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MELISSA

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1. - INTRODUCTION

The successful operation of the MELISSA (Micro-Ecological Life Support System Alternative) loop concept as a life support system relies in the connection of its compartments through different gas and liquid flows. This requires to successfully test the continuous operation of its four different bioreactors connected sequentially. In the following paragraphs the main characteristics of these compartments are summarised.

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

The anoxygenic phototrophic compartment II metabolises 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*) immobilised 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 development of compartments II, III and IV has been successful during a number of years of isolated operation. However, as mentioned before it is required to evaluate the effect of its interconnected operation either in its stationary and transient states. In the present technical note, the connection at bench scale, of compartments II, III and IV is reported.

As a first step in the evaluation of its interconnected operation, the bioreactors were set to run in predetermined fixed conditions. For the heterotrophic compartment II a 2.5 l. continuous stirred tank reactor was used. The effluent of this reactor, free of biomass after centrifugation and filtration steps, was fed into a 0.5 l packed bed column used to represent the nitrifying compartment III. The filtered effluent of compartment III was directly fed into a 3 l. airlift reactor used as the photoautrophic compartment IV as in this case very few cells are released from the immobilised cell reactor.

The initial culture media used was designed in order to have all the nutrients required in each compartment in such a way that the carbon source would be completely consumed in compartment II, avoiding the entrance of volatile acids in compartment III.

Further studies about the effect of different changes in the carbon source or operational conditions on the operation of the different compartments will be performed in the next phase of the project.

2. - EXPERIMENTAL SET UP

The experimental set up consisted in three different bioreactors (compartments II, III and IV) and the necessary separation units to connect them. A detailed description of each compartment and the different steps required in their connection are given in this section. A diagram of the experimental set up can be found in figure1.

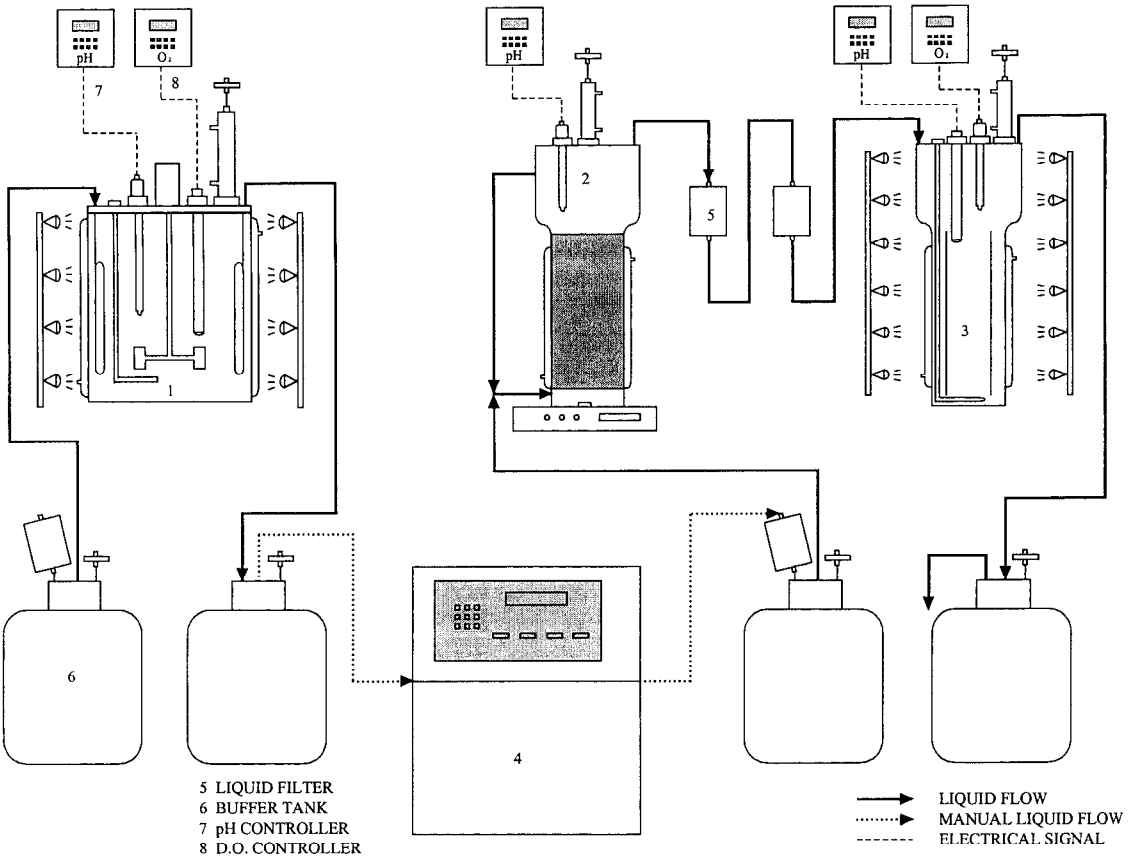

Figure 1. - Diagram of the experimental set up for the connection of compartments II, III and IV of MELISSA loop at bench scale.

