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

MELISSA

Memorandum of Understanding
ECT/FG/CB/95.205 (After jan 1997 ECT/FG/MMM/97.012)

Contract Number : ESTEC/CONTRACT11549/95/NL/FG

TECHNICAL NOTE : 37.410

Operation of the Nitrifying Pilot reactor

Step 1

Version : 1
Issue : 0

PÉREZ, J.; MONTESINOS, J.L. ; GÒDIA, F.

November 1997

Document Change Log

Version	Issue	Date	Observation
Draft	0	1/10/97	Original Version
1	0	13/10/97	

Table Of Contents

1.- Introduction

The third compartment of the MELISSA loop has been conceived as a packed bed reactor with cells (*Nitrosomonas* - *Nitrobacter*) immobilised on a solid support (Forler, 1994). The support material was selected in preliminary studies (Zeghal, 1992) and consist of polystyrene beads.

The previous work carried out in the MELISSA Pilot Plant with the third compartment bioreactor has focused on its physical characterisation and definition of the control system (TN 25.310, TN 25.330). Therefore the packed-bed reactor is prepared to start its operations. After sterilisation and inoculation, a phase for the attachment of cells on the beads surface for biofilm formation will be required before starting continuous nitrification.

The implemented control loops (temperature, ammonium concentration, liquid level, pH, dissolved oxygen and pressure) and the on-line monitoring of the nitrate concentration allow to maintain the operation conditions in the pilot reactor.

The configuration of the groups Nitrifying and Nitrifying II in the user and system station makes possible the communication between the process (third compartment) and the system station and to obtain data for the GPS (nitr.gps) (TN 25.320).

2.- The suspension cultures

The main goal of these cultures is to achieve enough amount of a mixed culture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* to inoculate the pilot reactor and the bench columns previously designed and constructed.

A general scheme of the strategy used to obtain the suspension cultures is described in **figure 1**.

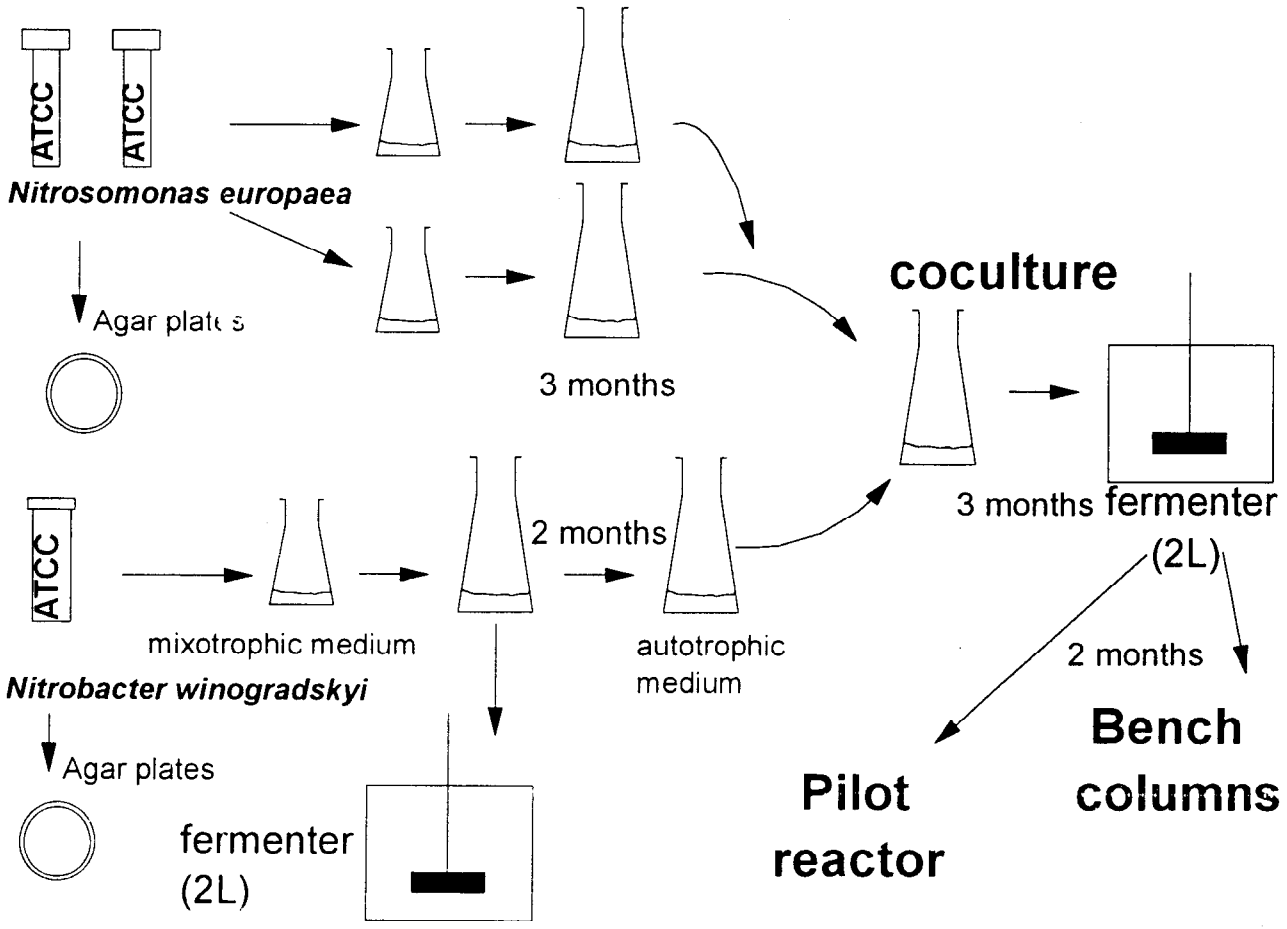

Figure 1.- General scheme of the suspension cultures of *Nitrosomonas* and *Nitrobacter*.

