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Tests With 3 Linked Bench Compartments Using 3 Different Carbon Sources

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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 IVa 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 this study has been successfully done in the connection of compartments II, III and IVa using acetic acid as the carbon source (Creus *et al.*, 1999; Creus *et al.*, 2001), a more realistic approach to the future operation of the whole connection of the loop had to be carried out.

The operation of the connection of compartments II, III and IVa using at the same time acetic, butyric and propionic acids as carbon sources is presented in this work. These 3 volatile fatty acids have been chosen as they are the major volatile fatty acids outcoming from compartment I.

2 SET-UP AND MATERIALS AND METHODS

2.1 SET-UP

As it is shown in figure 2.1 the experimental set up consists in three different bioreactors (compartments II, III and IVa) 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.

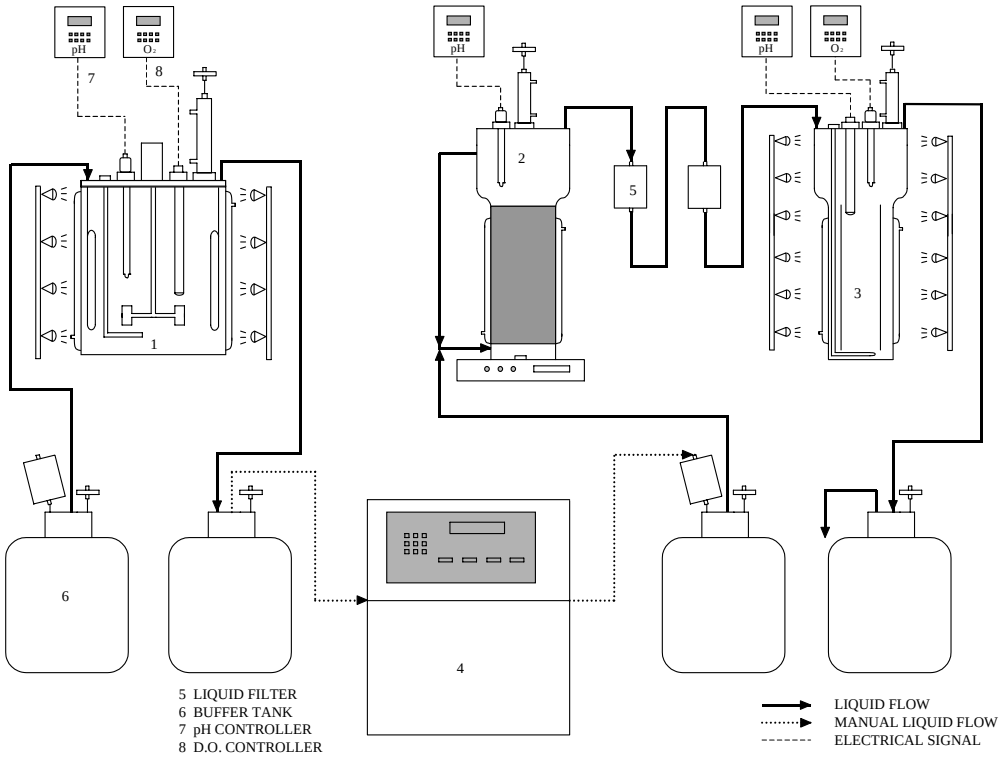

Figure 2.1. - Diagram of the experimental set up for the connection of compartments II, III and IVa of MELISSA loop at bench scale.

2.1.1 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.

2.1.2 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.

2.1.3 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.

2.1.4 Connection between compartments III and IVa

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.

2.1.5 Compartment IVa

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

2.2.2.1 Connection of compartments II, III and IVa

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.

The amount and of each volatile fatty acid finally used is presented in table 2.1. However, it is discussed in section 3.1. During these experiments CO_2 is bubbled into the fermentor as it is required in order to degrade butyric and propionic.

Product	**1 L**	
Acetic	0.313	g
Butyric	0.234	g
Propionic	0.078	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

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 elemets	
Compounds	*g/100mL*
Iron citrate	0.300
$MnSO_4$·H_2O	0.002
H_3BO_3	0.001
$CuSO_4$·5 H_2O	0.001
$(NH_4)_6MoO_{27}$·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.

2.2.2.2 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*
$EDTA\text{-}Na \cdot 2\ H_2O$	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

2.2.3.1 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.

2.2.3.2 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.

2.2.3.3 Acetic, butyric and propionic concentrations

Acetic, butyric and propionic acids concentrations are 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.

Alternatively gas chromatography (Hewlett Packard 5890) was used.

2.2.3.4 Axenicity control

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

2.2.3.5 Metals concentration determination

- Solid samples

0.3 g of sample are pre-digested during 12 h using 6 mL of HNO_3. Then the digestion takes place in an analytic microwave first heating the sample to 170ºC. The heating is done gradually and the desired temperature is reached after 4 minutes. Then a second gradual temperature increase, that takes 3.5 minutes, is done up to 180ºC. Temperature that is maintained 10 more minutes. The obtained solutions are diluted with milli-Q water up to 100mL and then they are filtered. 5 elements (Na, K, Mg, Ca and P) are directly analyzed from these solutions using induced coupled plasma emission spectroscopy (ICP-OES). The other elements (B, Mn, Fe, Ni, Cu and Zn) are analyzed from 2/10 (2/100 in case of Fe) dilutions using induced coupled plasma mass spectroscopy (ICP-MS). External patrons are used in order to quantify the samples.

- Liquid samples

Liquid samples are filtered previous analysis. Na, K and Ca are analyzed from 1/10 or 1/100 dilutions using ICP-OES and the other elements (Mg, B, P, Mn, Fe, Ni, Cu and Zn) are analyzed using ICP-MS after diluting 1/100 the sample. External patrons are used in order to quantify the samples.

3 RESULTS AND DISCUSSION.

3.1 Evaluation of the behavior of compartment II when its carbon source is a mixture of acetic, butyric and propionic acids.

Different acetic, butyric and propionic acids concentrations are added in the inlet media of compartment II (described in section 2.2.2) and its effect in the evolution of this compartment along different operational conditions is studied. The different operational conditions as well as the different volatile fatty acids concentrations used are presented in table 3.1. This experiment is done with compartment II disconnected from the loop. Its purpose is to determine the operational conditions in which compartment II can consume all the entering volatile fatty acids.

The initial acetic, butyric and propionic acid concentrations were chosen in order to have around 0.5 gC/L in the inlet media, as previous experiments showed that *R. rubrum* could consume at different light intensities 0.5g C/L, coming from acetic acid, at quite high dilutions rates ($D=0.08h^{-1}$) TN47.5 (Creus *et al.*, 2001).

In order to simulate real conditions, the proportion between the different VFA was established to be the one outcoming from compartment I, TN 41.2 (Hermans, V. and Demey, D., 1998).