Compartment II

Cultures corresponding to the second compartment were done in a 3.L stirred tank bioreactor (APPLIKON). An autonomous controller (METTLER TOLEDO PH2100) was used to regulate the pH of the culture media to 6.9, by addition of the acid (HCl 0.5M) or base (NaOH 0.5M). The corresponding pH probe was calibrated before autoclaving following a standard protocol. The temperature was controlled by an external water jacket.

To maintain anaerobic conditions for the culture of *Rhodospirillum rubrum*, He, was bubbled inside the reactor. The absence of oxygen was monitored by measuring the DO (dissolved oxygen) with a oxygen analyser (INGOLD O_2 amplifier Type 170% O_2). The calibration of the O_2 probe was made, using operational conditions before starting the experiment, following a standard protocol.

Mechanical stirring (APPLIKON ADI 1012) was used assuring the perfect mixing.

Illumination of the bioreactor was 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 provided the necessary electrical power. Light supplied to the reactor was modified by changing the voltage supplied to the lamps. A calibration of the light intensity in W/m^2 supplied in the middle of the reactor as a function of the voltage is given in Appendix 1. In figure 2 a picture of compartment II set up is presented.

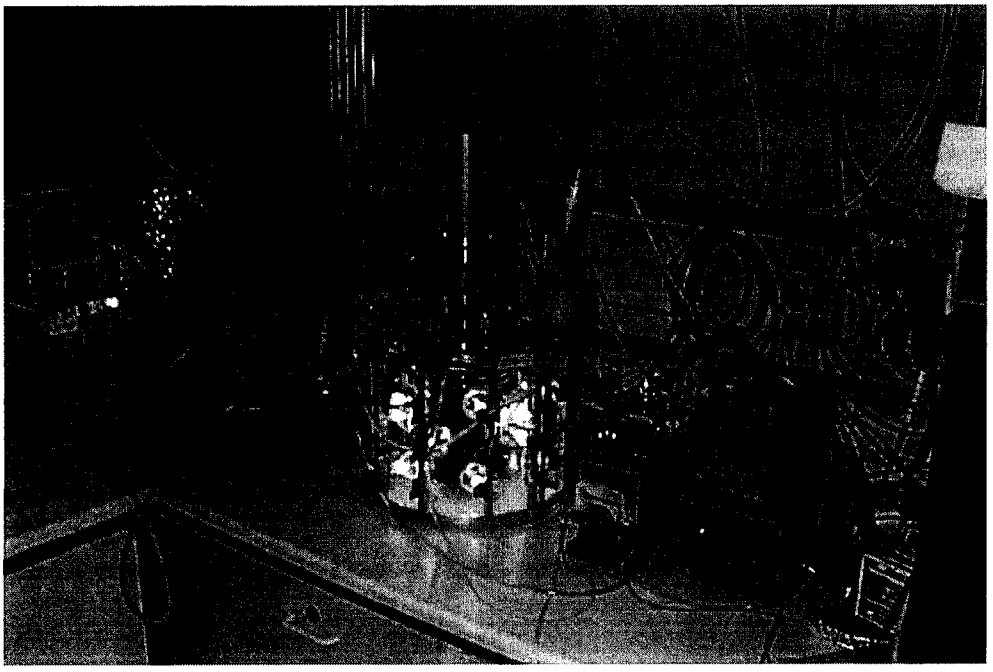

Figure 2. - Compartment II set-up. 2 l. stirred tank reactor with external illumination for *Rhodospirillum rubrum*.

Connection between compartment II and III

Due to the fact that the outlet of compartment II contained biomass, a removal step was necessary. To this purpose a centrifugation step was 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, were too small to use a continuous centrifuge. For this reason a batch centrifuge (BECKMAN J2-21 M/E) (10000rpm, 4°C, 20min) was used. As the centrifugation operation was a discontinuous process, two buffer tanks, one for the outlet of compartment II and the other for the inlet of compartment III were required. This allowed collecting the liquid effluent for centrifugation and storing the biomass free medium after the centrifugation step. This centrifugation step was 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 for compartment III, sterilisation before introducing it to the input storage tank for the third compartment was required. To this purpose two filtering steps using liquid filters (MILLIPORE OPTICAP™ 4") of .022µm were introduced.

Compartment III

Implementation of compatment III was done using a packed-bed column, as described previously (Pérez et al. 1998, TN 37.510).

The pH was controlled by an autonomous controller (CRISON pH/mV 252). Regulation of the pH, was achieved by means of acid (CO_2) or base (Na_2CO_3 40g/l) addition. The calibration of the pH probe was made before starting the experiment following a standard protocol.

Temperature was controlled by an external water jacket. Aeration was done through a gas sparger. To achieve a higher conversion, part of the effluent of the bioreactor was recirculated. To ensure the perfect mixing between the inlet and the recirculation a magnetic stirrer was used. In figure 3 a picture of compartment III is shown.

