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RHODOSPIRILLUM RUBRUM CARBON LIMITATION TESTS

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1 Introduction

The development of compartment II, and particularly the heterotrophic subcompartment, is being performed at different levels, one of those being the characterization of the photoheterotrophic bacteria used in that compartment. In order to characterise the growth of *Rhodospirillum rubrum* cells, several studies were performed previous to the present one. The preliminary studies were done using different volatile fatty acids as carbon source at different light intensities (Lenguaza *et al.* 1997). These tests were batch cultures carried out using monodimensional illumination. Those preliminary tests were followed by a range cultures were the growth rate was evaluated using two different kinds of light source, halogen and incandescent lamps (Cabello *et al.* 1999). Finally, several continuous culture experiments were carried out using acetic acid as carbon source at different light intensities (Cabello *et al.* 2001). Complementary data on the behaviour of this compartment using different carbon sources was also obtained during the bench loop interconnection tests (Creus *et al.* 2002a and Creus *et al.* 2002b).

The original tasks assigned in the contract to this workpackage had the main objective to test the light control law for this compartment developed by other MELISSA partners. However, at present time this control law is still in progress due to the difficulties found in its development. Due to this delay it was agreed with ESA to modify the original task in order to collect experimental data complementing the previously obtained ones. The experiments proposed had to provide results emphasizing the carbon limiting conditions. Due to the limited data available in those conditions but also to limit the number of experiments to perform (that is manpower and budget allocation to the already assigned ones), it was agreed to select three different carbon sources among the main ones produced in compartment I. For each carbon source experimental data should be obtained for at least two light intensities and two dilution rates. The experiments should be performed trying to approach, as much as possible, the conditions were the culture changes from light limiting conditions to carbon limiting conditions as those are considered the most interesting points. While performing the tests, biomass composition should be analysed at each steady state and any characteristic trend showing variation in the measured composition remarked.

Consequently in this work package several tests have been carried out, using acetic, propionic and butyric acids as carbon sources, under different carbon limitation conditions. These tests have been performed in continuous culture mode using two different photobioreactors (2.4 L and 8 L) at different illumination conditions and at different dilution rates.

The present results complement previously obtained experimental data, already reported in TN 37.7 (Cabello *et al.* 2001). These results, as the previous ones, indicate some relationship between the ratio of available light/available carbon and the performance of the culture. The empirical data indicate that when this ratio is low (low light intensity and/or high carbon concentration), the *R. rubrum* cells start to accumulate granules of reserve polymers (such as poly-β-hydroxy-butyrate, poly-β-hydroxy-valerate and sometimes glycogen) inside the cell, changing its morphology and physiology.

The obtained results complement the other obtained ones and contribute to the progress towards the building or verifying mathematical models that will be later used in the planning, sizing or development of the control of this compartment.

2 Material and methods

The bacterial strain used was *Rhodospirillum rubrum* (ATCC 25903) and was obtained from the American Type Culture Collection. The strain was received freeze-dried and was revived using R8AH medium (ATCC medium 550). This medium was also used for periodical subculture of the backup inoculum cultures.

The culture medium used during the tests was based on the basal salt mixture formulated by Segers & Verstraete. The carbon and electron sources used in the tests were acetic acid, propionic acid, and butyric acid and biotin was used as the only vitamin. The culture medium composition is detailed in Appendix 1.

Acetic acid tests were carried out in a 8 L *Bioengineering* photobioreactor (figure 1). The bioreactor vessel is an illuminated cylindrical glass vessel with stainless steal head and bottom sections where the probes are located. Because of the metallic sections, especially due to the head section size, the illuminated volume fraction (IVF) is equal to 0.52 (IVF is the fraction of the bioreactor total volume that is externally illuminated by lamps (4.16 L) previously measured filling the cylindrical glass vessel with water). The bioreactor is surrounded by a set of 48 halogen lamps (Sylvania professional BAB 38º 12V 20W, improved version, cool beam, UV filtered (green box) code type 215). The bioreactor is mechanically stirred (300 rpm) by a central stirrer with five propellers, as it is described in TN 47.1 (Cabello *et al.*, 2000). The temperature was controlled at 30 ºC by means of a thermostatic bath, which pumps cool water through the external glass jacket of the photobioreactor, located in the glass vessel. The temperature of the gas condenser was maintained low by means of a water flow at 4 ºC. The pH was maintained at 6.9 by means of an auxiliary control unit, which added HCl (1.5 M) or NaOH (1.5 M) according to the deviation of the pH from the set point value. Anaerobic conditions were maintained by filling the headspace with Argon gas.

Propionic and butyric acid tests were performed in a 2.4 L Applikon photobioreactor (figure 2). This reactor consists in a 2.4 L cylindrical glass vessel mechanically stirred by a Rushton turbine (300 rpm). The external surface of the reactor was completely illuminated by either 15 or 30 halogen lamps (Sylvania professional BAB 38º 12V 20W, improved version, cool

beam, UV filtered (green box) code type 215). The temperature was controlled at 30 ºC by means of a thermostatic bath, pumping cool water through the external glass jacket of the photobioreactor. The temperature of the gas condenser was maintained low by means of a water flow at 4 ºC . No gas was added through the internal sparger and the CO_2 requirements of the culture were provided by the concentration of $NaHCO_3$ in the fresh medium. The pH was maintained at 6.9 +/- 1 by means of an auxiliary control unit, which added HCl (1.5 M) or NaOH (1.5 M) depending on the deviation of the pH from the set point value.

In all of the tests, biomass concentration was determined measuring the dry weight. The dry weight was determined by filtering the sample through a 0.22 μm Millipore filter, dried until constant weight in a microwave oven (20 min., 150 W) and cooled to room temperature in a desiccator. Dry weight value is calculated as the arithmetical mean of three samples. Carbon source concentration was analyzed by gas chromatography analysis. The carbon concentration analysis method is described in appendix 2.

Samples were collected from the output flow of the photobioreactor by filling a 10 or 25 mL sterile tube immersed in ice for each steady state. The time to fill the tubes depends on dilution rate. The procedure is repeated until enough liquid or biomass is collected for the required analysis.

- For liquid samples each tube is centrifuged (14000 rpm, 20 minutes, 4ºC) for initial biomass separation. Liquid sample is collected from the supernatant and frozen at -80 ºC until analyzed.
- For biomass samples each tube is centrifuged (14000 rpm, 20 minutes, 4ºC) for initial biomass separation. Supernatant is discarded and biomass resuspended in saline solution (0.4M NaCl, 50 mM $MgSO_4$). The centrifugation step is repeated at the same conditions and biomass resuspended to reach approximately a concentration between 5- 10 g/L. The resuspended sample is freeze dried and stored in sealed vials until required for analysis. For freeze drying, immediately after centrifugation the biomass suspension is quickly frozen by immediate immersion in liquid nitrogen for 5 minutes. Up on freezing, biomass is introduced into the freeze drier compartment and vacuum conditions are applied overnight.

Figure 1: Bioengineering 8L photobioreactor

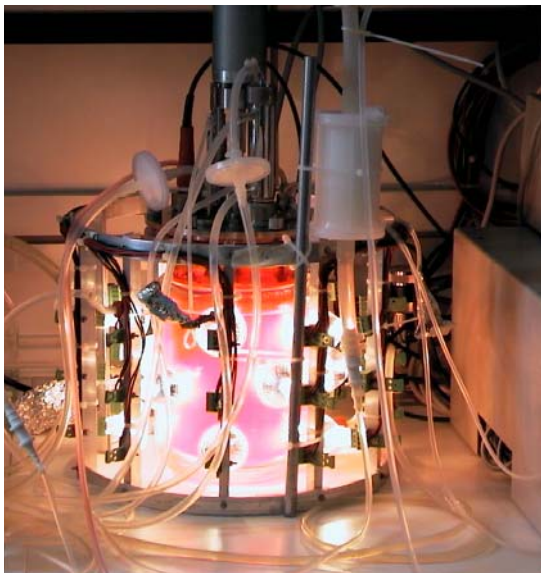

Figure 2: Applikon 2.4L photobioreactor

3 Experimental results and discussion

Photoheterothophic organisms, and particularly the *Rhodospirillaceae* genera, use the carbon source as both carbon and electron source for the photosynthetic process. This allows them to synthesize the building blocks that are later used for biomass growth. Consequently, there is a direct link between the light energy supplied and the carbon source consumed. In the MELISSA loop those microorganisms are used to remove the volatile fatty acids present in the output flow of compartment I.

For the design, improvement and control of the MELISSA loop it is necessary to understand and be able to predict the behaviour of those bacteria from the key variable values of the culture such as the type of carbon source or the amount of light energy supplied. This objective is usually fulfilled by means of mathematical models able to reproduce the bacteria behaviour according to the values of those key variables. In order to be able to build those models it is necessary to obtain experimental data of the bacterial growth for different values of the key variables. This is the main purpose of the tests described in this technical note.

Compartment I can produce a range of different volatile fatty acids depending on operational conditions. The volatile fatty acids used in this work package are the main volatile fatty acids produced by compartment I in the previous tests, namely acetic, propionic and butyric acids. Accordingly, the tests performed and described in the following consist on continuous cultures using either acetic, propionic or butyric acids as the only carbon source. As main goals it required to obtain, for each carbon source, at least one steady state where the carbon source is completely consumed (this allows to assume carbon limiting conditions), and modify the conditions until the carbon source is partially consumed and light energy limitation can, with a high probability, be assumed. A new steady state in those conditions of carbon accumulation is sometimes not possible to be obtained and is also an objective to obtain data in those non stable conditions. Due to the limited data available at the time of requesting the tests, the exact values of the main variables to use were not initially fixed. It was agreed that they had to be fixed during the tests in order to fulfil the mentioned carbon or light energy source limiting conditions. The results of the different experiments performed are

grouped according to the carbon source used and preliminary discussion on the results is performed in a later section including the results of all biomass analysis.

3.1 Acetic acid tests

Based on the previous preliminary tests using acetic acid (TN 37.7, Cabello *et al.* 2001) it was known that the light energy provided by this bioreactor allowed to completely consume an amount of carbon source around 0.5 g C/L at 0.04 h^{-1}. (gC/L: grams of acetic acid carbon per litre). It was therefore decided to start the continuous cultures using a concentration of 0.4 g C/L of acetic acid and a dilution rate or 0.04 h^{-1} at the maximum light intensity. This should allow to reach a steady state were all the carbon source was consumed.

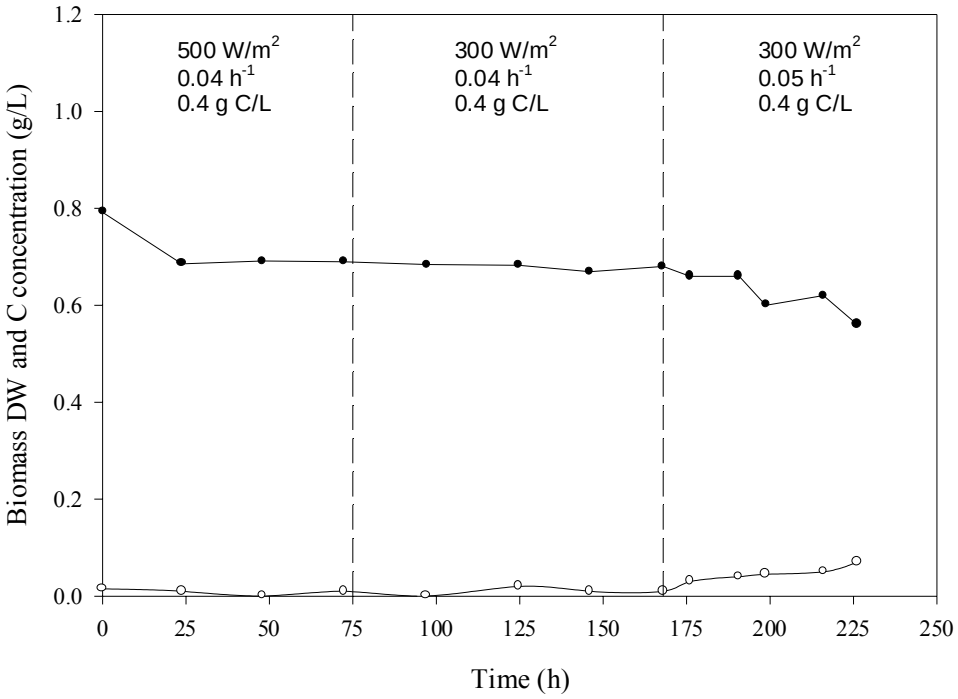

Figure 3: Step down in the incident light intensity and step up in the dilution rate. Cell attachment appeared at 0.05 h^{-1} using 300 W/m^2 and 0.4 g C/L (acetic acid).

As can be seen in the first part of figure 3 a steady state was attained at 0.04 h^{-1} and 500 W/m^2 with stable values of dry weight around 0.68 g/L of biomass, confirming the previous assumptions.

One the steady state of total carbon source consumption was obtained, culture conditions had to be changed in order to drive the culture to a new state were the carbon source accumulates (that is, to pass from a carbon limitation situation to a non carbon limitation one so that the threshold conditions are delimited). This can be obtained either by means of decreasing the light energy supply or by means of increasing the dilution rate. As this culture was started at the maximum light intensity possible and to confirm that the light intensity was not initially the main limiting factor, it was decided as a first step to decrease the light intensity.

When the step down in the incident light intensity was carried out (Figure 3, second phase), the dry weight concentration was not significantly modified from the previously reached level. Therefore it was confirmed that the light intensity used in these initial culture conditions was not the growth limiting factor and consequently the cells were growing under carbon limitation. The fact that the residual carbon concentration is very low during the 0.04 h^{-1} test supports also this conclusion. It also indicates that this bioreactor might presumably sustain higher growth rates and in order to reach the limit of the growth rate that can be sustained at the provided light intensity, the dilution rate was increased. When the dilution rate is increased from 0.04 h^{-1} to 0.05 h^{-1}, the cells started to attach to the internal wall of the glass vessel. The cell attachment was the main reason to stop the culture. This effect was already previously reported in TN 37.7 (Cabello *et al.* 2001). The present result confirms the emergence of this behaviour in acetic acid cultures, when light energy is the limiting factor for growth and the carbon source concentration accumulates surely due to the fact that the carbon source supply exceeds the consumption for growth. Therefore, at the end of the tests reported in figure 3, it can be said that the incident light intensity became the limiting factor when the dilution rate was increased from 0.04 h^{-1} to 0.05 h^{-1}, at an incident light intensity of 300 W/m^2.

When dilution rate is increased it could initially be expected that the biomass concentration would decrease until reaching a new steady state where the increased average light intensity would allow to sustain an increased growth rate. In the present case however the cells aggregate and attach to the bioreactor walls, further decreasing the light intensity in the centre

of the bioreactor and impeding a new steady state to be reached. Those kinds of conditions would rend the MELISSA loop unstable and therefore have to be located, studied, and avoided.

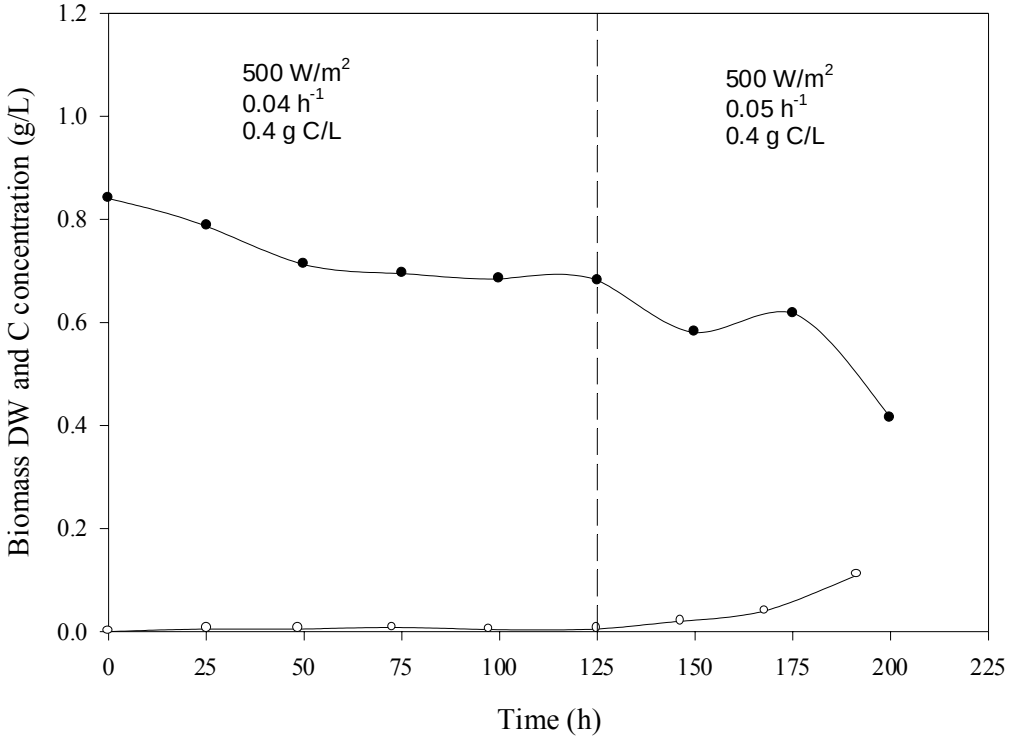

Figure 4: Step up in the dilution rate after the stable conditions of the previous test were reached. Cell attachment appeared at 0.05 h^{-1} using 500 W/m^2 and 0.4 g C/L (acetic acid).

In the test that followed, the same increase in dilution rate (from 0.04 to 0.05 h^{-1}) done in the previous test (figure 3) was performed but using the maximum light intensity the bioreactor can supply (500 W/m^2). The purpose was to further evaluate if a dilution rate of 0.05 h^{-1} can be obtained in this bioreactor for this type and amount of carbon source. The results obtained are reported in figure 4. It can be seen that in the first phase the culture tends to stabilization at the same biomass levels previously obtained. After increasing the dilution rate, and identically as in the previous test at 300 W/m^2, the culture started a strong wash out dynamics while biomass aggregated and attached to bioreactor walls.

Different hypothesis could be proposed to explain the results. According to a Monod type of kinetic model, increasing a dilution rate requires an increase in the limiting nutrient to allow for a corresponding increase in the growth rate. In the case of a carbon source limitation this increase is only measurable for high dilution rates as the affinity for the carbon source is usually high. Any increase in the carbon source in the liquid medium at steady state results in an immediate proportional decrease of the steady state biomass level. If this were the case a new steady state would have occurred at a lower amount of biomass level.

Another point to consider is that, when a culture also depends on light intensity, any increase in the growth rate requires that the light energy conditions allow for the increase in growth rate. Usually the minimum levels of light energy required to sustain a certain growth rate increase faster than the levels of carbon in the liquid medium. That is, for the same increase in growth rate, a culture under light limiting conditions is expected to decrease its steady state biomass level than the same culture under carbon limiting conditions[1]. This fact also means that increasing the dilution (\growth) rate can result in passing from the carbon limiting conditions to the light limiting conditions. Light limitation can be compensated by the increased light levels[2] reached at a lower biomass concentration at steady state which also would result into an accumulation of the carbon source in the liquid medium. This situation would not be a stable one, if the increased carbon source level induced a decrease in the growth rate (substrate inhibition). That is, the accumulated carbon would decrease the growth rate down to values below the existing dilution rate forcing a washout and increasing the carbon accumulation and further inhibition. One way to decrease this amount of accumulated carbon is to decrease the carbon concentration in the input medium so that lover carbon source levels are reached when biomass decreases. This was tested in the following test.

