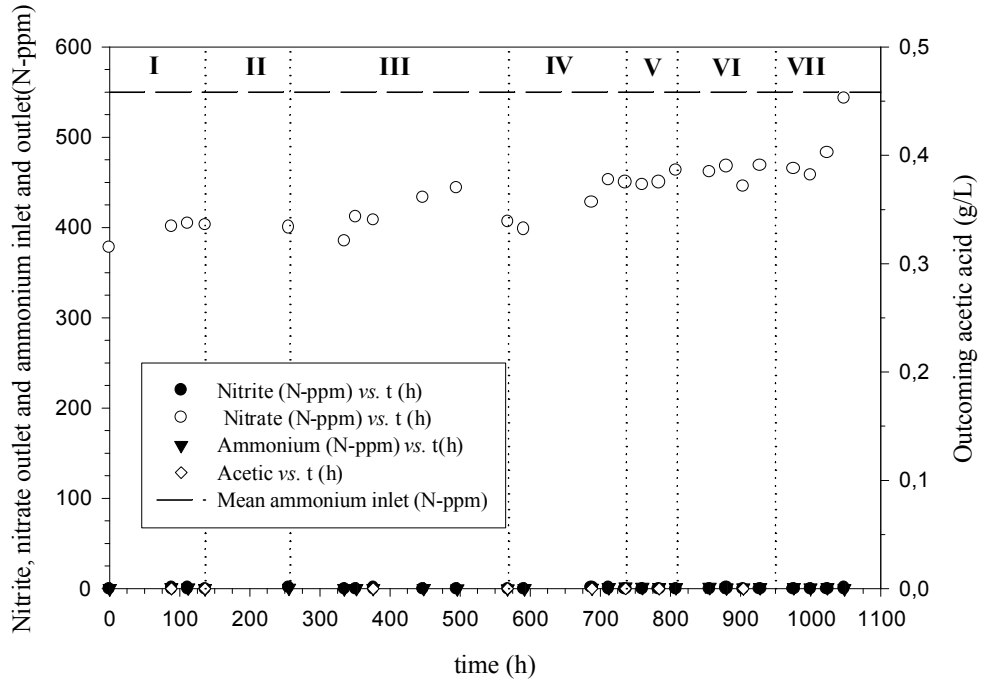

Figure 3.9.- Evolution of compartment III

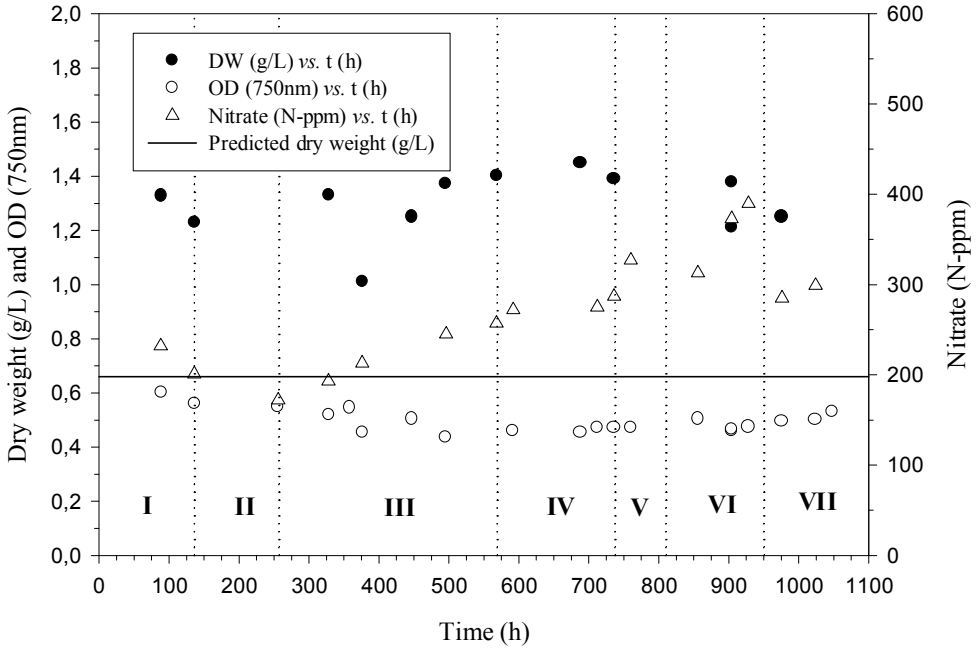

Figure 3.10.- Evolution of compartment IV

As expected, when a step up in the dilution rate or a step down in the light intensity is made, acetic acid is found in excess in compartment II and enters in compartment III. The evolution of the three compartments under these conditions is discussed in this section.

As it has been seen in section 3.1.1 and it is observed in figures 3.3, 3.6 and 3.9 compartment III is not disturbed by the presence of acetic acid. Thus, all the ammonium is converted to nitrate. No acetic acid is found in the outlet of this compartment, being

all the acetic acid entering in this compartment completely consumed. Fact that has been already discussed in section 3.1.1. As can be seen in figures 3.2, 3.6 and 3.9 the nitrogen balance is not closed. There is always a gap of about 50 N-ppm between the ammonium (N-ppm) outcoming from compartment II and incoming in compartment III and the nitrate (N-ppm) outcoming from compartment III. Possible explanations for these results are a possible ammonium loose during the centrifugation and filtration steps which are found between compartments II and III in order to separate the biomass from the medium, some ammonium can be also assimilated by the biomass and some ammonium can be also washed out from the culture through the gas phase due to its conversion to ammonia at a high pH.

Compartment IV has a complete stable situation during all the different experiments, as can be seen in figures 3.4, 3.7 and 3.10. No ammonium, neither nitrite, neither acetic are entering in this compartment. Thus, it is working at its optimal conditions. As can be seen in the graphics, the experimental dry weight does not match with the predicted one by PHOTOSIM 2 (Cornet, 1993). This fact can be due to the difficulty in calculating the average light intensity supplied to this compartment as it has two different radiuses and also to the high exopolysaccharide production observed, that can affect the dry weight measurements.