2.1.- Nitrosomonas europaea

The strain *Nitrosomonas europaea* (ATCC 19718) was grown in erlenmeyer flasks to obtain axenic cultures. The erlenmeyers were incubated at 30 °C (in darkness) and the composition of the medium used is described in **table 1**.

Component	Mass (g) per L of demineralised water
$(NH_4)_2SO_4$	1.32
$FeSO_4 \cdot 7H_2O$	0.0025
$CuSO_4 \cdot 5H_2O$	$1.2 \cdot 10^{-4}$
Na_2HPO_4	0.71
$NaH_2PO_4 \cdot 2H_2O$	0.78
$MgSO_4 \cdot 7H_2O$ *	0.052
$CaCl_2 \cdot 2H_2O$ *	$7.4 \cdot 10^{-4}$

Table 1.- Medium for the cultures of *Nitrosomonas europaea* . The pH was adjusted at 8.1 with Na_2CO_3. (* Compounds added after sterilisation by microfiltration, 0.22 µm). Adapted from Wijffels, 1994.

The ammonium and nitrite concentrations were periodically analysed to determine the frequency of the additions of fresh medium. Once the culture reached a suitable volume and concentration of cells, it was possible to start a coculture in erlenmeyer.

2.2.- *Nitrobacter winogradskyi*

The strain *Nitrobacter winogradskyi* (ATCC 25391) was grown in erlenmeyer flasks to obtain axenic cultures. The erlenmeyers were incubated at 30 °C (in darkness) and initially, the medium was mixotrophic (**table 2**) to accelerate the growth velocity. When this strain grow chemolithotrophically decreases the generation time from a range of 70-100 h to a range of 8-14 h (Staley *et al.*, 1988).

Component	Amount per L of demineralised water
$NaNO_2$	0.691 g
NaCl	0.584 g
KH_2PO_4	0.150 g
KCl	0.075 g
Sodium pyruvate ($NaC_3H_3O_3$)	0.550 g
Yeast extract	1.5 g
Peptone	1.5 g
$MgSO_4 \cdot 7H_2O$ *	0.049 g
$CaCl_2 \cdot 2H_2O$ *	0.147 g
Solution D	0.5 ml

Table 2.- Composition of the mixotrophic medium used for the cultures of *Nitrobacter winogradskyi*. The pH was adjusted with Na_2CO_3. (* Compounds added after the sterilisation by microfiltration, 0.22 µm). Solution D: **table 2.2**. Adapted from Hendrikus *et al.*, 1992.

Component	Amount
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	0.08 g
$ZnSO_4\cdot 7H_2O$	0.1 g
$CuSO_4\cdot 5H_2O$	0.02 g
$CoCl_2\cdot 6H_2O$	0.002 g
$MnCl_2\cdot 4H_2O$	0.2 g
denineralised water	1000 ml

Table 2.2.- Composition of the micronutrients solution used for the cultures of *Nitrobacter winogradskyi*.

The nitrite and nitrate concentrations were periodically analysed to determine the frequency of the additions of fresh medium.

When an appropriate concentration of cells was obtained the mixotrophic medium was substituted for an autotrophic medium (**table 3**). This fact decreased the growth velocity, and when the cells were adapted to the new composition of the medium, the beginning of a coculture in erlenmeyer was possible.

Component	Mass (g) per L of demineralised water
$NaNO_2$	1.00
KH_2PO_4	0.68
$Na_2HPO_4\cdot 12H_2O$	1.79
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	0.177
$ZnSO_4\cdot 7H_2O$	$4.3\cdot 10^{-6}$
$CuSO_4\cdot 5H_2O$	$4\cdot 10^{-6}$
$NaHCO_3$	0.0244
$MgSO_4\cdot 7H_2O$ *	0.0517
$CaCl_2\cdot 2H_2O$ *	$7.34\cdot 10^{-4}$

Table 3.- Composition of the autotrophic medium used for the cultures of *Nitrobacter winogradskyi*. The pH was fitted at 8.2 with Na_2CO_3 (* Compounds added after the sterilisation by microfiltration, 0.22 µm). Adapted from Wijffels, 1994.

After rather two months this coculture had enough volume to be transferred into a laboratory fermenter.

2.3.- Cocultures

From the suspension cultures of *Nitrosomonas* and *Nitrobacter* previously obtained, a mixed culture was initiated in erlenmeyer flask. The composition of the medium is detailed in **table 4**. The culture was kept at 30 °C (in darkness).The analysis of NH_4^+, NO_2^-, NO_3^- allow to decide the frequency of the additions of fresh medium

(**table 5**). The samples were also used to check for any cellular contamination by means of microscopic observations (**figure 2**).