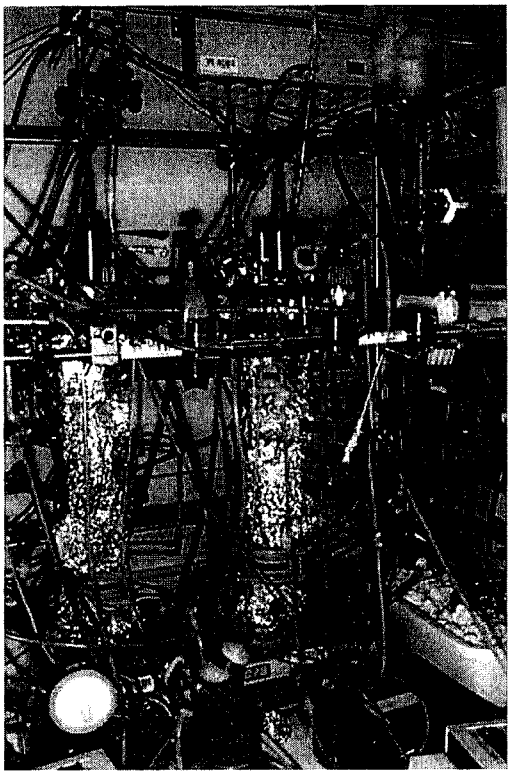

Figure 3. - Compartment III set-up. 475 ml column reactor with *Nitrosomonas europaea* and *Nitrobacter winogradskyi* immobilised onto Biostyr beads.

Connection between compartments III and IV

The connection between these two compartments could have been done by direct connection. However, due to the fact that the outlet of compartment III contained some biomass, two filtering steps through liquid filters (MILLIPORE OPTICAP^{TM} 4") of 0.22μm were included. One in the output of compartment III and another one at the input of compartment IV. In this way, the two compartments were isolated allowing a disconnection in case of malfunction in one of them.

Compartment IV

In this case the bioreactor used was 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 cause 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) regulated the acid (HCl 0.5M) or base (NaOH 0.75M) addition to control the pH. Calibration of the pH probe was done before starting the experiment following a standard protocol.

The oxygen was monitored by measuring the DO (dissolved oxygen) with a polarographic oxygen probe (METTLER TOLEDO $O_2$4100). The calibration of the O_2 probe was done following a standard protocol before starting the experiment at experimental conditions. Temperature was controlled by an external water jacket.

Radial illumination of the bioreactor was done using a set of 20 halogen lamps (*SYLVANIA* Halogen –Professional de luxe-, 12V, 20W, ∅ 50mm.) distributed in 5 columns containing one or two lamps successively. One 15V–40A power supply provided the necessary electrical power. Variation of the light supplied to the reactor was done by changing the voltage supplied to the lamps. A calibration of the light intensity in W/m^2 supplied in the middle of the reactor as a function of the voltage is given in Appendix 2. In figure 4 an illustration of compartment IV is presented.

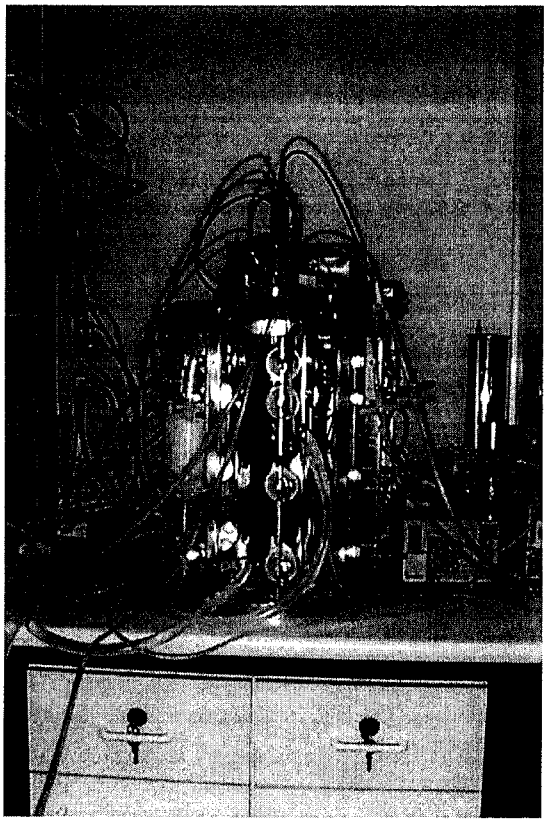

Figure 4. - Compartment IV set-up. 2.75 l. air-lift reactor with external illumination for *Spirulina platensis.*

3. - MATERIALS AND METHODS

Compartment II

The strain used to colonise this compartment was *Rhodospirillum rubrum* (ATCC 25903) obtained from the American Type Culture Collection. It was revived and the subcultures were done using their recommended medium. The inoculum volume was fixed as the 10% of the working volume (2.4 l).

The continuous culture medium was based on the basal salts mixture of SEGERS & VERSTRATE as described by Suhaimi (Suhaimi et al 1987), using acetic acid as a carbon source and biotin as the only vitamin. Phosphate concentration was decreased to the following levels: KH_2PO_4 0.49 g/l, K_2HPO_4 0.52 g/l. The pH was adjusted to 6.9.

Flow rate was set at 0.724 ml/min, which corresponds to a dilution rate of 0.0181 h^{-1} and a residence time of 2.3 days for our working volume. The temperature was fixed at 30°C, pH at 6.9, the agitation rate at 300 rpm and the dissolved oxygen was maintained at 0%. The light intensity chosen was 135 W/m^2.