Figures 3.2, 3.5 and 3.8 present the evolution of compartment II. When the steps up in the dilution rate or the steps down in the light intensity are done compartment II behaves as usual, all acetic acid is consumed and R. *rubrum* moves in the fermentor broth, until acetic acid is present in excess, then *R. rubrum* cells attached to the solid surfaces. As graphics show, when there is no acetic acid in the outlet of this compartment all the incoming acetic acid is assimilated to biomass and thus, biomass concentration is maintained constant. Once some acetic acid is left, the biomass concentration decreases, *R. rubrum* changes its morphology, this fact is observed in figure 3.11, and no steady states can be reached. Even keeping the same conditions, more and more acetic acid is left and cells are washed-out.

.

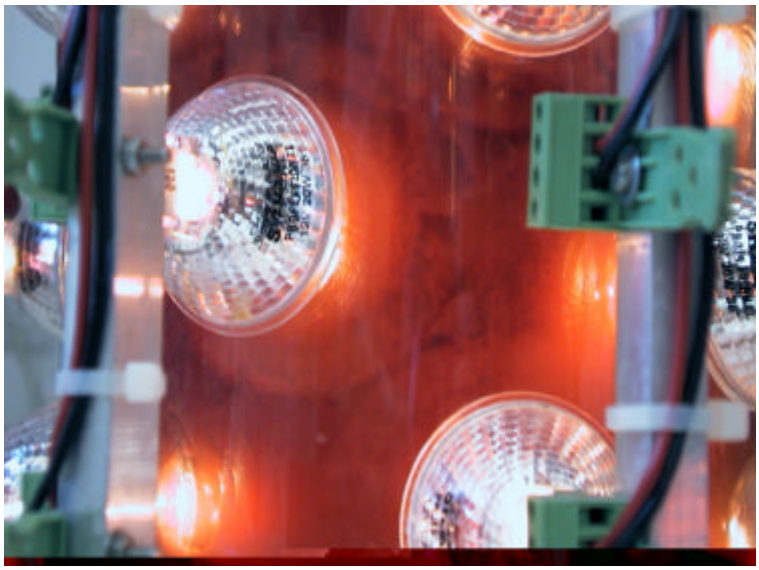

Figure 3.11.- Cell attachment to the walls when acetic acid is found in excess in compartment II

The evolution of *R. rubrum* during these experiments is followed by microscopy. Figures 3.12 and 3.13 show the morphology of R. *rubrum* when acetic acid is the limiting factor of compartment II and when it is found in excess.

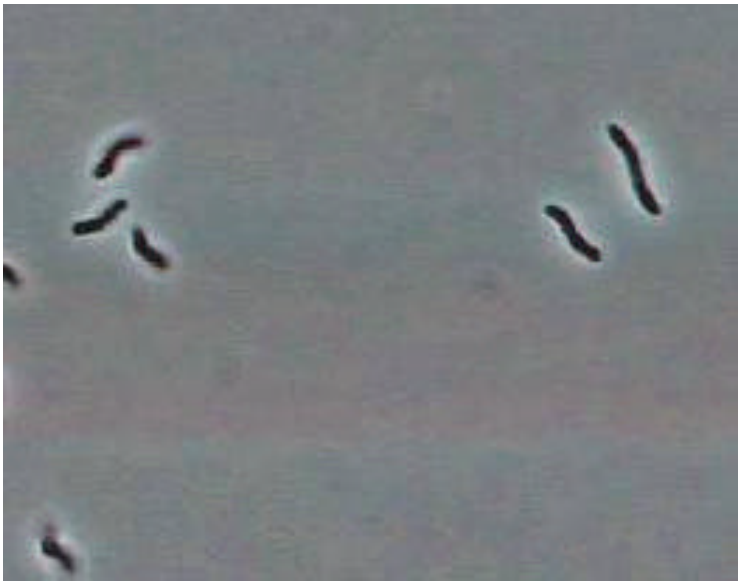

Figure 3.12.- Morphology of *R. rubrum* when acetic acid is the limiting factor

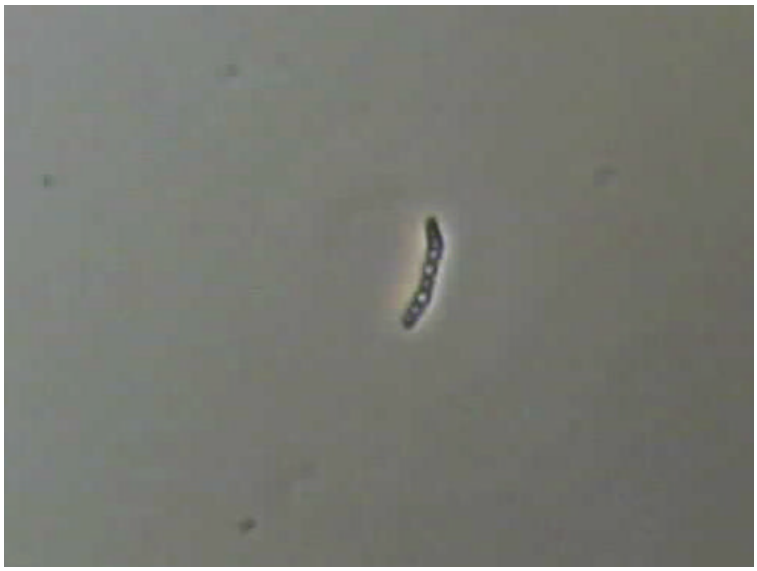

Figure 3.13.- Morphology of *R. rubrum* when acetic acid is found in excess

As it is seen in figures 3.12 and 3.13 when the acetic acid concentration increases in compartment II, *R. rubrum* accumulates intracellular granules, loosing its mobility, attaching to the solid surfaces and having cell division problems.

Two different reserve polymers: glycogen and poly-hydroxybutyrate (PHB), can be accumulated by *R. rubrum*. Its synthesis basically depends on the carbon source. The major carbon sources leading to a glycogen accumulation are pyruvate, malate, succinate and lactate and the major carbon sources leading to a PHB accumulation are acetic and butyric acids (Merrick, 1978). Taking into account that in these experiments

acetic acid is the only carbon source used, accumulation of PHB is quite probable. Thus, PHB analyses of the biomass are done in each steady state. These results are presented in table 3.5.