Component	Mass (g) per L of demineralised water
$(NH_4)_2SO_4$	1.32
$FeSO_4 \cdot 7H_2O$	0.0025
$CuSO_4 \cdot 5H_2O$	$4 \cdot 10^{-6}$
Na_2HPO_4	0.71
KH_2PO_4	0.68
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.177
$ZnSO_4 \cdot 7H_2O$	$4.3 \cdot 10^{-6}$
$MgSO_4 \cdot 7H_2O$ *	0.052
$CaCl_2 \cdot 2H_2O$ *	$7.4 \cdot 10^{-4}$
$NaHCO_3$ *	0.8

Table 4.- Detailed composition of the autotrophic medium used in the coculture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*. (* Compounds added after the sterilisation by microfiltration, 0.22 µm). Adapted from Wijffels, 1994.

Days of culture	g $N\text{-}NH_4^+$ / L	g $N\text{-}NO_3^-$ / L	
0	0.451	0.081	
8	0.383	0.134	Addition of fresh medium
8	0.244	0.057	
30	0.259	0.136	
50	0.216	0.158	Addition of fresh medium
50	0.169	0.085	
58	0.180	0.092	
84	0.151	0.114	

Table 5.- Evolution of ammonium and nitrate concentrations in the mixed culture.

The nitrite concentration was lower than 30 ppm in all the samples analysed, due to the dilution factor of the analysis, the nitrite concentration was kept under the detection limit of the analysis.

Days of culture	g $N\text{-}NH_4^+$ / L	g $N\text{-}NO_3^-$ / L	g total N / L
0	0.451	0.081	0.532
8	0.383	0.134	0.517
30	0.259	0.136	0.395
50	0.216	0.158	0.374
50	0.169	0.085	0.254
58	0.180	0.092	0.272
84	0.151	0.114	0.265

Table 6.- Evolution of the nitrogen balance in the mixed culture. (g total N / L = g $N\text{-}NH_4^+$ / L + g $N\text{-}NO_3^-$ / L).

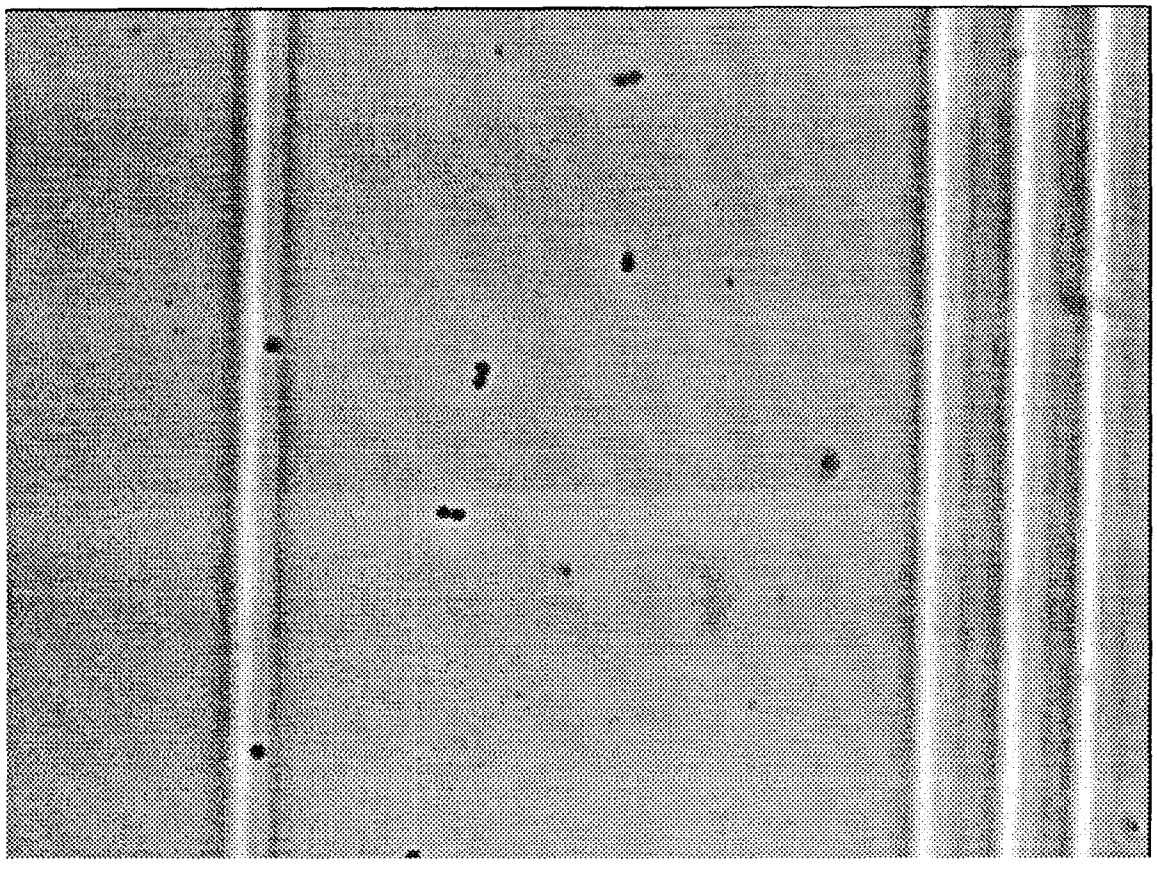

Figure 2.- Microscopic examination of a coculture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*.