Evolution of the culture was followed by measuring biomass concentration, acetic acid concentration and ammonia concentration. Biomass concentration was determined by optical density at 700nm (UVIKON) and by measuring dry weight. To determine biomass dry weight the sample was filtered through a 0.22μm pre-weighted filter, dried until constant weight in a 100°C oven and cooled down in a dessicator. Acetic acid concentration was determined by a High Performance Liquid Chromatography (HPLC) (Hewlett Packard 1050 with a ionic exchange column Aminex HPX-87H and a index refraction detector HP 1047A). Ammonia was measured by means of LCK 305 ammonia analysis kits (Dr. Lange Nitrax).

The bioreactors broth and the input media were checked for bacterial contamination by direct observation using a microscope (ZEISS AXIOSKOP).

Compartment III

The strains used were *Nitrosomonas europaea* and *Nitrobacter winogradskyi*.

The co-culture used to inoculate the column (*Nitrosomonas europaea* and *Nitrobacter winogradskyi*) was obtained from a preculture in a Biostat B (BRAUN) (Pérez, et al., 1997, TN 37.510). The inoculum volume was approximately 150 ml.

Culture medium was based in a mixture of an addapted media for *Nitrosomonas europaea* (Wijffels, 1994) and an addapted media for *Nitrobacter winogradskyi* (Hendrikus et col., 1992) and (Wijffels, 1994). This media is described in (Pérez J. *et al,* 1997). For this pre-cultures an ammonia concentration of 400 N-ppm, was used.

The flow rate was set at 0.656 ml/min, which corresponds to a dilution rate of 0.083 h^{-1} and a residential time of 12.05 hours for our working volume of 0.475 l. The temperature was fixed at 30°C, pH at 8.2, the magnetic agitation rate at 30 rpm, the recirculation flow at 8.3 ml/min and the aeration rate at 75 ml/min.

The evolution of the culture was followed by measuring inlet ammonia, outlet ammonia, nitrite and nitrate concentrations. Ammonia, nitrite and nitrate were measured using ammonia, nitrite and nitrate analysis kits (Dr. Lange Nitrax, LCK 305, LCK 341 and LCK 339).

The bioreactor outlet and the input media were checked for bacterial contamination by direct observation using a microscope (ZEISS AXIOSKOP).

Compartment IV

The strain used in the cultures was *Spirulina platensis*. Maintenance subcultures and bioreactor tests were done using the Zarrouk salt mixture media (Zarrouk, 1996).

The inoculum volume was fixed as the 10% of the working volume (3.75 l).

Flow rate was set at 0.656 ml/min , which represents a dilution rate of 0.016 h^{-1} and a residential time of 2.29 days for the bioreactor working volume and the base addition (≈250 ml/day). The temperature was fixed at 36.5°C, pH at 9.5 and the aeration (≈ 0.4l/min) and CO_2 flow rates were manually regulated. The light intensity used was 94.37 W/m^2.

The evolution of the culture was followed by measuring biomass and nitrate concentrations. Biomass concentration was determined by optical density at 700nm

(UVIKON) and by measuring dry weight. To determine cell dry weight the sample was filtered through a 0.22μm pre-weighted filter, dried until constant weight in a 100°C oven and cooled down in a dessicator. Nitrate was measured by means of LCK 339 nitrate analysis kits (Dr. Lange Nitrax).

The bioreactors broth and the input media were checked for bacterial contamination by direct observation using an optical microscope (ZEISS AXIOSKOP).

General culture media

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*, was designed. The media used finally was based in a combination of the above-described media for each individual compartment. Some modifications were done because some of the nutrients were in excess and others were not necessary due to this particular experimental procedure. The final composition of the used media can be found in table 1.

Product	1 l. media	
CH_3COONa	3.4179	g
EDTA-Na·2 H_2O	0.1000	g
$MnCl_2$·2 H_2O	0.0082	g
$FeSO_4$·7 H_2O	0.0325	g
KH_2PO_4	0.4000	g
$NaHCO_3$	0.2500	g
$MgSO_4$·7 H_2O	1.2001	g
$CaCl_2$·2 H_2O	0.0907	g
Trace element	1.0000	ml
Biotin	1.0000	ml
$(NH_4)_2SO_4$	2.7280	g
$CuSO_4.5H_2O$	4.0000E-06	g
Na_2HPO_4	0.4890	g
$ZnSO_4.7H_2O$	4.3000E-06	g
$(NH_4)_6Mo_7O_{27}.4H_2O$	0.1770	g
K_2SO_4	0.5500	g
A5	1.0000	ml
B6	1.0000	ml

Table 1. - Media composition for the preliminary test of connection of compartments II, III and IV of MELISSA.

Where the trace element solution, biotin solution, A5 solution and B6 solution were prepared as previously described. The most important characteristics of this media

are the ammonia concentration which is set to give a 400 ppm ammonia concentration in the outlet of compartment II and is expected to be transformed to NO_3^- in compartment III in the reactor, and the acetic concentration which is supposed to be consumed in compartment II. Carbon source in compartment IV is given by CO_2 bubbling.

4. – RESULTS AND DISCUSSION

4.1 - START-UP PROCEDURE

To start up the operation of the linked bioreactors and before proceeding to the connection of the different compartments, each bioreactor was started-up separately.