D (h^{-1})	F_R(W/m^2)	C_o (g/L)	PHB (%)	Std dev.
Experiment I				
0.08	136	0.5	0.94	0.145
	92		0.98	0.204
	59		6.37	1.275
	34		5.37	0.502
	18.1		10.50	1.045
Experiment II				
0.08	260	0.5	0.756	0.274
0.12			4.51	0.099
Experiment III				
0.12	260	0.4	1.85	0.175
	224		6.24	0.076
	192		9.80	0.472
	138		4.52	0.184
	92		8.84	0.092
	58		13.81	1.847

Table 3.5.- PHB concentration of the biomass in each steady state

In table 3.5 a PHB accumulation when a light step down or a dilution rate step up is done, is observed. The only point that does not follow this tendency is when, having a dilution rate of 0.12 h^{-1}, a step down in the light intensity from 192W/m^2 to 138 W/m^2 is produced. Fact that can be explained taking into account that the inlet media had contamination problems and had to be changed. Thus, the reactor was left in batch mode for a short period. This fact also shows that the PHB accumulation is reversible. When no more acetic is incoming in compartment II, *R. rubrum* uses the accumulated PHB.

3.2.- Effect of an ammonium and nitrite inlet in compartment IV

A potential deviation from the steady state or a decrease in the oxygen concentration in compartment III would cause an ammonium and nitrite inlet increase in the income of compartment IV. In order to study this effect two different experiments are performed.

3.2.1.- Effect of an inlet of ammonium and nitrite in compartment IV having compartments III and IV interconnected

As compartment II was being used for other purposes, the firsts tests of an inlet of ammonium and nitrite in compartment IV are performed only with compartments III and IV interconnected and using the media described in section 2.2.2.

In order to evaluate the inlet of ammonium and nitrite in compartment IV a step up in the dilution rate is done. The operational conditions of both reactors during this experiment are found in table 3.6.

	Conditions 1		**Conditions 2**	
	Compartment III	*Compartment IV*	*Compartment III*	*Compartment IV*
T (ºC)	30	36.5	30	36.5
pH	8.2	9.5	8.2	9.5
V_{liquid} *(L)*	0.475	2.5	0.475	2.5
Light (W/m^2)	0 (photoinhibited)	11	0 (photoinhibited)	11
t (days)	0.79	4.13	0.41	2.17
D (h^{-1})	0.053	0.01	0.1011	0.0192
Q_L *(mL/min)*	0.42	0.42	0.8	0.8

Table 3.6.- Summary of the operation conditions of compartment III and IV during this experiment

The evolution of compartments III and IV along this change are presented in figures 3.14 and 3.15.

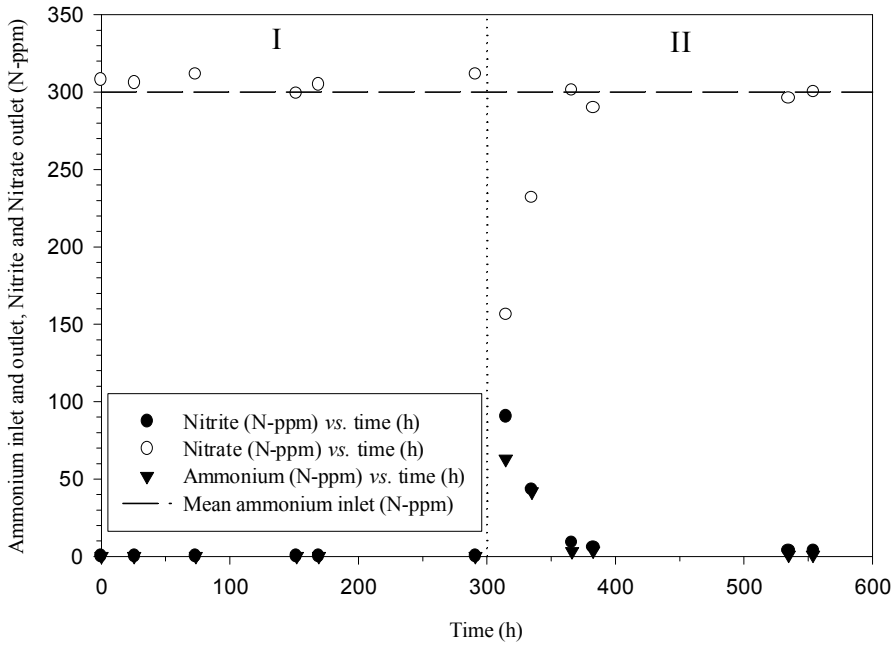

Figure 3.14.- Evolution of compartment III along the time (h) when the step up in the dilution rate is done.

As it is seen in figure 3.14 when the step up in the dilution rate is done, this compartment is not able to degrade all the ammonium to nitrate and, thus, ammonium

and nitrite enter in compartment IV. This situation is reversed in about 72 h, and then the column has created enough biofilm to convert all the entering ammonium to nitrate.

Previously published data indicate that even though nitrates are the main nitrogen source for *S. platensis*, ammonium and nitrite can also be assimilated (Ciferri, 1983; Richmond, 1986; Becker, 1994). When *S. platensis* cells grow using nitrate as the nitrogen source, its growth rate is not significantly decreased until a high nitrate concentration, of about 16800 N-ppm (Ciferri, 1983), is reached. Ammonium can also be assimilated if its concentration does not exceed 100 N-ppm (Richmond, 1986). It has been observed that ammonium is first consumed when nitrate and ammonium are simultaneously used (Guerrero and Lara, 1987). Nitrite is also assimilated even though it is toxic at high concentrations (Becker, 1994), although the toxic limit concentration is not clearly defined in the literature. In a culture using both nitrate and nitrite as nitrogen sources, a toxic effect when the nitrite concentration was over 18 N-ppm has been reported (Soyer, 1992). Nevertheless, in another work where the growth rate of *S. platensis* cells was studied, using different nitrogen sources, similar growth rates were obtained using concentration values of 28 N-ppm of nitrate or nitrite (Filali and Dubertret, 1996).