The nitrogen balance closure is difficult to determine in this culture, because nitrite concentration has not been determined, because the volumes of the additions of fresh medium are not perfectly known and there were possible evaporation losses. Moreover, although the nitrogen balance was not the main goal of this culture, in some cases a first approach can be made (**table 6**), taking into account the culture concentrations of nitrite usually are low.

After approximately three months, the volume and the concentration of cells of the coculture were enough to inoculate a culture of 750 ml in a fermenter (Biostat B, from Braun Biotech International, 2 L of working volume).

The inoculum volume used was 100 ml, and the operation conditions in the bioreactor are detailed in **table** 7.

The analysis of ammonium and nitrate determined the frequency of the addition of fresh medium until reaching the maximum capacity of the fermenter, 2 L of volume. Regular microscopic observations were made along the process (**figure 3**).

After reaching the maximum capacity of the reactor, culture medium was renovated periodically by withdraw of 0.5 L of spent medium, and addition of 0.5 L of new medium. By this operation mode the risk of accidents is much lower being such consideration a very important fact having into account the culture have to operate

during long periods. In **table 8** and **figure 4**, the evolution of the pH and the concentration of ammonium and nitrate are presented and the additions of ammonium and fresh medium are detailed.

Variable	Range	Control
Temperature	28 °C	automatic
pH	7.80-8.20	automatic (CO_2)/manual (Na_2CO_3, 30 g/L)
Dissolved oxygen	60-100 %	without automatic control
Stirring	300 r.p.m.	automatic
Light conditions	darkness	

Table 7.- Coculture conditions in the reactor Biostat B.

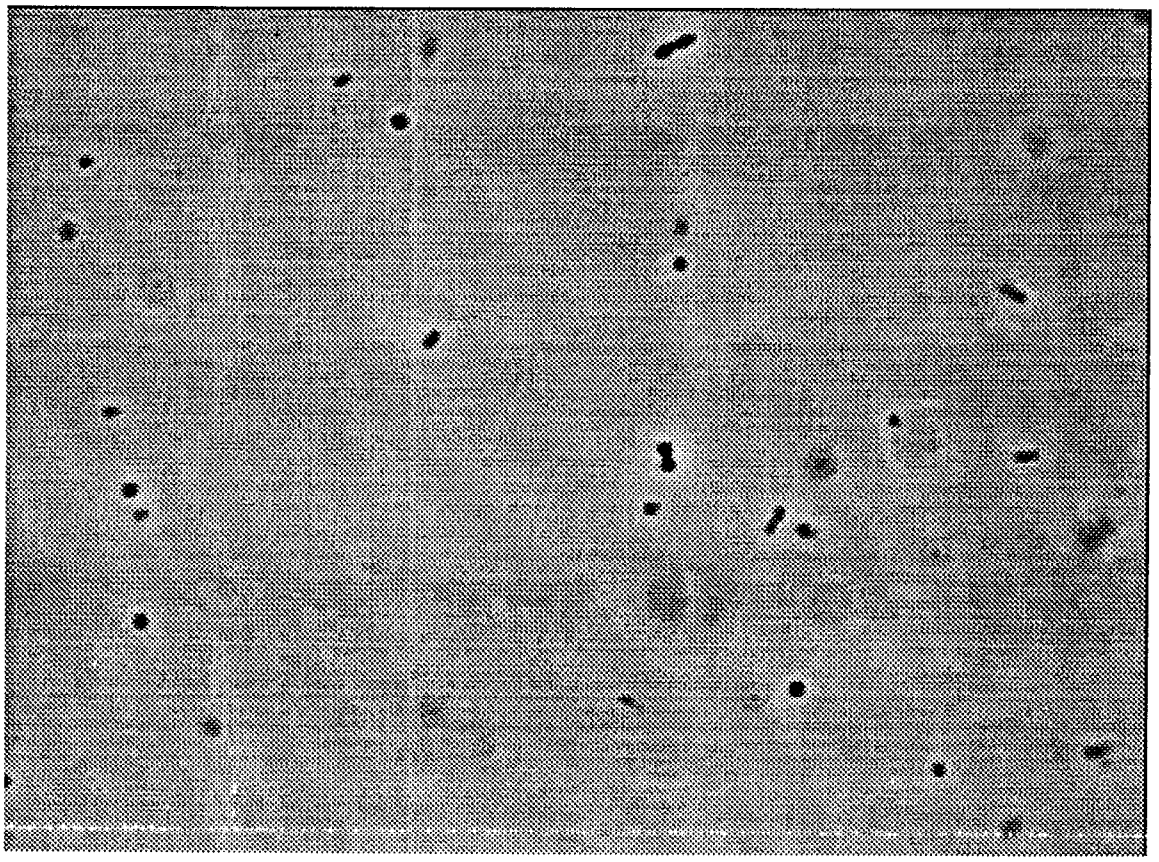

Figure 3.- Microscopic examination of a coculture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*.