Compartment II was heat sterilised, inoculated and operated in batch mode until enough biomass concentration was obtained. Afterwards, a continuous operation was started using the general media.

The start-up of compartment III was done as it is described in (Perez et al. 1997, TN 37.510) and (Perez et al. 1997, TN 37.520). The above-described media was used to initialise the continuous operation.

Compartment IV was heat sterilised, inoculated and operated in batch mode until enough cell growth was achieved. Afterwards, a continuous operation was started using the above-described media.

Once the second compartment had reached a steady state (after waiting for 5 residential times, that is, when a constant concentration at its outlet is obtained) its outlet was manually connected to the inlet of compartment III. Culture media was centrifuged (10000rpm, 4°C and 20min) and filtered before being introduced in the next compartment. When the third compartment had reached the new steady state, its outlet was directly connected to the inlet of compartment IV. Connection was done through two filter units as explained before. Having the third compartment reached its new steady state, it could be said that the connection between compartments had been successfully realised.

Evolution of compartments II, III and IV versus time can be followed in figures 5, 6 and 7.

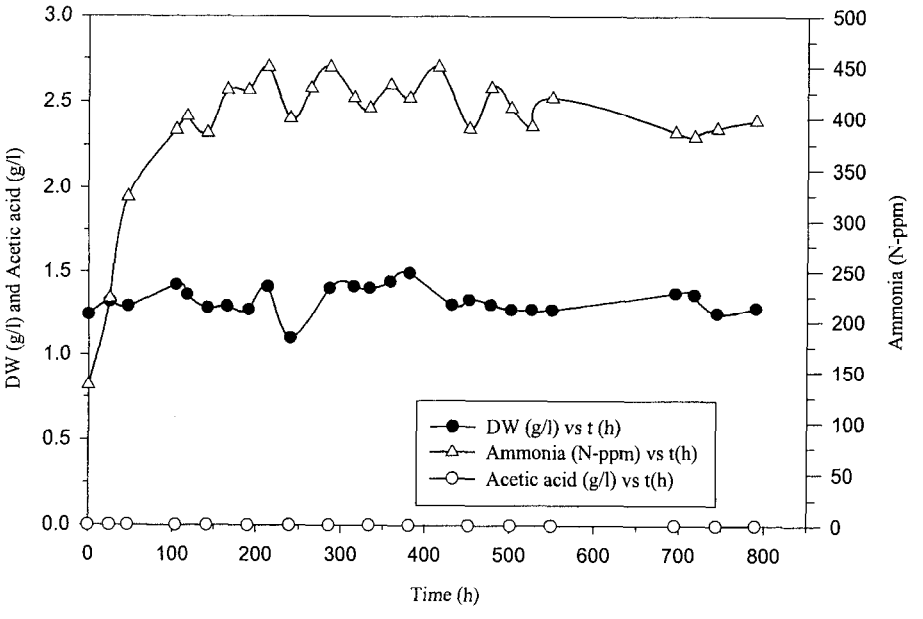

Figure 5.- Evolution of D.W and, acetic and ammonia concentration versus time in compartment II.

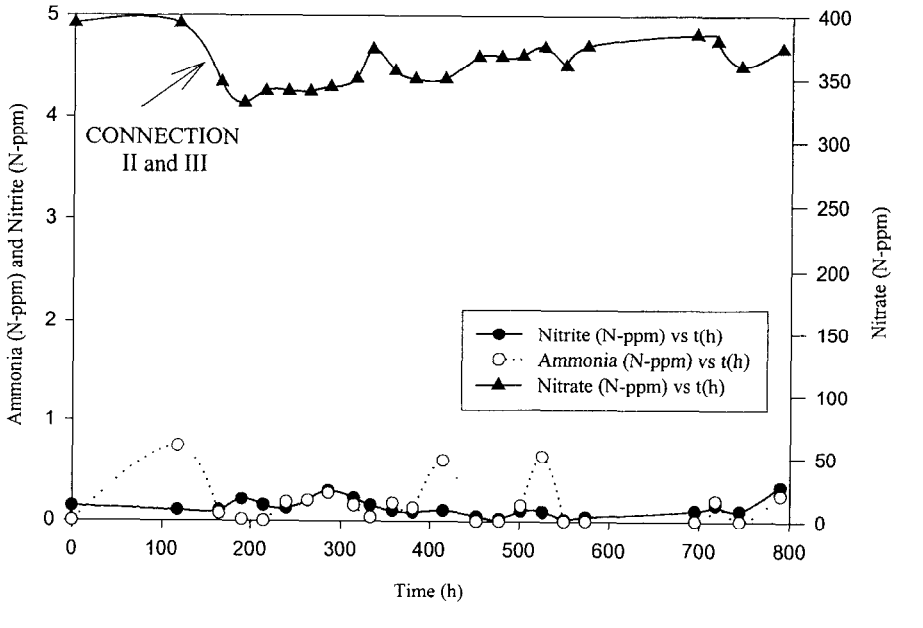

Figure 6.- Evolution of ammonia, nitrate and nitrite concentration versus time in compartment III.

