Université Blaise Pascal LGCB
Laboratoire de Génie Chimique et Biochimique

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TESTS OF NTE BIOMASS SCAN SYSTEM SENSOR

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TABLE OF CONTENT

1. Introduction

Determination of bacterial cell mass concentration is an important measurement for fermentation scale up and production control. The ability to monitor cell mass concentration is critical to the optimization of the fermentation process. The most routine method of measuring bacterial cell mass concentration was typically relied on off-line, intermittent measurements of optical density with the use of an external spectrophotometer used after sampling procedures followed by dilutions. In fermentation processes, the cell concentration is typically measured off-line via optical density (OD) measurements. Samples are drawn from the fermenter and the optical density is measured in a bench-top spectrophotometer. For higher concentrations, it is necessary to dilute the sample in order to get reasonable results. Because samples are drawn from the fermenter and require dilution, the OD method proves to be time consuming and does not provide a continuous picture of cell growth throughout the fermentation. It is therefore hardly used for automatic on-line control.

Since 1990, different on-line biomass measurements were tested using, either optical fibre technology or capacitance measurements. The advantages of on-line biomass measurements are multiple (Combs and Bishop, 1993):

- This enables process automation and fingerprinting ;
- This decreases risk of contamination ;
- This improves time, cost and labour efficiency ;
- This avoids dilution technique errors associated with off-line measurement ;
- This avoids volume loss during off-line sampling ;
- This eliminates aerosols produced during sampling.

1.1. Optical fibre sensors

Some manufacturers do offer in-line or continuous OD measurement systems which do provide a continuous picture of the fermentation. However, these systems suffer from the same limitations as the off-line measurements, i.e. a fixed optical path length. A fixed optical path length often results in incorrect biomass correlation during stages of fermentation – especially at high cell concentrations.

Optical fibre sensors provide an alternative to traditional in-line OD measurement limitations. METTLER TOLEDO, Switzerland, sensors utilize backscattered light to depict true cell mass levels throughout the entire fermentation. Backscattered light technology and the use of optic fibre cables enable a sensor design with uniform, unbroken surface structure. These sensors are able to meet the toughest demands placed on optical sensors with respect to freedom of fouling and easy cleanability.

1.2. Capacitance measurements

Harris *et al*. (1997) designed and built an instrument working which measures the dielectric permittivity of microbial suspensions. At low frequencies (0.1- 1.0 MHz), the microbial cell membrane behaved as a capacitor, and became charged by the so-called ξ-dispersion effect. The magnitude of this $\tilde{\xi}$-dispersion was linearly proportional to the membrane-enclosed volume fraction, which means that the dielectric permittivity is linearly proportional to the volume fraction (Kell *et al.*,1987). Since the dielectric permittivity is directly proportional to the capacitance, an on-line measurement of capacitance was linearly proportional to the biomass concentration.

Cells with intact plasma membranes in a yeast slurry can be considered to act as tiny capacitors under the influence of an electric field. The non-conducting membrane allows a build up of charge. The resulting capacitance can be measured; it is dependent upon the cell type and is directly proportional to the concentration of these viable cells.

ABER INSTRUMENT LTD develops a unique 25mm probe (named "Biomass Monitor") which incorporates four electrodes and is used to apply a radio-frequency to the yeast slurry. Electronic processing of the resulting signal produces an output which is an accurate measurement of the concentration of viable cells.

The system is sensitive to viable cells and is insensitive to cells with leaky membranes, wort, gas bubbles and non yeast solids. Fehrenbach *et al.* (1992) has used this probe to follow the biomass concentration on-line, in bioreactors from 20 to 2000 L total volume. Microbial cultures of *Saccharomyces cerevisae*, *Pichia pastoris* and *Streptomyces virginiae* were grown in batch and fed-batch culture in both defined and complex media in order to demonstrate the wide dynamic operating range of the instrument.

Different authors have used this probe to improve their analytical methods (Stoicheva *et al.,* 1989, Sarra *et al*., 1996). Simple but accurate models for loss of linearity between dielectric increment and biomass content at high volume fractions (yeast and bacteria) were developed by Davey *et al*. (1992). Salter *et al*. (1990) describe the use of the "Biomass Monitor" as a convenient mean for assessing the rate of biomass accumulation on-line and in real time in immobilised cell systems. Woodley *et al*. (1992) have demonstrated the decrease in capacitance of *S. cerevisiae* following octanol addition.

In a similar approach, NTE Company specialised in the development of new technology for space, has developed another sensor (Figure 1). The sensor was originally developed as a side project of MELiSSA. In the framework of Technology and Transfer Program of ESA, this sensor is used by Freixenet Company, for improving control of fermentation vessels for the production of the sparkling white wine, Cava. Wine is made by fermenting grapes with yeast to produce alcohol and carbon dioxide. To produce good quality wine, it is important to control the growth of the yeast. The result is a better-controlled fermentation process and hence improved quality and constancy of the wine.

Sensors determining the concentration of yeast or other microorganisms in a liquid have, until now, relied on measuring the intensity of light shone through the liquid. Such methods, however, have been demonstrated to be inaccurate at high concentrations and when air bubbles or clumps of microorganisms are present. This new sensor overcomes such problems by measuring the electrical capacitance rather than optical properties of the fermenting wine to derive the concentration of yeast.

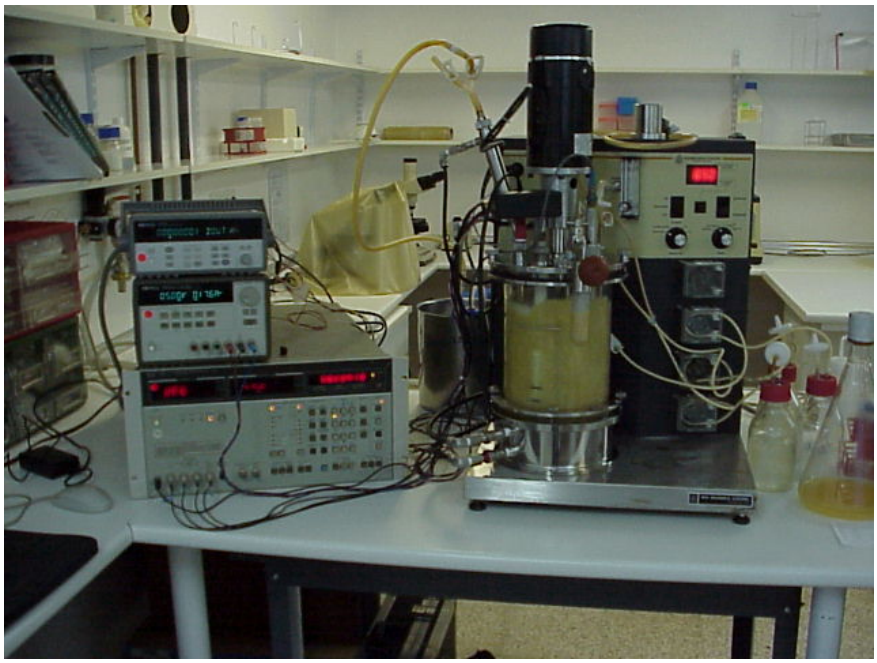

Figure 1: NTE experimental configuration of the setup

1.3. Proposed experimental approach

1.3.1. Objectives

The aim of this work is to evaluate the performances of the Biomass Scan System (NTE probe) on three MELiSSA strains: *Rhodospirillum rubrum* (compartment II) and *Arthrospira platensis* (Compartment IVa) and *Fibrobacter succinogenes* (fiber degradation). The main problem to solve concerns the sensitivity of the probe and of the amplifying system for assessing low concentrations of active micro-organisms. It is particularly important for the two PBRs, namely compartments II an IVa, because the control system requires reliable on-line estimation of biomass concentrations for the command of the light energy supply. The normal biomass concentrations are typically in the range of 1 $g.L^{-1}$ which is the low limit of validity of this measuring technique. Moreover, it is particularly important to prove that the system is operative on photosynthetic organisms.

1.3.2. MELISSA strains selected

Purple non-sulfur bacteria (*Rhodospirillaceae*) are, apart MELiSSA use, very promising tools for many industrial purposes, especially when cultivated in photoheterotrophic conditions in closed, artificially illuminated and fully controlled photobioreactors. Among a wide variety of processes, their main applications include waste-water treatment of industrial effluents (Kobayashi et al., 1979). The production of biodegradable polymers such as β-polyhydroxybutyrate (PHB) (Brandl et al., 1991), the production of single cell proteins or high-value products (Cornet et al., 1998) such as pigments, growth factors, chemical entities of therapeutic interest, and the photo-production of hydrogen (Tsygankov et al., 1994) are also potential applications of purple bacteria cultures.

The second test bacteria is *Spirulina* which has also a wide spectrum of applications, mainly for producing a variety of important industrially products or for pharmaceutical and food industries. The feasibility of mass production depends heavily on the optimal utilization of solar energy and of elements such as carbon, phosphorus, nitrogen and trace elements. Although considerable attention has been directed to understanding the inorganic nutrition of cyanobacteria with respect to phosphate, nitrate and major cations, little information is available regarding trace elements.

The last test bacteria is *Fibrobacter succinogenes*, a strictly anaerobic bacterium isolated from the rumen, which has large potentials in degradation of lignocellulosic residues and specially with highly lignified material (Bryant and Burkey, 1953). Pure cultures of Fibrobacter succinogenes digest more cellulose from intact forages than other cellulolytic rumen bacterial species (Dehority, 1993). The enzymatic machinery of *Fibrobacter succinogenes* allows to explain these specific performances. This bacterium degrades

cellulose by a very efficient complex cellulolytic system. Cellulose is depolymerized at the bacterial surface by different cellulases and the released cellodextrins are hydrolysed into glucose and cellobiose in the periplasm. It produces ferrulic acid esterase, acetylxylane esterase, and arabinofuranosidase that are necessary to cleave the ester bonds linking hemicelluloses to lignin or debranching xylans. Several different xylanases and α-glucuronidase complete the cellulolytic system (Matheron et al, 1998).