[1] from this point of view the situation is similar to the classic case when increasing the dilution rate of one heterotrophic culture under aerobic carbon limiting conditions and the culture passes from the carbon limiting conditions to an oxygen limitation.
[2] It should also be noted though that if light intensity increases above a certain limit, it usually also has inhibitory effects.

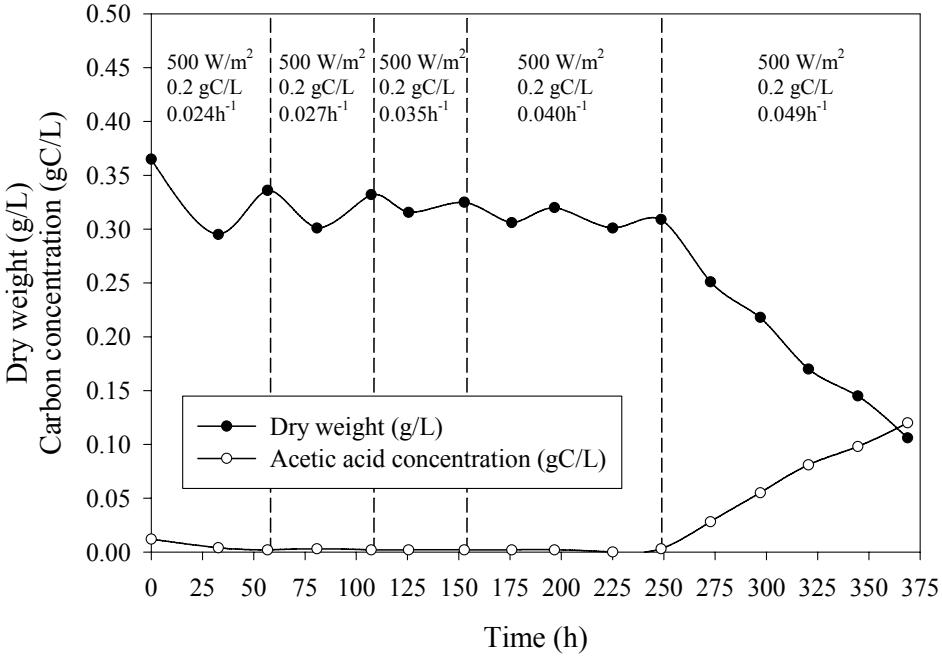

Figure 5: Tests carried out under carbon limitation at 500 W/m^2 increasing dilution rate up to 0.049 h^{-1}.

Since the incident light intensity could not be increased (500 W/m^2 is the maximum for this photobioreactor), decreasing the carbon source concentration in the input tank must result in a lower biomass concentration in the reactor at the steady state with a corresponding increase in average light levels for the biomass. The increased available average light energy levels should allow to increase the maximum dilution rate the culture can sustain before the whashout.

To reach the desired culture conditions an initial new batch culture was performed to obtain the necessary biomass and subsequently the dilution rate was progressively increased up to the previous stable levels. In figure 5 the results of the continuous culture tests in this new conditions are reported using 0.2 g C/L (acetic acid) in the fresh medium and 500 W/m^2 of incident light intensity. As expected, biomass concentration the culture tends to stabilize is at about half of the one obtained in figures 3 and 4 while dilution rate is being progressively increased from 0.02 to 0.04h^{-1}. This allows to verify the acetic acid yield at a different average light intensity. This biomass concentration was maintained during around 250 hours while the dilution rate was increased. This fact also confirms that the biomass growth is not inhibited by

the increased available light intensity resulting from the lower biomass and shows that, in the previous experiments using 0.4gC/L of carbon source, when the dilution rate was increased and biomass levels decreased, the progressive increase in light levels were not causing a light inhibition at least until the biomass levels reached equivalent levels to the ones in this experiment.

When the dilution rate was again increased to 0.049 h^{-1}, the cells started to aggregate and attach to the bioreactor walls sililarly as occurred in the previous attempts to increase dilution rate over this limit. The cells remaining in the culture were washed-out. The possibility to increase the dilution rate in a smaller step was also considered but had to excluded because the input pump used could only be increased in discrete steps and the applied increase already corresponded to one step.

The initial conclusion in this case was that either the result of the carbon source accumulation (or the combined effect of this fact with the increased average light intensity) resulted in a unstable condition, probably due to an inhibition effect, driving the culture to a washout. As a steady state was obtained at two biomass concentrations (that is for the two different carbon source concentrations at 0.2 and 0.4 gC/L) it can be followed that, a light inhibition does not exist for biomass concentrations higher than 0.3 gDW/L and 500 W/m^2 of incident light intensity in this bioreactor.

Consequently, should this hypothesis be confirmed, it can be said that in this photobioreactor, to attain a steady state at a dilution rate higher than 0.04 h^{-1}, and assuring at the same time that the carbon concentration is the limiting factor (that is no accumulation) will be highly difficult in the best case. It could be argued that such conditions could perhaps be obtained at lower biomass concentrations using lower input levels of carbon source. However those lower biomass concentrations present multiple experimental inconcoveniences (increase the culture instability, requirement of higher sample volumes for analysis, requirement of longer times to fill the sampling tubes,…) making them undesireable and unconvenient as experimental conditions.

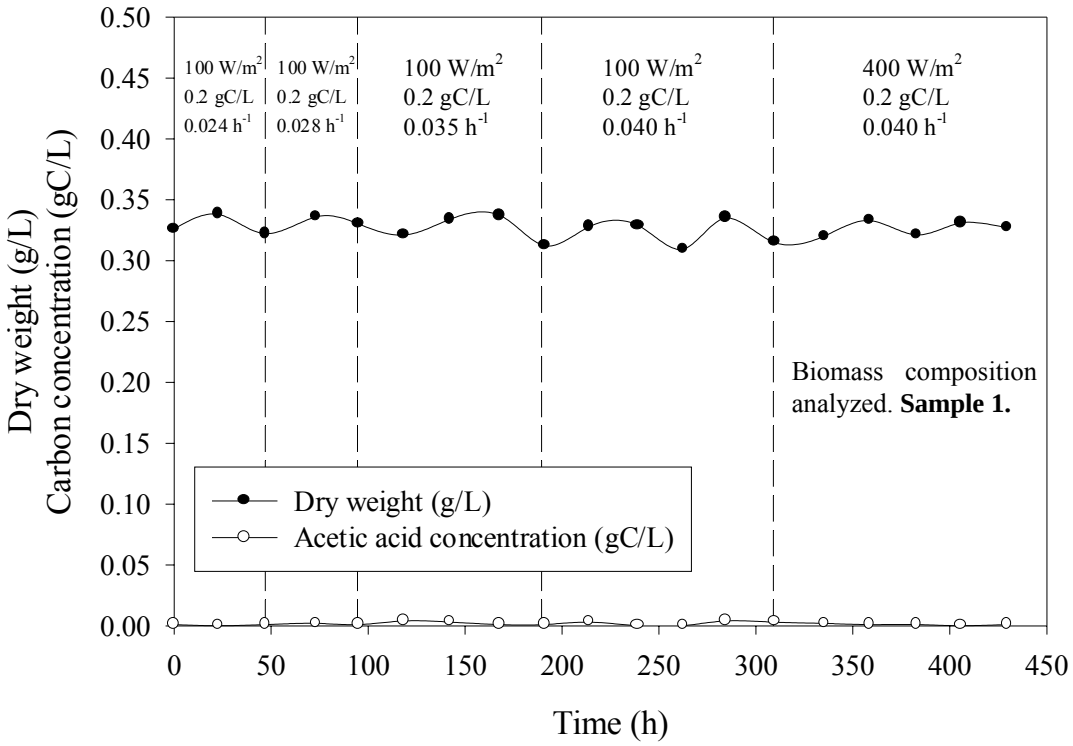

Figure 6: In order to check that there were no light limitation conditions at 0.04 h^{-1}, a light intensity step up was carried out.

In the previous tests, light intensities used were high enough so as to assure that light was not a limiting factor. After the obtained results (using 500 W/m^2 and 300 W/m^2 of incident light intensity) it could be presumed that, for dilution rates up to 0.04 h^{-1}, the same steady states could probably be reached using lower values of the incident light energy and still assure a non light limiting condition. In order to test this hypothesis a continuous culture was set up at 100 W/m^2 of incident light intensity and the dilution rate was also increased progressively (figure 6).

The tests reported in figure 6, confirm the previous values of biomass steady state under carbon limiting conditions, while at the same time using lower energy levels. When a steady state is achieved at 0.04 h^{-1} and 100 W/m^2 using 0.2 g C/L, an incident light intensity step up was carried out up to 400 W/m^2. The dry weight in the new steady state does not differ significantly from the previous one in the same run nor from the previous test using the same input carbon source concentration, further confirming the carbon limiting conditions.

Finally, under these last experimental conditions, a significant amount of biomass was collected and freeze-dried in order to be able to perform biomass composition analysis as described in a later chapter. At this point it must be mentioned that in order to decrease the cost of the analysis for this work package fewer number of biomass samples were analyzed for their macromolecular composition in the acetic acid cultures since this had already been done in TN 37.7 (Cabello et al. 2001). Nevertheless, one sample was analysed for comparison purposes.

A summary of the experimental conditions and major variables values for the acetic acid cultures can be found in table 1.

Table 1: Summary of experimental conditions and major variables in acetic acid cultures. [C] Concentration of carbon source in gC/L.

Steady state		F_R (W/m^2)	D (h^{-1})	Carbon source	$[C]_{INLET}$ (g C/L)	DW (g/L)	$[C]_{CULTURE}$ (g C/L)
Figure 3	I	500	0.04	Acetic	0.4	0.689 ± 0.002	0.007 ± 0.003
Figure 3	II	350	0.04	Acetic	0.4	0.679 ± 0.003	0.010 ± 0.004
Figure 4	I	500	0.04	Acetic	0.4	0.685 ± 0.004	0.005 ± 0.003
Figure 5	I	500	0.035	Acetic	0.2	0.324 ± 0.005	0.002 ± 0.0005
Figure 5	II	500	0.04	Acetic	0.2	0.309 ± 0.004	0.002 ± 0.0005
Figure 6	I	100	0.024	Acetic	0.2	0.329 ± 0.005	0.001 ± 0.0005
Figure 6	II	100	0.028	Acetic	0.2	0.329 ± 0.005	0.001 ± 0.0005
Figure 6	III	100	0.035	Acetic	0.2	0.327 ± 0.005	0.002 ± 0.001
Figure 6	IV	100	0.040	Acetic	0.2	0.321 ± 0.004	0.002 ± 0.001
Figure 6	V	400	0.040	Acetic	0.2	0.325 ± 0.001	0.001 ± 0.0005

These obtained results allow to conclude:

a) The tests performed at an input carbon source concentration of 0.2 g C/L and 0.4 g C/L and different dilution rates up to 0.04 h^{-1} (0.024, 0.028, 0.035,0.04 h^{-1}) and using 3 different incident light energy levels (100, 300 500 W/m^2) were performed under carbon limiting conditions.

b) The biomass yield values from the carbon source obtained previously are confirmed. What is more the biomass yields from the carbon source obtained for different average light intensities are not significantly different (1.66 ±0.06 gDW/gC). Any differences, should they exist, are within the experimental measurement error.

c) Incident light energy levels of 100 W/m^2, are sufficient for maintaining an steady state at dilution rates up to 0.04 h^{-1} and input acetic acid concentrations of 0.2 g C/L . The increase in average light energy of the 0.2 gC/L tests performed at 500 W/m^2 compared with those performed at 100 W/m^2, do not indicate the existence of any light inhibiting condition at those conditions.

d) Increasing the dilution rate above 0.04 h^{-1} in the used culture conditions drives the culture to an unstable state similar to the ones observed in previous TN using acetic acid.

A definitive explanation for the last fact is difficult to give with the presently available data. Nevertheless, all the data collected up to now indicate that, whithin a range of different continuous culture conditions, an increase in the carbon source concentration in the culture medium is observed while the cells change their metabolic response and enter in an unstable state. It is difficult to assure if the change in the metabolic state generates the carbon accumulation or if the carbon accumulation forces the metabolic change but the increase in the culture carbon source levels appear as the most probable candidate for the metabolic change. It can be remembered here that in previously reported acetic acid tests this behaviour of the biomass was characterized by a decrease in cell division with cell elongation and accumulation of the carbon source in form of PHA (TN 37.7, Cabello *et al* 2001). Similar instabilities using acetic acid as carbon source have also been found by other MELISSA groups (Favier-Teodorescu *et al.* 2003 TN 49.2), confirming that this is not an isolated effect.

Taking into account the data already obtained in previous work packages, using acetic acid as carbon and electron source (and also because the requested minimum number of tests in these conditions had been done: 2 dilution rates and 2 light intensities) it was decided to continue the tests using the remaining carbon sources.

3.2 Propionic acid tests

In previous experiments (Cornet & Albiol 2000, Creus *et al.* 2002), it was observed that the average light energy required for *R. rubrum* cultures to obtain the same growth rate, using propionic acid, butyric acid or valeric acid as carbon sources, was higher than the energy required if instead acetic acid is used at the same culture conditions. This indicates that the maximum incident light energy provided by the 8 L Bioengineering photobioreactor, where the previous experiments were done, is not sufficient to carry out continuous tests using propionic acid as the carbon source except perhaps for very low flow rates. Therefore, propionic acid tests were carried out in the 2.4 L Applikon photobioreactor, since this reactor can supply higher radiant energy per unit of culture volume (up to 250 W/litre instead of 120 W/litre in the 8 litres bioreactor). This reactor does not have a non illuminated volume other than the one generated in the centre of the bioreactor (the 8 litres bioreactor has an illuminated volume fraction (transparent illuminated volume/total volume) of 0.52). It is also more easy to start-up and operate.

The amount of carbon source provided to these cultures was of 0.2 g C/L in order to provide the same amount of carbon as in previous cultures. After the required initial batch culture, the continuous culture was started using the maximum incident light intensity available with the 15 lamp set-up (Fr around 230 W/m^2) and at a low flow rate in order to increase it progressively. As it can be observed in figures 7 and 8 stable steady states can be obtained at dilution rates ranging from 0.009 h^{-1} up to 0.015 h^{-1}.

However, the obtained results also indicate that 0.015 h^{-1} is the maximum dilution rate that can be maintained before washing-up the culture in the Applikon reactor using propionic acid as the only carbon source and the 15 lamps set up. For this reason the light supply capability of the photobioreactor was increased using the maximum number of lamps. That is, the number of lamps was increased from 15 to 30 lamps and as a consequence the maximum light intensity, reached with this new set up at the surface, was of 394 W/m^2.

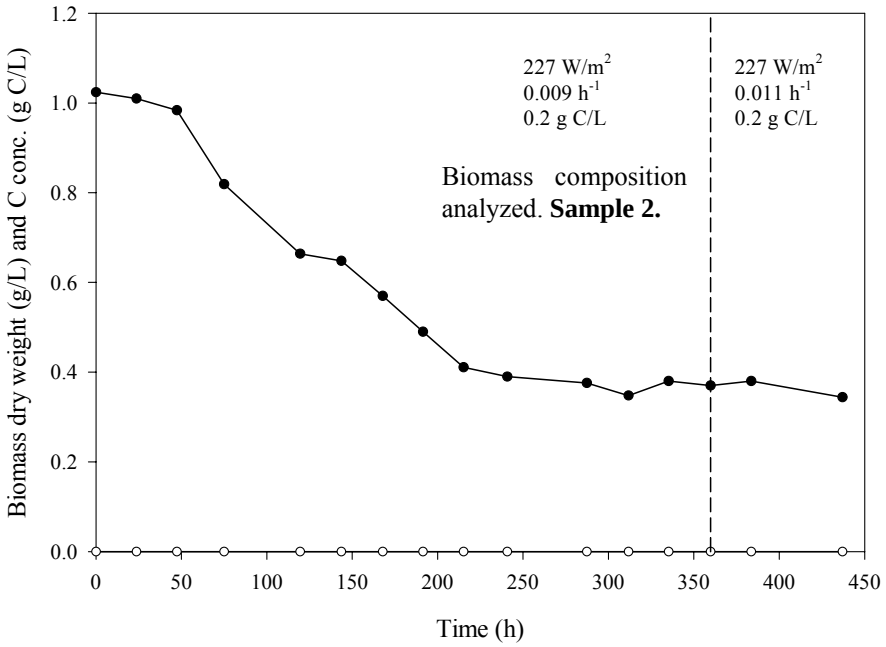

Figure 7: Biomass concentration evolution in the 2.4 L *Applikon* reactor at 0.009 h^{-1} and 0.0114 h^{-1} using propionic acid (0.2 g C/L) as the only carbon source.

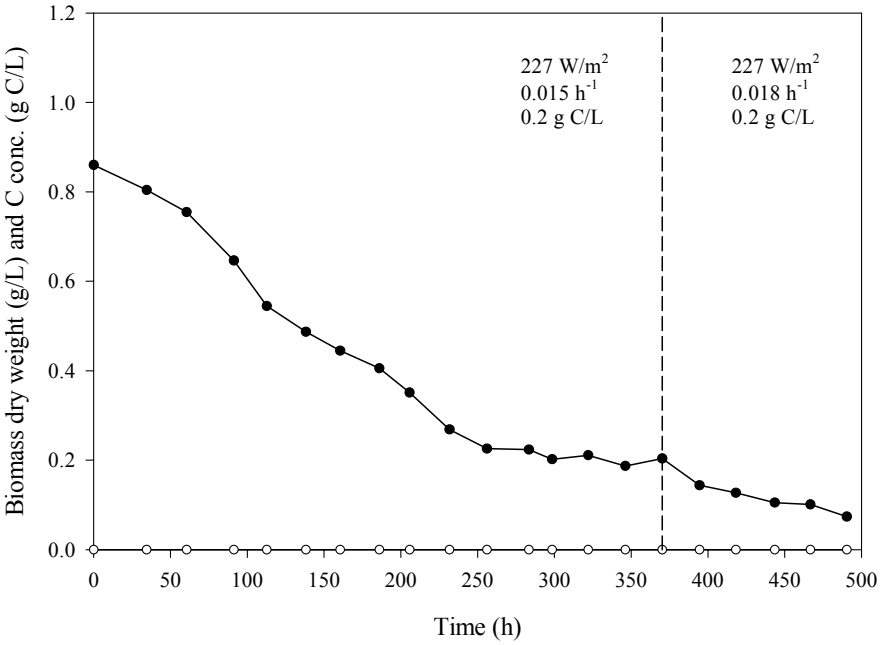

Figure 8: Biomass concentration evolution in the 2.4 L *Applikon* reactor at 0.015 h^{-1} using propionic acid (0.2 g C/L) as the only carbon source. When the dilution rate was increased up to 0.018 h^{-1} the culture was washed-out.

Using the upgraded configuration, two replicate tests cultures were carried out (figures 9 and 10) using the incident light intensity of 394 W/m^2, a carbon concentration in the fresh medium of 0.2 g C/L and at a dilution rate of 0.005 h^{-1} which complements the previous data. These tests corroborate the biomass steady state value obtained in the previous tests and confirm that no light inhibition exist for this biomass concentration and growth rate.