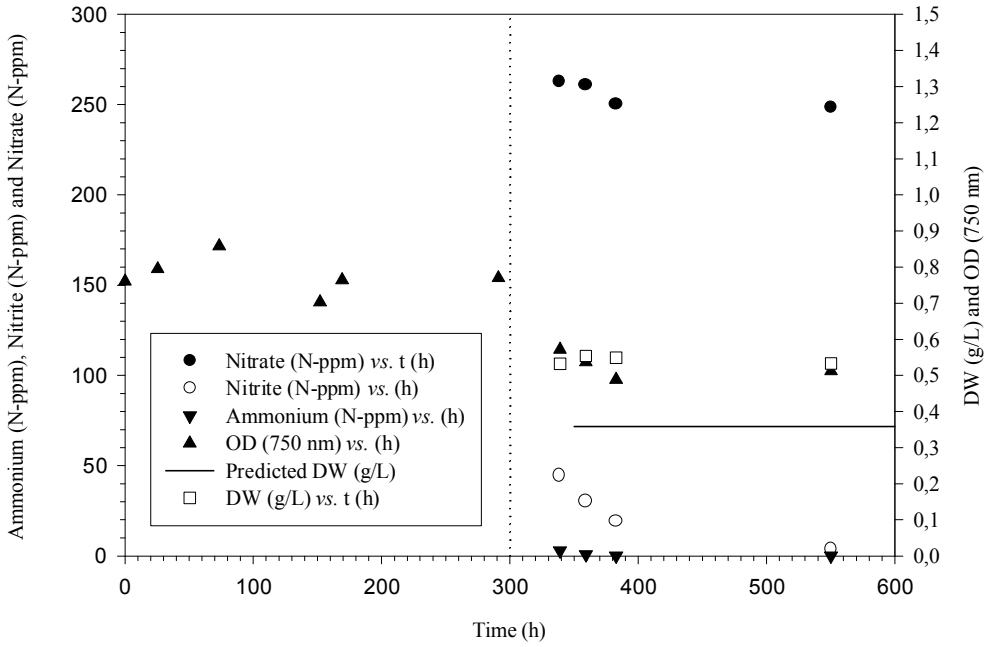

Figure 3.15.- Evolution of compartment IV along the time (h) when the step up in the dilution rate is done.

In the evolution of compartment IV, a decrease in the biomass concentration is observed and a new steady state is reached. This change is due to the step up in the dilution rate. If this biomass decrease was related to the presence of nitrite, it should be partially recovered when no more nitrite is present in compartment IV, and, this is not observed. Thus, no toxic effect is observed when nitrite is present in these concentrations in compartment IV. As can be seen in the figure, the experimental dry

weight does not match with the predicted one by PHOTOSIM 2 (Cornet, 1993). This fact has been also observed in chapter 3.1.2.

It can also be observed that ammonium is first uptaken when having ammonium, nitrate and nitrite as nitrogen sources. Immediate ammonium assimilation in compartment IV is shown in figure 3.15. Having an inlet ammonium peak similar to the nitrite peak, no ammonium is left in the outlet of compartment IV. Thus, ammonium is consumed in this compartment. An increase in the nitrate consumption in compartment IV is also observed when a decrease in the ammonium inlet is produced. This fact is explained by the lower ammonium availability. Therefore, the preference of *S. platensis* cells in consuming ammonium in front of nitrate is demonstrated. However, ammonium is also partially washed-out from the fermentor through the gas phase due to its conversion to ammonia at high pH. Higher nitrite concentrations than the above mentioned toxic levels, taken from literature (Soyer, 1992; Filiali and Dubertret, 1996), are found not to be toxic in compartment IV. In order to see whether nitrite is partially consumed in compartment IV, the experimental nitrite concentrations in compartment IV are compared to the theoretical wash-out curve of nitrite in this compartment as it is seen in figure 3.16.

To calculate the nitrite wash-out curve, the nitrite inlet concentration in compartment IV is not considered as it can be neglected if compared with the existent nitrite concentration in compartment IV at that moment. Making this assumption and considering the air-lift reactor perfectly mixed, the nitrite wash-out curve is described by equation 3.1.

$$\left[NO_2^-\right] = C_0 \exp^{-(t-t_0)/t} \qquad (3.1)$$

where: $[NO_2^-]$ is the nitrite concentration (N-ppm)
t_0 is the initial time (h)
t is the time (h)
ι is the residential time (h)
C_0 is the initial concentration (N-ppm)

Substituting the operational conditions of compartment IV in equation 3.1 equation 3.2 is obtained.

$$\left[nitrit\right] = 29839.85 \exp^{-0.0192\ t} \qquad (3.2)$$

where the nitrite concentration is expressed in N-ppm and the time in hours.

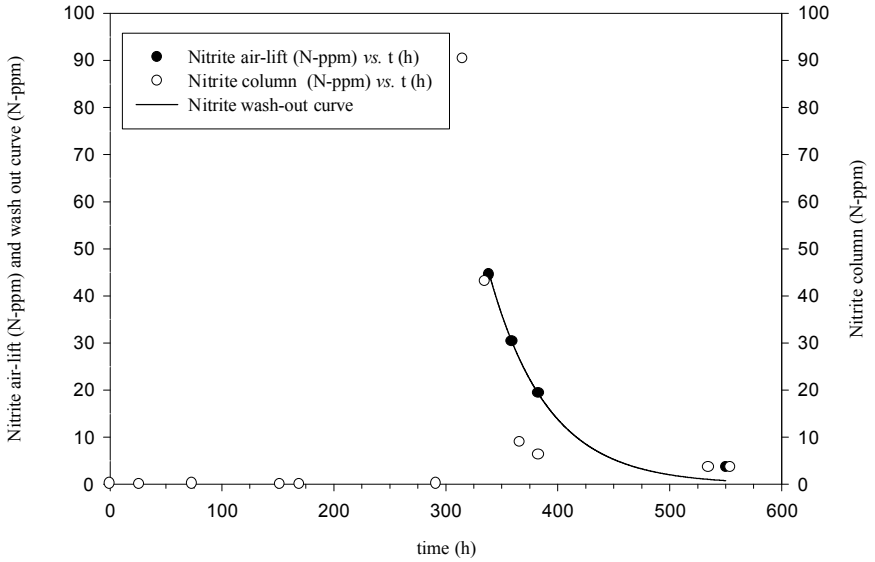

Figure 16.- Nitrite evolution in the outlet and inlet of compartments III and IV respectively and theoretical nitrite wash-out curve in compartment IV