2. Materials

2.1. VIAMASS sensor (NTE probe)

The VIAMASS Sensor is a device that, using Electrical Impedance Spectroscopy techniques is able to measure the viable biomass suspended in a medium.
The VIAMASS Sensor has been adapted for use in the MELiSSA's Compartment 1 (CI) bioreactor, according to the agreements stemming from R2 and R3. The VIAMASS system conceptual scheme is shown in Figure2.

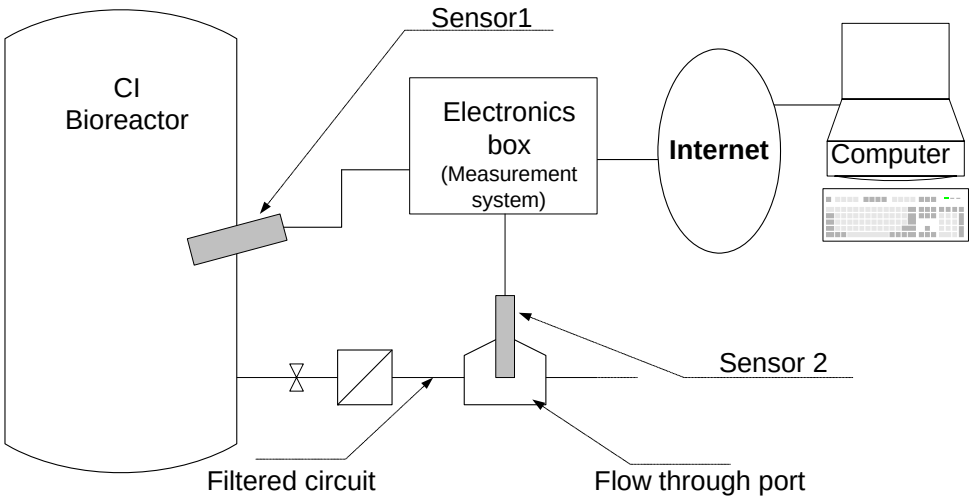

Figure 2: VIAMASS System scheme.

Sensor 1 contains the electrodes and the front-end electronics. The sensor has been encapsulated so that it is compatible with an INTRACK 777 retractable housing by METTER TOLEDO. Sensor 1 is in direct contact with the process of the bioreactor. Sensor 1 is associated to the measurement Channel 1. From electrical point of view, Sensor 2 is identical to the Sensor 1. Sensor 2 has been mechanically adapted so that is

can be placed in the filtration loop, external to the CI bioreactor, and used for calibration purposes. Sensor 2 is associated to the measurement Channel 2. The electronics box contains the electronic circuitry in charge of generating the measuring signal at the defined frequencies, driving the front-ends, and reading and temporary storing the measurement data. These electronics are commanded by a microprocessor that also handles the communication between the sensors and the box electronics and the communication with the PC, either directly or through Internet. This box also includes the power conditioning electronics and the connector interfaces for the sensors and the PC/Internet.

There are two SW modules associated with the sensor, namely: VIAMASS_SW_10.exe and TRANSLATOR2T.exe.

The VIAMASS_SW_10.exe SW runs on the PC and it is the actual VIAMASS SW manipulated by the user. It controls the system and manages the communication between the sensor and the PC through Ethernet. This program issues commands to measure the medium impedance at the commanded frequency and channel and receives data packages with the measurement values obtained at these commanded frequencies and channels. One measurement consists in the injection of 32 signals at different frequencies in the range from 1kHz to 10MHz, organized in 16 consecutive pairs of signals, each pair containing one low (LF) and one high frequency (HF). The values of the frequencies are fixed. For each injected signal the system returns the measured impedance of the medium plus three temperature readings, obtained from three different temperature sensors located in the medium zone, the electronics box volume and the electronics in the electronics box. These measurement values are stored in a text file created by the SW. The program allows the selection of the measurement channel and of a time delay between the issue of the measurement command and the actual measure. Measurement data are stored in a text file created by the user at the beginning of the session. A measurement sweep is completed once 16 pairs of signals at different frequencies are issued. The SW allows selecting the time delay between measurements. This feature permits to accommodate the measurement rate to the kinetics of the biological process under measurement.

This SW is presented to the user on a window with two selectable tabs. The Control tab permits the actual configuration of the SW for the intended measurement. The Test tab displays information related to the measurement status. This tab is mainly oriented to troubleshooting purposes.

The control page is shown in Figure 3.

Figure 3: Appearance of the Control tab of the VIAMASS2T3.exe program.

The displayed items are as follows:
Port: Selects the port for the communication with the sensor. It is 1100 by default. It must be written before the start of the program, but it is susceptible to be changed.
Address: It is the IP address of the sensor. It must be written before starting the measurement.
Channel: Allows the user to select Channel 1 (process measurement) or Channel 2 (calibration).
Start: Click on this button to start the measurements.
Stop: Click on this button to stop the measurements.
Measure Delay: It allows selecting the time delay between measurements (batch of 16 frequency pairs) from 0 (continue) to 60 minutes in steps of 5 minutes. The option selected by default is continue.
Request Delay: It allows selecting the time delay (in seconds) for the execution of the adaptive routines before requesting the measurement to the sensor. The delay options are 5, 10, 15 and 20 seconds. The option selected by default is 5 s.

The Test page is shown in figure 4.

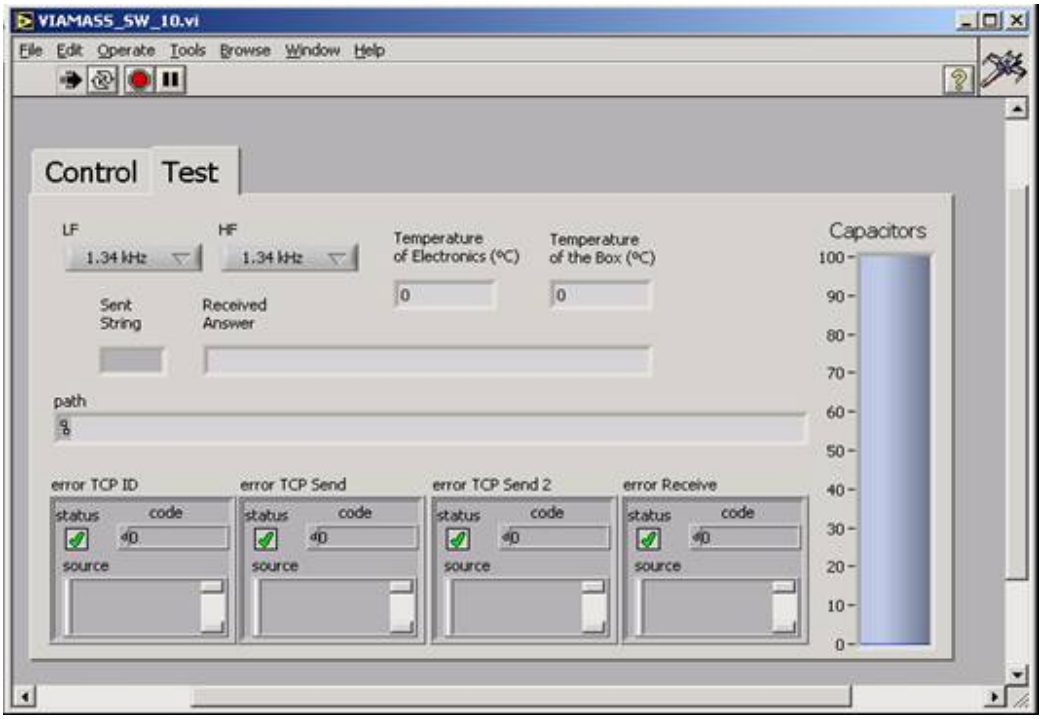

Figure 4: Appearance of the Test tab of the VIAMASS2T3.exe program.

The displayed items are as follows:
LF: It displays the lower frequency of the injected signal pair
HF: It displays the higher frequency of the injected signal pair.
Temperature of the Box: It displays the temperature (in ºC) inside the box of the system.
Temperature of Electronics: It displays the temperature (in ºC) of the electronic boards inside the box of the system.
Sent String: It is the sequence sent to the sensor. It contains the selected frequencies, the selected channel and other control bits.
Received Answer: It is the received data frame. It contains the frequencies and the impedance and temperature data in hexadecimal format.
Path: It displays the path of the text file where the measurements data are stored.
Errors: They display the TCP errors during the running (valuable information for troubleshooting)
Capacitors level: It displays the charge level of the capacitors.

The TRANSLATOR2T.exe is an auxiliary SW also resident in the PC. This tool reads the measured data stored in the text file, and after some sorting and re-formatting stores them in another text file with a convenient format. With this SW, the user can process, analyze and plot the measurement data easily.

2.2. Biomass sensor adapted for test at UBP

The biomass sensor adapted for test at UBP is represented in figure 5. It is a simplified version of the VIAMASS system described previously. It consists in:

- Electronic box with identical components and functionality as for the VIAMASS case. This box also housed the front end electronics.
- Electrodes: there are four wire electrodes located at the bottom of a prismatic PVC container for the dilutions.
- Cabling: power cable and crossover Ethernet cable.