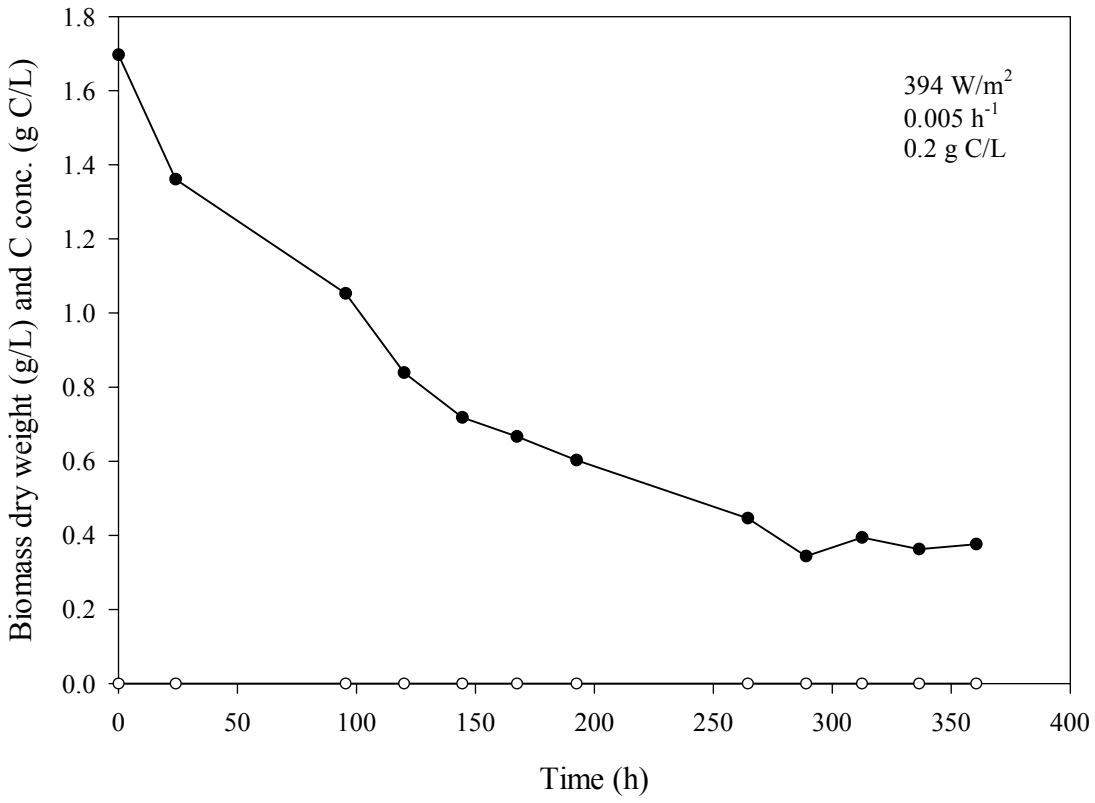

Figure 9: Biomass concentration evolution in the 2.4 L *Applikon* reactor at 0.005 h^{-1} using propionic acid (0.2 g C/L) as the only carbon source. This test was carried out using 30 lamps, providing 394 W/m^2 of incident light (F_R).

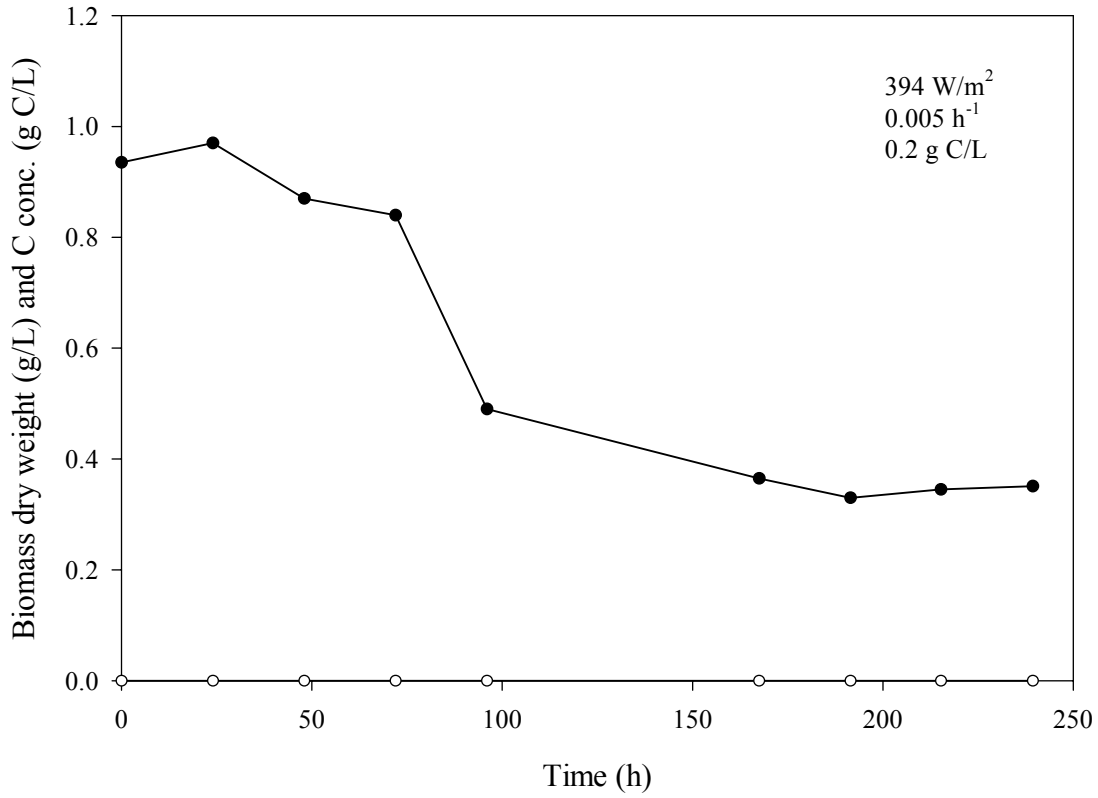

Figure 10: Biomass concentration evolution in the 2.4 L *Applikon* reactor at 0.005 h^{-1} using propionic acid (0.2 g C/L) as the only carbon source. This test was carried out using 30 lamps, providing 394 W/m^2 of incident light (F_R).

In the following tests it was decided to decrease progressively the light intensity down to the point were the carbon source started to accumulate. The carbon source accumulation would allow to see if the same effects found in the acetic acid tests (changes in the metabolic response of the cells) occur when propionic acid is used. It was also decided that the transition zone from carbon to light limitation could probably be more clearly seen wile working at higher biomass concentrations. In those cases, as soon as carbon source starts to accumulate, biomass concentration steadily decreases and practical biomass concentration measurements are less precise. Also at very low biomass concentrations any small flow instability quickly washes out the biomass. With the purpose of increasing the biomass concentration, the carbon concentration in the fresh medium was increased from 0.2 g C/L to 0.5 g C/L for the following tests.

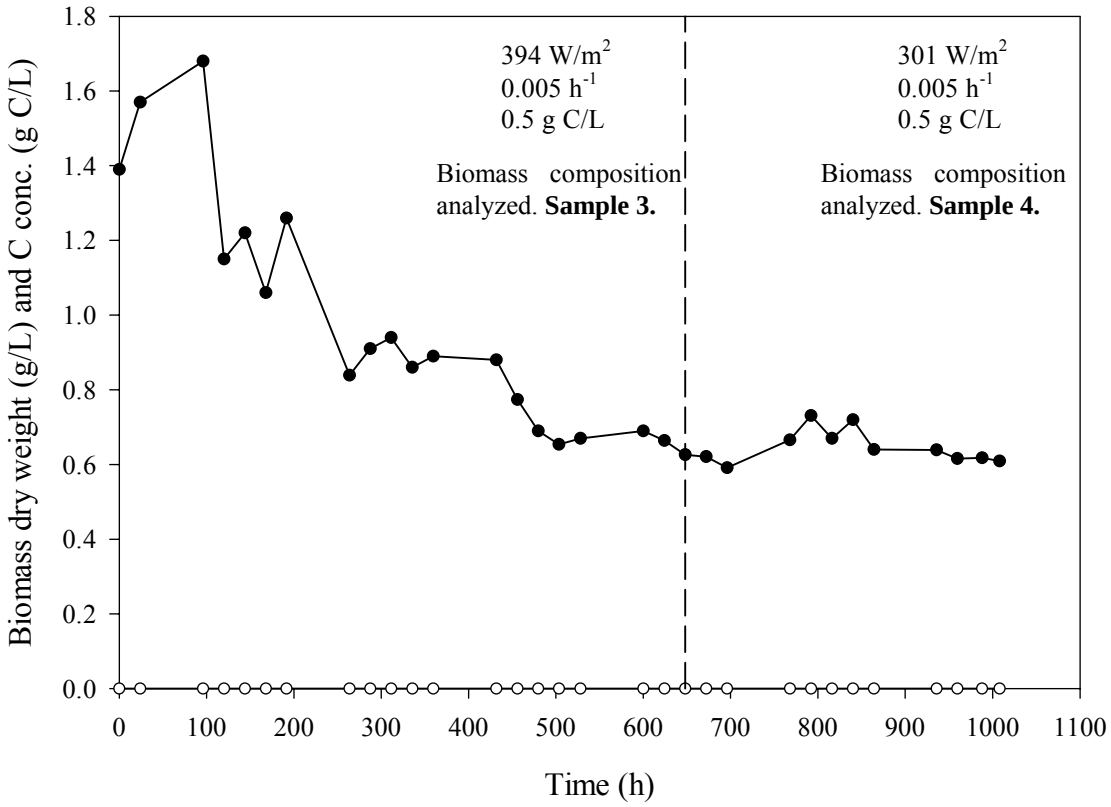

Figure 11: Biomass concentration evolution in the 2.4 L Applikon reactor. Steady states achieved at 0.005 h^{-1} using propionic acid (0.5 g C/L) as the only carbon source in a step down in the incident light intensity (F_R) from 394 W/m^2 to 301 W/m^2.

Figure 11 shows that for light intensities at the surface of the bioreactor of 394 W/m^2 and 301 W/m^2 it was possible to consume the entire carbon source and maintain equivalent biomass levels at steady state (carbon limitation). In order to see if the same metabolic changes effects appeared using this carbon source it was decided to further decrease the light intensity to approach light limitation and induce the accumulation of the carbon source.

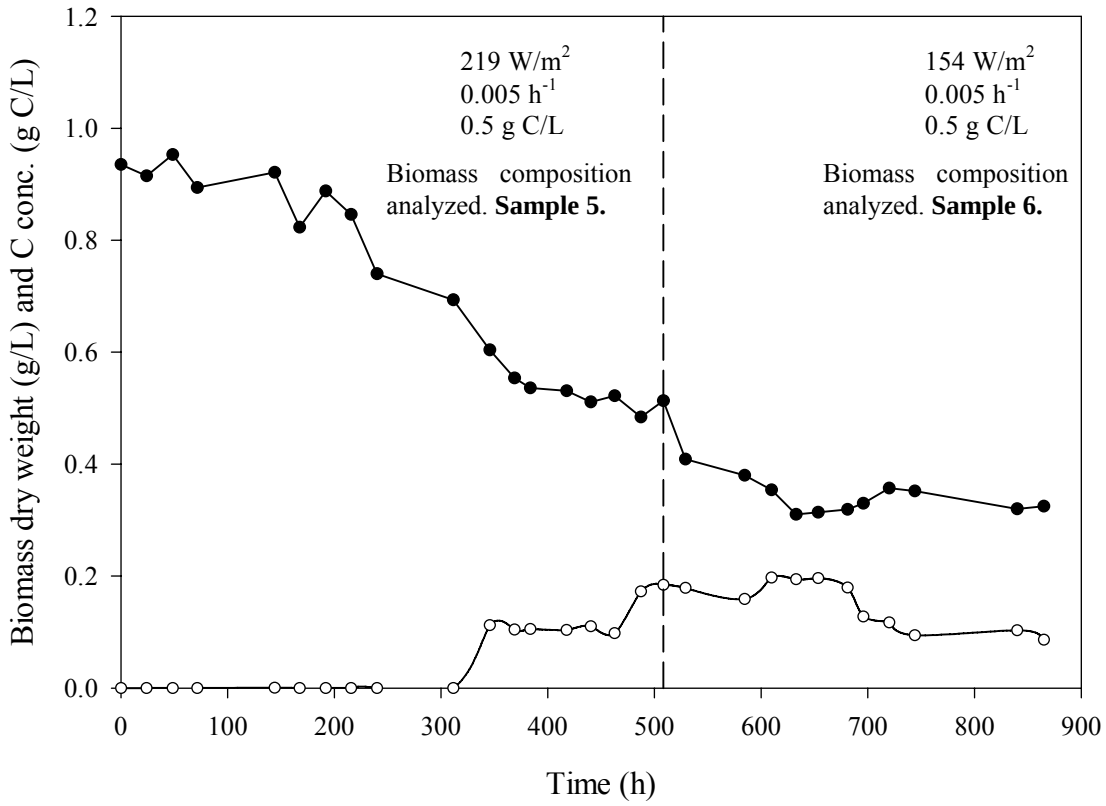

Figure 12: Biomass concentration evolution in the 2.4 L Applikon reactor. Steady state achieved at 0.005 h^{-1} using propionic acid (0.5 g C/L) as the only carbon source after a step down in the incident light intensity (F_R) from 219 W/m^2 to 154 W/m^2.

According to the previous results, the following test was started at a lower light intensity value of 219 W/m^2. As shown in figure 12 at this light intensity the accumulation of carbon source is noticeable. However at none of the two light intensities any sign of instability was detected as was previously seen in the acetic acid experiments.

For this reason, and due to the importance of verifying any kind of inhibition, it was decided to repeat the experiment and start the culture at lower incident light intensities. Figure 13 shows the results obtained at the new conditions. In this case it is remarkable that the biomass obtained after 5 residence times is higher that the one obtained in the previous experiment and consequently that no carbon source remains to be consumed. At this point what can be said is either that we are in front of a system exhibiting a bifurcation, in the sense that the steady state obtained depends on the way to reach it and more than one steady states are possible for

the same culture conditions. That seems possible after the results obtained using acetic acid as a carbon source (Cornet *et al.* 2003, TN 49.2 Favier-Teodorescu *et al.* 2003, TN 37.7, Cabello *et al.*, 2001,). Alternatively the carbon source accumulation in the previous experiment might also be an artifact of that particular experiment. This issue will have to be addressed in future tests. In any case, the behaviour of this particular experiment was further verified by further decreasing light intensity and it was confirmed the fact that the transient state is critic to define de steady state.

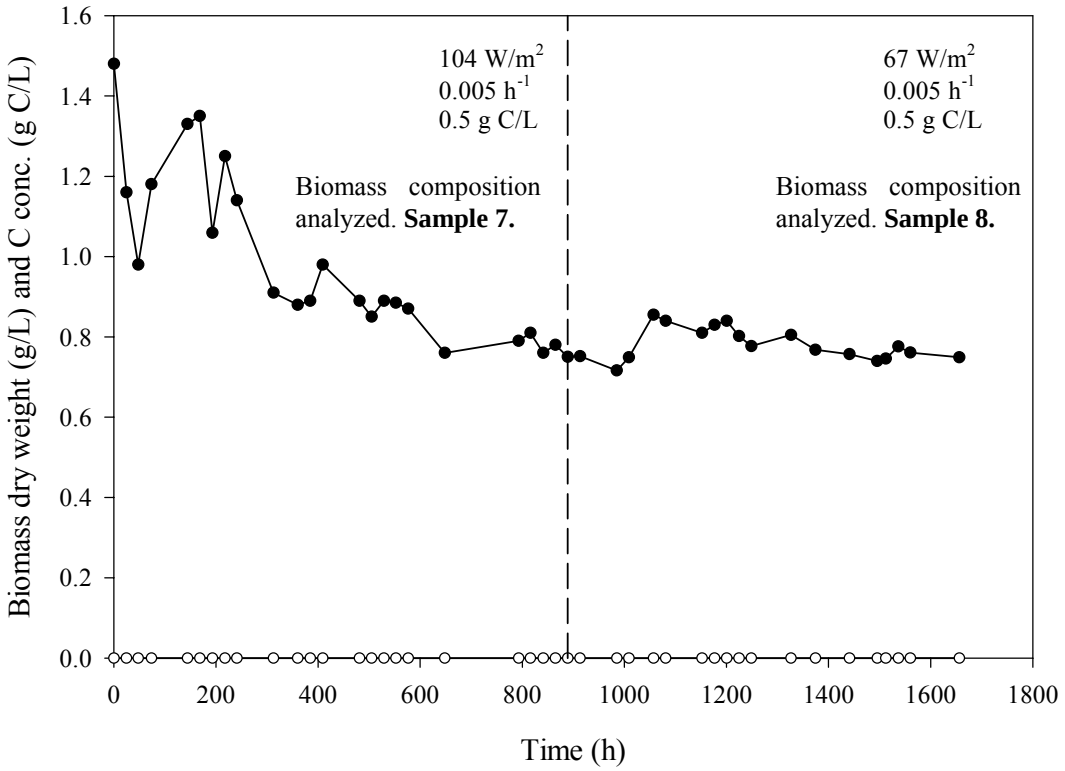

Figure 13: Biomass concentration evolution in the 2.4 L Applikon reactor. Steady states achieved at 0.005 h^{-1} using propionic acid (0.5 g C/L) as the only carbon source in a step down in the incident light intensity (F_R) from 104 W/m^2 to 67 W/m^2.

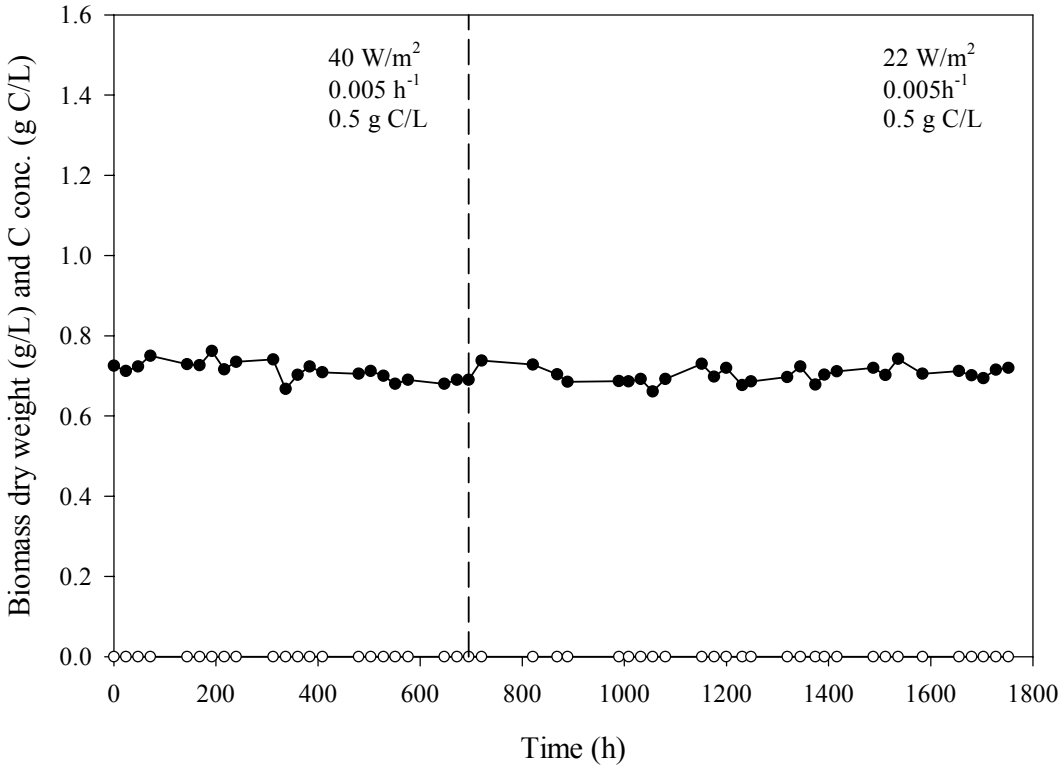

Figure 14: Biomass concentration evolution in the 2.4 L Applikon reactor. Steady states achieved at 0.005 h^{-1} using propionic acid (0.5 g C/L) as the only carbon source in a step down in the incident light intensity (F_R) from 40 W/m^2 to 22 W/m^2.