	Compartment II				
	I	**II**	**III**	**IV**	**V**
Q_L (L/day)	0.850	BATCH	0.5	0.850	1.225
ι (days)	2.82	BATCH	4.80	2.82	1.96
D (h^{-1})	0.0148	BATCH	0.0087	0.0148	0.0212
Acetic (g/L)	0.625	BATCH	0.625	0.313	0.313
Butyric (g/L)	0.469	BATCH	0.469	0.235	0.235
Propionic (g/L)	0.156	BATCH	0.156	0.081	0.081
Light (W/m^2)	250	250	250	250	250

Table 3.1.- Experimental conditions

In figure 3.1 the evolution of compartment II during this experiment is presented.

As can be observed in figure 3.1 when conditions I are used, the reactor is washed. Thus, it is left in batch until all the volatile fatty acids are consumed and

therefore, the biomass concentration increases, conditions II. Then, the continuous mode, using a lower dilution rate, is reestablished, conditions III. Using conditions III all the entering volatile fatty acids are consumed, thus the optimal conditions are reached. However, the dilution rate used is too low. If it is considered that a steady state is reached after 5 residence times, reaching a steady state would require around 25 days. In order to increase the dilution rate, the volatile fatty acids concentration is decreased, conditions IV. Using conditions IV the reactor is operating again at optimal conditions. Once the steady state is reached, the dilution rate is increased, conditions V, in order to see the dilution rate range in which the reactor is working at optimal conditions.

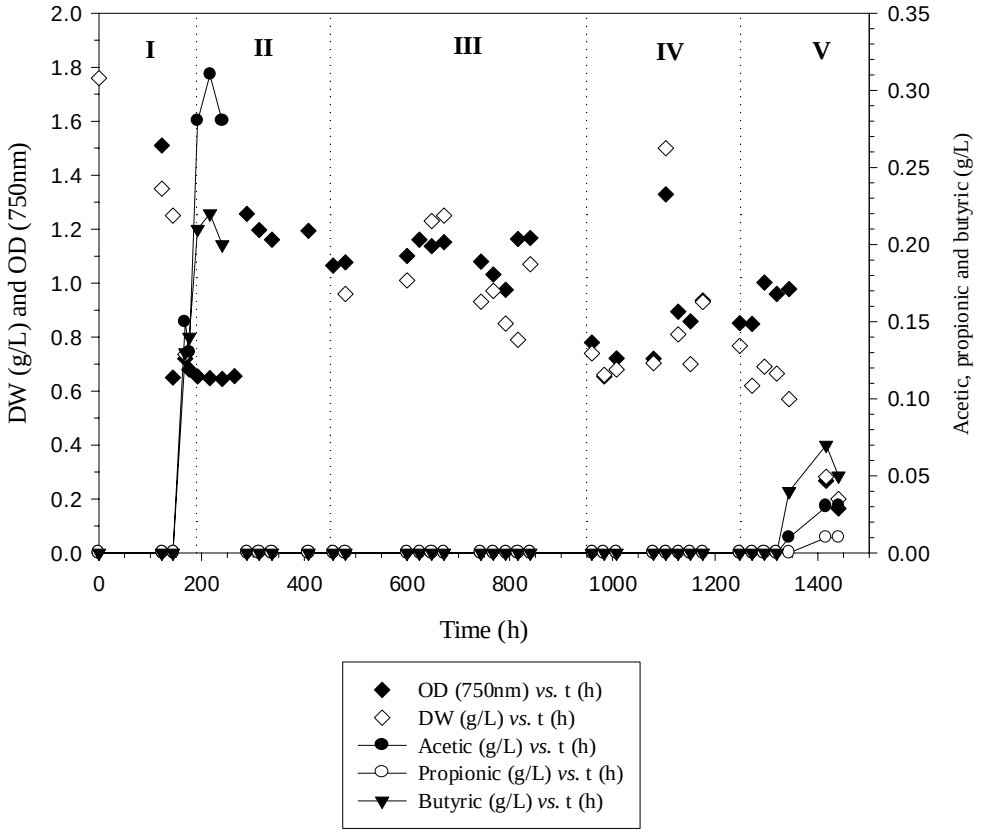

Figure 3.1.- Evolution of compartment II

The carbon balances of this compartment during the steady states of conditions III and IV are presented in table 3.2. The stoichiometric equations used to develop these balances are the following ones, TN45.4 (Favier-Teodorescu, L. *et al.*, 1999):

- **Acetic acid** + 0.4115 NH_3 + 0.0299 H_3PO_4 + 0.0066 H_2SO_4 → 1.8550 $CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161}$ + 0.1450 CO_2 + 1.1843 H_2O

- **Propionic acid** + 0.7201 NH_3 + 0.0523 H_3PO_4 + 0.0116 H_2SO_4 + 0.2462 CO_2 → 3.2462 $CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161}$ + 1.5726 H_2O

- **Butyric acid** + 1.0287 NH_3 + 0.0748 H_3PO_4 + 0.0165 H_2SO_4 + 0.6374 CO_2 → 4.6374 $CH_{1.6004}O_{0.3621}N_{0.2218}S_{0.0036}P_{0.0161}$ + 1.9609 H_2O

	Carbon balance			
	II		*IV*	
	Inlet (gC/L)	*Outlet (gC/L)*	*Inlet (gC/L)*	*Outlet (gC/L)*
Acetic	0.250	-	0.125	-
Butyric	0.254	-	0.127	-
Propionic	0.080	-	0.040	-
CO_2	0.046	0.02	0.023	0.01
cells	0	0.55	0	0.34
TOTAL	0.63	0.57	0.32	0.35

Table 3.2.- Carbon balances of the steady states of compartment II. Conditions II and IV.

As seen in table 3.2 the carbon balances can be considered closed . If a 10% of experimental error is accepted, the inleting carbon matches with the outleting carbon.

Once the operational conditions of compartment II are established the connection between compartments II, III and IVa can be done.

3.2 Evaluation of the behavior of the connection when its carbon source is a mixture of acetic, butyric and propionic acids.

The connection of compartments II, III and IVa is carried out using a mixture of acetic, butyric and propionic acids as the carbon source of compartment II. The operational conditions used along this experiment are presented in table 3.3. Initially, no volatile fatty acids are wanted to be in the outlet of compartment II, thus, compartment II has to operate at optimal conditions.