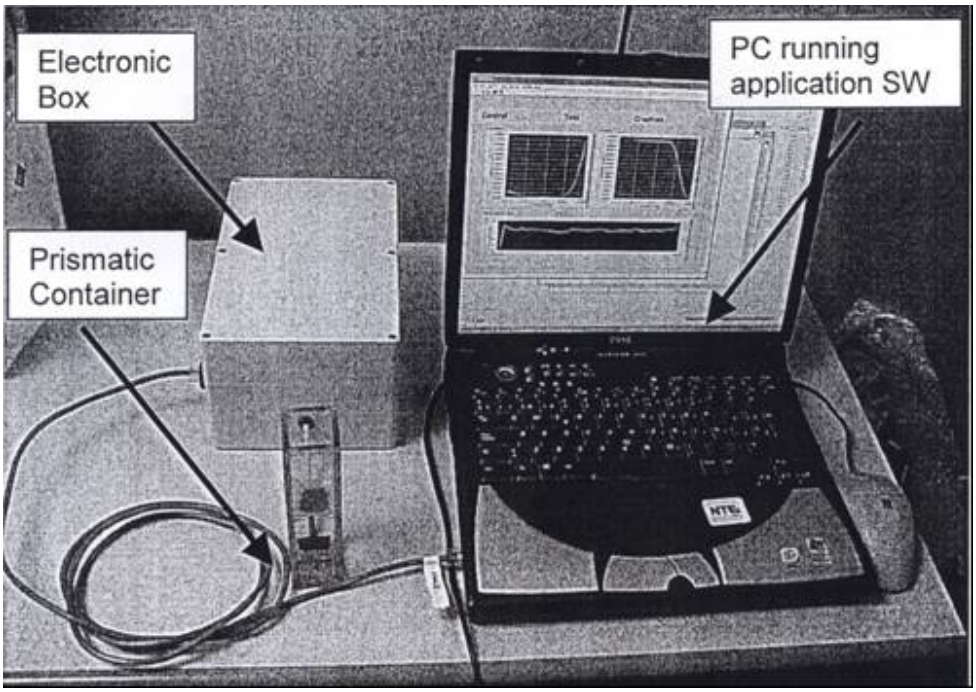

Figure 5: Connection of the BIOMASS sensor

The installation of the sensor is straight forward and does not require special tools.
The electrodes in the prismatic container are connected to the sensor through a ca. 10cm, 4 wires cable that goes with the electronic box. This cable must be connected to the connector at the bottom of the prismatic container (Figure 6).

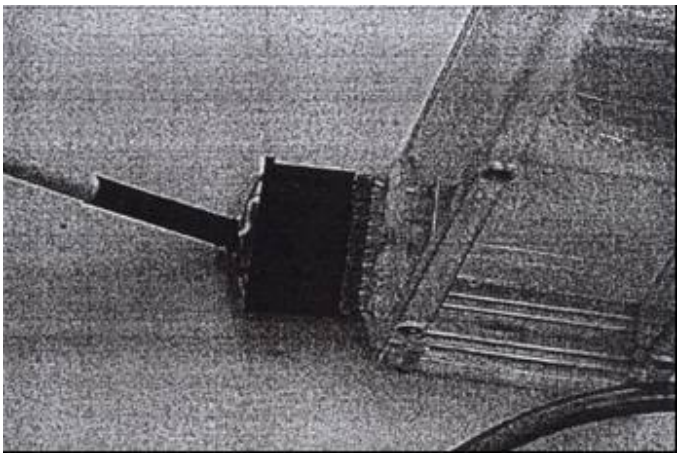

Figure 6: Connector of the prism

The Ethernet cable must be connected on one end to the Ethernet connector located in the lateral side of the electronic box and to the PC's Ethernet port on the other end (Figure 7).

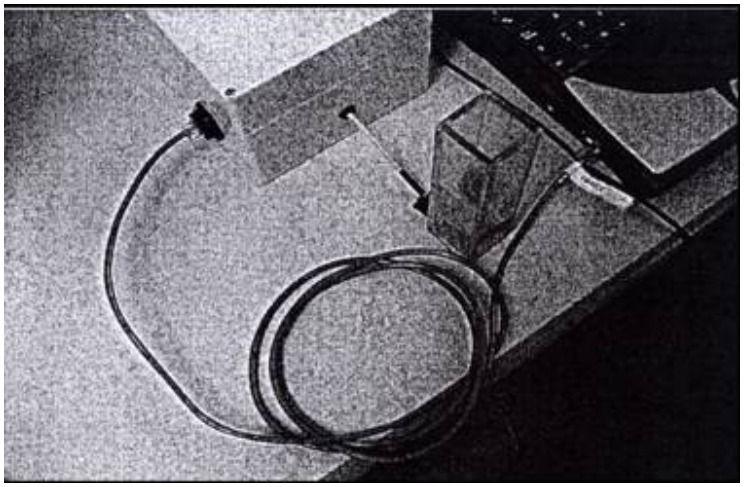

Figure 7: Connection of the Ethernet cable

The electronic box is connected to the mains through the power cable and power connector. The sensor is turned on through the switch next to the connector.

The biomass sensor system is completed with a specific SW application that can run on a PC platform. This application module is named UBP32.exe. The UBP32 SW controls the system, manages the communication between the sensor and the PC through Ethernet and provides the user interface. This program is adapted from VIAMASS3T2 program, so the measurements are the same and it allows the same things as previously like the measurement channel, the time delay between the issue of the measurement command and the actual measure…. But The TRANSLATOR2T program is not necessary with the UBP32 program.

This SW is presented to the user on a window with three selectable tabs. The Control tab permits the actual configuration of the SW for the intended measurement. The Test tab displays information related to the measurement status. This tab is mainly oriented to troubleshooting purposes. The graphic tab shows the obtained modulus, phase and temperature graphics of each sweep of measurement.

The control tab and the test tab are the same as described previously for VIAMASS2T3.exe program (figure 8 and 9).

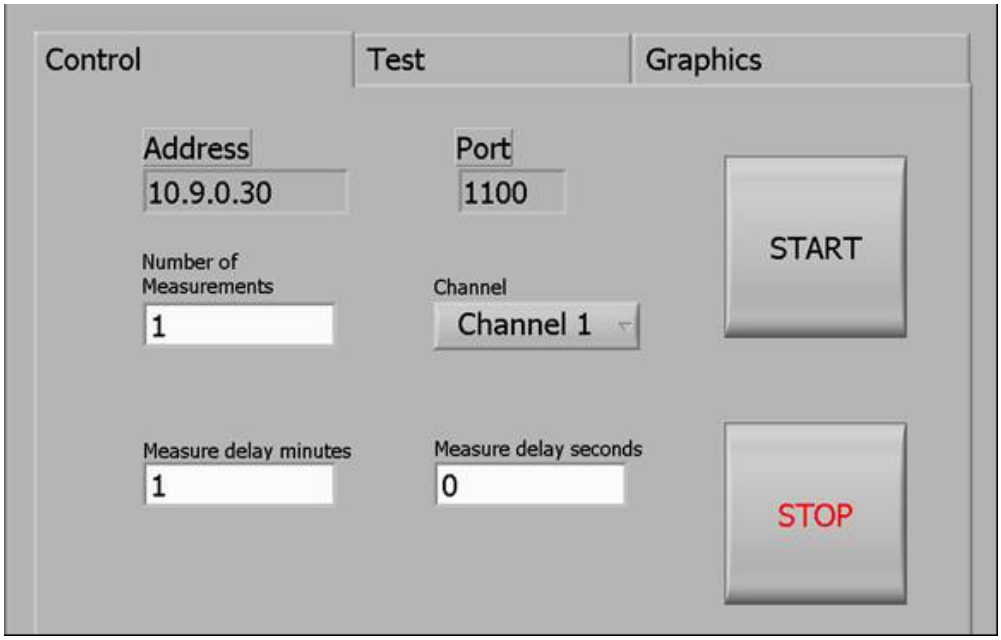

Figure 8: Appearance of the Control tab of the UBP2.exe program.

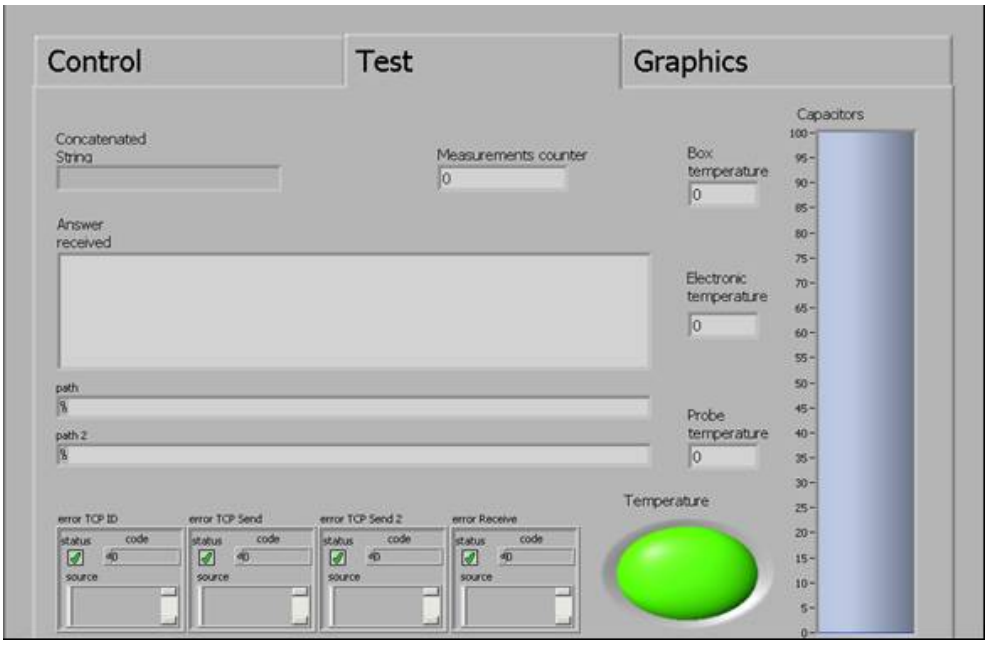

Figure 9: Appearance of the Test tab of the UBP2.exe program.

The graphic tab is shown in figure 10. The displayed items are as follows:

- Modulus: it displays the modulus of the impedance upon completion of a frequency sweep. The vertical axis displays counts (i.e. digital values). The horizontal axis displays a logarithmic frequency range. The display is refreshed at every frequency sweep.
- Phase: it displays the phase of the impedance upon completion of a frequency sweep. The vertical axis displays counts (i.e. digital values). The horizontal axis displays a logarithmic frequency range. The display is refreshed at every frequency sweep.
- Probe temperature: it displays the evolution of the front end electronics through the course of the measurement time. The vertical axis displays °C and the horizontal axis displays the measurement time from the start.