The results obtained by further decreasing the light intensity can be seen in figure 14. In this case, the experiment is a continuation of the previous one (figure 13) (that is, no previous reinoculation and batch culture performed). It can be seen that even at very low incident light intensities such as 24 W/m^2 the culture maintains its growth rate and the biomass consumes the entire carbon source provided.

As this experiment has been running for about 3600 hours (more than 21 residence times), at different light intensities, it can be assured that this behaviour is not a transitory state.

The data obtained in those two different runs can be further compared by plotting the biomass concentrations obtained at the different steady states versus the light intensity used during the

experiment, as shown in figure 15. Both runs were done after starting a batch culture and progressively decreasing the light intensity supplied. The behaviour of the two runs was different. One of them exhibiting a consistent decrease in the steady state biomass obtained and showing an incomplete carbon source consumption. The other one shows a more stable biomass level with complete carbon source consumption. The composition of the biomass obtained in both runs is discussed in the second part of this technical note.

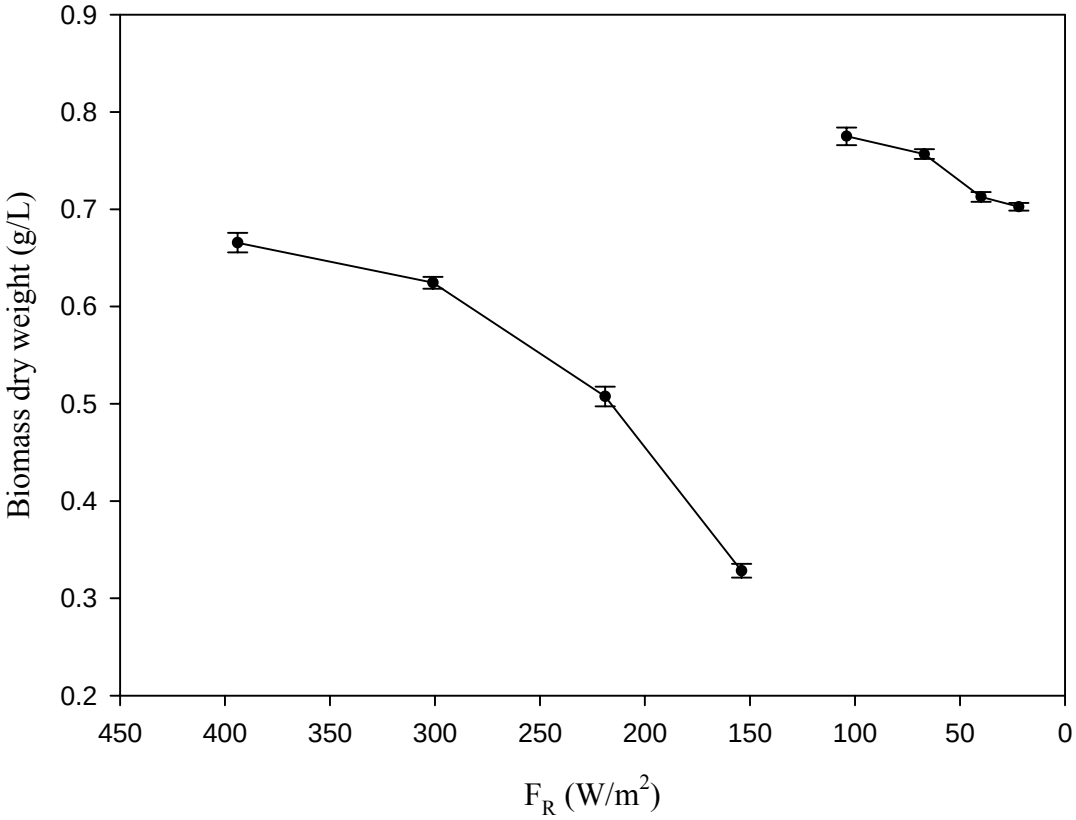

Figure 15: Biomass dry weight (g/L) obtained at different the steady states depending on the incident light intensity (W/m^2). Each line corresponds to continuous culture steady states initiated from two different batch cultures. Dilution rate 0.005 h^{-1}.

Several reasons could explain these results. It is possible that the photosynthetic systems of *R. rubrum* cells became saturated at high light intensities and therefore operate inefficiently. To cope with this, the cells would modify the light collection systems on the internal membranes (for example decreasing the amount of pigments to collect light energy, with

maybe a slow characteristic time). This could explain that when starting the continuous cultures (using the cells after a batch growth, and therefore adapted to high biomass concentrations and low average light intensities), cells perform better if a lower initial light intensity is used. This effect has been proposed as an explanation of the different lag times when a fresh culture is started using a stock inoculum. This effect should disappear as the cells readapt their photosynthetic system and the speed of adaptation will depend on the characteristic time of the pigment synthesis process.

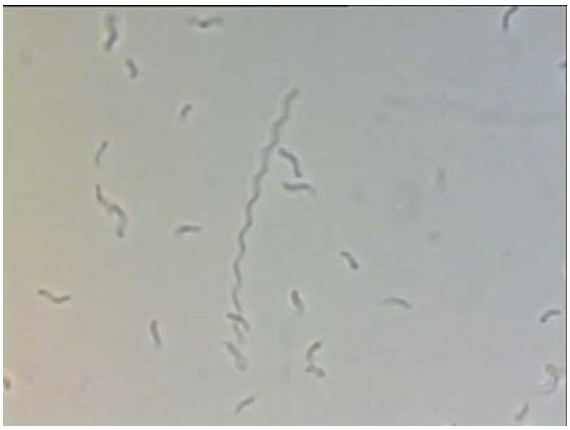

Figure 16.a.

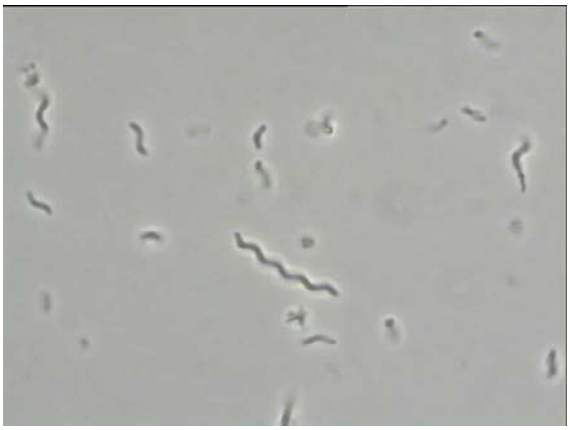

Figure 16.b.

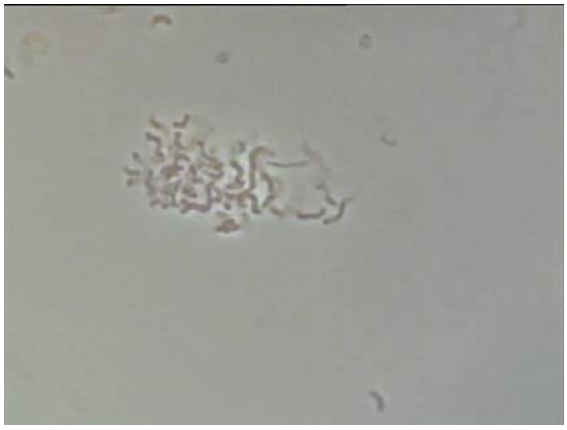

Figure 17.a.

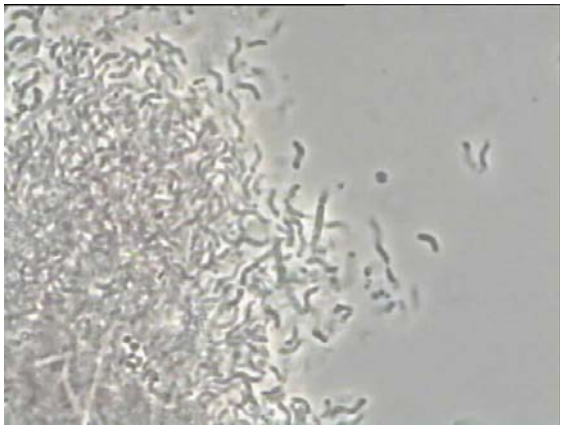

Figure 17.b.

Another feasible possibility could be that if the light intensity is lowered and the carbon source accumulates in excess, the cells start to accumulate internal polymeric carbon reserves. In this case the dry weight increases, not due to real cell growth and division, but as a result of the increase in the mass of the cells due to the accumulation of reserve polymers. A

combination of this effect with a decrease in growth rate due to the previous effect was observed in the previous cultures using acetic acid as a carbon source. Nevertheless this polymer accumulation was not confirmed in the biomass composition analysis (see below).

As it was described in TN 37.7 (Cabello *et al.* 2001) for acetic acid cultures, when the carbon source is in excess in the medium, regarding to the light energy available, the cells change its morphology and physiology. Before starting to accumulate reserve polymers, the cells change its morphology and become longer as they had lost the capability to divide into two new cells. This change in the morphology (figures 16.a. and 16.b.) was verified in these experiments in the continuous culture grown at 104 W/m^2. The cell elongation, the intragranules of polymers and the starting of cell aggregation (figures 17.a. and 17.b.) were observable microscopically in the continuous culture grown at 67 W/m^2.

Another possibility could be simply that to describe a steady state the previous history of the culture has to be taken into account. If the previous culture conditions had an effect in internal variables with long characteristic times or showing hysteresis in their dynamics, this could act as a memory system affecting the subsequent behaviour of the culture. In those cases obtaining exactly the same steady state would require to repeat identically a number or previous steps.

In conclusion, with only those two runs it is not possible to ascertain the real motivation of the different behaviours observed. Nevertheless, in both cases the biomass and carbon levels in the bioreactor were maintained along several residence times. This suggests that it is possible that both behaviours result from the different starting point conditions, especially after the previous results, obtained in different labs, on the behaviour of this species using acetic acid.

In future propionic acid experiments it will be addressed the possibility that starting the continuous cultures from different initial conditions, (p.ex. using relatively high or low incident light intensities) can drive the cultures to two different steady states.

Table 2 summarizes the different results of biomass and carbon source input/output conditions and experimental results obtained in the previous experiments for 4 dilution rates (0.005,

0.009, 0.011, 0.015 h^{-1}) and 9 light intensities (22, 40, 67, 104, 154, 219, 227, 301, and 394 W/m^2). Results of the biomass composition analysis are discussed in a following chapter.

Table 2: Summary of experimental conditions and major variables in propionic acid cultures. [C]cult.: Concentration of carbon source in the culture as gC/L. (B.D.: Below detection limit of the gas chromatograph. S.E.: Standard error of the mean.)

Steady state		F_R (W/m^2)	D (h^{-1})	Carbon source	$[C]_{INLET}$ (g C/L)	DW±S.E. (g/L)	$[C]_{CULT.}$ ±S.E (g C/L)
Figure 7	I (S2)	227	0.009	Propionic	0.2	0.379 ± 0.009	B.D.
Figure 7	II	227	0.011	Propionic	0.2	0.36 ± 0.01	B.D.
Figure 8	I	227	0.015	Propionic	0.2	0.201 ± 0.005	B.D.
Figure 9	I	394	0.005	Propionic	0.2	0.378 ± 0.009	B.D.
Figure 10	I	394	0.005	Propionic	0.2	0.348 ± 0.007	B.D.
Figure 10	I (S3)	394	0.005	Propionic	0.5	0.67 ± 0.01	B.D.
Figure 10	II (S4)	301	0.005	Propionic	0.5	0.624 ± 0.006	B.D.
Figure 12	I (S5)	219	0.005	Propionic	0.5	0.508 ± 0.008	0.12 ± 0.01
Figure 12	II (S6)	154	0.005	Propionic	0.5	0.328 ± 0.006	0.100 ± 0.007
Figure 13	I (S7)	104	0.005	Propionic	0.5	0.775 ± 0.009	B.D.
Figure 13	II (S8)	67	0.005	Propionic	0.5	0.757 ± 0.005	B.D.
Figure 14	I	40	0.005	Propionic	0.5	0.712 ± 0.005	B.D.
Figure 14	II	22	0.005	Propionic	0.5	0.703 ± 0.004	B.D.

3.3 Butyric acid tests

Using Butyric acid as carbon and energy source requires a higher amount of energy than the one required using acetic acid (Cornet & Albiol 2000, Creus *et al.* 2002). Following the previous experiments and taking into account the higher light energy supply capacities of the 2.4 L Applikon photobioreactor and its easier manipulation, butyric acid tests were also carried out using this bioreactor.

To allow comparison with the previous experiments the cultures were started at the same carbon source concentration and light intensity used in the initial experiments with propionic acid in this reactor. With respect to the dilution rate, experiments were started at low dilution rates and increased progressively as in previous tests.

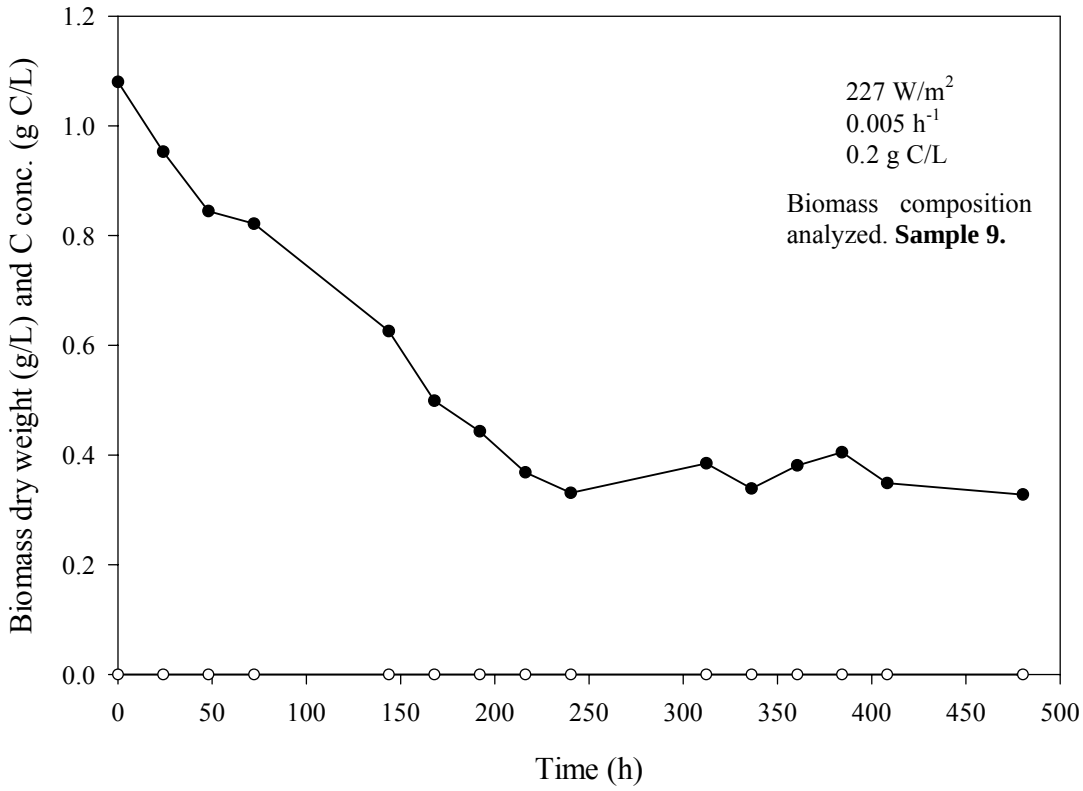

Figure 18: Biomass concentration evolution in the 2.4 L Applikon reactor. Steady states achieved at 0.005 h^{-1} using 227 W/m^2 and butyric acid as the only carbon source (0.2 g C/L).

After the usual batch culture the continuous culture run was started at a dilution rate of 0.005 h^{-1}. As can be seen in figure 18 the experimental results show a stabilization during the last 200 hours of culture (one residence time) and this was taken as a stable point. Biomass was collected for composition analysis.

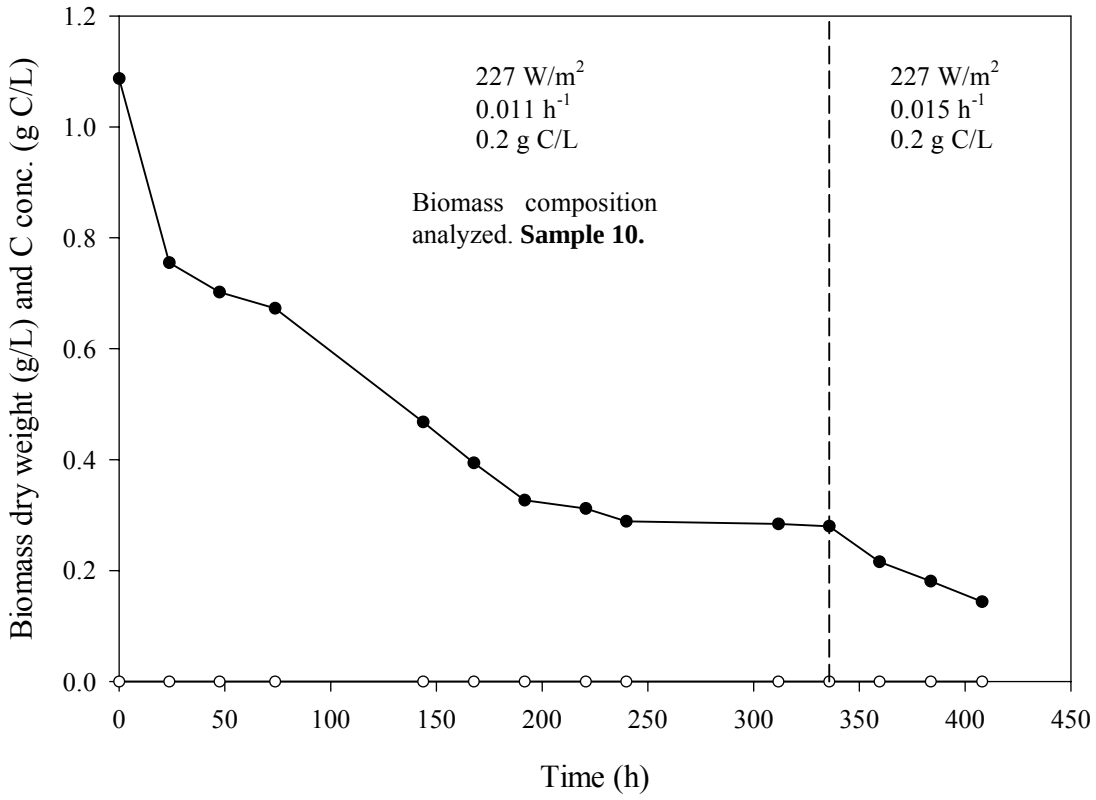

Figure 19: Biomass concentration evolution in the 2.4 L Applikon reactor. Steady state achieved at 0.011 h^{-1} using 227 W/m^2 and butyric acid as the only carbon source (0.2 g C/L). When the dilution rate was increased up to 0.015, the culture was washed-out.

For the second test, the continuous culture was started at a higher dilution rate 0.011 h^{-1} following the progressive increase approach. Results are reported in figure 19. In this case during the last residence time (last 91 hours), a new dry weight steady state is achieved. Consequently, after the corresponding biomass collection, the dilution rate was further increased to 0.015 h^{-1}. In this second case, the culture was washed out. As discussed before one possibility could be that the light energy supply was insufficient to sustain the increased growth rate. For this reason, the following test had to be at a higher light intensity.