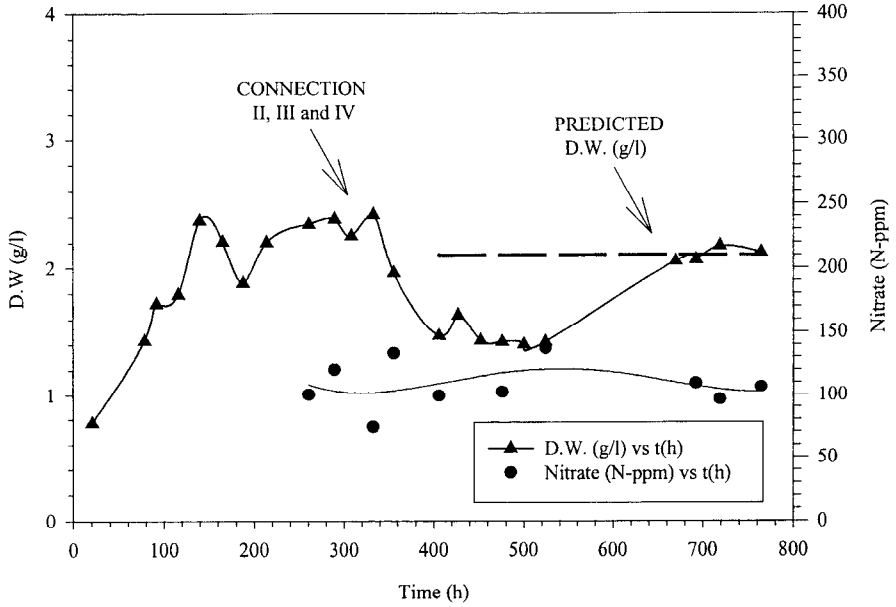

Figure 7. - Evolution of D.W. and nitrate concentration versus time in compartment IV.

4.2 - GENERAL EVOLUTION OF THE TEST

Operational conditions in the three reactors (that is inlet flow rates) were basically fixed by compartment III. Being the smaller one (only 0.5 l by the 2.5 l and 2.75 l of the other compartments) its dilution rate would fix the dilution rate of the others. The operational dilution rate was fixed at the highest dilution rate known from previous experimentation that would give a complete ammonia conversion to nitrate. Once the inlet and outlet flow rates of compartment III were established, the dilution rates of compartments II and IV were fixed, in order to be able to connect the flows of the three compartments.

In principle, it is assumed that the volatile fatty acids have to be consumed completely in the second compartment. However, it could be possible that a small amount of these compounds could be consumed in the other compartments. Nevertheless, there is at present time no previous experimental results allowing knowing the effect of those volatile fatty acids on the other compartments. Therefore, all acetic acid had to be consumed in compartment II. As can be seen in figure 5, this objective was accomplished, as there was no detectable acetic acid concentration in the

outlet of the second compartment. In this case, the second compartment was operating under carbon limitation.

Ammonia concentration in the outlet of compartment II was approximately 400 N-ppm as can be seen in figure 5. This value was expected from the media design and the calculation of the nitrogen consumed by *Rhodospirillum rubrum*. However, the nitrate concentration in the outlet of compartment III was only of approximately 370 N-ppm as it is shown in figure 6. That means, that from the outlet of compartment II to the outlet of compartment III there was an unbalance of 30 N-ppm. Measurements of the ammonia concentration after the centrifugation and the two filtering steps gave also a concentration of 370 N-ppm, the same value that was observed in the outlet of this compartment. A possible explanation for this result might be that due to some salt precipitation problems, ammonia salts were lost in these separation steps.

Carbon and nitrogen balances in compartment II are presented in table 2. Inlet ammonia and acetic acid concentrations were measured from the media giving the following values: 580 N-NH_4^+ppm and 2.60 g/l acetic acid. Cell composition was assumed to be $CH_{1.5945}O_{0.3707}N_{0.2142}S_{0.0037}P_{0.0134}$ (Poughon, L. 1995, TN23.3) as the C/N is approximately 1.75 containing 9.8% of ash in the dry weight (Albiol, J., 1994).

	INLET	**OUTLET**
Acetic acid	1.04 gC/l	0 gC/l
CO_2	0 gC/l	0.07 gC/l
Cells	0 gC/l	0.62 gC/l
TOTAL	**1.04 gC/l**	**0.69 gC/l**
Ammonia	580 N-ppm	410 N-ppm
Cells	0 N-ppm	163.8 N-ppm
TOTAL	**580 N-ppm**	**573.8 N-ppm**

Table 2.- Carbon and nitrogen balances in compartment II.

From this table it can be seen that the nitrogen balance can be considered closed, (within the normal experimental error) with a 97% nitrogen recovery.

On the other hand, the carbon balance cannot be considered closed. In previous experiments, the same results have been obtained. Several possibilities could be responsible for this fact. Production of an extracellular and not measured by-product, change in the biomass composition, for example with PHB (polihydroxibutyrate) accumulation, which would modify the biomass elemental composition, or an excess of carbon dioxide production. Such possibilities will be studied in further studies.

The ammonia conversion to nitrate achieved in compartment III was approximately of 100% as can be seen in figure 6.