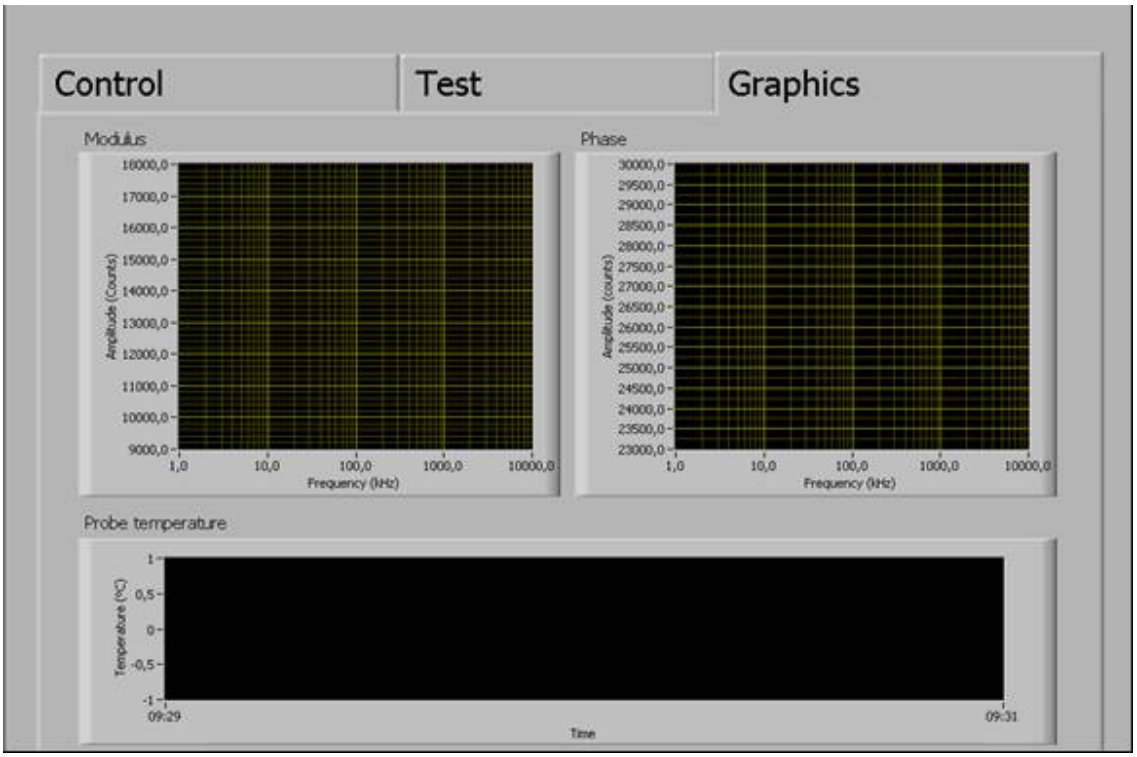

Figure 10: Appearance of the graphic tab of the UBP2.exe program.

Once the program is active, the port, the channel, an IP address, and the delays parameters in the control tab must be entered. After that, the START button is pushed to command the measurement process. Two windows like that shown in figure 11 will pop up consecutively. The first window asks the user to enter a filename for the text file where the measured data are stored in native format. The second window asks the file name that stores the data in a pre-processed format that allows to perform some graphical analysis using programs like MSExel.

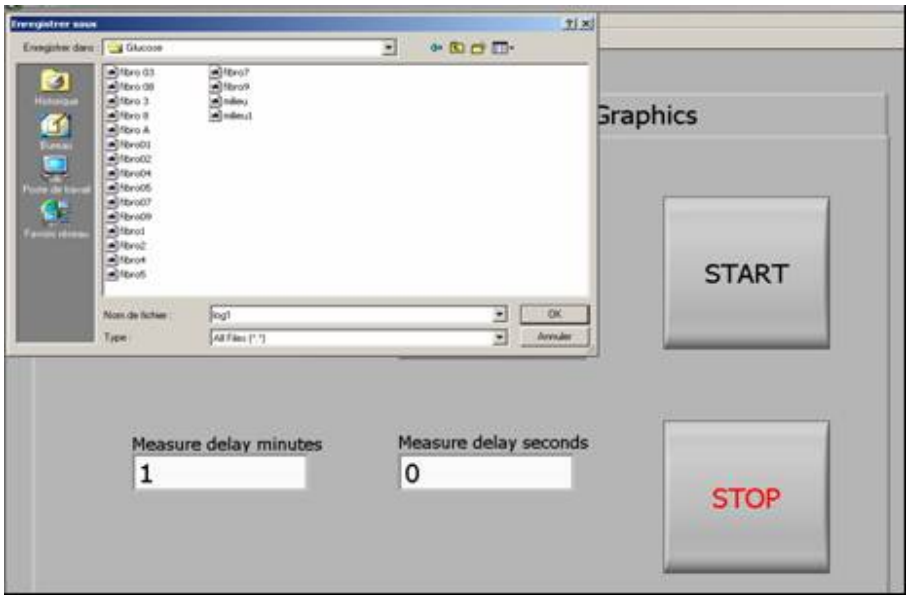

Figure 11: Window to choose the file to translate.

After defining the filenames the measurement process starts and the frequency sweeps continue to take place at the selected rate until the STOP button is pushed. At this point all data are stored in the previously selected text files. Data are organized in a matrix structure. Rows provide time information and columns provide frequency-related measurements. The following table (figure 12) shows partially (only 7 columns) the data recording format.

log2.txt 20/06/2006
Channel 1

Time	Module 1.34kHz	1.78kHz	2.37kHz	3.16kHz	4.22kHz	5.62kHz	7.5kHz
09:33:37		28123	28122	28120	28095	28067	28057
09:34:01		28109	28089	28104	28127	28150	28155
09:35:01		28125	28141	28151	28157	28164	28153
09:36:01		28047	28085	28110	28117	28132	28126
09:37:01		28093	28116	28129	28145	28149	28146
09:38:01		28050	28080	28093	28105	28116	28121
09:39:01		28047	28070	28086	28096	28101	28107
09:40:01		28062	28089	28099	28108	28116	28121
09:41:01		28057	28080	28095	28104	28115	28123
09:42:01		28062	28085	28098	28110	28118	28121
09:43:01		28079	28107	28115	28129	28140	28143
09:44:01		28058	28076	28094	28104	28116	28123
09:45:01		28106	28117	28118	28126	28157	28171
09:46:01		28070	28093	28078	28061	28064	28071
09:47:01		28038	28057	28070	28083	28096	28105

Figure 12: Data recording format

2.3. METTLER TOLEDO sensor

The dual optical fiber sensor InPro8200 (figure 13) combines high resolution at low turbidity ranges with high linearity in the mid turbidity range. Backscattered light technology and the use of fiber optic cables enable a sensor design with uniform, unbroken surface structure. Therefore InPro8200 sensors are able to meet the toughest demands placed on optical sensors with respect to freedom of fouling and easy cleanability. A system is completed with a Trb8300 transmitter.

The LGCB uses a prototype sensor wavelength-modified by Mettler considering the specific optical properties of *R. rubrum*. One optical fiber give a 950 nm signal and the other measures light quantity which is backscattered by the turbid medium 500 times per second. The commercial probe operates at 880 nm which is the ISO normalized wavelength for turbidity measurements.

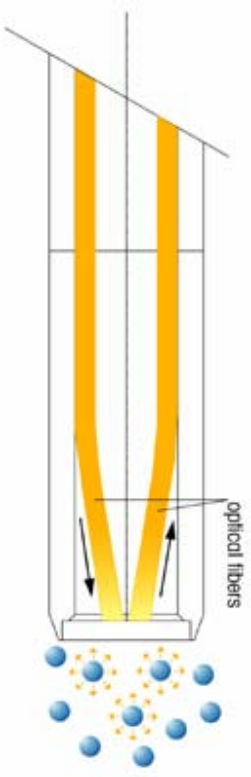

Figure 13 : Scheme of the Mettler Toledo probe principle

3. Protocol measurement

3.1. Global approach

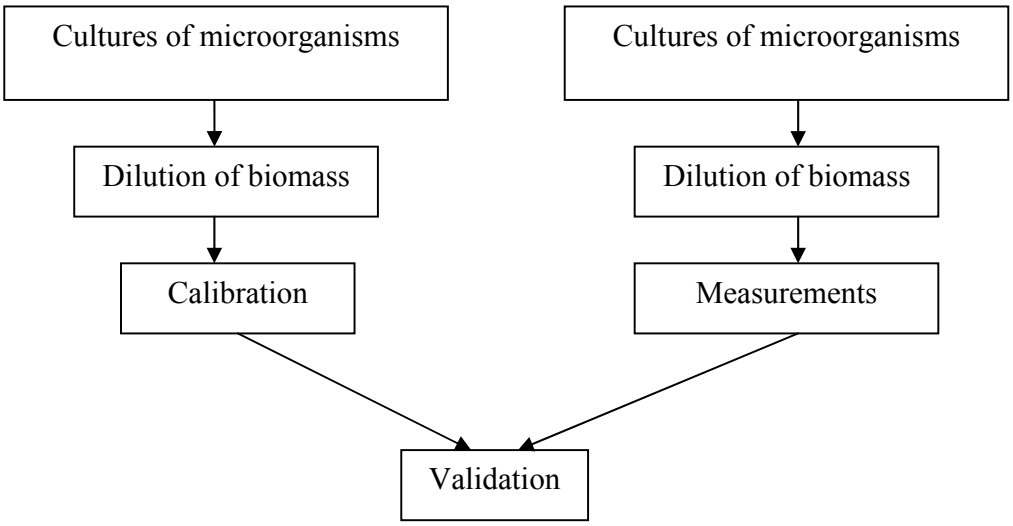

Figure 14 : Experimental protocol

3.1.1. Calibration

Calibration was performed using Erlen cultures of fresh microorganisms as concentrated solutions. Sets of diluted solutions were prepared.
The aim of calibration is to obtain three correlation curves:

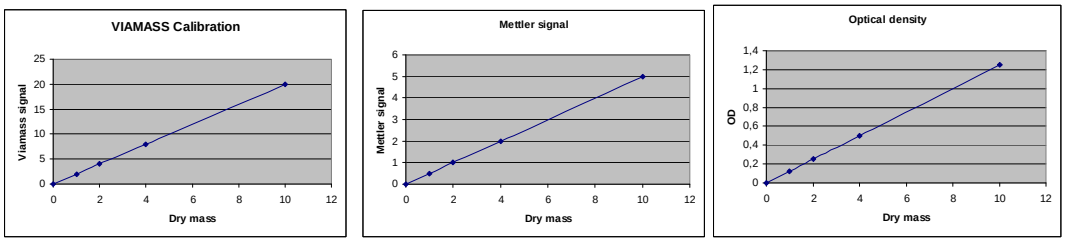

Figure 15 : Theoritical calibration curves

3.1.2. Measurements

From unknown diluted sample, we determine three values: $DM_{viamass}$, $DM_{mettler}$ and $DM_{spectro}$ using correlation curves obtained by the calibration step.