As described before, using the 30 lamps set up the maximum light intensity in the 2.4 L Applikon bioreactor is around 394 W/m^2. The following experiment was performed using this maximum light intensity and maintaining the rest of the culture conditions as in the previous experiments. That is, dilution rate at 0.01 h^{-1}, and carbon source at 0.2 g C/L.

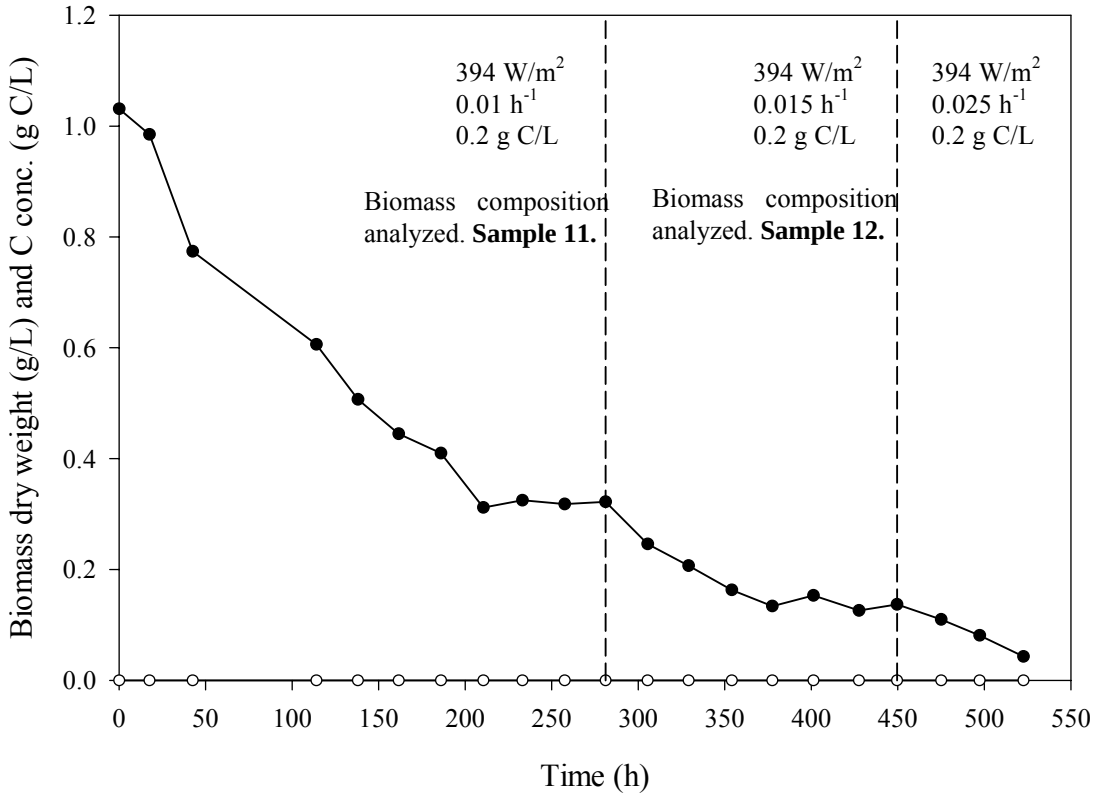

Figure 20: steady states achieved at 0.01 h^{-1} and 0.015 h^{-1} using 394 W/m^2 and butyric acid as the only carbon source (0.2 g C/L). When the dilution rate was increased up to 0.025, the culture was washed-out.

Once the first steady state was reached (figure 20), the biomass was collected and the dilution rate was increased up to 0.015 h^{-1}. At this point, another steady state was achieved, after verifying the stability of the biomass concentration during the last residence time (66 hours). At this point, and after biomass collection, the dilution rate was once more increased up to 0.025 h^{-1}. This time the culture was washed out.

As a stable biomass concentration was obtained at a higher dilution rate than in the previous case (with only 227 W/m^2), it can in principle be assumed that it would be necessary to

increase the light energy supply to allow for a continuous tests at dilution rates higher than 0.015 h^{-1} in this photobioreactor using butyric acid as the carbon source.

In both propionic and butyric acid cultures most of the measures of carbon concentration at the steady state were below the detection limit of the gas chromatograph. Nevertheless, it should be kept in mind that some carbon source was also being lost via de gas phase, because of the thermodynamic gas-liquid equilibrium (see Poughon 1995 TN 23.1). In none of the tests it was observed a carbon source accumulation in the culture medium nor the starting of the morphology change and aggregation effect.

The strong decrease in biomass concentration when increasing the dilution rate from 0.011 h^{-1} to 0.015 h^{-1} would indicate that the culture is passing from a carbon limiting situation to a light limiting one indicating the crossing of the threshold conditions. This represents a limitation in this bioreactor anticipating the washout of the bioreactor at the next increase in dilution rate given the low levels of biomass reached.

Table 3 summarizes the experimental conditions and main experimental variables measured during the butyric acid tests for 3 dilution rates (0.005, 0.011 and 0.015 h^{-1}) and 2 light intensities (227 and 394 W/m^2). Results of the biomass composition analysis are discussed in a following chapter.

Table 3: Summary of experimental conditions and major variables in butyric acid cultures. [C] Concentration of carbon source in gC/L. (B.D.: Below detection limit of the gas chromatograph.)

Steady state		F_R (W/m^2)	D (h^{-1})	Carbon source	$[C]_{INLET}$ (g C/L)	DW (g/L)	$[C]_{CULTURE}$ (g C/L)
Figure 18	I (S9)	227	0.005	Butyric	0.2	0.36 ± 0.01	B.D.
Figure 19	I (S10	227	0.011	Butyric	0.2	0.284 ± 0.003	B.D.
Figure 20	I (S11)	394	0.01	Butyric	0.2	0.319 ± 0.003	B.D.
Figure 20	I I (S11)	394	0.015	Butyric	0.2	0.138 ± 0.006	B.D.

4 Biomass analysis

To complement the range of data available from the previous experiments and to better monitor and ascertain the modification of behaviour observed along the previous experiments biomass composition analysis were performed. These data is necessary for a later, more in depth, metabolic analysis and can provide clues on the reasons for the metabolic changes occurred. Besides any other future analysis that might be done using these data, some major characteristic traits on the different behaviours can already be seen. Those are later discussed were appropriate.

A summary of experimental conditions at which each biomass sample was obtained can be found in table 4.

Table 4: Summary of conditions and biomass concentrations at which each biomass sample was obtained (B.D. below detection limit).

	Figure	F_R (W/m^2)	D (h^{-1})	Carbon source	$[C]_{INLET}$ (g C/L)	DW (g/L)	$[C]_{CULTURE}$ (g C/L)
Sample 1	6 (V)	400	0.040	Acetic	0.2	0.325 ± 0.001	0.001 ± 0.0005
Sample 2	7 (I)	227	0.009	Propionic	0.2	0.379 ± 0.009	B.D.
Sample 3	11 (I)	394	0.005	Propionic	0.5	0.67 ± 0.01	B.D.
Sample 4	11 (II)	301	0.005	Propionic	0.5	0.624 ± 0.006	B.D.
Sample 5	12 I	219	0.005	Propionic	0.5	0.508 ± 0.008	0.12 ± 0.01
Sample 6	12 II	154	0.005	Propionic	0.5	0.328 ± 0.006	0.100 ± 0.007
Sample 7	13 I	104	0.005	Propionic	0.5	0.775 ± 0.009	B.D.
Sample 8	13 II	67	0.005	Propionic	0.5	0.757 ± 0.005	B.D.
Sample 9	18 I	227	0.005	Butyric	0.2	0.36 ± 0.01	B.D.
Sample 10	19 I	227	0.011	Butyric	0.2	0.284 ± 0.003	B.D.
Sample 11	20 I	394	0.01	Butyric	0.2	0.319 ± 0.003	B.D.
Sample 12	20 II	394	0.015	Butyric	0.2	0.138 ± 0.006	B.D.

4.1 Protein determination

The biomass protein percentages were determined as described in Appendix 3. Each sample was analysed in triplicate. For each sample, the mean value of the three analyses and its standard error is reported in table 5.

Table 5: Results of the protein determination. (data given in % DW).

Sample No.	Protein content (% DW)	Standard Error
1	67.52	4.12
2	62.80	1.65
3	65.88	1.69
4	65.06	2.17
5	64.44	0.87
6	63.86	1.56
7	60.28	1.40
8	60.44	1.44
9	64.58	0.42
10	66.83	1.50
11	64.22	1.90
12	64.36	2.57

4.2 Lipid determination

The determination of the lipids content in the biomass was done as described in Appendix 4. The main lipid components found in *R. rubrum* samples were palmitic acid, palmitoleic acid, mirisitic acid, linoleic acid, oleic acid, elaidic acid, and estearic acid. The percentage value determined for these lipids as well as the dispersion of the analyses is reported in table 6.

Table 6: Results of the lipid determination. (data given in % DW).

Sample No.	Miristic acid (C14:0)	Palmitoleic acid (C16:1)	Palmitic acid (C16:0)	Linoleic acid (C18:2)
1	0.029	0.056	0.193	0.063
3	0.205	0.077	1.424	0.024
4	0.070	0.114	0.625	0.078
5	0.020	0.073	0.182	<0.1
6	0.014	0.006	0.126	0.026
7	0.054	0.055	0.333	0.054
8	0.254	0.766	0.986	1.016
10	0.074	0.025	0.573	0.072
12	0.006	0.048	0.107	<0.1
Sample No.	**Oleic acid (C18:1)**	**Elaidic acid (C18:1)**	**Estearic acid (C18:0)**	**Total lipids**
1	0.135	0.478	0.161	1.116
3	0.226	0.139	1.889	3.984
4	0.117	0.249	0.901	2.154
5	0.011	0.740	0.147	1.173
6	0.076	0.090	0.121	0.459
7	0.120	0.233	0.320	1.169
8	0.612	0.458	0.874	4.966
10	0.088	0.121	0.844	1.797
12	0.006	0.397	0.086	0.650

4.3 RNA determination

The RNA content of the biomass was performed as described Appendix 5. Each sample was analyzed in triplicate. The mean value of the three analyses as well as the calculated deviation is reported in table 7.

Table 7: Results of the RNA determination. (data given in % DW).

Sample No.	RNA content (% DW)	Standard Error
1	3.40	3.40
2	2.90	0.04
3	6.45	0.18
4	6.65	1.37
5	3.26	0.84
6	5.10	0.12
7	7.91	0.05
8	6.78	0.06
9	4.19	0.23
10	5.46	0.11
11	4.72	0.15
12	6.08	0.08

4.4 DNA determination

The DNA determination has been carried out as is explained in Appendix 6. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the calculated deviation is reported in table 8.

Table 8: Results of the DNA determination. (data given in % DW).

Sample No.	DNA content (% DW)	Standard Error
1	0.96	0.39
2	0.35	0.14
3	0.70	0.29
4	0.93	0.38
5	0.33	0.14
6	2.08	0.85
7	1.31	0.58
8	2.33	1.04
9	1.69	0.75
10	0.79	0.32
11	2.39	0.97
12	0.93	0.38

4.5 β-Polyhydroxybutyrate (PHB) and β-Polyhydroxyvalerate (PHV) determination

The major polyhydroxy alcanoates (PHA) present in *R.rubrum* are β -Polyhydroxybutyrate (PHB) and β-Polyhydroxyvalerate (PHV). PHB and PHV determination has been carried out as described in Appendix 7. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the calculated deviation is reported in table 9.

Table 9: Results of the PHA determination. (data given in % DW). n.d.: not determined.

Sample No.	PHB content (% DW)	Standard Error	PHV content (% DW)	Standard Error
1	2.52	0.10	0.47	0.10
2	2.52	0.20	4.83	n.d.
3	0.26	0.06	1.30	0.11
4	0.33	0.08	2.22	0.98
5	1.90	0.03	4.11	0.07
6	2.35	0.08	4.56	0.14
7	2.83	0.01	3.88	0.22
8	2.82	0.08	4.44	0.32
9	2.38	0.04	0.91	0.09
10	2.12	0.15	0.96	0.08
11	2.40	0.44	0.00	n.d.
12	2.20	0.41	0.82	0.21

4.6 Carbohydrate determination

The total carbohydrates determination was performed as described in Appendix 8. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the calculated deviation is reported in table 10.

Table 10: Results of the total carbohydrates determination. (data given in % DW).

Sample No.	Carbohydrates content (% DW)	Standard Error
1	15.50	0.79
2	14.42	0.65
3	19.10	0.79
4	19.78	2.00
5	16.36	2.30
6	14.69	0.66
7	15.60	0.92
8	11.34	0.31
9	18.42	1.65
10	16.73	2.07
11	17.06	0.33
12	14.41	0.12

4.7 Glycogen determination

The glycogen determination has been carried out as explained in Appendix 9. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the calculated deviation is reported in table 11.

Table 11: Results of the glycogen content determination. (data given in % DW).

Sample No.	Glycogen content (% DW)	Standard Error
1	2.06	0.17
2	5.50	0.41
3	2.56	0.12
4	6.75	0.17
5	4.56	0.10
6	4.25	0.07
7	5.06	0.08
8	3.19	0.10
9	3.69	0.08
10	4.50	0.09
11	5.19	0.25
12	5.25	0.06

4.8 Pigment determination

The pigment determination has been carried out as is explained in Appendix 10. Each sample has been analysed three times, and for each sample, the mean value of the three analyses as well as the calculated deviation is reported in table 12.

Table 12: Results of the pigment content determination. (data given in % DW).

Sample No.	Pigment content (% DW)	Standard Error
1	2.08	0.01
2	2.03	0.03
3	1.98	0.02
4	1.46	0.00
5	1.97	0.03
6	1.98	0.02
7	1.89	0.08
8	2.07	0.06
9	1.86	0.01
10	2.10	0.09
11	1.95	0.10
12	2.07	0.02

4.9 Elemental composition

The elemental composition analysis was performed as described in Appendix 11. The mean value for C, N, H, S and P and the corresponding deviations are reported in table 13.

Table 13: Results of the elemental composition determination (w/w %). Percentages do not add to 100% due to the lack of determination of oxygen and ashes.

Sample No.	C (%)	Std. Error	H (%)	Std. Error	N (%)	Std. Error	S (%)	Std. Error	P (%)	Std. Error
1	47.68	0.03	7.12	0.07	11.39	0.03	0.34	0.02	1.42	0.01
2	48.05	0.03	6.99	0.12	10.93	0.05	0.26	0.03	0.83	0.00
3	36.80	0.13	5.93	0.09	9.08	0.02	0.26	0.02	1.03	0.01
4	41.00	0.48	6.54	0.01	9.57	0.02	0.33	0.01	2.53	0.01
5	40.87	0.13	6.13	0.09	9.08	0.04	0.94	0.07	1.66	0.01
6	41.14	0.38	6.82	0.12	10.16	0.02	0.25	0.01	2.01	0.06
7	46.31	0.03	7.45	0.11	10.11	0.18	0.20	0.01	1.70	0.03
8	46.28	0.13	7.65	0.06	10.68	0.03	0.20	0.01	1.70	0.02
9	35.42	0.09	4.97	0.12	7.12	0.03	0.21	0.01	2.13	0.01
10	46.49	0.17	6.95	0.06	10.18	0.05	0.34	0.01	1.22	0.04
11	41.39	0.13	6.81	0.08	9.14	0.03	0.16	0.01	2.19	0.01
12	39.68	2.18	6.18	0.13	9.16	0.06	0.23	0.02	2.02	0.23

4.10 Summary and discussion of biomass composition results

The values of the biomass composition analysis performed for the tests included in this technical note will be more deeply analyzed in the future as more experimental data becomes available. Nevertheless, the results presently obtained allow to already describe some general response characteristics.

Table 14: Summary of the macromolecular composition results

Sample No.	Proteins		Lipids		RNA		DNA		PHB	
	% DW	Std. error	% DW	Std. error	% DW	Std. error	% DW	Std. error	% DW	Std. error
1	67.52	4.12	1.12	--	3.40	3.40	0.96	0.39	2.52	0.10
2	62.80	1.65	--	--	2.90	0.04	0.35	0.14	2.52	0.20
3	65.88	1.69	3.98	--	6.45	0.18	0.70	0.29	0.26	0.06
4	65.06	2.17	2.15	--	6.65	1.37	0.93	0.38	2.40	0.44
5	64.44	0.87	1.17	--	3.26	0.84	0.33	0.14	2.20	0.41
6	63.86	1.56	0.46	--	5.10	0.12	2.08	0.85	2.35	0.08
7	60.28	1.40	1.17	--	7.91	0.05	1.31	0.58	2.83	0.01
8	60.44	1.44	4.97	--	6.78	0.06	2.33	1.04	2.82	0.08
9	64.58	0.42	--	--	4.19	0.23	1.69	0.75	2.38	0.04
10	66.83	1.50	1.80	--	5.46	0.11	0.79	0.32	2.12	0.15
11	64.22	1.90	--	--	4.72	0.15	2.39	0.97	6.30	0.42
12	64.36	2.57	0.65	--	6.08	0.08	0.93	0.38	3.23	0.22
Sample No.	**PHV**		**Carbohydrates**		**Glycogen**		**Pigments**		**Total**	
	% DW	Std. error	% DW	Std. error	% DW	Std. error	% DW	Std. error	% DW	Std. error
1	0.47	0.10	15.50	0.79	2.06	0.17	2.08	0.00	93.56	8.92
2	4.83	0.00	14.42	0.65	5.50	0.41	2.03	0.03	89.85	2.72
3	1.30	0.11	19.10	0.79	2.56	0.12	1.98	0.02	99.65	3.14
4	0.00	0.00	19.78	2.00	6.75	0.17	1.46	0.00	98.43	6.35
5	0.82	0.21	16.36	2.30	4.56	0.10	1.97	0.03	90.57	4.80
6	4.56	0.14	14.69	0.66	4.25	0.07	1.98	0.02	95.08	3.43
7	3.88	0.22	15.60	0.92	5.06	0.08	1.89	0.08	94.87	3.27
8	4.44	0.32	11.34	0.31	3.19	0.10	2.07	0.06	95.19	3.31
9	0.91	0.09	18.42	1.65	3.69	0.08	1.86	0.01	94.03	3.19
10	0.96	0.08	16.73	2.07	4.50	0.09	2.10	0.09	96.80	4.33
11	0.00	0.00	17.06	0.33	5.19	0.25	1.95	0.10	96.64	3.87
12	2.70	0.49	14.41	0.12	5.25	0.06	2.07	0.02	94.43	3.87

In order to facilitate this initial evaluation, the results of the biomass macromolecular composition have been collected in table 14 and experimental conditions in table 4.

As a preliminary task, the dry weight contribution of each macromolecular fraction was added (Table 14) in order to ascertain that the major biomass components were taken into account. The glycogen content is not added to the total composition, as glycogen is already included in the total carbohydrates content. The result of the addition of the biomass components gives values close to the 100% being the differences within the experimental precision of this kind of analysis. It can be considered therefore that all the major components were taken into account.