As it is shown in figure 16 the evolution of the nitrite concentration in compartment IV is entirely in agreement with the nitrite wash-out curve. Therefore, it can be concluded that when having in the fermentor an excess of ammonium or nitrate, nitrite is not consumed and it is washed out from the bioreactor. This fact will have to be taken into account when the use the out coming water from this compartment will be considered for watering the plants of the higher plant compartment.

3.2.2.- Effect of an inlet of ammonium and nitrite in compartment IV due to a decrease in the oxygen concentration of compartment III, having compartments II, III and IV interconnected.

The operational conditions of this experiment are presented in table 3.7. The three reactors are interconnected and the aeration flow rate of compartment III is decreased.

	Compartment II	***Compartment III***			***Compartment IV***
		I	**II**	**III**	
Light (W/m^2)	260	0	0	0	20
D (h^{-1})	0.04	0.07	0.07	0.07	0.017
Q_L (mL/h)	96	33.33	33.33	33.33	41.6
t (days)	1.04	0.59	0.59	0.59	2.5
Aereation (mL/min)	0	≈20	≈10	75	1200

Table 3.7.- Summary of the operation conditions of compartment II, III and IV during this experiment. (*) This flow rate is bigger than the flow rate in compartment III due to base addition

The evolution of the connection during this experiment is presented in figures 3.17, 3.18 and 3.19.