In figure 7 some dispersion in dry weight measurements as well as in nitrate concentrations is observed. Dispersion in dry weight results can be attributed to two different facts, some biomass attachment problems appeared and the inlet carbon source concentration was not precisely controlled. Dispersion in nitrate outlet concentration can be attributed to the different inlet composition as well as to the variations in the gaseous carbon source inlet flow (CO_2 flow rate could not be measured and controlled precisely). However, taking into account that pH constantly decreased in the reactor, due to CO_2 addition, and that there was still nitrate in the outlet concentration it can be said that compartment IV was operating under light limitation conditions. It is relevant to say that, as can be seen in figure 7, the experimental dry weight data has a really good correlation with the predicted one using the photosim 2.0 model (Cornet J.F. *et al*, 1993).

Elemental balances in compartment IV have not been done because the inlet carbon flow was unknown and that biomass attachment in the walls of the bioreactor impeded an homogeneous sampling. Therefore, the average dry weight concentration could not be properly measured.

7.- CONCLUSIONS

In summary it can be said that compartments II, II and IV have been successfully connected. Using a global media, the three compartments can be connected and, after waiting for more than 5 residential times in each one, they worked properly. No toxic or deleterious effects were observed during the time while the three of continuous operation of the reactors fed one to each other. Until now compartments II and III have been connected during 4 weeks and compartment III and IV during 3 weeks.

Once the connection has been achieved different experiments varying the light intensity in the photosynthetic bioreactors, the media compositions, etc. have to be done in order to evaluate the performance and behaviour of the cluster of the three bioreactors as a unit, and the effects of the changes on the mass balances and the dynamic behaviour on of the connection.

In future experiments, the inlet carbon source flow in compartment IV will be measured using electronic CO_2 flowmeter allowing to better asses the carbon dioxide supply. In addition, attachment problems in compartment IV and media precipitation will be investigated.

8. – REFERENCES

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APPENDIX I. -LIGHT CALIBRATION OF COMPARTMENT II

For the operation of photobioreactors the light availability determination is of key importance. Determination of light availability at any point of the bioreactor can be done provided the light intensity at the bioreactor surface is known.

Determination of the light intensity at the bioreactor's surface is done 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 can be done using the following equation:

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

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

As the available light measured by the sensor is given in units of $\mu mols/m^2s^2$. 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 has been 0.425, which was 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 were measured in the empty bioreactor and with water circulating through the external jacket.

Light intensity measurements were done at different at different vertical positions and for different voltages supplied to the lamps. The results of these measurements are plotted in figure 8.

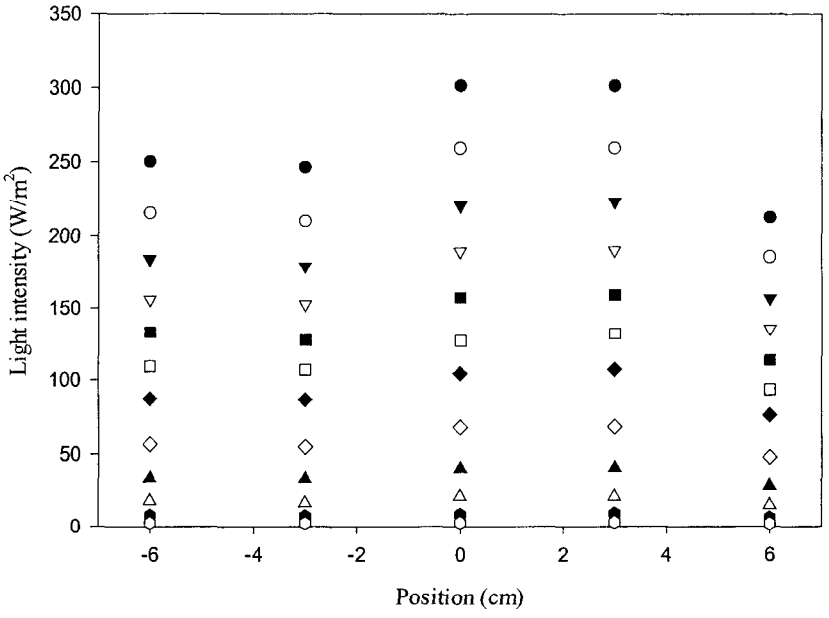

Figure 8.- Light intensity at different vertical positions supplying different voltages (assuming 0cm the centre of the reactor).

The measurements obtained for each voltage at different vertical positions were averaged to obtain a light intensity value for each voltage supplied to the lamps. The light intensity values measured by the sensor in $\mu mols/m^2s^2$, were converted to Fr values using the above mentioned formula and conversion factor. As a result of this measurements a relationship between the voltage supplied to the lamps and the Fr of the bioreactor was obtained as can be seen in figure 9.