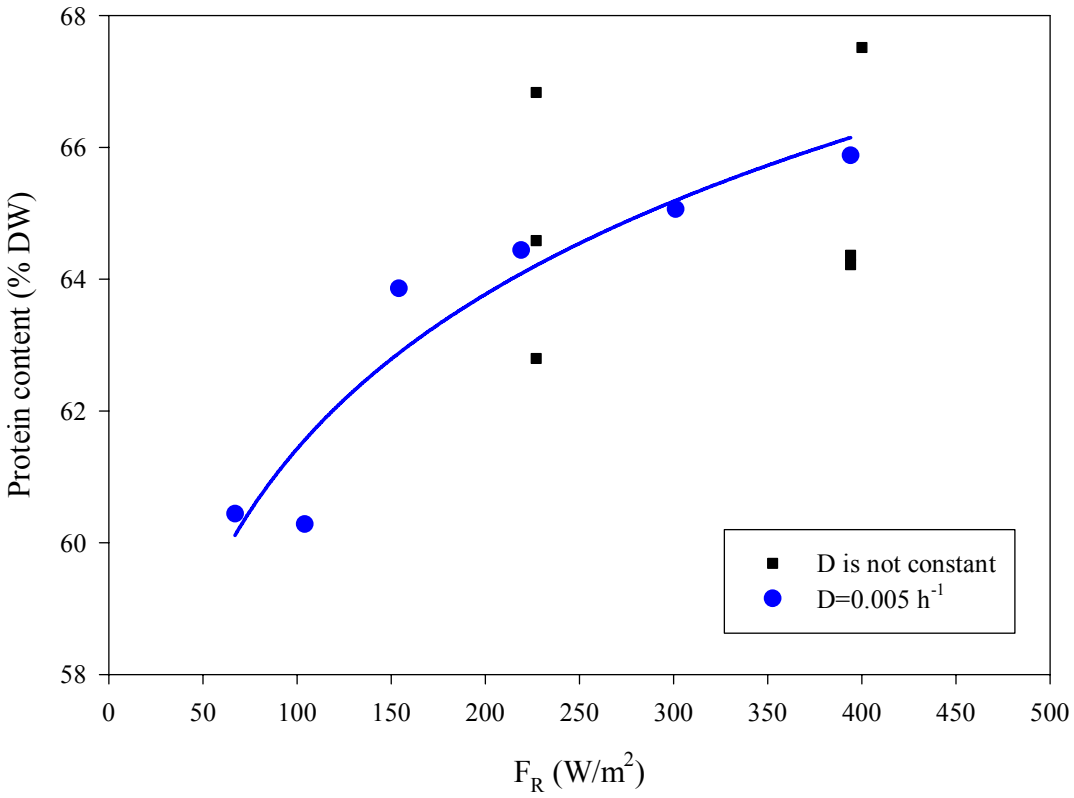

Figure 21: variation in the protein content depending of the incident light intensity.

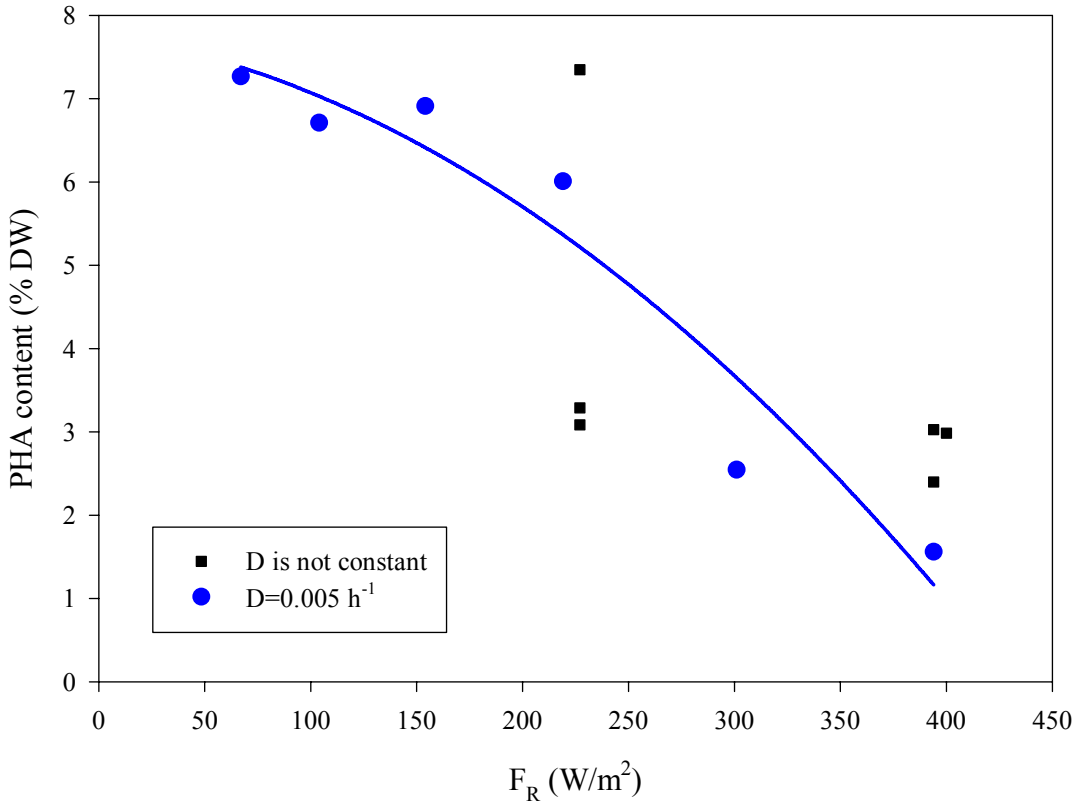

Figure 22: variation in the PHA content depending of the incident light intensity.

As light energy is one of the key parameters for this mode of growth an obvious possibility is that light intensity should be one of the key parameters influencing biomass behaviour. The major components of the biomass, for example the protein or the polyhydroxyalcanoates (PHA, polyhydroxybutyrate and polyhydroxyvalerate) content are represented versus the incident light intensity (Figures 21 and 22). As a result an initial trait can be observed. Observing the biomass composition for the same growth rate (D=0.005 h^{-1}) (blue dots in figures 21 and 22) it appears evident that higher light intensities can be positively correlated with higher a protein content and lower amount of storage polymers (PHA). The PHA content is inversely correlated with the increase in the incident light intensity, verifying the experimental results reported in TN 37.7 (Cabello *et al.* 2001). Plotting in the same graph the data obtained at different dilution rates (black squares) indicate however that other factors may be of importance. In general ad for similar incident light intensities, more active biomass

growing farter and at lower biomass concentrations (higher average light intensities) shows lower levels of PHA.

Another observation that can be made after observation of the biomass composition analysis is that the source of carbon has an influence on the type of the reserve polymer that is accumulated. When the source of carbon is acetic acid or butyric acid, the polymer preferentially accumulated is polyhydroxybutyrate (PHB). At the opposite, when the carbon source is propionic acid, the polymer mainly accumulated is polyhydroxyvalerate (PHV). This tendency is well reported in the bibliography (Satoh H. *et. al.* 1992, Lemos PC, *et. al.* 1998 and Lemos PC, *et. al.* 2003) and it can be observed in figures 23 and 24. In other words, when the source of carbon has an odd number of carbon atoms, polyhydroxyvalerate is the major reserve polymer accumulated while when the carbon source has an even number of carbon atoms the polyhydroxybutyrate is the reserve polymer accumulated in higher quantity.

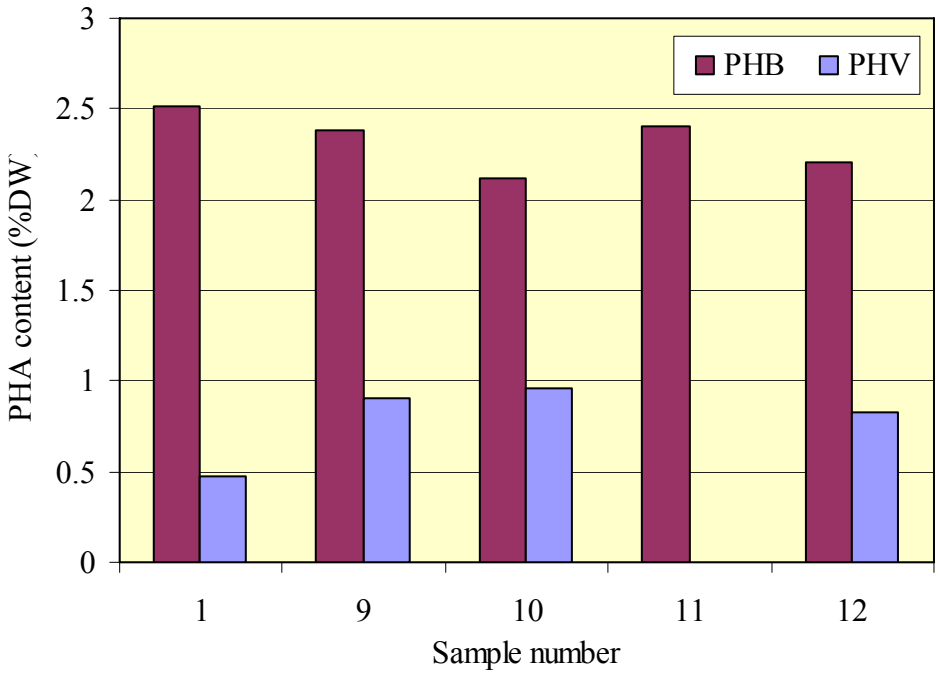

Figure 23: PHB and PHV content using acetic or butyric acid as the C source.

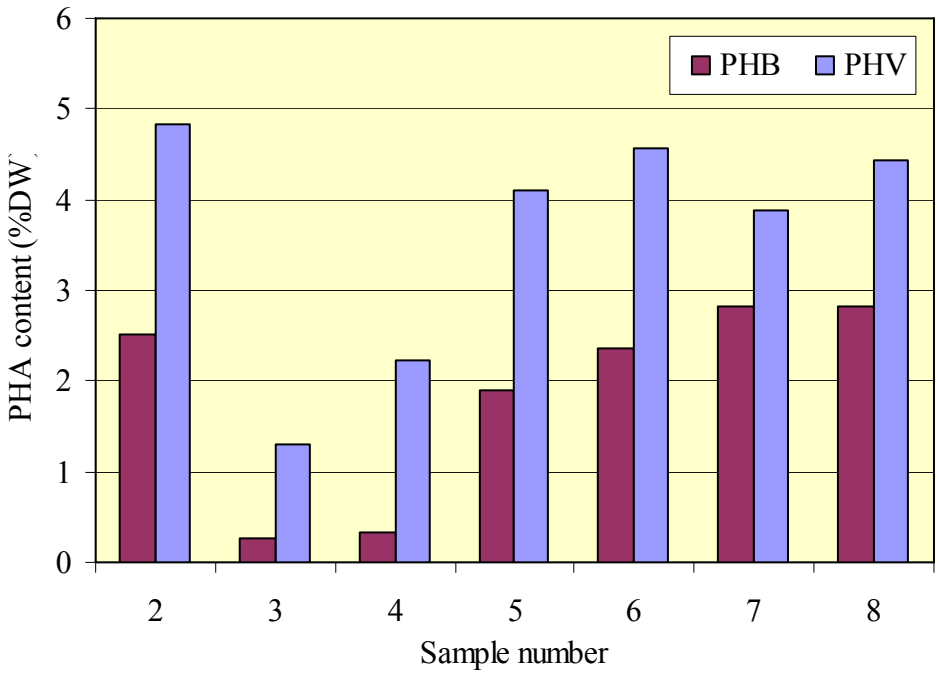

Figure 24: PHB and PHV content using propionic acid as the C source.

5 Conclusions

In the tests reported in the TN 37.7 (Cabello *et al.* 2001), it was observed that for a range of culture conditions, such as dilution rate, light intensity and carbon source concentration (acetic acid), *R. rubrum* cells response departed from the common behaviour. Non-consumed carbon source accumulated in the bioreactor while cells changed their morphology, delaying cell division and showing elongated forms. Biomass heavily attached to the bioreactor walls and consequently internal average light further intensity decreased starting and unstable process. According to that data, one possible explanation could be that, below a certain level of light energy availability, cells modify the metabolic response increasing the internal amount of carbon source storage polymers, such as PHA or glycogen, while decreasing growth rate. On the other hand, in previous tests using mixtures of acetic, propionic and butyric acids, cell aggregates were rarely observed even under conditions of carbon source accumulation due to the low light energy available (Creus et al. 2002a and 2002b, (TN 52.6 and TN 75.2)) which indicates that, in the real MELISSA conditions where a mixture of VFAs is expected, the appearance of those problems might be marginal.

In the present report propionic, butyric or acetic acids were used as unique carbon sources instead of mixtures. In the range of culture conditions of light energy and dilution rate tested, carbon source accumulation in the culture media was observed only in propionic acid cultures at light intensities of 219 and 154 W/m^2 at a growth rate of 0.005 h^{-1}. The modification in cell morphology was observed further decreasing the light energy supply, for the same growth rate, down to 104 and 67 W/m^2. In this case, carbon source accumulation in the liquid phase was not observed. One possibility is that some carbon source was being extracted via the gas phase due to the thermodynamic gas liquid equilibrium. However, measurements of the gas phase composition were not available to confirm this hypothesis. Future experiments will require the measurement of the carbon source both in the liquid and gas phases during all the experiments. It will also be required to consider this in the design of the upgraded C-II pilot bioreactor. In any case, incorporation of the carbon source into biomass at very low culture medium concentrations implies a high affinity for the substrate. This would require either an

active transport or a highly displaced internal reaction consuming the substrate after crossing the cell membrane. Any of those processes would require important energy expenditure, which, in the lack of oxygen, must come from the light energy supplied and therefore adversely affected by the lack of light energy.

Cell composition analysis of the collected biomass samples verifies that the intracellular granules, observed by means of optical microscopy examination, are generally formed by the polihydroxyalcanoates (PHA), β-polyhydroxybutyrate (PHB) and β-polyhydroxyvalerate (PHV), although in certain cases glycogen has been also accumulated.

As shown in figures 21 and 22 the accumulation of carbon source storage polymers increases while decreasing light energy availability. This effect was also reported in previous technical notes. On the other hand, it has been confirmed that the ratio of PHB/PHV is modified depending on whether the number of carbon atoms of the carbon source is even or odd, being the major polymer produced and accumulated correspondingly one or another. These effects have already been described in other bacteria.

With the data presently available from different sources, it can be said that decreasing light energy availability limits the generation of active biomass while the consumed carbon source is incorporated into storage polymers as far as possible. The storage polymer in this case is preferentially PHA, which also acts as an electron sink.

According to previous data and to the behaviour of other bacterial species, the accumulation of PHA also takes place under strong nitrogen limiting conditions, which limit protein and nucleic acid generation. It can be expected that under nitrogen limiting conditions, (besides the type of VFA used and assuming carbon dioxide/bicarbonate and light energy were also available), an increase in glycogen content could also have been observed and is proposed to be analyzed in the future.

In summary, when biomass generation is limited, for example by a shortage of energy or by a shortage of nitrogen source, cells decrease active biomass generation and division and, if possible, accumulate carbon sources as polymers. When the effects are strong enough, cells cease to divide and the increase in dry weight is the result of storage polymers accumulation.

Those metabolic changes appear to be reversible. However, they require a modification of culture conditions for recovery. On one side an increase in energy availability is required if the process has to be produced at a measurable rate. On the other side, due to the reduced nature of the carbon source stored, an electron acceptor is required. Under anaerobic conditions, carbon dioxide or bicarbonate can be used together with light energy to reverse conditions. However, under microaerobic conditions the minute amounts of oxygen supplied could provide both requirements.

The data provided in the present report complement previous reports. On one side, it confirms the general behaviour described and delimits the conditions were those effects appear while on the other side provides further numerical data about the level of those observed effects, under different culture conditions.

Future metabolic analysis, using a wider amount of experimental data will allow to numerically reproduce this behaviour and therefore to use the information for an improved prediction and control of the MELISSA loop behaviour.

6. References

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7 Appendixes

Appendix 1: Culture medium composition

Reactant Number	Component			g/L medium		
1	CH_3COOH	CH_3CH_2COOH	$CH_3CH_2CH_2COOH$	2.500	1.029	0.367
2	EDTA-Na · 2 H_2O			0.100		
3	$MnCl_2$ · 2 H_2O			0.008		
4	$FeSO_4$ · 7 H_2O			0.033		
5	$(NH_4)_2SO_4$			2.728		
6	$CuSO_4$· 5 H_2O			$4.0 \cdot 10^{-6}$		
7	$ZnSO_4$· 7 H_2O			$4.3 \cdot 10^{-6}$		
8	$(NH_4)_6Mo_7O_{27}$ · 4 H_2O			0.177		
9	KH_2PO_4			0.400		
10	Na_2HPO_4			0.489		
11	K_2SO_4			0.550		
12	$NaHCO_3$			0.250	0.500	1.500
13	$MgSO_4$· 7 H_2O			1.200		
14	$CaCl_2$· 2 H_2O			0.091		
15	Trace element solution			1.00 mL/L medium		
16	Biotin solution			1.00 mL/L medium		

Dissolutions	
Trace element solution (g/L solution)	
$NiSO_4$· 6 H_2O	0.500
$MnCl_2$ · 4 H_2O	0.500
$FeSO_4$· 7 H_2O	0.500
$ZnSO_4$· 7 H_2O	0.100
$CoCl_2$ · 2 H_2O	0.050
$CuSO_4$· 5 H_2O	0.005
H_3BO_3	0.100
Na_2MoO_4· 2 H_2O	0.050
Biotin solution (g/L solution)	
Biotin	0.015

The medium preparation consists on adding the total amount of reactants 1 to 8 –both included– (amounts calculated for the total volume of medium) in 0.9 L of distilled water, with magnetic stirring. Next, the pH is adjusted to 6.9 +/- 0.1. Once the pH has been adjusted, reactants 9 to 12 are added. Verify the pH and correct if necessary. When more than 1 litre has to be prepared (up to 20 litres), the concentrations involved are increased proportionally. Once this concentrated solution has been prepared, it is transferred to a bigger tank able to

hold the final volume if necessary and water is added up to approx 90% of the final volume under magnetic stirring. At this point, the rest of reactants (13 to 16) are added. Once added the pH is verified and readjusted. Finally the total volume is completed.

After preparation the medium is immediately pumped to the inlet tank of the bioreactor through the 0.22µm liquid sterility filters.

Appendix 2: VFA concentration determination method

This method allow the analysis of saturated and unsaturated fatty acids from two to five carbon atoms, corresponding to acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid (ordered by retention time).

The range of concentrations that can be determined is 0.0625-4.000 g/L. For the analysis, 50 µL of sample are used. Samples were analyzed by triplicate.

Instrumentation

- Liquid chromatograph Hewlett Packard 5890, equipped with capillary column and an automatic injector.
- FID detector
- Integration software Millenium 3.20

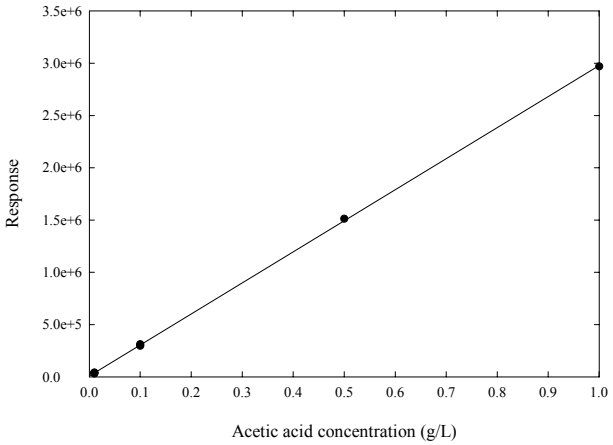
Analysis physical conditions

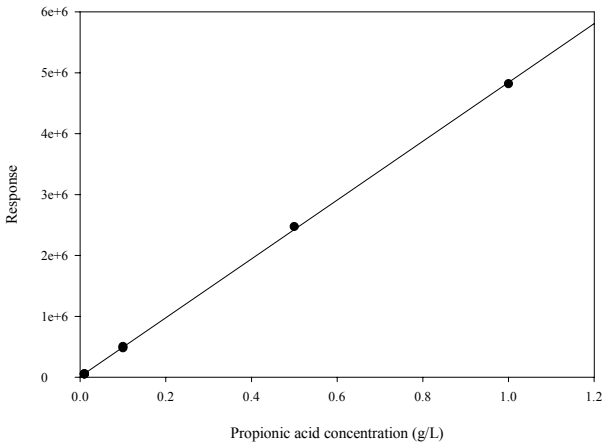
- Column: HP-InnoWax 30m x 0.53mm x 1.00 µm
- Carrier: helium
- Flow rate: 49.0 mL/min
- Injection volume: 1 µL
- Analysis time: 19 minutes
- Injector temperature: 260 ºC
- Detector temperature: 280 ºC
- Oven temperature: start 1 min. 80ºC. ramp 5 ºC/min up to 150 ºC, ramp 20 ºC/min up to 230 ºC.