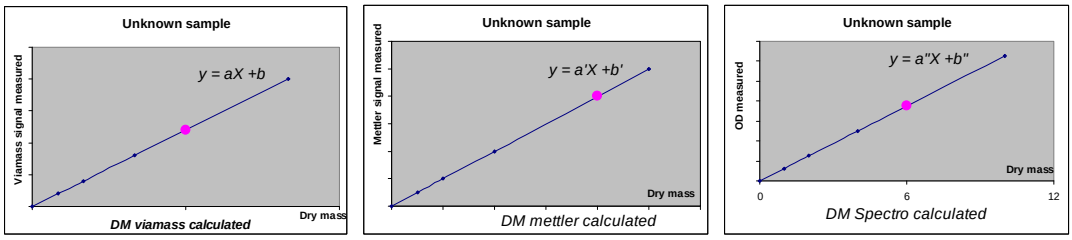

Figure 16: Definition of DM measurements

3.1.3. Validation

Validation is obtained by comparison of the three values of DM, the classical OD vs DM correlation being considered as a reference for the discussion of the results.

3.2. VIAMASS protocol

The VIAMASS probe has been designed to perform continuous electrical impedance spectrum measurement on dilution samples with the purpose to perform a qualitative validation of this technology in detecting viable biomass and its variation over time.
This technology requires that the sensor is connected to the prismatic container with the medium at least for 20 to 30 minutes. Once the system is trained, actual measurements can start. If the probe could not be left for 20 to 30 minutes in the cell suspension prior to any measurement due to the characteristics of the target solution, the best procedure is to use firstly a saline solution with the same conductivity as the target solution and left the system train by performing several initial sweeps. Then, the acquisition must be stopped and the saline solution must be substituted by the target cell suspension.

In order to obtain meaningful results the system must be thermally stable. This implies time schedule is important when the sequence of measurement is planned. A measurement timeline example is illustrated in figure 17.

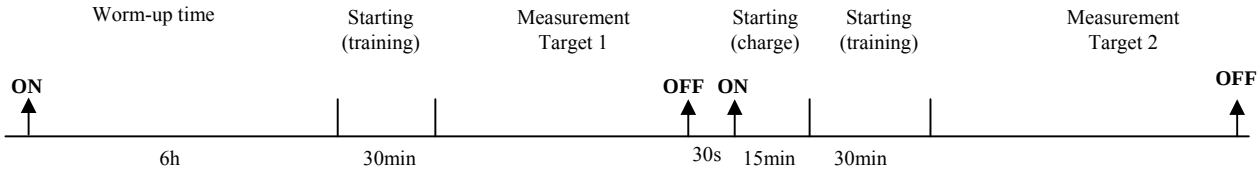

Figure 17: Measurement timeline example

After a warm-time of at least six hours for the probe, modulus spectra of solutions are measured during at least 20min, frequency range being swept every minute.

An average measurement is calculated from 10 last sweeps for each solution. To prevent calibration from being sensitive to temperature and some other physical parameters, concentration will be correlated with the difference of two impedance modulus: one for a low-frequency and another one for a high-frequency. The resulting estimator is obtained in the arbitrary system counts scale

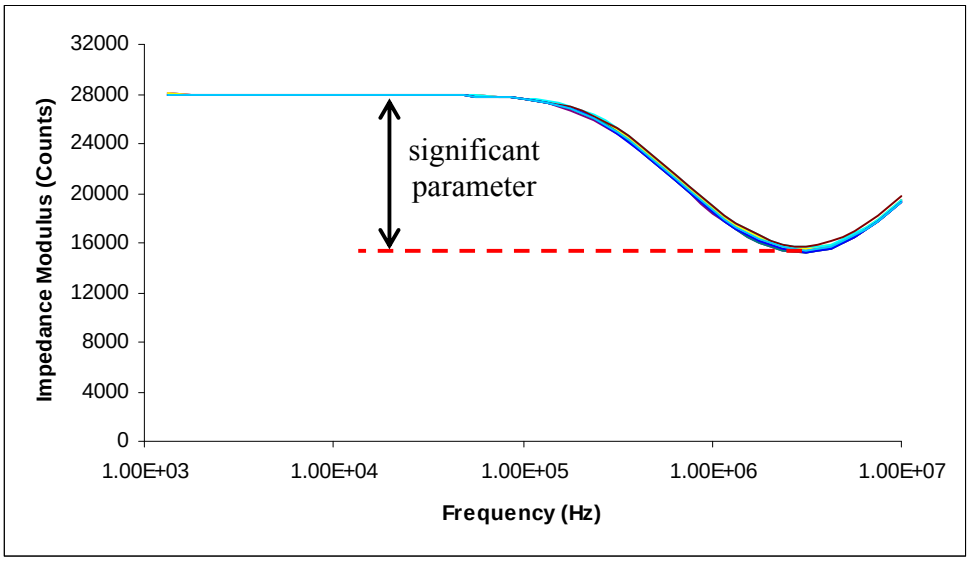

Figure 18 : Theoritical curve of viamass result

To be sure choosing the most significant difference between low and high frequencies, every possibilities were calculated and statistical error calculations were applied (table 1). Cell density estimation can be obtained through the difference between the impedance magnitude at high frequency (HF) and low frequency (LF) value. A calibration or a regression can be used to obtain the correspondence with the cell counting or dry weight units.

	Model	Parameters	Standard deviation	Absolute error
Average	$y = a$	$\bar{y} = \frac{\sum_i y_i}{n} = a$	$\sigma(a) = \frac{\sigma_{y,n-1}}{\sqrt{n}}$	$\Delta a = \tau_{(n-1)}\sigma(a)$
Linear correlation (1 parameter)	$y = ax$	$a = \frac{\sum_i x_i y_i}{\sum_i x_i^2}$	$\sigma(a) = \sqrt{\frac{\sigma_y^2}{\sum_i x_i^2}}$	$\Delta a = \tau_{(n-1)}\sigma(a)$
Linear correlation (2 parameters)	$y = ax + b$	$a = \frac{\sum_i y_i (x_i - \bar{x})}{\sum_i (x_i - \bar{x})^2}$ $b = \frac{\sum_i y_i}{n} - a\bar{x}$	$\sigma(a) = \sqrt{\frac{\sigma_y^2}{\sum_i (x_i - \bar{x})^2}}$ $\sigma(b) = \sqrt{\sigma_y^2 \left(\frac{1}{n} + \frac{\bar{x}^2}{\sum_i (x_i - \bar{x})^2} \right)}$	$\Delta a = \tau_{(n-2)}\sigma(a)$ $\Delta b = \tau_{(n-2)}\sigma(b)$

Table 1 **:** Statistical error formula for average and linear correlation.

4. Measurements

4.1. *Fibrobacter succinogenes*

4.1.1.Experimental set-up

Two tests were performed with *Fibrobacter succinogenes* cultures grown on glucose. *Fibrobacter succinogenes* has been grown anaerobically under 100% CO_2 in a basal medium that contained (per liter) : 450 mg KH_2PO_4, 450 mg K_2HPO_4, 900 mg NaCl, 1.8 g $(NH_4)_2SO_4$, 90 mg $MgSO_4$, 90 mg $CaCl_2$, 3 mg $MnSO_4$,6 H_2O, 0.3 mg $CoCl_2$,6 H_2O, 8 mg $FeSO_4$,7H_2O, 0.25 mg biotin, 0.005 mg para-aminobenzoic acid (PABA), 0.01 mg hemin, 8 g carbon substrate and a mixture of volatile fatty acids (Gaudet et al., 1992).
400 mL of this media was introduced in erlen and sterilized (20', 121°C). Cystein (0.5 g/l) and Na_2CO_3 (4 g/l) were introduced in the hot medium to decrease redox potential (–350 mV) and increase pH (6.0-6.5). Then, as *Fibrobacter succinogenes* is a strictly anaerobic organism, erlen were flushed with CO_2 during 3 hours in order to completely fill in the atmosphere with this gas. After these preparation steps, the erlen were inoculated with an overnight preculture (10 mL) grown on cellobiose (8g/l) and then were incubated 48h at 39°C.

After the worm-up during one night with *F.s.* medium and the training during 30 min. in cell suspension, measurements were performed with several dilutions from the cultures in erlen. In the same time the optical density of dilutions was measured in order to establish a correlation with the impedance obtained with the BIOMASS sensor.
We also realized tests with cell suspension in the basal medium containing 10g/L of wheat straw.

4.1.2.Results

4.1.2.1. *Medium without wheat straw*

The system stores all the measurement data in the log files created by the user. The successive sweeps can be averaged frequency per frequency.