The ammonium additions were done in order to maintain the ammonium concentration in the reactor about 0.28 g $N\text{-}NH_4^+$/L. The level of dissolved oxygen was constant about 90 %, for this reason it was not automatically controlled, although after some of the additions the level of dissolved oxygen decreased to 60 % during several hours.

Days of culture	pH	g $N\text{-}NH_4^+$ / L	g $N\text{-}NO_3^-$ / L	Observations
0				inoculation
1	7.90	0.130	0.073	
3	7.50	0.117	0.063	
5	7.90	0.108	0.077	
8	7.20	0.102	0.088	fresh medium added
8	7.82	0.113	0.077	
12	8.13	0.084	0.111	
15	8.14	0.054	0.151	
18	8.20	0.037	0.163	
20	8.19			0.21 g $N\text{-}NH_4^+$ added
22	7.95	0.280	0.169	
27	8.10	0.138	0.301	
32	8.04	0.128	0.318	
36				0.8 g Na_2CO_3 added
38	8.17	0.087	0.342	
42		0.025	0.393	
43				0.8 g Na_2CO_3 added
45	8.07	0.111	0.286	
50	8.36	0.088	not determined	fresh medium added ; 0.28 g $N\text{-}NH_4^+$ added
60	8.24	not determined	not determined	
62	8.31	0.113	0.191	
63				fresh medium added; 0.26 g $N\text{-}NH_4^+$ added
63		0.264	0.185	
66		0.033	0.418	
69				0.5 L extracted, 0.5 L of fresh medium added; 0.42 g $N\text{-}NH_4^+$ added
69	8.17	0.250	0.314	
71				continuous operation
73		0.258	0.248	continuous operation stopped
78	8.04	0.113	0.337	
85		0.006	0.483	0.5 L extracted, 0.5 L of fresh medium added
96	8.85	0.007	0.497	
105				0.5 L extracted, 0.5 L of fresh medium added
113				0.5 L extracted, 0.5 L of fresh medium added
120	8.38	0.003	0.360	

Table 8.- Evolution of the coculture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* in the reactor Biostat B.

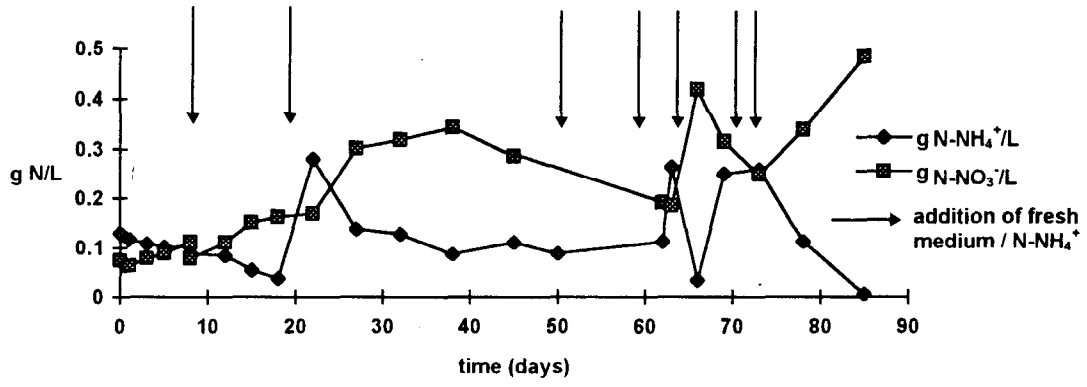

Figure 4.- Evolution of the ammonium and nitrate concentrations of the coculture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* in the reactor Biostat B.

The nitrogen balance can not be totally closed for the same reasons discussed about the analysis of table 5. Nevertheless, in the major part of the cases the balance closure could be considered satisfactory, having in mind the points already explained in the erlenmeyer co-culture. Hence, adding the ammonium and nitrate concentration of the table 8, the obtained values of the total nitrogen are presented in (**table 9**).

Days of culture	g $N\text{-}NH_4^+$ / L	g $N\text{-}NO_3^-$ / L	g total N / L
1	0.130	0.073	0.203
3	0.117	0.063	0.180
5	0.108	0.077	0.185
8	0.102	0.088	0.190
8	0.113	0.077	0.190
12	0.084	0.111	0.195
15	0.054	0.151	0.205
18	0.037	0.163	0.200
22	0.280	0.169	0.449
27	0.138	0.301	0.439
32	0.128	0.318	0.446
38	0.087	0.342	0.429
42	0.025	0.393	0.418

Table 9.- Evolution of the nitrogen balance in the mixed culture. (g total N / L = g $N\text{-}NH_4^+$ / L + g $N\text{-}NO_3^-$ / L).

3.- Start-up procedure for the pilot nitrifying column

In order to maintain the sterility of the cultures of the pilot reactor, a sterilisation process was proposed by Forler (1994). The main problem of the process is the sterilisation of the polystyrene beads, due to the fact that this support does not resist the high temperature and pressure of the conventional steam sterilisation processes (120 °C, 2,2 atm). Therefore, an sterilisation procedure based on acid/base treatment had been designed (Forler, 1994).