	Compartment II	***Compartment III***			***Compartment Iva***		
		I	**II**	**III**	**I**	**II**	**III**
Light (W/m^2)	250	0	0	0	20	20	20
D (h^{-1})	0.015	0.031	0.031	0.053	0.0074	0.0074	0.0120
Q_L (L/day)	0.875	0.35	0.35	0.6	0.4	0.4	0.65
τ (days)	2.74	1.36	1.36	0.79	5.63	5.63	3.46
Connection		III-IVa	3 comp	3 comp	III-IVa	3 comp	3 comp

Table 3.3.- Operational conditions of the 3 compartments along this experiment

As it is presented in table 3.3, compartment II is operating always at the same conditions. Initially, in conditions I, compartments III and IVa are interconnected but they are not connected to compartment II. The connection between the three compartments starts in conditions II. Taking into account that using conditions II a long time is required in order to reach a steady state in compartment Iva, the dilution rate of compartments III and IVa is increased, conditions III. The flow rate in compartment IVa is higher than the one in compartment III, this is due to the base inlet used to adjust the pH. The pH decreases in this compartment as there is a constant bubbling of CO_2, its carbon source, which is present in excess.

The evolution of compartments II, III and IVa along this experiment is presented in figures 3.2, 3.3 and 3.4.

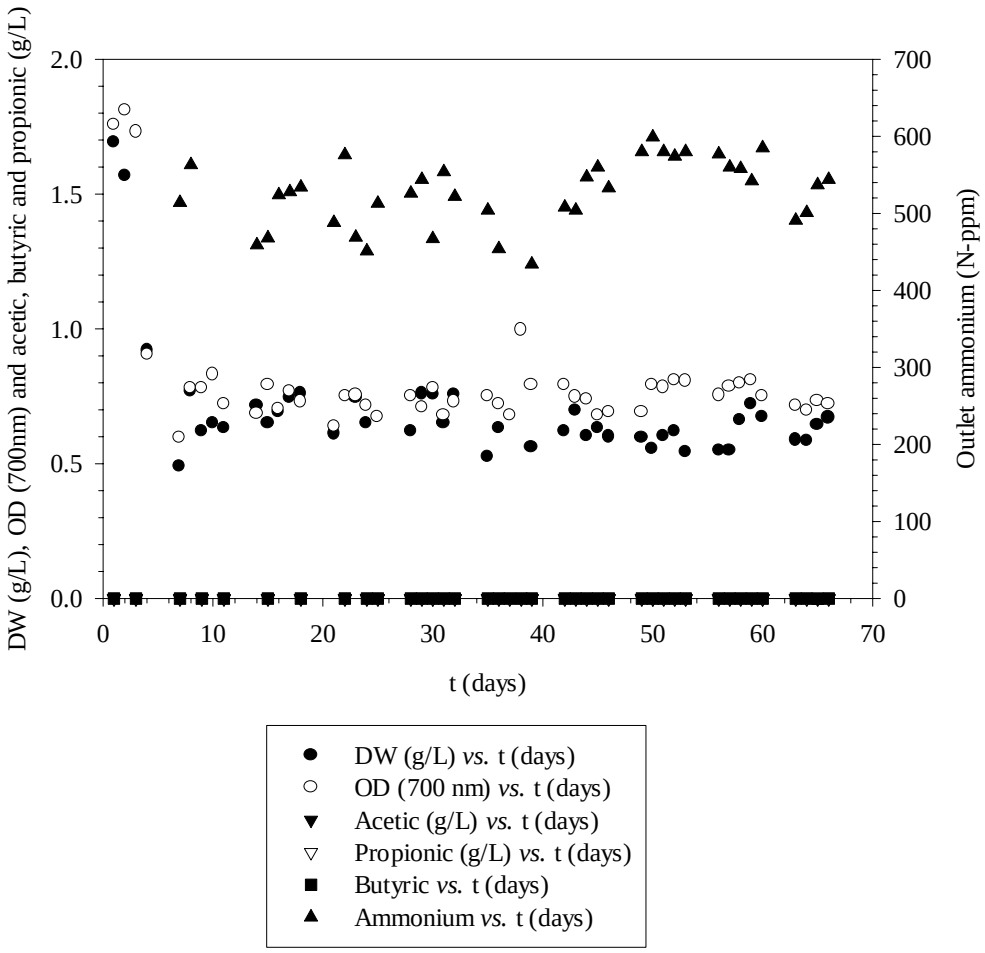

Figure 3.2.- Evolution of compartment II

As desired, and as can be observed in figure 3.2, compartment II is operating at optimal conditions. Thus, no volatile fatty acids are found in the outlet of this compartment. The carbon and nitrogen balances of this compartment are found in table 3.4 and 3.5. The stoichiometric equations used to develop these balances are presented in section 3.1, TN 39.1 (Poughon, L., 1998).

As seen in table 3.4 and 3.5 the carbon and nitrogen balances can be considered closed . If a 10% of experimental error is accepted, the inleting and the outleting carbon and nitrogen match.

	Carbon balance	
	Inlet (gC/L)	*Outlet (gC/L)*
Acetic	0.125	-
Butyric	0.127	-
Propionic	0.040	-
CO_2	0.023	0.01
cells	0	0.32
TOTAL	0.32	0.33

Table 3.4.- Carbon balance of compartment II.

	Nitrogen balance	
	Inlet (N-ppm)	*Outlet (N-ppm)*
Ammonium	600	530
cells	0	81
TOTAL	600	611

Table 3.5.- Nitrogen balance of compartment II

As presented in figure 3.3, all the entering ammonium is converted to nitrate in compartment III during conditions I and II. However, when the dilution rate is increased, conditions III, ammonium and nitrite are found in the outlet of this compartment. The ammonium and nitrite concentrations decrease along the time as more biofilm is formed and the column increases its degradation capacity.

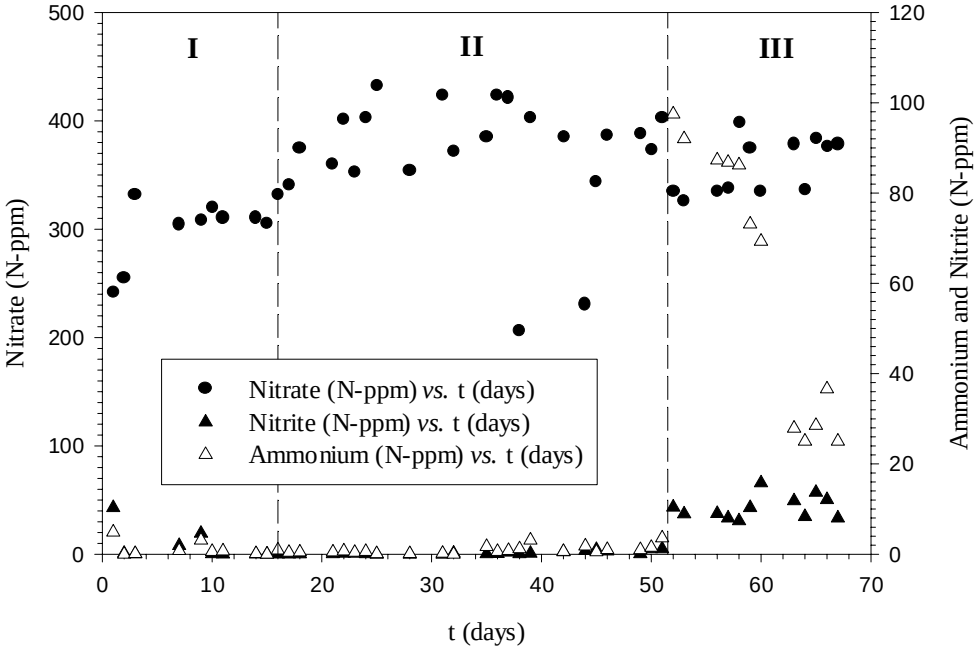

Figure 3.3.- Evolution of compartment III

A nitrogen unbalance of about 100 N-ppm is observed if the outleting ammonium concentration from compartment II is compared to the outleting nitrate concentration of compartment III. This unbalance can be due to an error observed on the nitrate analysis kits measuments. It has been seen that they allways give a lower measure than the real nitrate concentration, TN 52.21 (Montras, A., 2001).