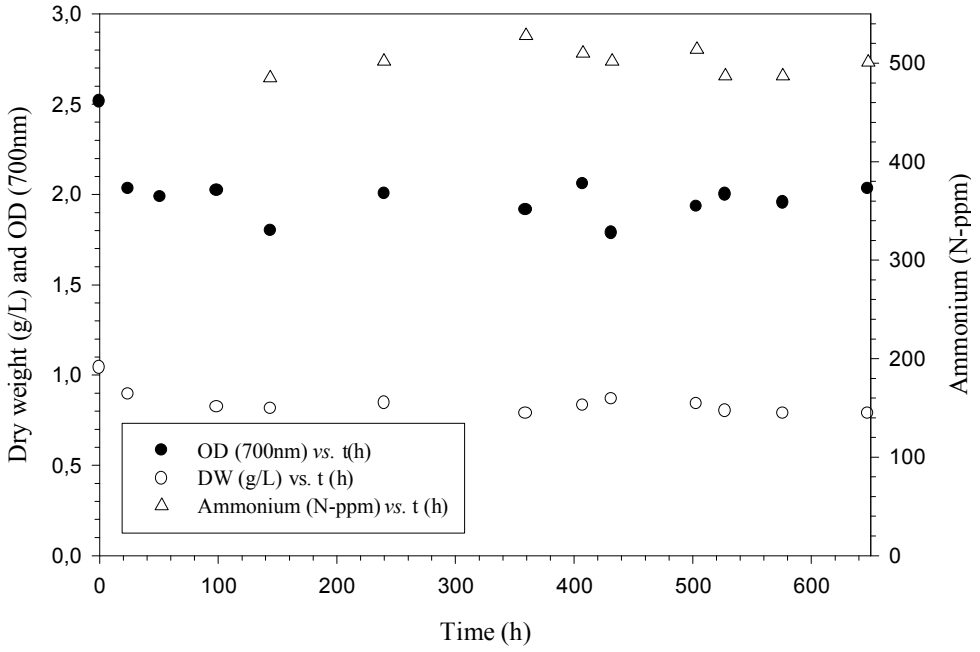

Figure 3.17.- Evolution of compartment II

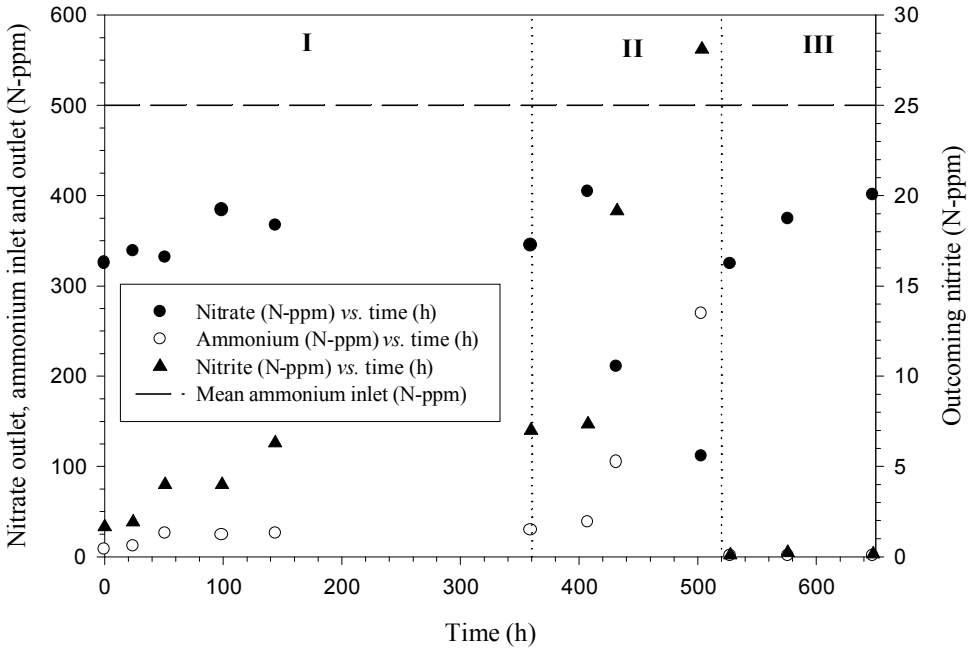

Figure 3.18.- Evolution of compartment III

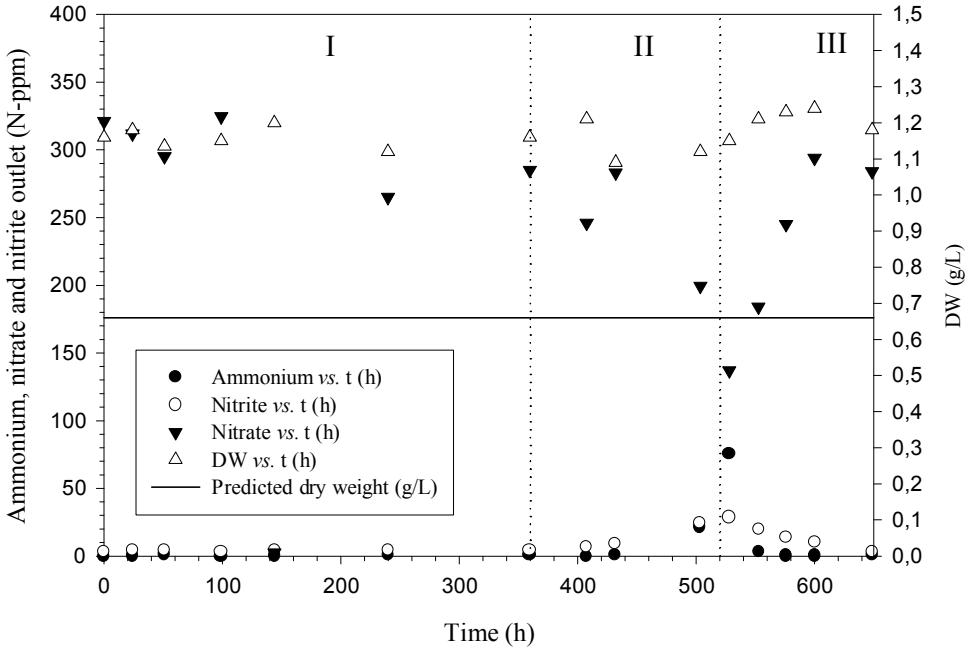

Figure 3.19.- Evolution of compartment IV

As expected and can be seen in figure 3.17, compartment II is completely stable during all experiment. No changes are done in the operational conditions of this compartment and thus no change is appreciated.

When the oxygen is limiting in compartment III ammonium is not completely converted to nitrate. Then nitrite and ammonium enter in compartment IV. This fact can be seen in figure 3.18.

As noticed in section 3.2.1, the preference to consume ammonium in front of nitrate and nitrite is also observed in figure 3.19. There is an ammonium pick even higher than the nitrite pick and almost no ammonium is found in the outlet. Ammonium is only found in the outlet of this compartment when it is present in excess and biomass does not need to assimilate any more nitrogen.

The non consumption of nitrite is also observed in figure 3.19. All nitrite entering in this compartment is found in the outlet. Due to the new operation conditions established in compartment III (compartment III has enough oxygen to degrade all the incoming ammonium) no more nitrite is entering in compartment IV. In figure 3.20 the nitrite wash-out curve is compared with the nitrite concentration decrease obsreved in this compartment. The nitrite wash-out is obtained by substituting the present operational conditions in equation 3.1.

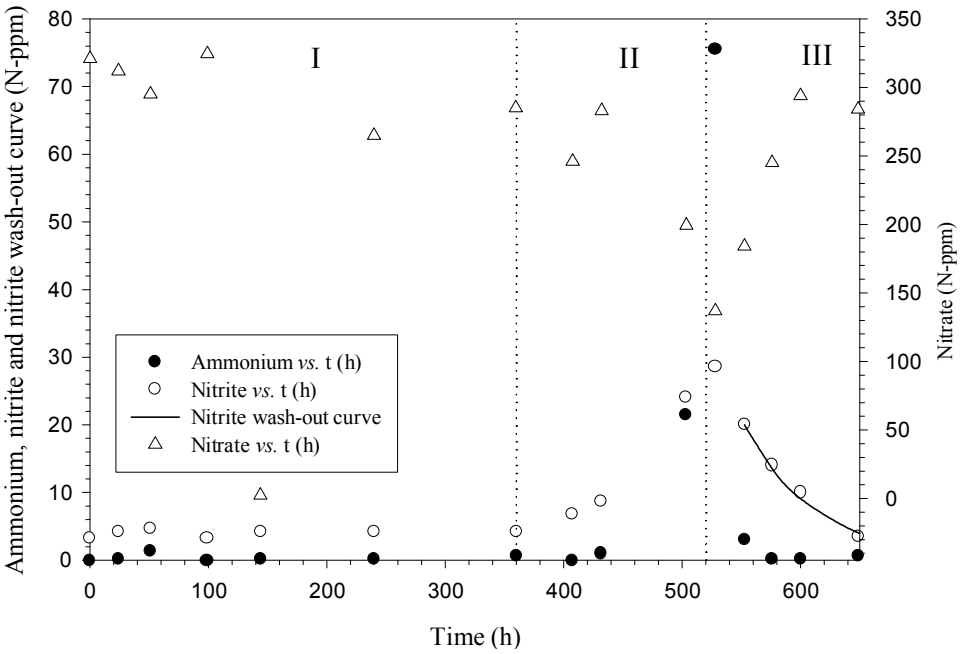

Figure 3.20.- Nitrite concentration compared to the nitrite wash-out curve in compartment IV

As it is observed in figure 3.20 the nitrite wash-out curve completely matches with the nitrite experimental concentrations. Thus, nitrite is wash-out of the fermentor.