3.1.- Sterilisation process

The sterilisation process consist mainly in two steps :

- With the pilot reactor empty of beads, start the phase of flowing steam (without pressure). The position of the valves in this step is described in the **figure 5**, and the **table 10**. The duration of this process is about 2 hours (in this period of time the pressure is maintained at 1.1 atm and the temperature over the 100 °C).
- Once the vessel and the gas loop are sterile, the next step is the filling of the bed with the support (the polystyrene beads are preliminary treated with acid/base solution and washed), then, after the reactor is again closed, the acid/base sterilisation process start :

    - reactor filling with acid (pH = 2.0), during about 6-7 hours ;
    - washing phase (demineralised sterile water until pH = 6-7) ;
    - reactor filling with base (pH = 12.0), during about 6-7 hours ;
    - washing phase (demineralised sterile water until pH = 6-7).

3.2.- Calibration of the probes

The pH probes were washed and calibrated before the beginning of the sterilisation process.

The temperature and the pressure sensors were checked for its proper sterilisation before to start the sterilisation of the pilot reactor.

3.3.- Problems found in the sterilisation process

During the process of sterilisation, specifically in the step of filling the reactor with base solution, the two teflon sections of the packed bed were damaged and cracking of the material occurred. The two sections of the reactor showed important cracks that

did not allow to follow the sterilisation process and, in consequence, the whole operation of the third compartment.

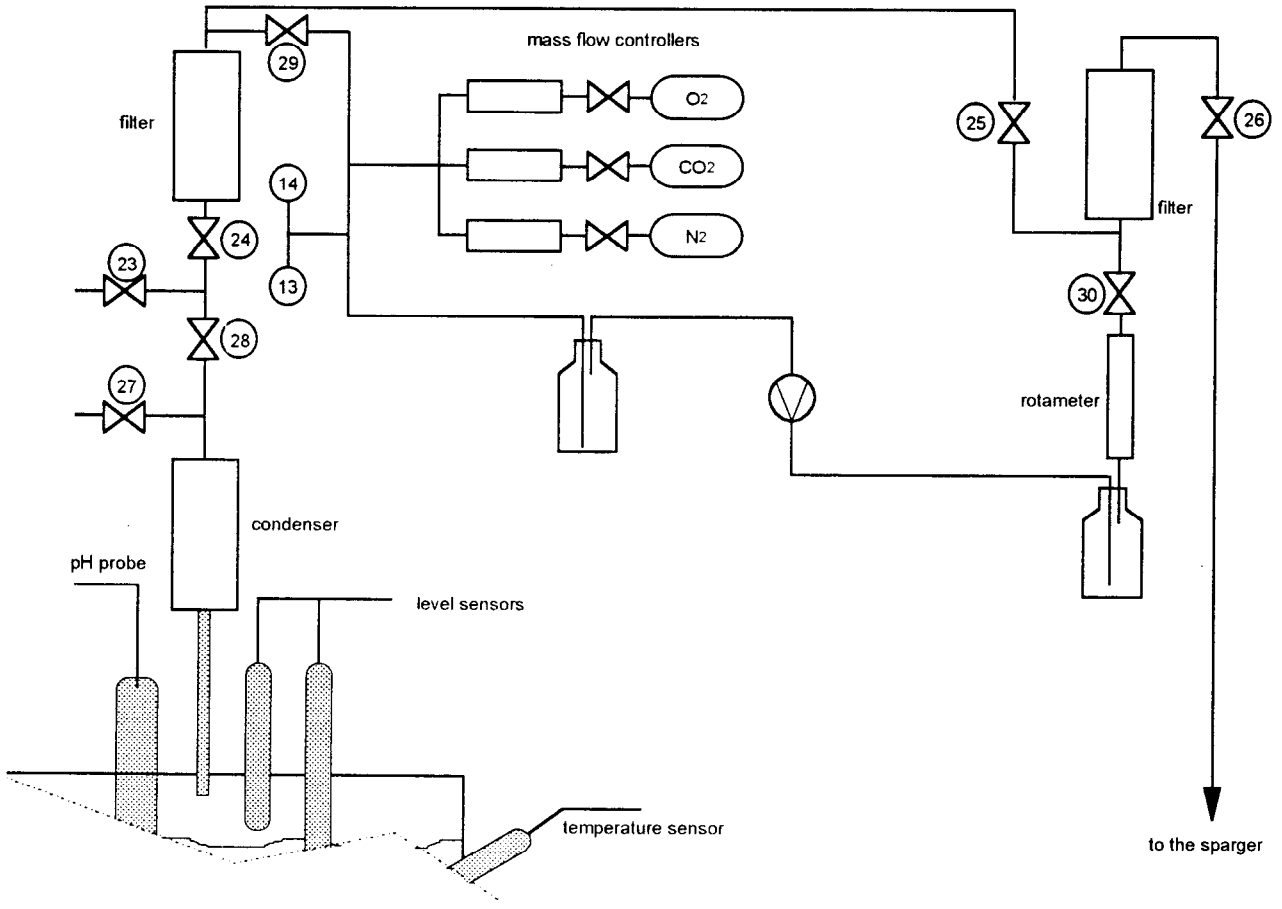

Figure 5.- General scheme of the gas loop in the pilot reactor of the third compartment.