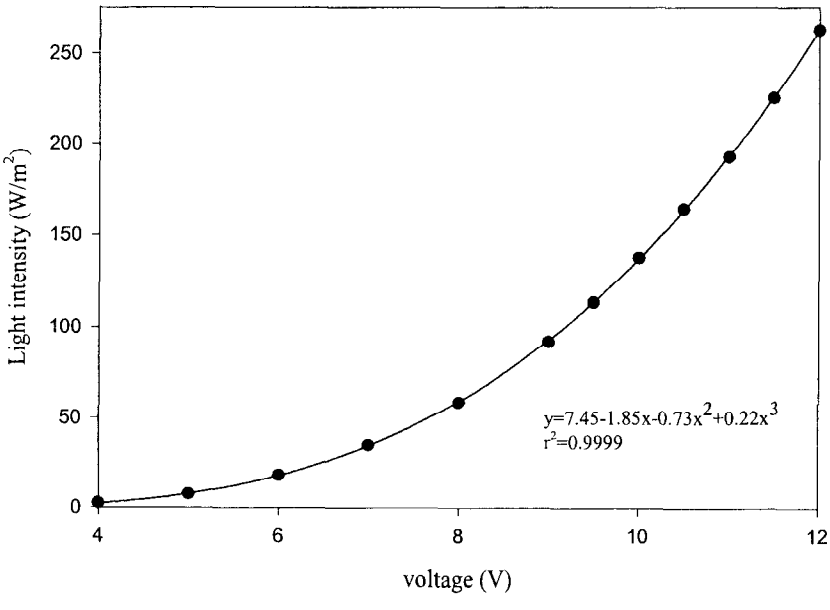

Figure 9 .- Average light intensity supplying different voltages.

APPENDIX II. -LIGHT CALIBRATION OF COMPARTMENT IV

The determination of the light intensity at the bioreactor's surface was 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 was 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 were measured with the empty airlift bioreactor, its internal draft tube dismounted, in order to allow the introduction of the spherical light sensor, and 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 10.

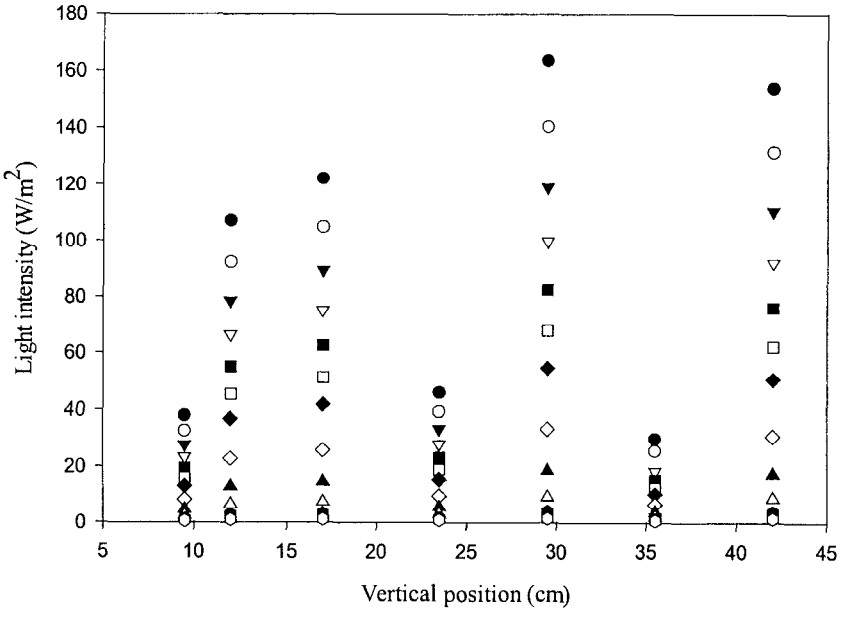

Figure 10 .- Light intensity at different vertical positions supplying different voltages (assuming 0cm the top part of the air-lift).

The average relationship between the voltage supplied to the lamps and the Fr of the airlift bioreactor obtained is presented in figure 11.

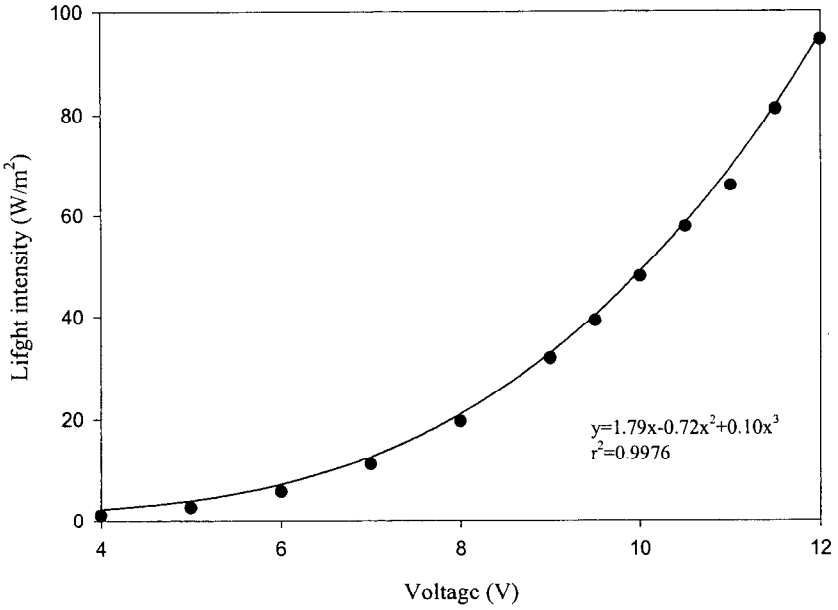

Figure 11.- Average light intensity supplying different voltages.