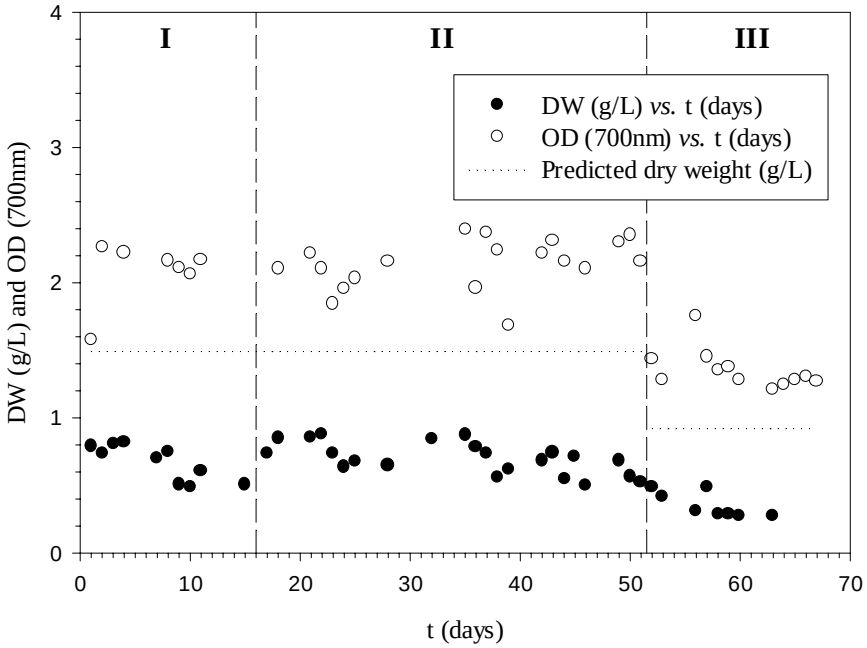

Figure 3.4.- Evolution of compartment IVa

In the evolution of compartment IVa, figure 3.4, when conditions I are changed to conditions II, no changes in the biomass concentration are observed. Thus, no relevant toxic effects are found due to the connection of this compartment to compartments II and III. A decrease in the biomass concentration is observed and a new steady state is reached when conditions II are changed to conditions III. This change is due to the step up produced in the dilution rate. As can be seen in the figure, the different experimental dry weights do not match with the predicted ones by PHOTOSIM 2 (Cornet, J.F., *et al*., 1992a, Cornet *et al*., 1992b). This fact has been also observed in TN47.5 (Creus *et al*., 2001).

Neither carbon nor nitrogen balances can be done as their evolution is not followed during this experiment.

As, during conditions III, ammonium, nitrite and nitrate are entering in compartment IVa, their evolution is followed, as seen in figure 3.5.

An immediate ammonium assimilation in compartment IVa is shown in figure 3.5. Ammonium is first uptaken when having ammonium, nitrate and nitrite as nitrogen sources, fact that has been previously observed TN 47.5 (Creus, N., *et al*., 2001). However, ammonium is also partially washed-out from the fermentor through the gas phase due to its conversion to ammonia at high pH.

In figure 3.5 a non consumption of nitrite is also observed. If the dilution factor, due to the base addition, is taken into consideration the nitrite inlet concentration is in agreement to the nitrite outlet concentration. Thus, no nitrite consumption is found when *S. platensis* cells have ammonium and nitrate, in excess, and nitrite as nitrogen sources. This fact has been previously described TN 47.5 (Creus, N., *et al*., 2001).

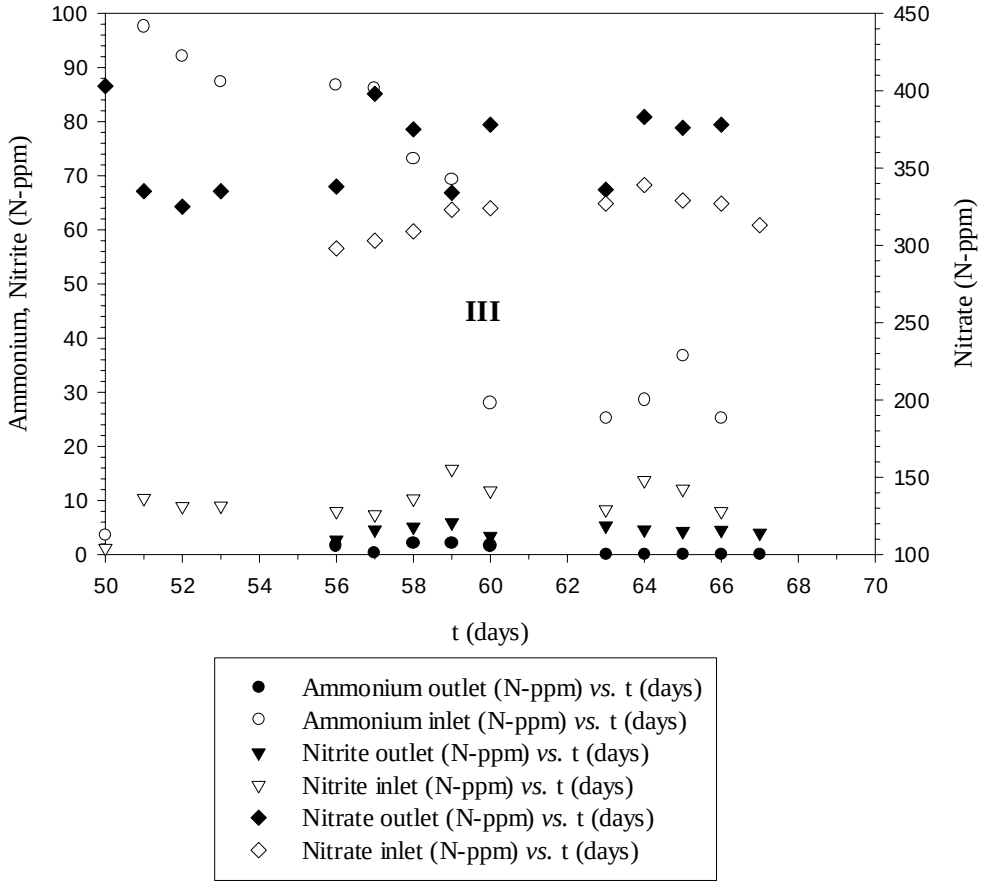

Figure 3.5.- Ammonium, nitrate and nitre evolution in copartment IVa during conditions III

A potential deviation from the steady state in compartment II would cause an acetic, butyric and propionic acids inlet in compartment III. In order to study this effect a light step down in compartment II has to be performed.