Ref. n°	Steam sterilisation	Acid/base sterilisation	Normal operating conditions
23	Open	Open	Closed
24	Open	Closed	Open
25	Open	Closed	Closed
26	Open	Closed	Open
27	Open	Closed	Closed
28	Closed	Open	Open
29	Closed	Closed	Open
30	Closed	Closed	Open

Table 10.- Positions of the valves of the gas loop for the sterilisation and for the normal operation

4.- Redesign of the new sections of the pilot reactor

As a consequence of the accident described the teflon parts of the packed bed have been replaced by stainless steal parts.

The replaced parts of the packed bed are made in stainless steal AISI-316, and only have slight differences with the original teflon sections. In **figure 6** the detailed design of these pieces is shown.

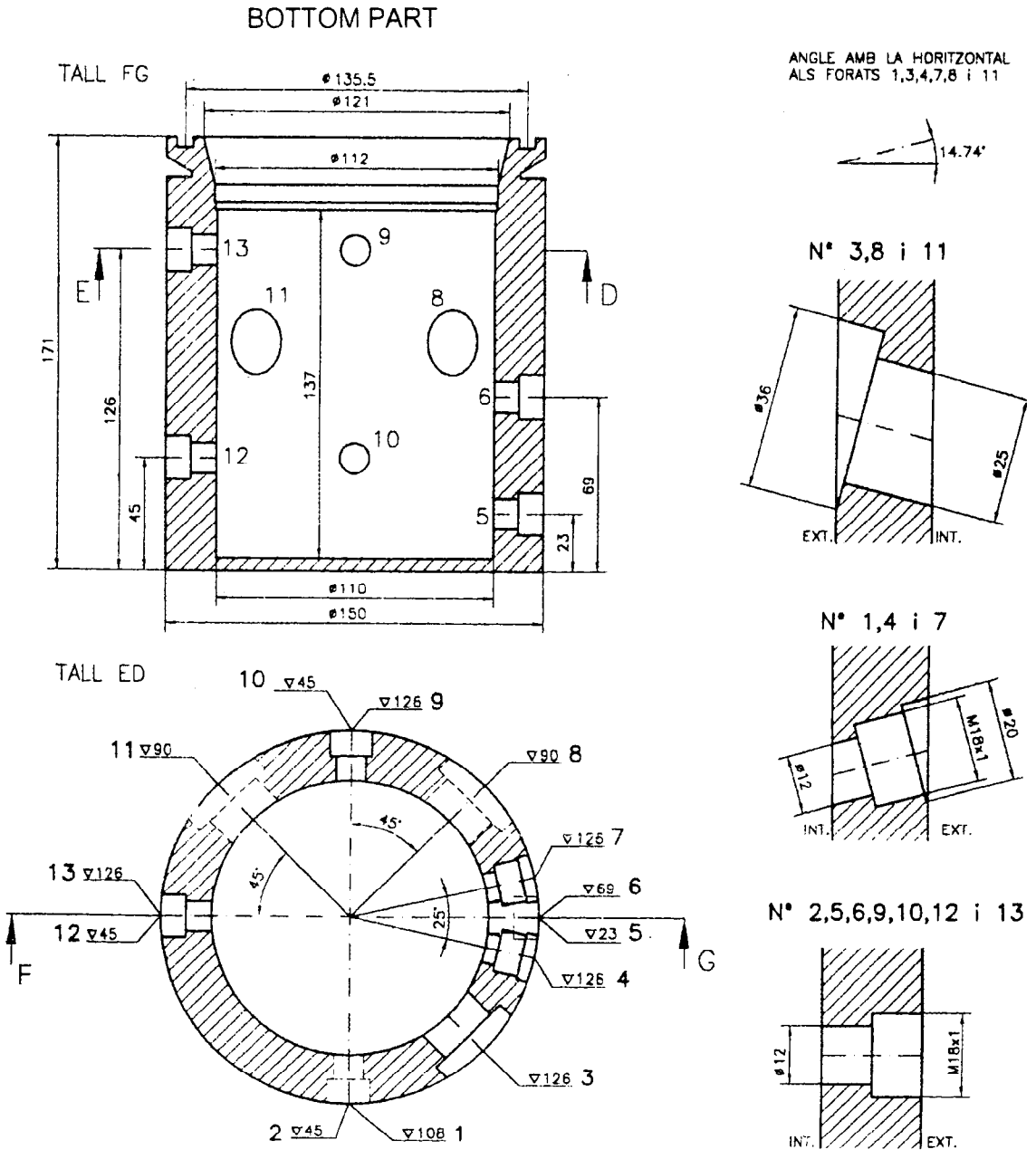

Figure 6a.- Detailed design of the new bottom part of the pilot reactor.