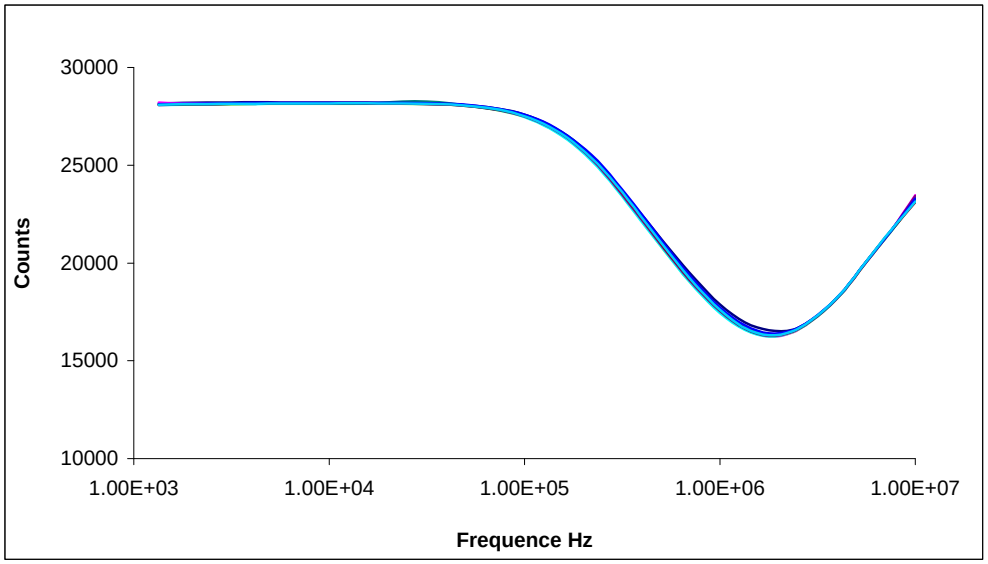

Figure 19: Example of graph obtained frequency per frequency for the first dilution tested

A graph identical to that presented in figure 13 is obtained for each dilution tested. Moreover counts for each frequency according to time can be represented for each dilution (figure 20).

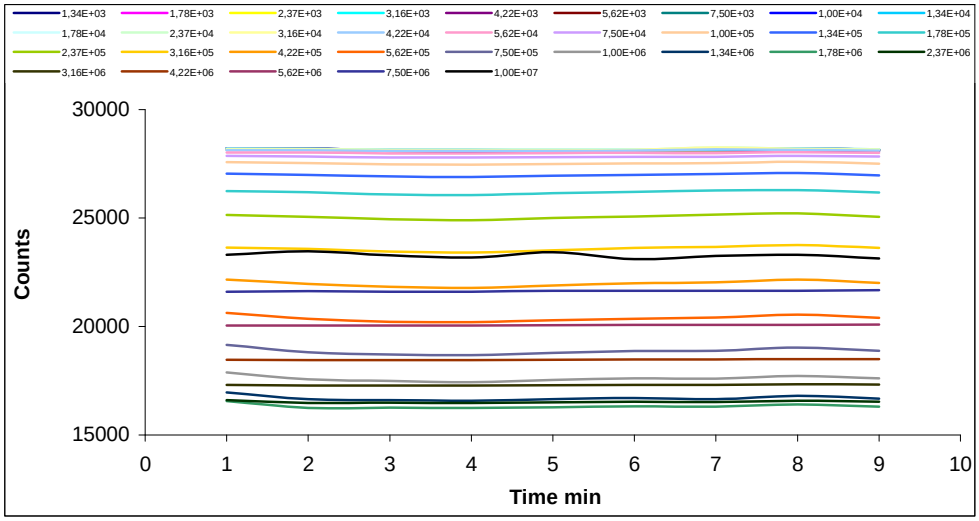

Figure 20: Example of graph obtained for each frequency according to time for the first dilution tested

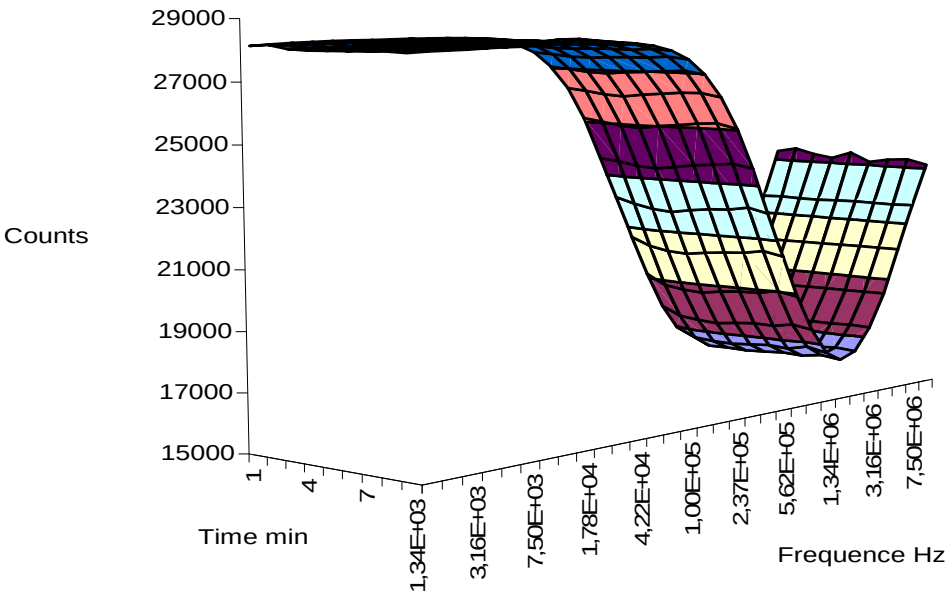

Figure 21 : 3D Combination from figures 20 and 21

Cell density estimation can be obtained through the difference between the impedance magnitude at high frequency (HF) and low frequency (LF) value (Delta counts). The resulting estimator is obtained in the arbitrary system counts scale. A calibration or a regression can be used to obtain the correspondence with the cell counting or dry weight units. Nevertheless, the best Delta counts estimator must be defined from the capacities measurements. Because we are seeking the best fit in dry mass and not the best frequency response, we decided, at the opposite of the NTE recommendation, to optimise the signal by testing all the couples of frequency possibilities in term of DM correlation. Thus, for each dilution tested and for each couple high frequency and low frequency, the difference LF-HF (Delta counts) was calculated and linear regressions with statistical treatment were performed. Among all the correlations (between 10 and 12 curves) the best result obtained in term of r^2 regression coefficient was retained in this study. Considering *F. succinogenes*, the resulting calibration curve is given in figure 22.

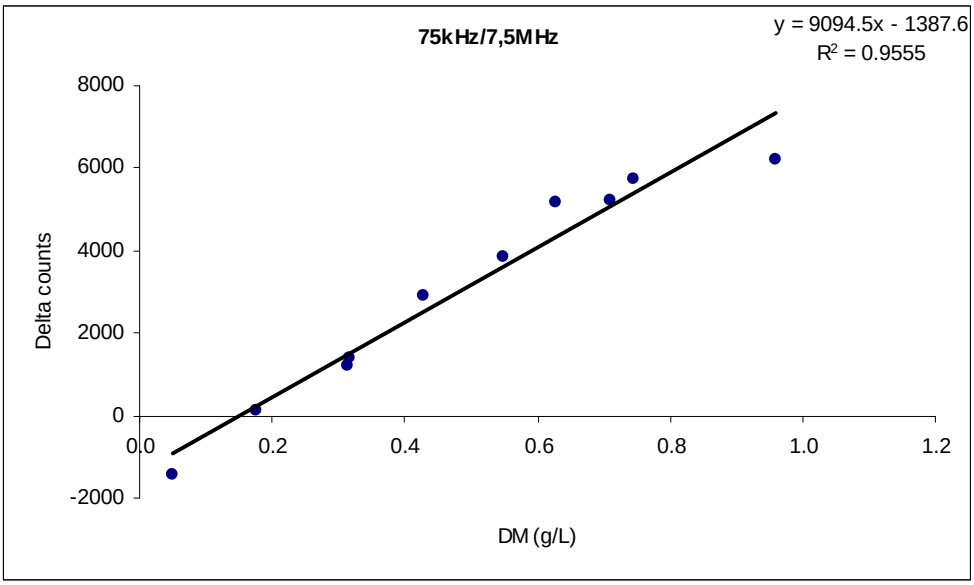

Figure 22: Correlation between concentration and delta counts for *F. succinogenes*

A statistical treatment of these results reveals the following standard errors for the slope and the offset:

$$\Delta\text{Count} = (9100 \pm 1600)\ DM - (1400 \pm 900)$$

The accuracy of the method may be increased by adding the offset for all the experimental points, giving the final relation:

$$\Delta\text{Count} + 1400 = (9100 \pm 700)\ DM$$

By this way, the expected accuracy is roughly $\pm 8\%$ for *F. succinogenes* calibration.

To check this correlation, other dilutions were performed, the impedance was measured with the probe, the same difference between LF and HF was performed and thanks to the correlation obtained previously the DM was calculated. In the same time the OD_{600} was measured and the DM obtained by the OD ($DM=0.37*OD_{600}$) and by the correlation were compared (Table 2).

Sample	$DM_{spectro}$ g/L	$DM_{viamass}$ g/L	Relative difference
1	0.958	0.828	13%
2	0.745	0.776	4%
3	0.709	0.737	4%
4	0.579	0.583	1%

Table 2: Comparison between **$DM_{spectro}$** and **$DM_{viamass}$** find with the correlation between **75kHz** and **7.5MHZ**

These results show that the $DM_{viamass}$ found with the sensor is very closed to the $DM_{spectro}$. As foreseen, the calculated mean relative error (6%) is within the range of the 8% obtained by the previous analysis.

The concentration of these same samples was measured with a probe of turbidity (Mettler) calibrated before for *Fibrobacter* and compared with results obtained with the spectrophotometer (Table 3).

Sample	$DM_{spectro}$ g/L	$DM_{mettler}$ g/L	Relative difference
1	0.958	1.02	6%
2	0.745	0.83	10%
3	0.709	0.67	5%
4	0.579	0.72	19%

Table 3: Comparison between **$DM_{spectro}$** and **$DM_{mettler}$** find with the Mettler probe of turbidity

Surprisingly, if this probe gave good results for concentrations higher than 0.7 g/L, we obtained a loss of accuracy for samples with concentrations lower than 0.5 g/L. This confirms the problems of calibration encountered for dilute turbid samples with *Fibrobacter*.

4.1.2.2. *Medium with wheat straw*

Samples of known concentration (50mL) mixed with 0.5g of straw were placed in the prism to measure the impedance. The same difference was performed and from the correlation previously established, *Fibrobacter succinogenes* concentration was estimated. Before measurement with cell suspension, a preliminary *Fibrobacter succinogenes* medium without cells was mixed with wheat straw and was placed in the prism in order to evaluate the background noise caused by the wheat straw. Indeed the measurement can be calibrated to the reference value (zero cells or minimum concentration suspension measurement) by subtracting the spectrum from the other measurement frequency per frequency. Table 4 gives the results obtained.