3.3 Effect of an inlet of volatile fatty acids in compartment III

In order to evaluate the effect of the presence of volatile fatty acids (VFA) in compartment III a media containing the sameVFA present in the outlet of compartment I, mantaining its own proportion, was prepared and fed to compartment III. The operational conditions of compartment III during this experiment are presented in table 3.6.

	Compartment III
pH	8.2
Light (W/m^2)	0
QL (L/day)	0.5
Volume (L)	0.475
D (h^{-1})	0.04
ι (days)	0.95
inlet NH_4^+ (N-ppm)	300
inlet VFA (gC/L)	0-0.7

Table 3.6.- Experimental conditions of compartment III

The volatile fatty acids compositions of the outlet of compartment I are presented in table 3.7.

VFA	Acetic	Propionic	Butyric	iso-Butyric	Valeric	iso-Valeric
g/L	0.77	0.27	0.15	0.10	0.17	0.15

Table 3.7.- VFA composition of the outlet media of compartment I

The amount of volatile fatty acids entering in compartment III was increased gradually, and its effect in the behaviour of this compartment is presented in figure 3.6.

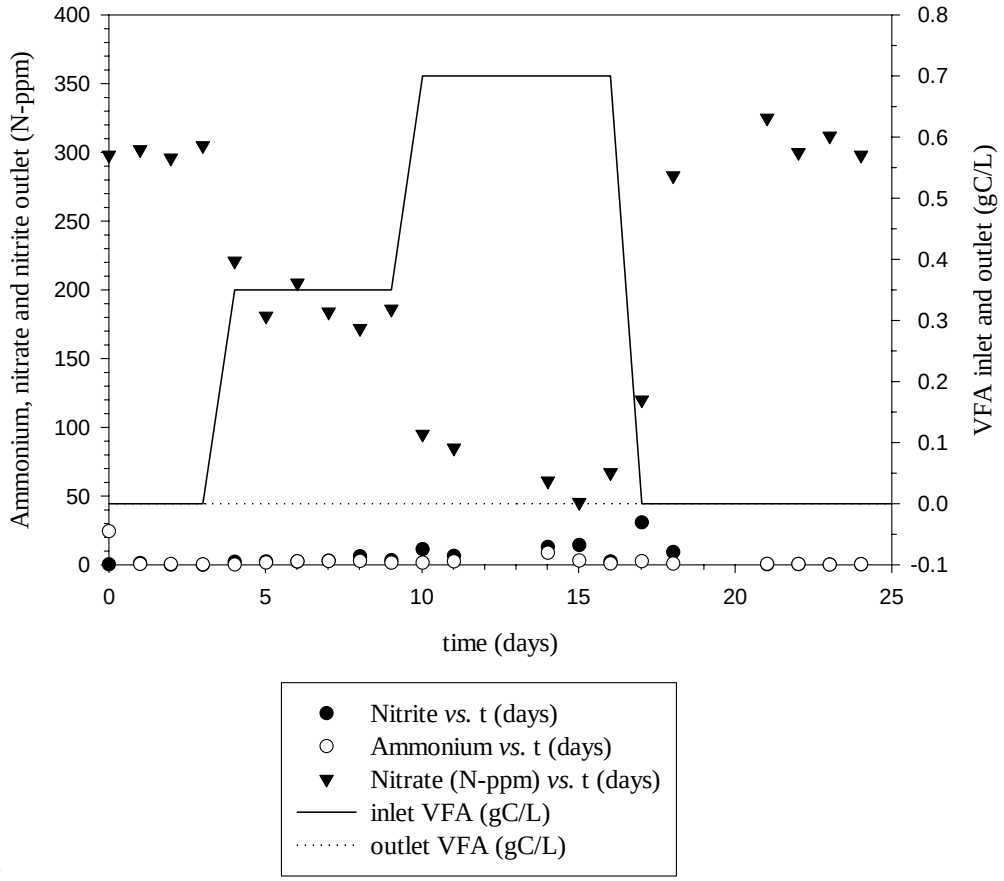

Figure 3.6.- Effect of the presence of VFA in compartment III

As can be seen in figure 3.6 the behavior of compartment III is not disturbed by the presence of VFA. Thus, neither ammonia nor nitrite are found in the outlet of this compartment. However, the nitrogen balance can be only closed when no VFA are present.

When VFA are entering in this compartment non are found in the outlet and a gap in the nitrogen balance is observed. This fact is probably due to biomass formation. The VFA assimilation rate is rather high to attribute it only to *Nitrosomonas europaea* and *Nitrobacter winogradskyi* growth (Creus, N**.,** *et al.;* 2001).

The column was operated during one day having the recirculation stream running backwards and at its maximum speed in order to detach some of its biofilm to be able to analyze it on the microscope. In the microscope observation of the detached biofilm microorganisms having a different morphology from *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were found.

3.4 3.4.- Elemental analyses of biomass and medium samples

11 different elements (Na, K, Mg, Ca, B, P, Mn, Fe, Ni, Cu and Zn) were analyzed from different samples of the incoming and outcoming medium and biomass of compartments II, III and IVa. These samples were obtained along the above-mentioned experiment. Their analyses are presented in table 3.8.

As can be seen in table 3.8 there is a high concordance between the results of the different samples of the same biomass or medium. The differences observed can be due to the experimental procedure. The outlet of compartments II and IVa have to be centrifuged and filtered or two times filtered in order to proceed with the analysis. Thus, if some precipitation occurs, some elements can be lost during these steps. Biomass has to be washed and freeze-dried. Differences in the washing procedure or non-homogenity of the sample can lead to differences in the analysis results.

Taking into account that the exact weighted products added to the media are not the theoretical ones, the theoretic elements concentration of the inlet media and the analyzed results are in high agreement, except with Zn and Na. The analyzed Zn concentration is higher than the theoretical one. The addition of Zn as impurities of other compounds and/or a possible interference of some other media element in the Zn analysis can be two possible explanations. The addition of NaOH in order to adjust the

media pH explains the differences in the Na analyzed concentration compared to the theoretical one.