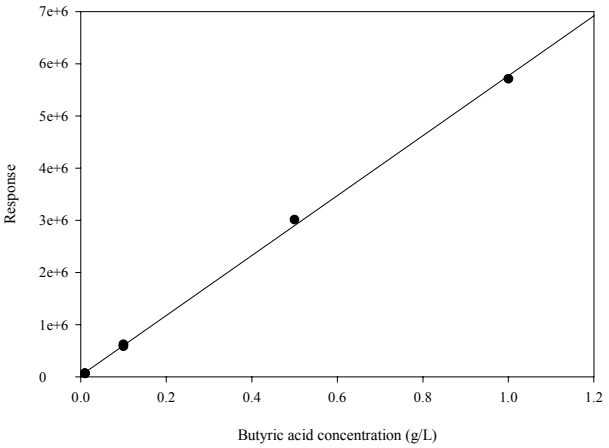
Sample preparation:

- Centrifuge (12000 g, 10 min, 8 ºC)
- Filter (0.22 µm membrane)

Calibration







Appendix 3: Protein determination method (Lowry modified method)

1.- Reactants:

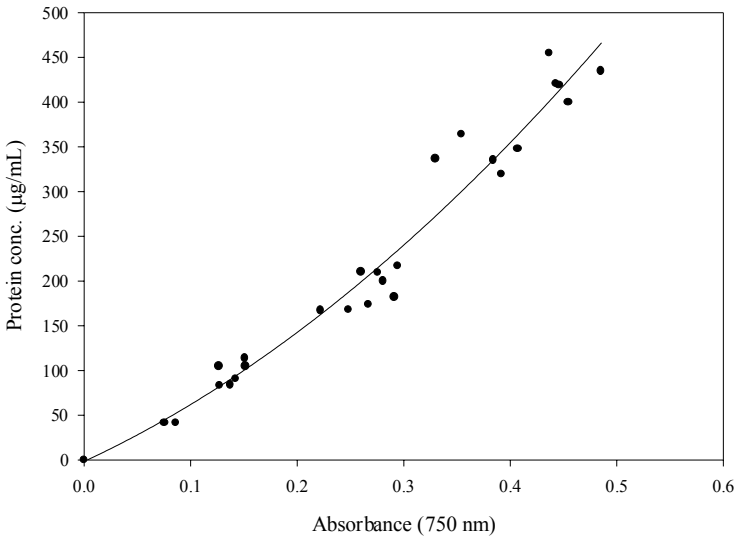
1.- Reagent A. 20 g of Na_2CO_3 dissolved in 1000 mL of double distilled water (DDW).
2.- Reagent B. 0.5 g $CuSO_4 \cdot 5H_2O$ and 1 g of Na-K tartrate dissolved in 100 mL of DDW.
3.- Reagent C. 50 mL of reagent A + 1 mL of reagent B. (This reagent cannot be conserved).
4.- Reagent D. Foling-Ciocalteus reagent, diluted 1:2 (v/v) in DDW.
5.- Albumin stock solutions in the range 400-40 µg prot./mL
6.- Freeze dried biomass solutions. 0.0025 g biomass/5 mL DDW (500 µg/mL).

2.- Prodecure:

1.- Prepare the following stock solutions of albumin:

400 µg/mL	0.04 g in 100 mL of DDW
320 µg/mL	20 mL solution 400 µg/mL in 25 mL
200 µg/mL	10 mL solution 400 µg/mL in 20 mL
160 µg/mL	10 mL solution 400 µg/mL in 25 mL
100 µg/mL	5 mL solution 400 µg/mL in 20 mL
80 µg/mL	5 mL solution 400 µg/mL in 25 mL
40 µg/mL	2 mL solution 400 µg/mL in 20 mL

2.- Prepare the biomass solutions.
3.- Take 0.5 mL of each stock solution, 0.5 mL of each biomass solution and 0.5 mL of DDW (in triplicate). (Prepare a blanc sample 0.5 mL of each stock solution and 1 mL DDW).
4.- Add up 0.5 mL of NaOH 1 M to every sample.
5.- Boil at 100 ºC for 10 minutes each sample. Cool in a water bath.
6.- Add 5 mL of reagent C to each sample. Shake and wait 10 minutes.
7.- Add 0.5 mL of reagent D to each sample. Mix. Keep in the dark for 30 minutes. Measure the absorbance at 750 nm..



Appendix 4: Lipid determination method

The lipid determination has been carried out by gas chromatography with a FID and a mass spectrometer as detectors. Analysis were performed by 'Serveis Cientificotècnics' Universitat de Barcelona.

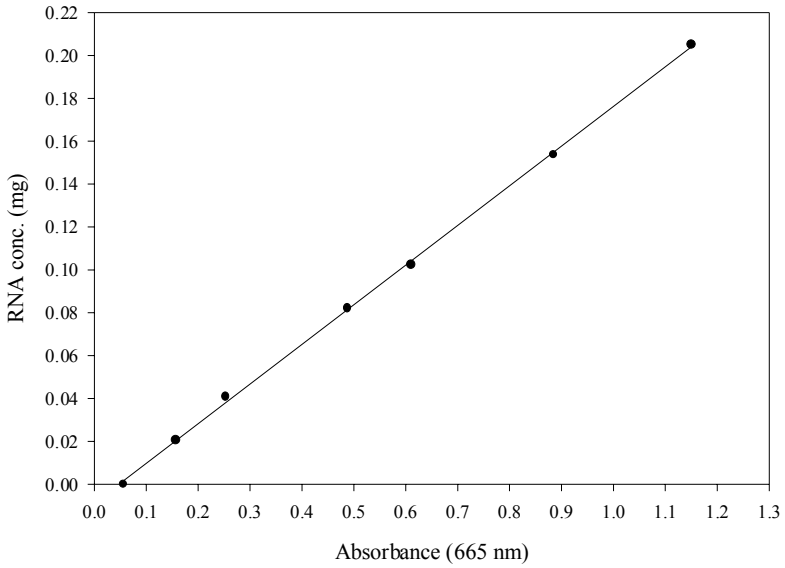
Appendix 5: RNA determination method

1.- Reactants:

1.- Reagent A. $HCLO_4$ 0.2 N
2.- Reagent B. Dissolve 2.5 g orcinol in 5 mL ethanol 95%
3.- Reagent C. Standard RNA. Concentration: 100 mg/mL (store at 4 ºC)
4.- Reagent D. 0.3 g of $CuCl_2 \cdot 2H_2O$/200 mL of conc. HCl (prepared the same day of the analysis)
5.- Reagent E. 2 mL reagent A and 100 mL of reagent C
6.- Reagent F. KOH 0.3 N
7.- Reagent G. 1 L chloroform + 500 mL methanol

2.- Prodecure:

1.- Add to 25 g of dry biomass sample 10 mL of reagent A. Keep 15 min. at 4 ºC. Centrifuge 5 min. at 6000 rpm. Discard de supernatant.
2.- Repeat step num. 1.
3.- Add to the pellet 10 mL of reagent G. Wait for 5 min. and centrifuge 5 min. at 6000 rpm. Discard de supernatant.
4.- Repeat step num. 3.
5.- Add to the pellet 2 mL of reagent F. Keep at 30 ºC during 18-24 h.
6.- Cool down and add conc. $HClO_4$ to reach pH=1
7.- Centrifuge at 6000 rpm for 6 min. Do not discard the supernatant.
8.- Wash the pellet with 1 mL of reagent A. Centrifuge at 6000 rpm for 6 min. Do not discard the supernatant.
9.- Mix both supernatants and take three samples (0.1 mL, 0.2 mL, and 0.3 mL). Take six samples of standard RNA solution (2 mg standard RNA in 20 ml chloroform) - 0.2mL, 0.4 mL, 0.8 mL, 1 mL, 1.5 mL, and 2 mL -. Add H_2O until 2 mL and 2 mL reagent G.
10.- Keep during 35 min. at 100 ºC. Cool down with water and measure the absorbance at 665 nm.



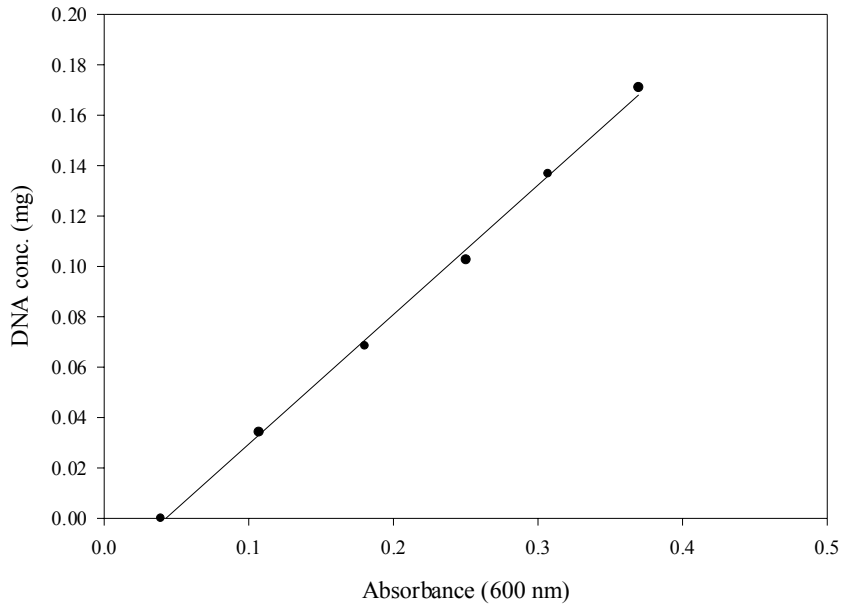
Appendix 6: DNA determination method

1.- Reactants:

1.- Reagent A. 15 g diphenylamine + 15 mL conc. H_2SO_4 + 1 L CH_3COOH
2.- Reagent B. 2 L $HCLO_4$ 0.2 N
3.- Reagent C. 1 L $HCLO_4$ 0.5 N
4.- Reagent D. 1 L chloroform + 500 mL methanol
5.- Reagent E. NaOH 5 mM

2.- Prodecure:

1.- Add to 25 g of a dry biomass sample in 10 mL of reagent B. Keep 15 min. at 4 ºC. Centrifuge 5 min. at 6000 rpm. Discard de supernatant.
2.- Repeat step num. 1.
3.- Add to the pellet 10 mL of reagent D. Wait for 5 min. and centrifuge 5 min. at 6000 rpm. Discard de supernatant.
4.- Repeat step num. 3.
5.- Add to the pellet 5 mL of reagent C. Keep at 70 ºC during 45 min. Centrifuge.
6.- Take three samples of the supernatant (0.5 mL, 1 mL, and 1.5 mL) and prepare 5 samples of the stock solution of DNA Na salt (0.1 g, 0.2 g, 0.3 g, 0.4 g, and 0.5 g). Add to all of the samples reagent C until 2 mL. Add 4 mL of reagent D and keep at 30 ºC during 16-24 h.
7.- Measure the absorbance at 600 nm.



Appendix 7: PHB-PHV determination method

1.- Reactants:

1.- Reagent A. Acidified methanol.
- 100.00 mg benzoic acid
- 30 mL H_2SO_4 (98%)
- Complete up to 1000 mL with methanol (99.8%)

2.- Reagent B. Chloroform

3.- Reagent C. Copolymer of 3-hydroxybutyric acid and 3-hydroxyvaleric acid (70:30), supplied by Fluka.

2.- Procedure:

1. Add to 40 mg of freeze dried biomass sample 4 mL of reagent A and 4 mL of reagent B.
2. Keep 3.5 h at 100 ºC. Centrifuge 5 min. at 5000 rpm.
3. Add 1 mL of distilled water
4. Shake during 5 min
5. Extract the chloroform (the heavy phase -3.5 mL-). Discard the light phase.
6. Centrifuge at 3000 g during 5 minutes
7. Filter using a 0.45 µm membrane (use filters for organic solvents).
8. Determine by gas chromatography

3.- Instrumentation:

- Gas chromatograph Hewlett Packard 5890, equipped with capillary column and an automatic injector.
- FID detector
- Integration software Millenium 3.20

4.- Analysis physical conditions

- Column: HP-InnoWax 30m x 0.53mm x 1.00 mm
- Carrier: helium
- Flow rate: 54.0 mL/min
- Injection volume: 1 µL, split 1:2
- Analysis time: 13 minutes
- Injector temperature: 220 ºC
- Detector temperature: 275 ºC
- Oven temperature: start 2 min. 70ºC. ramp 10 ºC/min up to 160 ºC, 2 min 160 ºC.

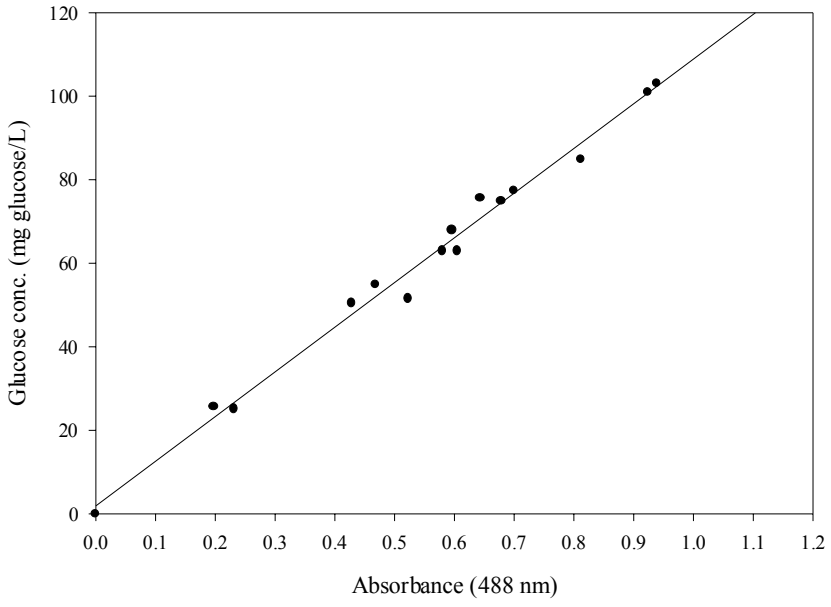
Appendix 8: Carbohydrates determination method

1.- Reactants:
1.- Concentrated sulphuric acid (96-97%).
2. -Phenol 5% (w/v)
3.- Glucose

2.- Sample treatment:
- Centrifuge 10 mL of culture suspension. Discard supernatant.
- Wash with DW (distilled water).
- Centrifuge in the same conditions. Discard the supernatant.
- Dissolve in DW.
(Alternatively dissolve a sample of freeze dried biomass in DW).

3.- Analysis
-Add to 1 mL sample
1 mL Phenol and mix carefully
5 mL of sulphuric acid. Mix carefully.
- Wait 10 min.
- Cool the tubes (15 min. in water 25 °C).
- Read absorbance of the sample and the Blanc at 488 nm, against DW.

4.-Results
-Prepare a calibration curve using glucose samples (0-100 mg/L).
Straight line fitted: Glucose conc. (mg/L) = -3.30 + 103.57 · Abs (488 nm) r^2=0.99
- Use the standard curve to calculate the concentration of the 1 mL sample by interpolation of the absolute absorbance.



Appendix 9: Glycogen determination method

1.- Take 20 mg of freeze dried biomass sample

2.- Add 10 mL of HCl 0.6 M

3.- Keep at a boiling water bath for 1 hour. Cool down.

4.- Filtrate through a 0.22 μm filter.

5.- Measure the glucose concentration with a glucose analyser (enzymatic analysis).

Appendix 10: Pigment determination method

1.- Reactants:

1.- Reagent A. Phosphate buffer solution Na_2HPO_4/NaH_2PO_4 (1:1)
2.- Reagent B. Acetone/Methanol solution (7:2 v/v)
3.-

2.- Sample treatment:

- Add to 5 mg of freeze dried biomass sample 5 mL of reagent A.
- Centrifuge at 15000 rpm during 10 minutes. Discard the supernatant.
- Add 10 mL of reagent B to the pellet.
- Redissolve the pellet using ultrasounds.
- Centrifuge at 15000 rpm during 10 minutes.
- Measure the absorbance at 772 nm and 475 nm

Appendix 11: Elemental composition determination method

The determination of C, N, H, and S composition is done with the sample combustion inside a Sn capsule with O_2 atmosphere in a furnace at 100ºC. P was determined by ICP. Analysis were performed by 'Serveis Cientificotècnics' Universitat de Barcelona.

A.- Combustion method

1. Basis:

   The combustion transforms organic sample components to the corresponding oxides, obtaining a gas mixture of CO_2, N_2, N_xO_y, H_2O, SO_2 and SO_3. The exothermic reaction, which turns Sn into SnO_2(s), releases heat that increases the capsule temperature till 1800 ºC. SnO_2 remains as a solid in the combustion zone. Inorganic sample components also remain as solid oxides in the combustion zone.

   The gases formed and the O_2 excess flow with He as a carrier gas to a reactor with WO_3, which produce the transformation of the gas mixture into the unique species for each element, obtaining CO_2, N_2, H_2O, SO_2 and the O_2 and He in excess. This mixture is transported to a reactor at 500 ºC containing CuO, which reacts with the O_2 in excess to give CuO(s).

   The remaining gases CO_2, N_2, H_2O, SO_2 are carried with He to a gas chromatograph (Porapak column, Waters Associates Inc.) where they are separated and measured using a thermal conductivity detector, which detects a signal proportional to the amount of component.

2. Sample preparation:

   The biomass sample is in freeze-dried form.

3. Results:

   The quantification is performed by interpolation on an appropriate reference curve.

B.- ICP Analysis

Sample (0.1 gDW) was digested with 2 ml HNO_3 and 2 ml H_2O_2 in a sealed reactor heated at 90ºC overnight. Digested sample was diluted with 15 ml distilled water 'Milli-Q' grade quality. Samples were digested in triplicate and 4 blanc samples added.
ICP determination was performed using an Induced Coupled Plasma machine 'Perkin Elmer' Optima 3200 RL. Calibration was performed using reference P samples digested with HNO_3 5%.

Appendix 12: Incident light calibration

In order to measure the available light energy inside the photobioreactor a spherical quantum sensor (LI-193SA) has been used, attached to an amplifier LI-250 (Lincoln, NE, USA), which measures the incident light in all directions.

This sensor measures the quantum radiation that arribes to its surface. To convert from quantum units ($\mu mol \cdot m^{-2} \cdot s^{-1}$) given by the sensor to radiometric units ($W \cdot m^{-2}$) a conversion factor must be calculated. This factor is obtained from the integration of the distribution of the radiation emission espectrum of the halogen lamps (at 12 V) in the range 350-950 nm. This range is wider than the one used with Spirulina cultures (350-750nm) as *R. rubrum* presents a light collection system able to use light from much wider spectrum. The obtained conversion factor is equal to 0.368 $(W \cdot m^{-2})/(\mu mol \cdot m^{-2} \cdot s^{-1})$ as it is described in TN 43.610 (Cabello *et al.*, 1999).