Sample	**$DM_{spectro}$** **g/L**	**$DM_{viamass}$** **g/L**	**$DM_{viamass}$ – background noise** **g/L**
1	0.970	2.204	1.358
2	0.783	2.152	1.306
3	0.694	1.697	0.851
4	0.688	1.950	1.104
5	0.602	1.539	0.693
6	0.430	1.292	0.446

Table 4: Comparison between **$DM_{spectro}$** and **$DM_{viamass}$** find with the correlation between **75kHz** and **7.5MHz**

The results obtained on the wheat straw with the BIOMASS sensor are different than those obtained from the OD. Indeed as the probes are at the bottom of the prism they are covered by the wheat straw and cannot measure the impedance because the wheat straw distorts the results. Contrary to the VIAMASS Sensor adapted for the MELiSSA's Compartment 1 which is in an agitated bioreactor, the BIOMASS sensor did not have a system of agitation, so it was impossible to evaluate the biomass among the wheat straw.

4.2. *Arthrospira platensis*

4.2.1. Experimental set up

Two tests were performed with *A.p.* cultures. *Arthrospira platensis* has been grown in a basal medium (Zarouk) that contained (per liter): 1g NaCl, 0.03g $CaCl_2$, 1g K_2SO_4, 0.2g $MgSO_4$, 7 H_2O, 0.5g K_2HPO_4, 2.5g $NaNO_3$, 16.8g $NaHCO_3$, 0.08g EDTA and 0.01g $FeSO_4$,$7H_2O$. To this mixture 1mL of a mineral solution containing (per liter) 0.23g $MnCl_2$, $4H_2O$, 0.11g $ZnSO_4$, 5 H_2O and 0.03g $CuSO_4$, 5 H_2O was added. 400 mL of this

media was introduced in erlen and sterilized (20', 121°C). Then, the erlen were inoculated with 20 mL of a previous culture and then were incubated one or two weeks at 37°C.
First, the medium without cells was placed under the prismatic container to do the worm-up and a cell suspension was placed to do the starting phase (30 min) and the measurement phase (30 min). Then several concentrations were tested (30min + 30min), the optical density of these solution were measured before the test.

4.2.2. Results

The system stores all the measurement data in the log files created by the user. The successive sweeps can be averaged frequency per frequency.

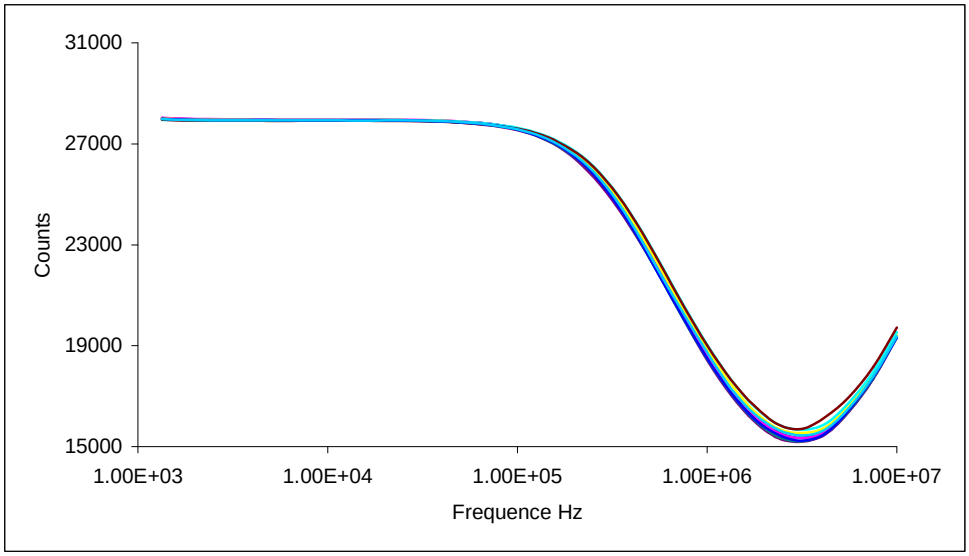

Figure 23: Example of graph obtained frequency per frequency for the first dilution tested

As for *Fibrobacter succinogenes*, a graph identical to that presented in figure 16 can be obtained for each dilution tested and counts for each frequency according to time can be represented for each dilution (figure 24).

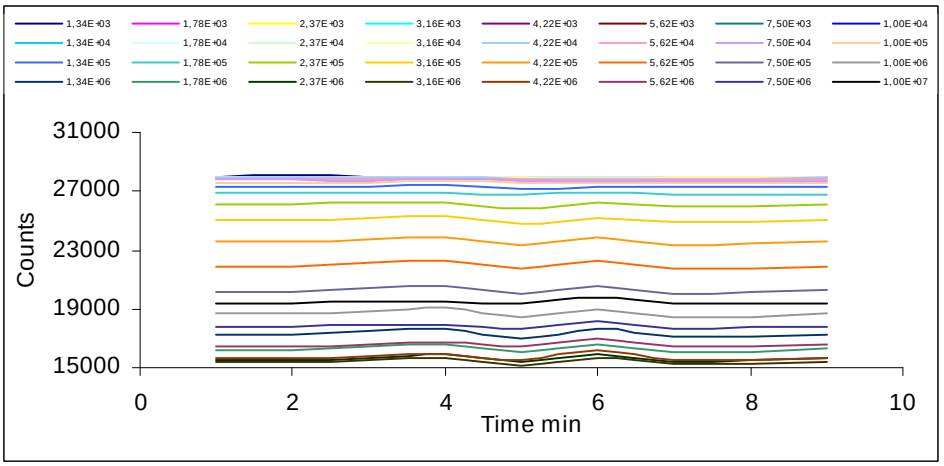

Figure 24: Example of graph obtained for each frequency according to time for the first dilution tested

Cell density estimation can be obtained through the difference between the impedance magnitude at high frequency (HF) and low frequency (LF) value. So for each dilution tested and for each high frequency and low frequency the difference LF-HF (Delta counts) was performed and graphs representing this difference according to the concentration were plotted and correlations statistically analysed.
A statistical treatment of the best retained results reveals the following standard errors for the slope and the offset:

$$\Delta\text{Count} = (15600 \pm 5100)\ DM - (1700 \pm 3000)$$

demonstrating that the offset is not significantly different of zero. The accuracy of the correlation is therefore increased by taking a one parameter linear relationship giving:

$$\Delta\text{Count} = (13500 \pm 3600)\ DM$$

The resulting accuracy is then 27% for *A. platensis*, and the corresponding experimental results are illustrated in figure 25. These results show that NTE probe is not as accurate with *A.Platensis* as with *F.succinogenes*, even if the frequency response amplitude is higher.

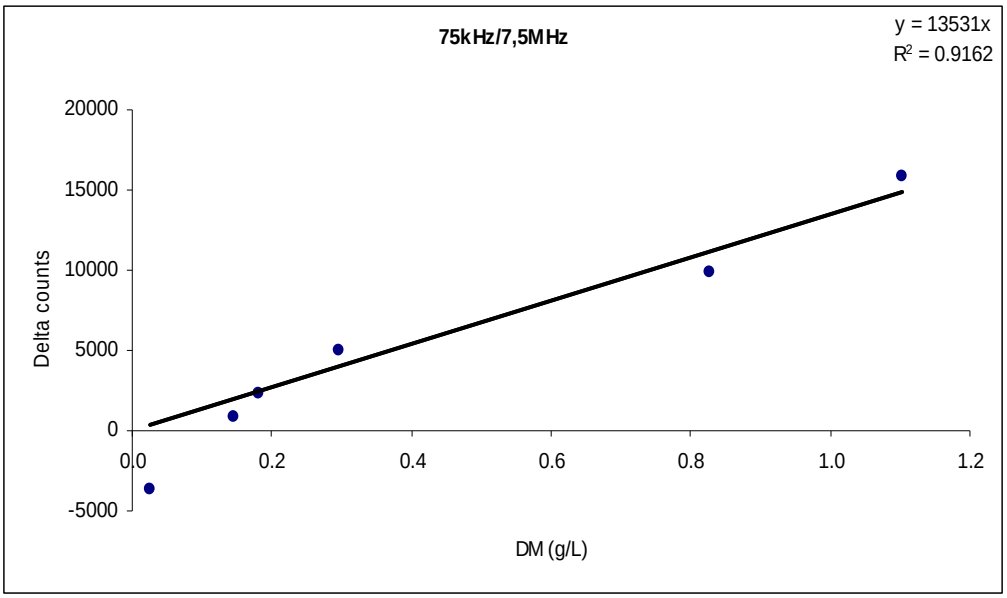

Figure 25: Correlation between concentration and delta counts for *A. platensis*

To check this correlation, other dilutions were performed, the impedance was measured with the probe, the same difference between LF and HF was performed (Delta counts). In the same time the OD_{750} was measured and the DM obtained by the OD (DM = 1,3958xDO_{750}) and by the correlation were compared (Table 5).

Sample	$DM_{spectro}$ g/L	$DM_{viamass}$ g/L	Relative difference
1	1.103	1,174	6%
2	0.147	1,08	635%
3	0.181	0,727	300%
4	0.296	0,371	25%

Table 5: Comparison between $\mathbf{DM_{spectro}}$ and $\mathbf{DM_{viamass}}$ find with the correlation between **75kHz** and **7.5MHz**

Surprisingly, the results of the test is not correct, except for the higher concentration, and the Viamass probe doesn't give as good results as expected with *A. platensis*. A possible statistical explanation could be that the postulated linear relationship was not correct, implying to reconsider the theoretical analysis of the measurements at low concentration.

The concentration of these same samples was measured with the Mettler probe of turbidity calibrated before for *Arthrospira platensis* and compared with results obtained from the OD_{750} (Table 6).

Sample	$DM_{spectro}$ g/L	$DM_{mettler}$ g/L	Relative difference
1	1.103	1,072	3%
2	0.296	0,307	4%
3	0.147	0,141	4%
4	0.181	0,177	2%

Table 6: Comparison between $\mathbf{DM_{spectro}}$ and $\mathbf{DM_{mettler}}$ find with the Mettler probe of turbidity

As attempted, these results show that the $\mathbf{DM_{mettler}}$ found with the sensor is very closed to the $\mathbf{DM_{spectro}}$ measured in this range of concentration.