4.- CONCLUSIONS

In this work:

- The effect of an excess of acetic acid in the outlet of compartment II has been tested concluding that:
  1. When acetic acid is found to be in excess in compartment II, *R. rubrum* accumulates PHB, looses its mobility attaching to any solid surface and presents cell division difficulties. These facts can lead to a wash out of the fermentor. Thus, in order to have a properly operation of the system non acetic acid has to be in excess in compartment II and, if it is, the situation has to be immediately reverted leading to a lower acetic acid concentration in this compartment. These facts will have to be taken into account in the modelization of this compartment.
  2. The presence of acetic acid in compartment III has not disturbed the operation of this compartment; all the income ammonium has been converted to nitrate.

- Increasing the dilution rate in compartment III, the ammonium and nitrite inlet in compartment IV has been studied. Concluding that:
  1. When having ammonium, nitrate and nitrite *S. platensis* preferably consumes first ammonium and then nitrate.
  2. Thus, Nitrite is found in the outlet of compartment IV. This fact has to be considered in case the outcoming water has to be used in, for example, compartment I or watering the HPC.

REFERENCES

Abbot, B. J. and Clamen, A. (1973) The relationship of substrate, growth rate and maintenance coefficient to single cell protein production. *Biotechnol. Bioeng.* **15**, 117.

Becker, E. W. (1994) Microalgae: biotechnology and microbiology. *Ed. Cambridge University Press*. Cambridge.

Bock, E. (1976) Growth of *Nitrobacter* in the presence of organic matter: II Chemoorganotrophic growth of *Nitrobacter agilis*. *Arch. Microbiol*. **108**, 305-312.

Creus, N., Albiol, J. and Gòdia, F. (1999) Preliminary connection between 3 compartments. Technical Note 43.8. ESTEC/CONTRACT11549/95/NL/FG.

Ciferri, O. (1983) *Spirulina*, the Edible Microorganism. *Microbiological Rev.* **47**, 551-578.

Comeau, Y., Kenneth, J.H. and Oldham W.K. (1988) Determination of Poly-β-Hydroxybutyrate and Poly-β-Hydroxyvalerate in Activated Sludge by Gas-Liquid Chromatography. *Applied and Environmental Microbiology*. **54**, 23325-2327.

Filali, R. and Dubertret, G. (1996) Growth capability of *Spirulina* on several nitrogen sources in the photosynthetic compartment of MELISSA. Technical Note 33.2. MELISSA. ESA-ESTEC/UNIVERSITE PARIS-SUD. *Res.* **9**, 865-871.

Guerrero, M. G. and Lara, C. (1987) Assimilation of inorganic nitrogen. In: The cyanobacteria. *Ed. Elsevier Science Publisher*. Amsterdam.

Hendrikus, J., Laambroek, H. J. and Gerards, S. (1992) Competition for limiting amounts of oxygen between *Nitrosomonas europaea* and *Nitrobacter winogradskyi* grown in mixed cultures. *Microbiology* **159**, 453-459.

Krümmel, A. and Harms, A. (1982) Effect of organicmatter on growth and cell yield of ammonia-oxidizing bacteria. *Arch. Microbiol*. **133**, 50-54.

Matin, A. (1978) Organic nutrition of chemolithotrophic bacteria. *Ann. Rev. Microbiol.* **32**, 436-468

Merrick, J.M. (1978) Metabolism of reserve material. In "The photosynthethic bacteria". *Ed. Clayton R. K. and Sistrom W.R. Plenum Press.* 199-219.

Pérez, J. (1997) Caracterización y puesta a punto de reactores de lecho fijo para su aplicación en procesos de nitrificación. Magister tesis. Universitat Autònoma de Barcelona.

Pérez, J., Montesinos, J.L. and Gòdia F. (1997) Operation of the bench nitrifying reactors. Technical Note 37.510. ESTEC CONTRACT 11549/95/NL/FG.

Pérez, J. (2000). Personal comunication.

Richmond, A. (1986) Principles of pond maintenance. In "Handbook of Microalgal Mass Cultures". *Ed. CRC Press*. Boca Raton.

Smith, A.J. and Hoare, D.S. (1977) Specialist phototrophs, litotrophs and methylotrophs: a unity among a diversity of prokaryotes? *Bacteriol. Rev.* **41**, 419-448.

Soyer, C. (1992) Étude d'une culture de *Spirulina platensis* sur un effluent du compartiment nitrifiant. Mémoire de DESS de microbiologie appliqueé et génie biologique. Université Paris VI.

Steinmüller, W. And Bock, E. (1976) Growth of *Nitrobacter* in the presence of organic matter: I. Mixotrophic growth. *Arch. Microbiol.* **108**, 299-304.

Wijffels, R. H. (1994) Nitrification by immobilized cells. Landbouwuniversitet Wageningen. PhD thesis.

APPENDIX I. -LIGHT CALIBRATION OF COMPARTMENT II

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 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:

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

Where: Fr is the light flux at the bioreactor's surface, Eb is the light intensity measured by the sensor, rb is the sensor's radius (30mm) and Rb is the bioreactor's radius (64mm).

The available light measured by the sensor is given in μmols/m^2s^2. For the application of the previously developed light transfer mathematical models, it is necessary to convert the units of the sensor to W/m^2. The conversion coefficient used is 0.425, which has been previously calculated by J.F.Cornet by integration of the used lamps spectra in the range 350–950nm used by *Rhodospirillum rubrum.*

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 and at different voltages supplied to the lamps. The results of these measurements are plotted in figure I.1.