The incident light intensity at the surface of the photobioreactor is calculated from the light energy measured locating the spherical sensor in the centre of the vessel and applying the following equation:

$$F_R = \frac{E_B \cdot r_B}{\pi \cdot R_B}$$

where:

F_R: light intensity at the surface of the bioreactor ($W \cdot m^{-2}$)

E_B: light intensity at the surface of the light sensor ($W \cdot m^{-2}$)

r_B: radious of the light sensor (30 mm)

R_B: radious of the photobioreactor (48 mm Bioengineering ; 64 mm Applikon)

Several measures were taken at different heights in the bioreactor and the final value was calculated as the average. Theses measures were repeated for different values of the voltage supplied to the lamps.

During the calibration proces, water was flowing through the double jacket of the photobioreactor. Measures were taken with the bioreactor vessel empty. It is known that when

the reactor is filled with water during the calibration process, and as a result of the effect of the refraction index on the light path when crossing different media, light intensities calculated with the bioreactor filled with water are higher than when the bioreactor is empty. This effect does not take place when biomass is in the liquid medium due to the scattering effects.

As an example of the measures, table 15 lists the light intensities measured by the sensor in six different positions, measured in the Applicon photobioreactor with 30 lamps, and the calculated average value depending on the voltage applied to the lamps.

Table 15: Incident light calibration experimental data in Applikon bioreactor (30 lamps)

Voltage		Positions of incident light measure (μmol/m^2/s)						Average	Eb	F_R
Lamps	Power supply output	1	2	3	4	5	6	(μmol/m^2/s)	W/m^2	W/m^2
4	4,23	80,1	84,7	88,7	120,1	119,6	100,8	99,0	36,4	**5,4**
5	5,25	212,6	222,6	238,2	323,8	327,2	275,2	266,6	98,1	**14,6**
5,5	5,78	322,3	338,1	359,3	493,0	504,3	421,4	406,4	149,6	**22,3**
6	6,28	456,2	479,1	507,1	696,4	711,1	603,1	575,5	211,8	**31,6**
6,5	6,79	627,4	656,6	698,9	956,1	978,5	831,7	791,5	291,3	**43,5**
7	7,30	840,5	878,6	931,9	1282,2	1307,9	1116,2	1059,6	389,9	**58,2**
7,5	7,82	1104,3	1143,7	1212,3	1673,1	1709,6	1458,5	1383,6	509,2	**76,0**
8	8,33	1390,8	1459,5	1536,6	2113,0	2180,0	1857,0	1756,2	646,3	**96,4**
8,5	8,85	1738,1	1827,6	1923,6	2643,0	2723,0	2327,0	2197,1	808,5	**120,6**
9	9,36	2131,0	2235,0	2359,0	3239,0	3344,0	2859,0	2694,5	991,6	**148,0**
9,5	9,88	2587,0	2705,0	2859,0	3928,0	4060,0	3469,0	3268,0	1202,6	**179,4**
10	10,38	3078,0	3208,0	3388,0	4651,0	4832,0	4137,0	3882,3	1428,7	**213,2**
10,5	10,88	3620,0	3775,0	3994,0	5472,0	5689,0	4867,0	4569,5	1681,6	**250,9**
11	11,40	4231,0	4413,0	4671,0	6413,0	6662,0	5715,0	5350,8	1969,1	**293,8**
11,5	11,90	4882,0	5091,0	5380,0	7401,0	7693,0	6587,0	6172,3	2271,4	**338,9**
12	12,41	5609,0	5860,0	6182,0	8493,0	8829,0	7565,0	7089,7	2609,0	**389,3**

Similar calculations were also done for the 8 L Bioengineering bioreactor and for the Applicon bioreactor with 15 lamps. The obtained calibration values are summarized in tables 16 and 17.

Table 16: Incident light calibration experimental data in Applikon bioreactor (15 lamps)

Lamp Voltage	F_R (W/m^2)
4	2.1
5	6.5
6	15.6
7	30.1
8	50.7
9	79.4
9.5	98.2
10	119.3
10.5	142.2
11	166.6
11.5	195.2
12	226.9

Table 17: Incident light calibration experimental data in Bioengineering bioreactor

Lamp Voltage	F_R (W/m^2)	Lamp Voltage	F_R (W/m^2)
3.1	2.2	9.64	259
3.8	5.7	9.85	278.6
4.54	13.1	10.06	298.6
5.02	20.4	10.28	321.6
5.34	26.8	10.5	344.3
5.67	34.6	10.72	369.7
6.18	49.3	10.94	395.6
6.54	61.6	11.16	422.9
6.9	75.9	11.38	450.3
7.26	92.8	11.61	477.6
7.64	112.3	11.84	510.6
8.02	133.7	11.96	527.3
8.42	159.7	12.07	544.4
8.82	189	12.19	569.9
9.22	222	12.3	597.3
9.43	241.5		

In figure 24 the average incident light, F_R in W/m^2, versus the voltage supplied to the lamps is shown for the three set-ups used in this report.

The experimental data have been fitted to a power law equation, obtaining an analytical relationship between the lamps voltage and the average incident light energy supplied to the culture. This relationship is expressed by means of the following equations:

Bioengineering 8 L: $F_R\ (W/m^2) = 0.0906 \cdot \text{Lamp voltage}^{3.4408}$

Applikon 2.4 L (15 lamps): $F_R\ (W/m^2) = 0.0251 \cdot \text{Lamp voltage}^{3.6688}$

Applikon 2.4 L (30 lamps): $F_R\ (W/m^2) = 0.0724 \cdot \text{Lamp voltage}^{3.4616}$

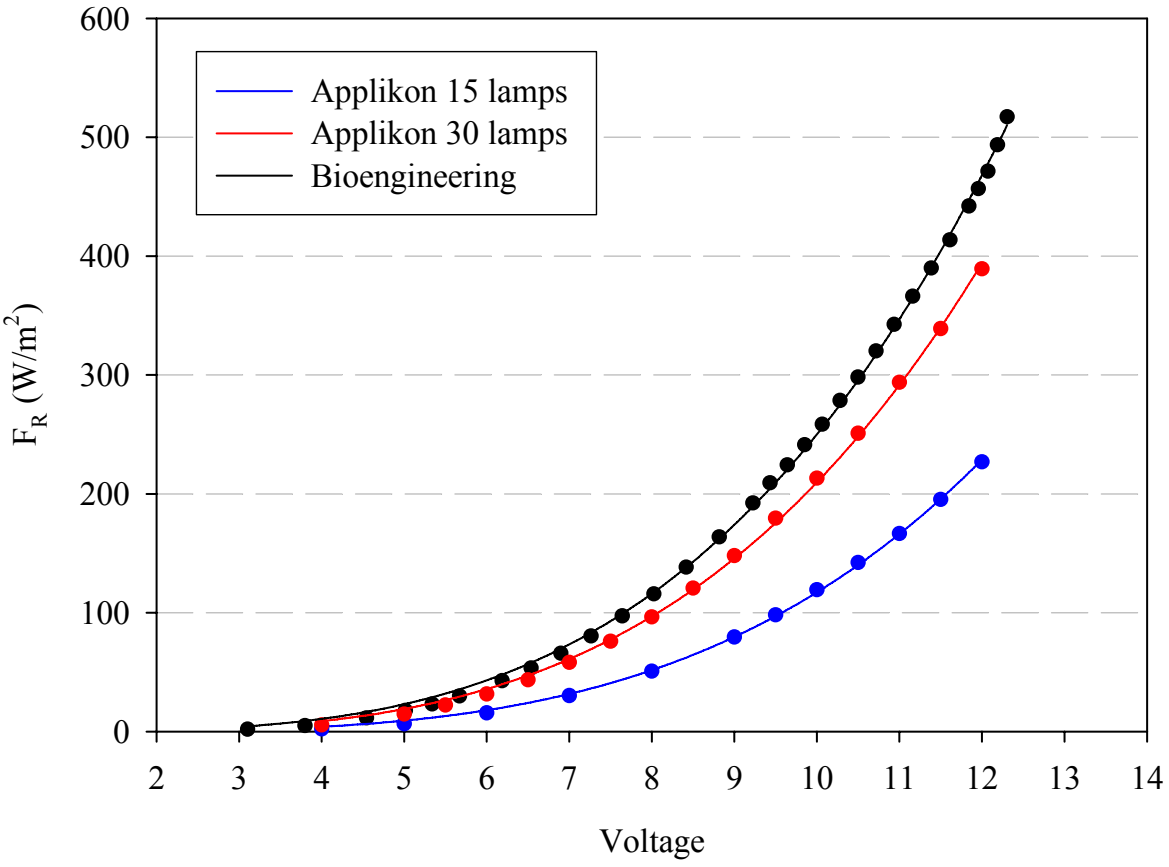


Figure 24: Incident light intensity and lamp voltage relationship for the three set-ups used.

Comments on TN 43.5
Rhodospirullum rubrum
Carbon limitation tests

General comments

This technical note is presenting a lot of experiments, i.e. a large effort has been put on the performance of experimental work.
However the reporting of this large experimental work could be quite improved by providing some missing basic information:

- Some information should be added with regards to sampling and analysis:
  - collected volumes, time to collect these required volumes, added
  - are liquid samples obtained according to the protocol mentioned in appendix 2 (i.e. owing to centrifugation and filtration)? In that case, how can we ensure that we don't modify the composition of this liquid phase (e.g. through adsorption on filters, through break of cells and release of some components…)? Due to the small size of the filter membrane, a modification of liquid composition due to adsorption, and taking into account VFA concentrations used should be negligible. The procedure of centrifugation and Freeze drying of biomass produces a minimum of cell breakage which is considered negligible as final dry weight of freeze dried biomass is in agreement with the filter dried biomass of the same steady state.
  - when you perform the biomass analysis, how do you prepare your biomass before (washing step?...) yes. Explanation added.
  - did you performed your analysis right after sampling? If no, how and how long did you store your samples? liquid medium analysis are done recovering frozen liquid medium samples. Biomass analysis are done using freeze dried biomass. Storage time is variable but usually spans a couple of months. In any case freezed liquid and freeze dried biomass should not be significantly affected by this storage time.
- Some values are given without any proper definitions. Added were necessary
- It would be of high benefit to include hydrodynamic datas (nature of the stirring equipment, rotation speed of the stirrer, presence of baffles) for both PBR used. Description was already included in TN 47.1 Cabello et al 2000. Nevertheless explanation in the text has been expanded and reference included.
- the logic of the overall test plan, aiming at the determination of carbon limiting conditions, is not obvious. Some assumptions are sometimes poorly supported. Can you please provide at the beginning of the TN the definition of carbon limiting conditions and give the overall approach of your test plan? In fact it is ESA test plan. This TN continues the one on light limiting conditions and was proposed to substitute a WP originally planned to test the control law which was not available for testing (to our knowledge still is not) during the period this old contract was still on. In this case the interesting point is to locate the transitions conditions while passing from carbon limiting to light limiting ones or the reverse and also obtain experimental data in carbn limitation. Carbon limitation is considered when carbon source is the main factor governing growth rate. For example if the carbon source in the input media of a continuous culture is decreased and it results in a decrease in the biomass concentration in the bioreactor at steady state. Or if light intensity is increased or decreased in a continuous culture and biomass concentration at steady state does not change. This fact usually is coincident with very low concentrations of the carbon

source in the bioreactor (below detection limit) but not necessarily always. Specially at high dilution rates. Assuming one has carbon limiting conditions the interesting point would be to determine for example at which light intensity the limitation is changed from carbon to light limiting conditions. In fact due to the close relationship between the carbon metabolism and light energy recovery mechanisms, ranges of values (carbon-light intensity) acting in co-limitation can be expected. In this framework ESA asked for a minimum number of experiments under carbon limitation and with a step of light as initial experiments in view to locate the transition points. That is as an initial screening. The results should allow to evaluate the location of the transition points and plan more specific future experiments if necessary. Of course the cost of the WP was not going to be changed and the number of test/time/manpower should be in agreement with those expected for the control law test previously planned. The experiments agreed were one steady state or each of 3 different carbon sources at 2 dilution rates and 2 light intensities. The values for the dilution rates and light intensities were decided during the corresponding progress meetings with ESA. Nevertheless a few more were added by UAB in an attempt to collect more information. Also the initial experiments foresaw only the use of the pilot reactor which had to be later changed due to the low light supply and the higher light energy requirements when using other carbon sources than acetic acid. This is explained in the text.

- Some additional bibliographic sources could be added this kind of data for R.rubrum does not exist except the data collected at LGCB which is only for acetic acid.

(minor remark: In the various tables, as you do it in table 2, it is better to state B.D than 0.) done

Detailed comments

p.5: 'stock strain'? Do you mean backup inoculum? Yes. The strain we have in stock to be used as inoculum. Updated to be more clear.
p.5: composition detailed in appendix 1: please detail in this appendix how the solution is prepared (volume, storage, various concentrations verified through regular analysis….) done
p.5 What is the definition of the "Illuminated volume fraction" ? How was the value 0.52 determined? Explanation has been added in the text. It is a characteristic of the bioreactor not to be confused with any 'dark area' calculated by any model.

p.6: No data are given on the system's hydrodynamic parameters. (Presence and number of baffles ? Stirrer rotation speed ? Type of stirrer ?) explanation and reference added in the text, see abowe.
p.6:the description of analysis methods should be updated according to the previous general comments. done

p.8: "it was known that the energy available allowed to completely consume an amount of carbon source around 0.5 gC/l at average growth rate." In continuous cultures, such a value is meaningless unless it is associated with a residence time or a dilution rate. The phrase is referred to previous experiments already done in the Pilot Plant and reported in previous TN and so the dilution rates or growth rates are known. For example 0.5 g C/l at 0.04 h^{-1}. in TN 37.7, Cabello *et al.* 2001). Tests are started in those previously known conditions if possible or slightly modified such as to increase carbon limitation. In any case explanation of those conditions such as growth rate has been added in the text.

p.10: as in the previous test…dilution rate: which tests are you referring to? Previous tests refers to the test previously described in this TN. The text has been clarified.
p.10: should we say biomass decrease or biomass concentration decrease? Concentration. Updated in the text.

p.10: The reasons invoked to explain the experimental results previously described are conflicting. The first reason would be "it is necessary an incident light higher than 500 W/m² to maintain the carbon limitation growth for the used input carbon source concentration.", i.e. light flux is too weak to provide enough energy for the microorganisms to grow. The second would be "the high average intensity, […], has inhibitory effect", i.e. the light flux induces photoinhibition. It was just an attempt to enumerate different a priori possibilities. The explanations have been modified.

p 10: "The high average intensity" should be defined and quantified. Whatever the model one uses to calculate the average light intensity, the average light intensity to which the cells are exposed will always increase if the biomass concentration is decreased and the incident light intensity is not modified. This is the only point it is intended to be made by the coment. Besides this point, the exact number will of course depend on the model used. At present time UAB has not received from LGCB any 'official' model for R.rubrum to evaluate this point for different carbon sources and light intensities, nor has been 'authorized' to officially use for MELISSA another model. As was previously done for Arthospira. Therefore the exact number was not calculated. In any case this can be done at any time.

p.10-11: The third reason invoked to explain the experimental results is not clear ("or any combination of those") it refers to the possibility of an existing co-limitation for more than one factor at the same time. That is if the results obtained for certain values of light intensity and carbon source are different when both actors are limiting (low levels) at the same time than if they are tested separately with only one being strongly limiting.

p.12: please explain what you call average light intensity which is in that case light/g of biomass Again, as previously said, if for the same operating conditions (incident light intensity, bioreactor, dilution rate) but lower carbon source concentration in the input tank it results, for example a half of the biomass concentration at steady state, the average light intensity to which the cells are exposed will of course be different, and in this case higher. As already said numeric calculation depends strongly on the model one applies. But the intention was merely to state the fact of the different light intensity the cell were exposed.
p.12: Concluding that "it is not possible to reach a steady state at a dilution rate higher than $0.04h^{-1}$" is doubtful, in so far as hydrodynamics were not considered. Although different behaviour has been described by LGCB for different agitation conditions, the information on the degree of effect for the Pilot Plant bioreactors used is not available. Therefore the phrase is of course based on the available information. One possibility is to plan those tests for the future. But what is true is that this bioreactor can only supply a low level of W/m3 compared to other bench bioreactors available at the P.P. this fact alone advises to use the other bioreactors, specially to test carbon sources which have higher energy requirements.

p.16: The amount of "maximum light energy" could be quantified. How are the values of radiant energy per unit volume of culture obtained? Which radiative model has been used? See previous comment. No model used. The comment refers to the maximum amount of

energy the lamps can supply (so Fr*surface/total_volume or total watts supplied/total volume.). As they are at the maximum voltage no more energy can be provided .

p.17 and followings: please add C concentration on the Y axis and associated legend on the various figures done
p.17: Steady states are not clearly defined on the following figures (from 8 to 20). Would it possible to indicate on the figures the time of change of dilution rates ? in figure 8, 11, 12, 13, 14, 19 and 20 it is already indicated by the dashed line. In figures 9, 18, the change is done at time zero.

p.18-19: The legends of figures 9 and 10 's caption conflict with the legends on the figures (470 W/m² et 394 W/m²) –corrected.

p.19: which inhibition effects with acetic acid are you referring to? As described in TN 37.7 we think that one strong possibility for the explanation of the different whashout effects observed along the numerous tests done up to now is due to an inhibition effect produced by accumulation of non consumed acetic acid in the culture medium. That is: as concentration of acetic acid increases in the culture medium, the growth rate feasible decreases.
p.20: "It was therefore necessary to further decrease the light intensity to see any accumulation of the carbon source". Nothing proves this assertion. It is obvious that light intensity can be decreased until light limitation forces to decrease the growth rate and so acetic acid consumption decreases and therefore it accumulates in the medium.

p.21: explain what you name a 'bifurcation' We mean the usual meaning of the term in non linear systems analysis. That is: for the same operating conditions 2 different stable steady states are possible.
p.21: "Which is possible after the results obtained by UAB and UBP", Giving the references may be needed. -added-

p.22: "In any case, the behaviour of this particular experiment was further verified by further decreasing light intensity" Then ? It means that in the following tests (figures 13 and 14) the test is repeated using lower light intensities and the stability of the biomass for the different light intensities indicates that this is a stable operational condition. It remains to be verified in the future if the case of stability with propionic acid accumulation is repeated.

p.23: since the batch……transient one: please rephrase? Done-
p.23: "Those new conditions" Which new conditions? They should be written in the corpus of the TN. done

p.26: Figures 16.a to 17.b are unreadable. –explanation is located in the text. Microscopic pictures are perfectly seen in our version. Nevertheless they have been expanded as possible.

p.31: the loss of VFA in the gas phase is valid for all VFA OK.

p.45: Figure 23 may be misunderstood. Maybe that dividing it in two different figures would make it easier to assimilate. Done. Figure was split in 2.

p.45: "If under nitrogen limiting conditions, and besides the VFA …" This phrase is not clear. Moreover, some conclusions are not clearly relevant with the scope of this TN. Why are aerobic conditions for *Rs. Rubrum* mentioned? – The mention of nitrogen is just an example of a condition that could decrease growth rate. Not in the present experiments but in general

with this bacteria. The mention to oxygen refers to the possibility that the equipment used does not completely avoid the entrance of oxygen from the ambient air even if the oxygen probe measures zero, as it does. This could generate microaerobic conditions in some parts of the reactor that would change the global stoichiometry. But it is mentioned just as a possibility.