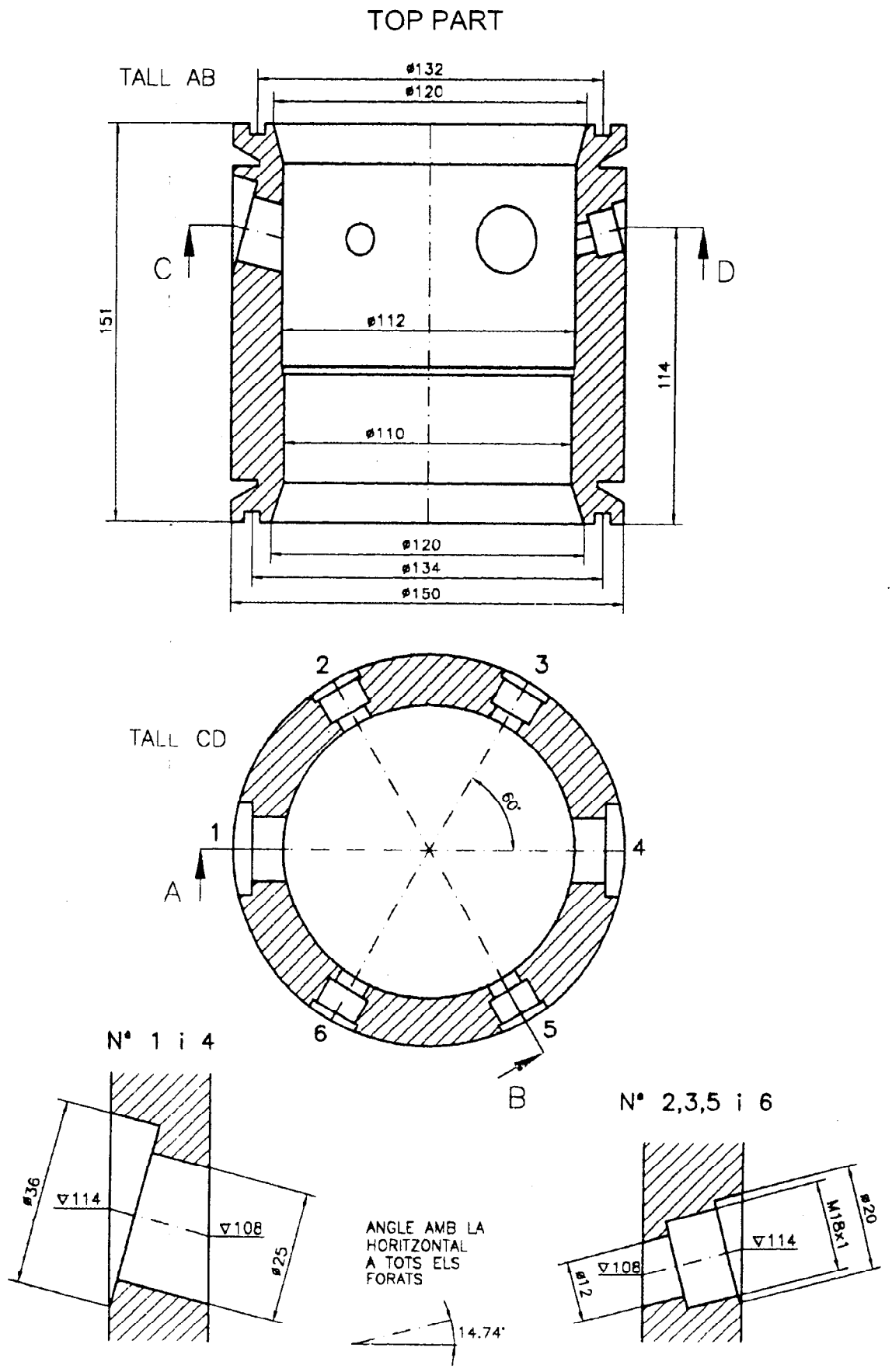

Figure 6b.- Detailed design of the new top part of the pilot reactor.

References

FORLER, C. ; (1994). MELISSA : development of a fixed bed pilot reactor for a continuous axenic coculture of *Nitrosomonas europae* and *Nitrobacter winogradskyi*. YGT ESA/YCL, X-997, 1994.

HENDRIKUS, J. ; LAAMBROEK, HJ ; GERARDS, S ; (1992). Competition for limiting amounts of oxygen between *Nitrosomonas europaea* and *Nitrobacer winogradskyi* grown in mixed cultures. Microbiology. Vol. 159, pp. 453-459.

PÉREZ, J. ; MONTESINOS, J.L. ; GÒDIA, F. ; (1996). Starting of the nitrifying reactor. TECHNICAL NOTE 25.310. ESTEC CONTRACT 11549/95/NL/FG.

PÉREZ, J. ; MONTESINOS, J.L. ; GÒDIA, F. ; (1996). Starting of the nitrifying reactor. TECHNICAL NOTE 25.320. ESTEC CONTRACT 11549/95/NL/FG.

PÉREZ, J ; MONTESINOS, J.L. ; GÒDIA, F. ; (1996). Starting of the nitrifying reactor. TECHNICAL NOTE 25.330. ESTEC CONTRACT 11549/95/NL/FG.

STALEY, J.T. ; BRYANT, M.P. ; PFENNING, N. and HOLT, J.H. ; (1988) Bergey's manual of Systematic Bacteriology. Williams & Wilkins. London. Vol.3 pp. 1814-1817.

WIJFFELS, R.H. ; (1994). Nitrification by immobilized cells. Landbouwuniversiteit Wageningen. PHD thesis.

ZEGHAL, S. ; (1992) Nitrification compartment. TN 1 ESA-MELISSA Contract PRF 310882.