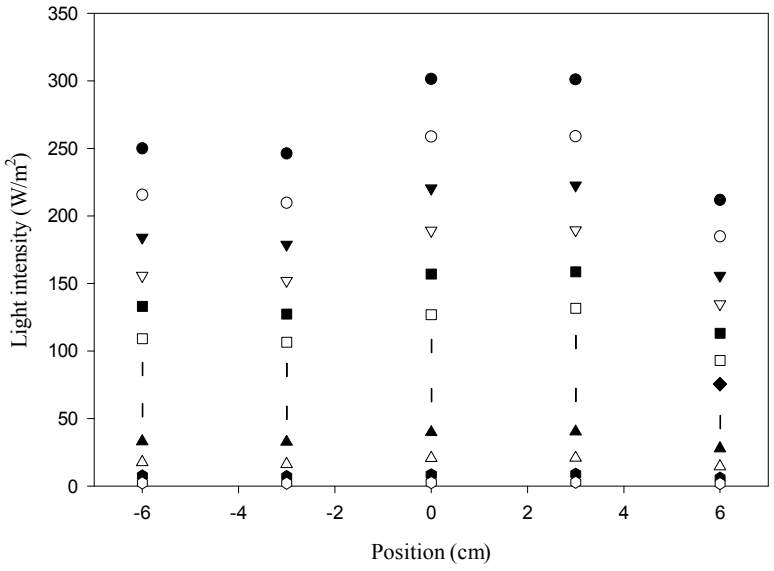

Figure I.1.- Light intensity at different vertical positions supplying different voltages (assuming 0 cm the centre of the reactor)

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

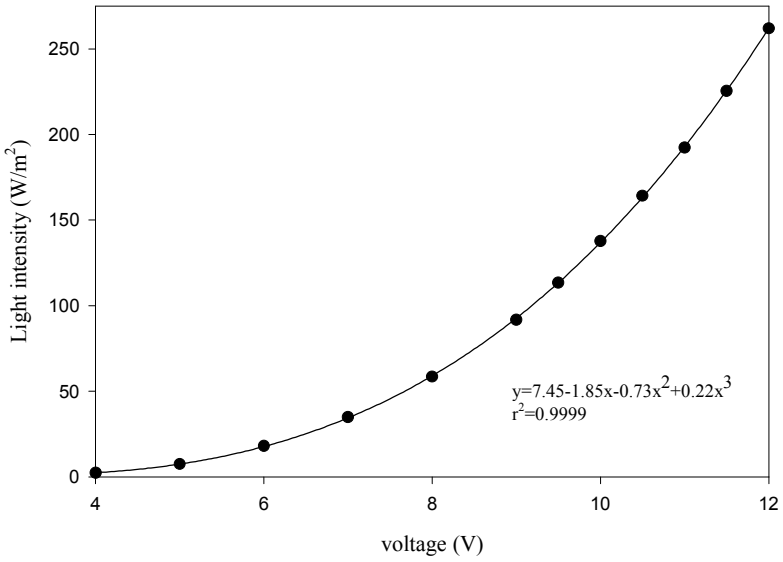

Figure I.2 .- Average light intensity supplying different voltages

APPENDIX II. -LIGHT CALIBRATION OF COMPARTMENT IV

The determination of the light intensity at the bioreactor's surface is done as previously described in Appendix I . In this case, the Rb (radius of the bioreactor) is 64 or 40mm, depending on the part of the column measured. The conversion factor is 0.291, which has been 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 airlift bioreactor with its internal draft tube dismounted, in order to allow the introduction of the spherical light sensor, and with water recirculating through the external jacket. The results obtained by measuring light intensity at different vertical positions and supplying different voltages to the lamps are plotted in figure II.1.

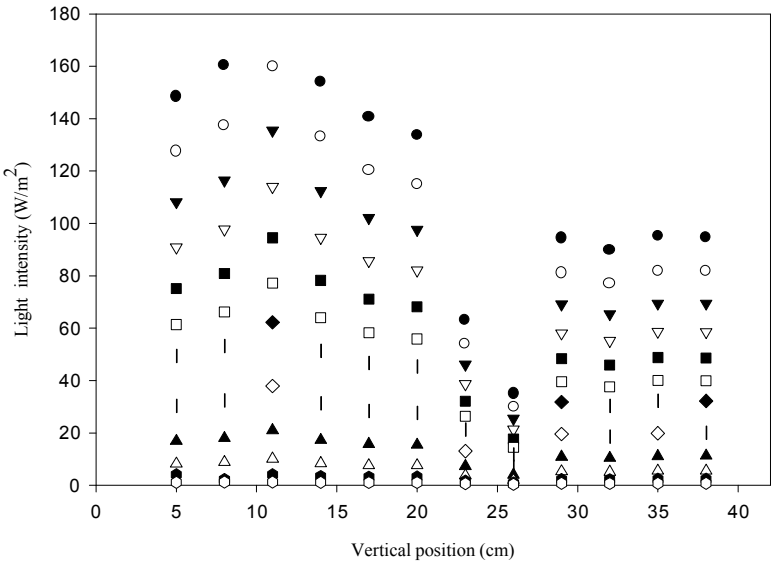

Figure II.1.- Light intensity at different vertical positions supplying different voltages (assuming 0cm the top part of the air-lift)

The average relationship between the voltage supplied to the lamps and the Fr of the airlift bioreactor obtained is presented in figure II.2.

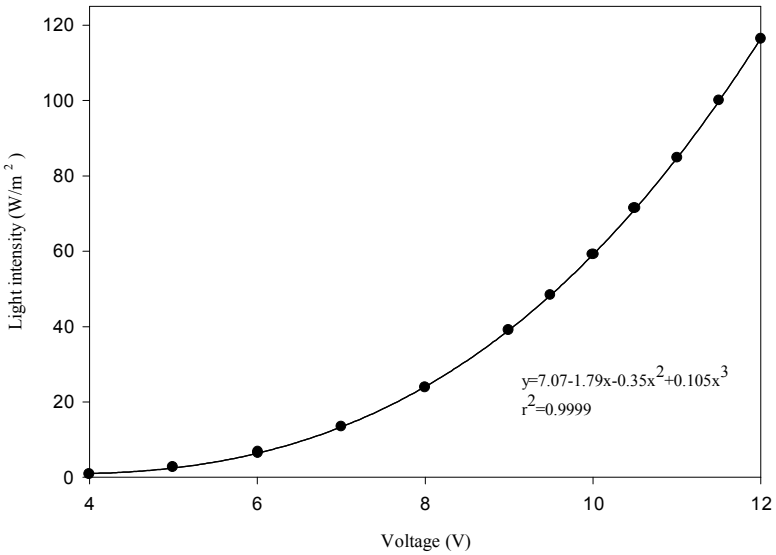

Figure II.2.- Average light intensity