An increase in the Na and B concentrations from the inlet media of compartment II to the outlet media of compartment IVa is observed in table 3.8. The Na concentration increase is possibly due to the NaOH addition used to control the pH. However, the B concentration results can be only explained if there is a possible interference of another media element in its analysis or if there are some B impurities in the acid or base bottles or in the bioreactor.

A balance of these elements during this experiment is found in table 3.9

The different elements balances do not match. The difference in the elements concentration between the inlet and outlet of each compartment is higher than the amount of each element required in order to produce the biomass. This fact is attributed to elements elimination during the filtration and centrifugation steps due to some precipitation and due to the dilution effect of the pH control.

	Date	Na	s.d.	K	s.d.	Mg	s.d.	Ca	s.d.	B	s.d.	P	s.d.	Mn	s.d.	Fe	s.d.	Ni	s.d.	Cu	s.d.	Zn	s.d.
S. plasmisis (mg/kg)	29/6	11543	*441*	13834	*256*	2931	*181*	860	*35*	184.9	*1.5*	9200	*500*	28.3	*0.2*	538	*2*	12.0	*0.3*	19.0	*0.3*	192.7	*0.4*
	06/8	9272	*459*	16220	*258*	3070	*182*	855	*37*	109.6	*1.1*	9864	*501*	33.9	*0.2*	708	*2*	7.3	*0.3*	7.0	*0.4*	120.5	*0.4*
	15/8	11713	*436*	13650	*253*	2417	*181*	1206	*34*	176.1	*1.4*	8396	*500*	27.6	*0.2*	577	*2*	5.8	*0.3*	7.8	*0.3*	193.5	*0.4*
	mean	**10843**	*1363*	**14568**	*1434*	**2806**	*344*	**973**	*201*	**157**	*41*	**9153**	*735*	**29.9**	*3.5*	**614**	*82*	**8.4**	*3.2*	**11.3**	*6.7*	**168.9**	*41.9*
R. rubrum (mg/kg)	27/8	2793	*18*	2640	*320*	4057	*183*	1961	*34*	21.9	*1.1*	16798	*479*	30.6	*0.2*	557	*3*	<1.9		22.2	*0.3*	31.1	*0.5*
	11/7	988	*23*	6123	*295*	7061	*172*	1714	*35*	28.2	*1.1*	21558	*456*	91.1	*0.2*	857	*2*	4.7	*0.4*	29.5	*0.3*	34.1	*0.5*
	6/8	801	*20*	4794	*340*	5021	*201*	1806	*39*	23.5	*1.3*	19541	*526*	176.0	*0.3*	2693	*3*	<1.9		26.7	*0.4*	33.3	*0.5*
	15/8	5716	*18*	5951	*290*	6337	*171*	1450	*35*	24.3	*1.1*	26012	*433*	49.2	*0.2*	505	*3*	<1.9		14.9	*0.3*	35.2	*0.5*
	mean	**2575**	*2279*	**4877**	*1604*	**5619**	*1340*	**1733**	*214*	**24.5**	*2.7*	**20977**	*3882*	**86.7**	*64.7*	**1153**	*1038*	**2.5**		**23.3**	*6.4*	**33.5**	*1.8*
media (mg/L)	**theoretical**	**233**		**362**		**118**		**24.7**		**0.5**		**221**		**3.4**		**6.6**		**0.1**		**0.02**		**1e-3**	
outlet C II (mg/L)	15/8	314	*13*	378	*3*	1133	*0.5*	25.3	*1.5*	0.7	*0.1*	175.2	*1.2*	3.6	*0.2*	5.2	*0.1*	<0.3		<0.1		0.31	*0.07*
	10/8	450	*13*	394	*3*	89.1	*0.5*	24.0	*1.5*	0.7	*0.1*	80.2	*1.0*	3.0	*0.2*	5.8	*0.1*	<0.3		<0.1		0.26	*0.07*
	17/8	418	*13*	361	*3*	84.7	*0.5*	24.0	*1.5*	1.0	*0.1*	88.9	*1.0*	3.4	*0.2*	5.5	*0.1*	<0.3		<0.1		0.30	*0.07*
	23/8	315	*13*	361	*3*	82.9	*0.5*	24.6	*1.5*	1.0	*0.1*	123.7	*1.0*	3.6	*0.2*	5.4	*0.1*	<0.3		<0.1		1.01	*0.07*
	mean	**394**	*71*	**372**	*19*	**85.6**	*3.2*	**24.2**	*0.4*	**0.9**	*0.2*	**97.6**	*23.0*	**3.3**	*0.3*	**5.6**	*0.2*	**<0.3**		**<0.1**		**0.28**	*0.42*
outlet C III (mg/L)	10/8	1462	*13*	351	*3*	79.5	*0.5*	20.1	*1.5*	1	*0.1*	79.6	*1.0*	3.1	*0.2*	4.8	*0.1*	<0.3		<0.1		0.29	*0.07*
	23/8	1559	*13*	355	*3*	97.1	*0.5*	25.9	*1.5*	1.6	*0.1*	98.6	*1.0*	3.0	*0.2*	4.5	*0.1*	<0.3		<0.1		0.30	*0.07*
	mean	**1511**	*69*	**353**	*3*	**79.5**	*12*	**23.0**	*4.1*	**1.3**	*0.4*	**89.1**	*13.4*	**3.1**	*0.1*	**4.7**	*0.2*	**<0.3**		**<0.1**		**0.30**	*0.01*
outlet C IVa (mg/L)	1/8	2531	*138*	216	*3*	32.7	*0.5*	14.6	*1.5*	3.3	*0.1*	70.1	*1.0*	2.2	*0.2*	1.7	*0.1*	<0.3		<0.1		0.26	*0.07*
	10/8	3368	*133*	298	*3*	31.4	*0.5*	11.7	*1.6*	2.9	*0.1*	46.9	*1.0*	1.9	*0.2*	1.8	*0.1*	<0.3		<0.1		0.25	*0.07*
	17/8	1790	*142*	339	*3*	69.4	*0.5*	22.9	*1.5*	2.7	*0.1*	72.2	*1.0*	2.0	*0.2*	1.9	*0.1*	<0.3		<0.1		0.35	*0.07*
	24/8	1730	*143*	335	*3*	89.5	*0.5*	12.4	*1.6*	2.8	*0.1*	105.8	*1.0*	2.3	*0.2*	1.5	*0.1*	<0.3		<0.1		0.28	*0.07*
	mean	**2255**	*768*	**297**	*57*	**55.8**	*29*	**15.4**	*5.2*	**2.9**	*0.3*	**73.8**	*24.3*	**2.1**	*0.2*	**1.7**	*0.2*	**<0.3**		**<0.1**		**0.29**	*0.05*

Table 3.8.-Elements analysis of the incoming and outcoming mediums and biomass of compartments II, III and IVa.