4.3. *Rhodospirillium rubrum*

4.3.1. Experimental set up

Rhodospirillum rubrum ATCC 25903 has been cultivated in the basal salt medium of Segers & Verstraete (1983) as described by Suhaimi *et al.* (1994) acetate and ammonium chloride as C and N sources, and biotin as the only vitamin. Acetate and NH_4^+ has been adjusted to avoid C and N limitation in the PBR, keeping a C/N ratio of 3. A phosphate buffer will be used (0.49 g L^{-1} KH_2PO_4 and 0.52 g L^{-1} K_2HPO_4) and pH adjusted to 6.9.

4.3.2. Results

The best calibration curve is obtained by substracting 75kHz frequency to 1.78MHz frequency with a higher correlation coefficient $r^2 = 0.64$.

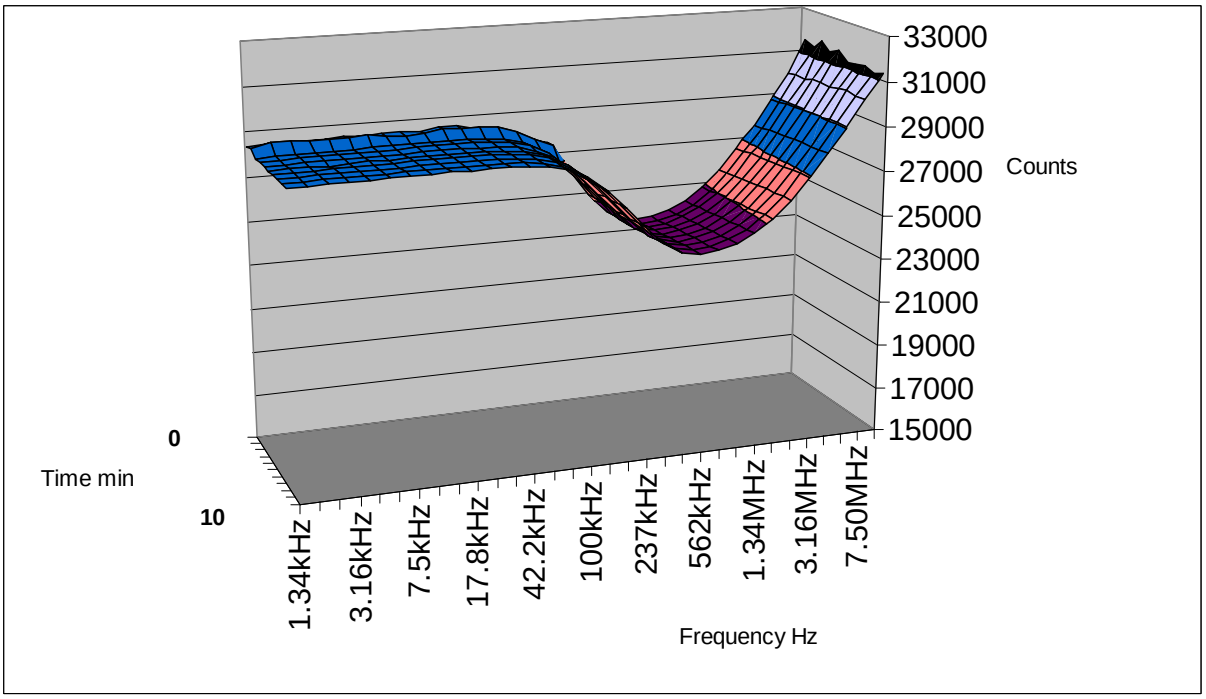

Figure 26 : Viamass results on *R.rubrum*

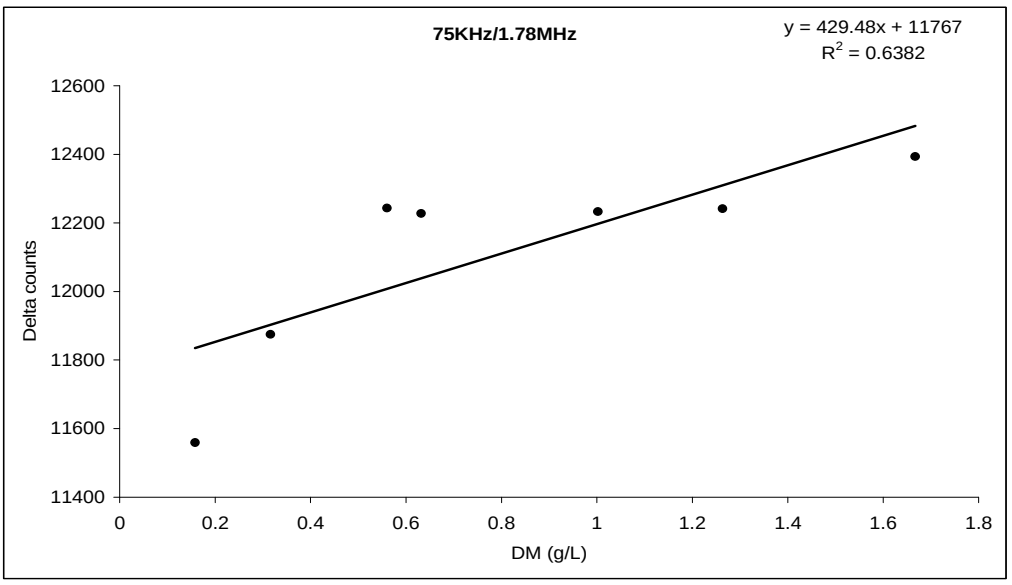

Figure 27: Correlation between concentration and delta counts for *R. rubrum*

A statistical treatment of these results reveals the following standard errors for the slope and the offset:

$$\Delta\text{Count} = (400 \pm 400)\ DM + (11800 \pm 400)$$

demonstrating obviously that, statistically speaking, the impedance measurement by the Viamass sensor was not sensitive in changing the *R. rubrum* biomass concentration in the range 0-2 g/L. In these conditions, the results of the tests comparing the biomass concentration obtained by the OD_{720} and by the Viamass sensor, and summarized in table 7 are not surprising. They confirm the lack of sensitivity for *R. rubrum* with errors that can reach 100%, even at high concentration.

Sample	$DM_{spectro}$ (g/L)	$DM_{viamass}$ (g/L)	Relative difference
1	0.12	0.15	25%
2	0.651	0.71	9%
3	0.987	0.69	30%
4	1.82	0.34	81%

Table 7: Comparison between $\mathbf{DM_{spectro}}$ and $\mathbf{DM_{viamass}}$ find with the correlation between **75kHz** and **1,78MHz**

At the opposite, the Mettler turbidity probe, whose wavelength had been modified to suit to *R. Rubrum*, is well-adapted to concentration measurement, as confirmed by the comparison with OD_{720} measurements in table 8.

Sample	$DM_{spectro}$ (g/L)	$DM_{mettler}$ (g/L)	Relative difference
1	0.12	0.113	6%
2	0.651	0.648	0.5%
3	0.987	0.992	1%
4	1.82	1.85	2%

Table 8: Comparison between $\mathbf{DM_{spectro}}$ and $\mathbf{DM_{mettler}}$ find with the Mettler probe of turbidity

5. Discussion

Concerning the backscattered light sensor from Mettler bought by the LGCB, as expected the results are consistent with the theory. The prototype sensor wavelength-modified by Mettler considering the specific optical properties of *R. rubrum* is well-adapted to low concentrations except for Fibrobacter under 0,5 g per liter. At the present time, we don't find a theoritical explanation. This point needs further experiments but it is out of the scope of this work.
In spite of good results, the most important default of this probe is the fact that it is a turbity probe without distinction between viable biomass and suspended particles.

On the contrary, electrical impedance spectroscopy technique is able to measure the viable biomass suspended in a medium but this measurement is known to be no noticeable at low biomass concentrations. To solve this problem, NTE probe makes a scan of frequencies which enables find a well-adapted couple of frequencies for each micro-organisms and with the best range.
The analytical results confirmed this expectation on three different micro-organisms:

- a bacteria, 1-2 µm
- a purpul photosynthetic bacteria, 1 per 5 µm
- a cyanobacteria, 7 per 350 µm

For this organisms, we observed an acceptable "deltacount" around 6000, 12000 and 15000 at 1g/L may be correlated to the size.
Surprisingly, the accuracy on dry mass correlations is not proportionnal to the amplitude of the signal. *Fibrobacter* is the best candidate in term of acceptability but it presents the lowest amplitude (Deltacount 6000). The good results obtained with this micro-organims on simple media should be confirmed on a complex substrate with vegetable wastes but unfortunately the prototype given by NTE is not adapted to this type of substrate.
On the opposite, *Rhodospirillum* which presents a better amplitude than *Fibrobacter* (deltacount 12000) gives results without any significant variation at low concentrations.
Results on *Rhodospirillum* and *Arthrospira* could be better with a logarithmic regression but this type of analysis raises doubts about the theory on biomass measurements.

6. Conclusion

The aim of this TN is to test the impedance sensor from NTE society on different micro-organisms and on different concentrations in biomass. The choice of micro-organisms is directed by the MELISSA project and the three organisms selected grow with an optimal biomass concentration which is low in comparison with classical micro-organisms like *Escherichia coli* or *Saccharomyces cerevisae*. This particularity present a very interressant challenge in term of biomass detection.

The results give the opportunity to confirm the scientific and commercial interest on this type of sensor which present the advantage to be based on impedance measurement and not on turbidity approach. As we say before, the sensor could only detected the living biomass.
It seems to be necessary to develop a theoretical approach to precise the link between dielectric properties of cells and impedance modulus on specific frequencies. For example, the results obtained on *Rhodospirillum* couldn't be explained by their conductive behaviour at 500THz.
It could be interesting to maintain this collaboration between LGCB and NTE with a real sensor adapted on a reactor to develop in parallel theoritical approach on low concentrations in biomass and experimental approach on photosynthetic micro-organisms and on complex medium including vegetables particles like in C1 compartment of MELISSA project. Scientifical and commercial impact is important.

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