			Na	K	Mg	Ca	B	P	Mn	Fe	Ni	Cu	Zn
CII	*inlet media*	ppm	314	378	113.3	25.3	0.7	175.2	3.6	5.2	<0.3	<0.1	0.31
	outlet C II	ppm	394	372	85.6	24.2	0.9	97.6	3.3	5.6	<0.3	<0.1	0.28
	Inlet-outlet	**ppm**	***-80***	**6**	**27.7**	**1.1**	***-0.2***	**77.6**	**0.3**	**-0.4**	**<0.3**	**<0.1**	**0.03**
	R rubrum 0.65 g/L	ppm	1.67	3.17	3.65	1.13	0.02	13.64	0.06	0.75	0	0.02	0.02
CIII	*outlet C II*	ppm	394	372	85.6	24.2	0.9	97.6	3.3	5.6	<0.3	<0.1	0.28
	outlet C III	ppm	1511	353	79.5	23.0	1.3	89.1	3.1	4.7	<0.3	<0.1	0.30
	outlets CII-CIII	**ppm**	***-1117***	**19**	**6.1**	**1.2**	***-0.4***	**8.5**	**0.2**	**0.9**	**<0.3**	**<0.1**	***-0.02***
CIVa	*outlet C III*	ppm	1511	353	79.5	23.0	1.3	89.1	3.1	4.7	<0.3	<0.1	0.30
	outlet C IVa	ppm	2355	297	55.8	15.4	2.9	73.8	2.1	1.7	<0.3	<0.1	0.29
	CIII-CIVa	**ppm**	***-844***	**56**	**23.7**	**7.6**	***-1.6***	**15.3**	**1**	**3**	**<0.3**	**<0.1**	**0.01**
	S platensis 0.4 g/L	ppm	4.34	5.83	1.12	0.39	0.06	3.67	0.01	0.25	0	0.01	0.07

Table 3.9.- Elements balances in each compartment.

3.5 Storage of biomass samples in order to perform genetic stability tests

Biomass samples from compartments II and IVa were stored periodically during this experiment in order to follow its genetic stability. It was not possible to take any sample from compartment III as, being a packed column, no biomass was found in the outlet. The storage protocol was accorded with Max Mergeay and Ruddy Wattiez as can be seen in the following e-mails:

from : Mergeay Max <MMERGEAY@SCKCEN.B>]
send : Fri, 22 Dec 2000
to : nuria.creus@uab.es
subject : Samples for the MELISSA genetic stability study.

Dear colleagues,
I am very happy with the idea to resume or to start a collaboration with you on the genetic fate and stability of the Melissa bacteria.
In this respect, I wish to convey you my sincere and enthusiastic congratulations for the connection of the three compartments: it is an achievment I couldn't seriously imagine in 1986/88 when we start with the concept.
Now, Christophe suggested me to let do some samplings from your cultures :
it is maybe somewhat difficult to organise it just at the Eve of Xmas but nothing should be impossible at the time of Christmas. I even guess it makes part of the rationale of Christophe when he sent me the message.

Now, I suggest to sample for two major purposes: to look at DNA (axeny, AFLP, REP-PCR, etc), to look at the proteins (a proteomic approach).
Samples for DNA study could be frozen in a minimal medium or buffer supplemented with 10% glycerol and stored in liquid nitrogen or in a good deepfreezer. Samples for the proteomic approach (for further analysis with a Maldi-Tof or under 2D gelelectrophoresis) have to resuspended in a special buffer I have not here and that should be provided by my colleague Ruddy WATTIEZ (wattiez@umh.ac.be)from the Université de Mons-Hainaut in Belgium.
Nevertheless, samplings without glycerol may be acceptable for a first try.
With my kindest regards and best wishes at the time of Christmas for a happy 2001 full of interesting human and scientific experiences,

Max Mergeay

from: Nuria Creus [mailto:Nuria.Creus@uab.es]
send: 12 March 2001 18:43
to: wattiez@umh.ac.be
subject: Samples for the MELISSA genetic stability study.

Dear colleague,
I will start collecting the samples in order to start the collaboration with you on the genetic fate and stability of the Melissa bacteria.
In this respect, and as you can read in the attached e-mail Max Mergeay sent us, he proposed to store the DNA samples in a minimal medium or buffer supplemented with 10% glycerol, in liquid nitrogen or in a good deepfreezer. He said that the samples for the proteomic approach (for further analysis with a Maldi-Tof or under 2D electrophoresis) have to be resuspended in a special buffer.
I wonder if you can give us the specific mediums and buffers in order to store properly the different kind of samples.

Thanks a lot.

from: wattiez@umh.ac.be]
send: 20 March 2001 18:43
to: Nuria Creus [mailto:Nuria.Creus@uab.es
subject: Samples for the MELISSA genetic stability study.

Dear colleague

for a proteomic approach, we have try different conditions and I proposed to
store the protein samples in liquid nitogen or in a -80 C without buffer,
just the bacterial pellet.

Thanks a lot

Ruddy

Thus, samples were taken from compartments II and IVa, they were centrifuged or filtered in order to obtain the biomass pelet and they were stored in a -80ºC freezer. All these steps were done in a sterile way.

For further experiments the MELISSA group devoted to genetic analysis visited the pilot plant in order to agree with the extraction samples protocol. Different protocols have been proposed and right now they are being discussed. A member from the genetic analysis group will come to the pilot plant to show how to proceed with the samples extraction.

The different proposed protocols are listed below:

3.6 Samples requirements

1) Axenicity:

Volume: 20-30 ml of fresh sample (taken directly from the reactor)

Frequency: 1/month-1/3months

Pretreatment: not required

Storage: 0-4 degC

CAUTION: SAMPLING HAS TO BE DONE AXENICALLY

2) Genomics:

Volume: 20-30 ml of fresh sample (3 times) (taken directly from the reactor)

Frequency: 1/3months-1/12months

Pretreatment:

- 1st option: immediate centrifugation after sampling, withdrawal of the supernatant and deep-freezing

- 2nd option: fixation with ethanol

Storage: deep-frozen

CAUTION: SAMPLING AND PRE-TREATMENT HAVE TO BE DONE AXENICALLY

3) Proteomics:

Volume: 20-30 ml of fresh sample (taken directly from the reactor)

Frequency: 1/3months-1/12months

Pretreatment for work with the supernatant:

- 1st option: immediate centrifugation after sampling, withdrawal of the cells and deep-freezing of the supernatant

- 2nd option: filtration with a 0.2 microns membrane and deep-freezing

- 3rd option: filtration/centrifugation, addition of enzymatic inhibitor and storage at 0-4 degC.

Pretreatment for work with intact cells (2-4 ml sample): immediate centrifugation and deep-freezing of the pellet

Storage: deep-frozen is preferred.

CAUTION: SAMPLING AND PRE-TREATMENT HAVE TO BE DONE AXENICALLY

If it is possible to have an immediate shipment (delivery in 24h maximum), it is possible to avoid the pre-treatment and send fresh samples kept cool (0-4 ºC)

4 CONCLUSIONS

In this work:

- The feasibility of the long-term connection of compartments II, III and IVa using the three main volatile fatty acids outcoming from compartment I, acetic, propionic and butyric acids, has been demonstrated. These three compartments have been working together for almost 2 months and no toxic effect has occurred.

- 11 different elements concentrations have been followed during this experiment in order to have an initial knowledge of these elements requirements in *R. rubrum* and *S. platensis* growth.

- An agreement in the samples extraction protocol to follow the microorganisms genetic stability has been achieved.

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6 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:

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

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.

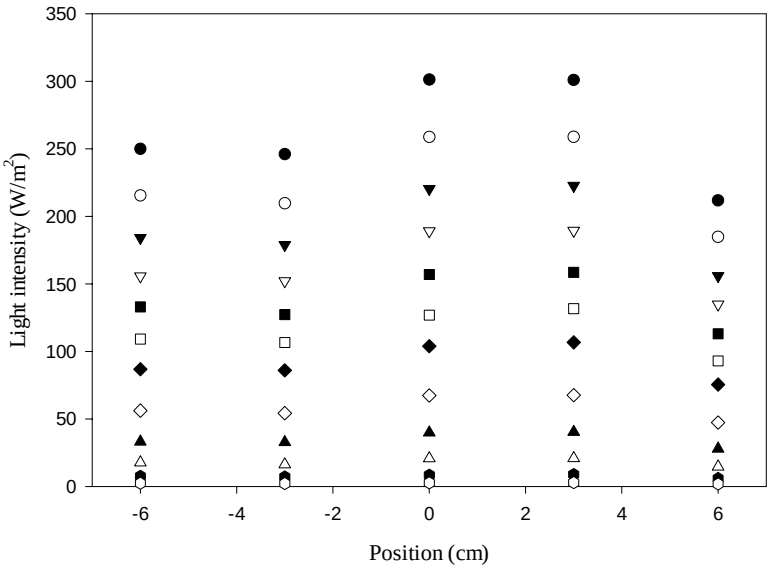

Figure I.1.- Light intensity at different vertical positions supplying different voltages (assuming 0 cm the centre of the reactor)

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 $\mu mols/m^2s^2$, 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.

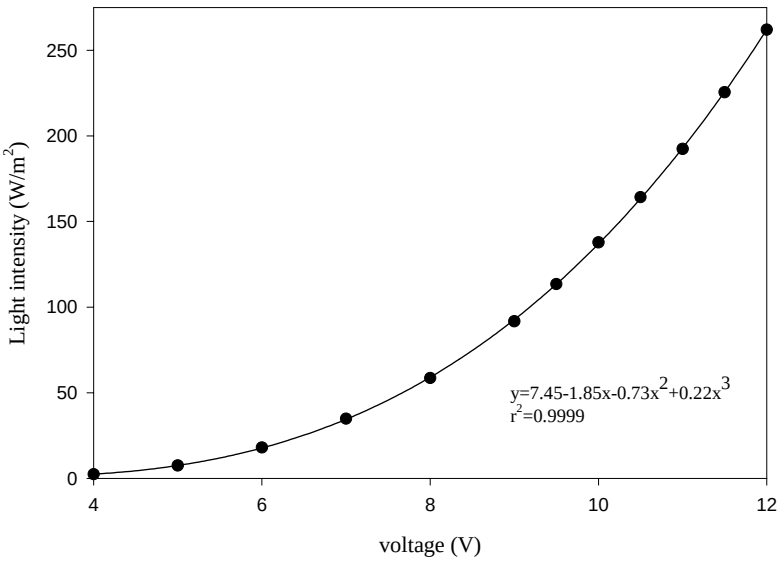

Figure I.2 .- Average light intensity supplying different voltages

7 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.

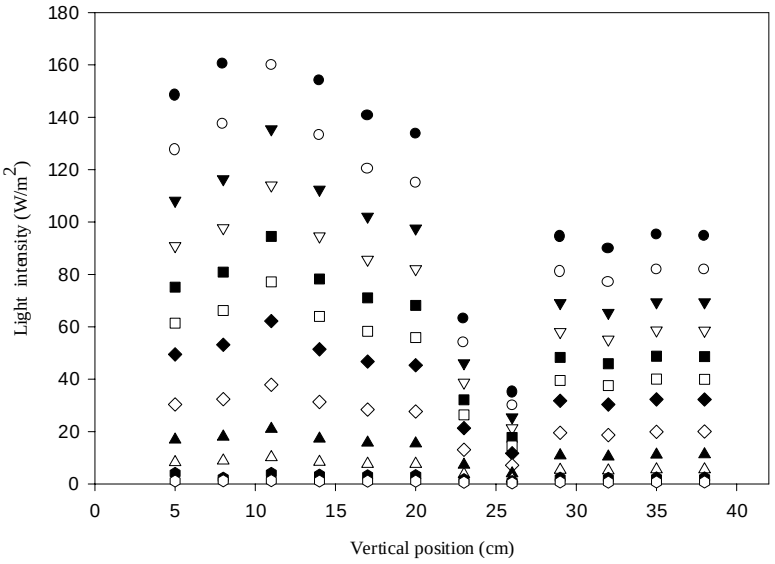

Figure II.1.- Light intensity at different vertical positions supplying different voltages (assuming 0cm the top part of the air-lift)

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

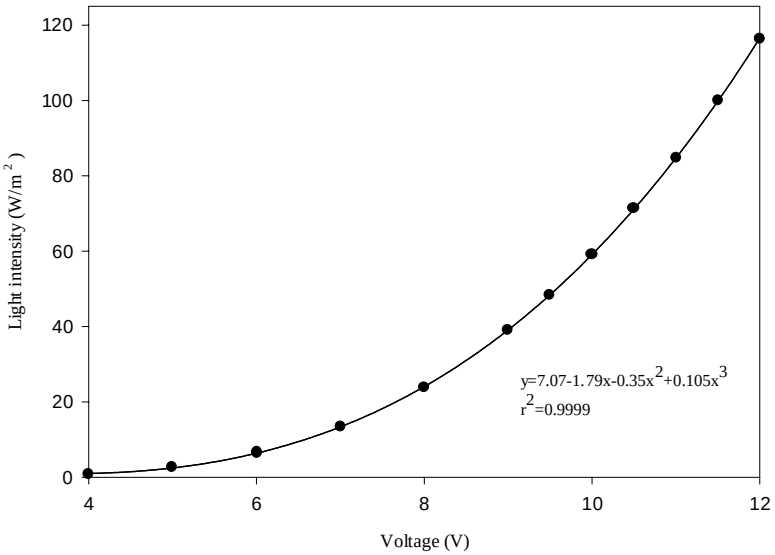

Figure II.2.- Average Light Intensity.