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FINAL BELISSIMA LOOP REQUIREMENTS

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Abbreviations

CI:	Compartment I - liquid waste compartment
CII:	Compartment II - photoheterotrophic compartment
CIII:	Compartment III - nitrifying compartment
CIVa:	Compartment IVa - photoautotrophic compartment
DM:	Dry matter
FU:	Filtration unit
HRT:	Hydraulic Retention Time
MELiSSA:	Micro-Ecological Life Support System
rpm:	rounds per minute
SRT:	Sludge Residance Time
T:	Temperature
TOC:	Total organic Carbon
VFA:	Volatile Fatty Acids

1 INTRODUCTION

BELISSIMA aims to study the behaviour of microcompounds in the MELiSSA loop. In TN80.11, 80.12 and 80.14 an overview was given of all potential study items, which were then ranked according to priority. In TN80.15, an overall research plan was developed taking into account some limiting factors and critical issues related to the microcompound study. In this TN the corresponding requirements for the BELISSIMA loop are listed to ensure that the final loop design will have the necessary features to accommodate for relevant microcompound studies over a long period of time.

2 REQUIREMENTS FOR GENERAL OPERATION OF MELISSA COMPARTMENTS

This first chapter reviews the requirements for general operation. All four compartments of the BELISSIMA loop are discussed separately. A distinction is made between general requirements, process and system hardware requirements and other issues. Hardware requirements will systematically refer to general operation, mechanical issues, on-line measurements and material selection. Additional issues are maintenance of reactors over longer periods, requirements during different operation modes, control, personnel safety, etc.

Part of the requirements per compartment are already adapted specifically for the BELISSIMA-study. However, some of the BELISSIMA-requirements will be described separately within chapter 3.

2.1 Compartment I

2.1.1 GENERAL REQUIREMENTS

Anaerobic digestion is a biological process that consists out of a series of reactions, which are carried out by a mixed group of bacteria (fermentative, acidogenic, acetogenic and normally methanogenic bacteria) in the absence of molecular oxygen. If partial digestion occurs, intermediate compounds may be produced such as alcohols, lactic acid and volatile fatty acids. The process of methanogenesis can be inhibited by maintaining a low pH. Nitrogen will be released as ammonium due to hydrolysis.
The thermophilic compartment I in the MELiSSA loop is responsible for the biodegradation of human faecal material, possibly urine, toilet paper and non-edible parts of crops. A membrane filtration unit will be required to separate the solid phase (i.e. biomass and non-biodegraded organic matter) from the liquid phase (containing VFA and ammonium) and to ensure the sterility of the liquid phase.

These specifications are translated in the general requirements for the waste compartment CI as listed in Table 1.

Table 1. General requirements CI

Parameter	Requirement
VFA	maximal production
NH_4-N	maximal production
CO_2	maximal production
Oxygen	anaerobic
CH_4	zero production
Temperature	55°C
pH	< 6,0
Filtration	maximal retention of the solid phase (biomass and non-biodegraded organic matter)

2.1.2 PROCESS REQUIREMENTS

The process requirements for optimal operation of the waste compartment and its filtration unit are summarized in Table 2.

Table 2. Process requirements CI

Parameter	Requirement	Comment
Oxygen	< 0,5%	anaerobic
Temperature	55 ± 0,5 °C	
pH	5,3 ± 0,15	
Suspended solids	< 50 g/l	for optimisation of FU
N-concentration	< 3 g/l	to avoid acidogenic bacteria inhibition
HRT	10 days ± 4 hours	The control response time should be less than one day
Bioreactor load	2,1 g DM/l.d ± 10%	The control response time should be less than one day
SRT	> 20 days	

In the BELISSIMA-study, the urine is intended to be added to the waste compartment. The addition of urine can pose a problem of toxicity to the culture. The total nitrogen concentration in the influent shall not exceed 3 g/l.

Sampling is needed for basic process follow-up and for the specific tests for microcompounds. It is proposed to use the same frequencies and volumes of sampling for the process follow-up as in MELiSSA (Table 3).

Table 3. Sampling requirements for general follow-up CI

Sampling point	Frequency	Volume
Reactor	3/week	80 ml/sample
Filtrate	3/week	100 ml/sample
Off-gas	3/week	50 ml/sample

Details on the sampling of micro-nutrients that will be studied in BELISSIMA are given in paragraph 3.4.2. Particular for CI, the bioreactor should be sized so that the maximum biomass sampling does not exceed the average drain.

2.1.3 SYSTEM REQUIREMENTS

This chapter reviews the system hardware requirements. A distinction can be made between the reactor and its filtration unit on one hand and the inlet and outlet buffering equipment on the other hand.

2.1.3.1 Compartment CI and its filtration unit (FU)

Table 4 reviews the basic system requirements for CI and the FU within MELISSA. If different from MELiSSA specific BELISSIMA requirements are included.

Table 4. System requirements CI and FU

	Detail	Requirements		Comment
		Reactor	FU	
Operation	Feeding	(semi-) continuous	continuous	
	Automation	intermediate	intermediate	control described further
	Sterility	no	yes	
	Gas closure	yes		
	Pressure	100±20 mbar		slight overpressure, to avoid contamination
	pH-correction	yes		
	Gas flow	N_2		for anaerobic conditions and overpressure
Reactor	Size	to be defined in TN80.21-23		
	Stirring	good homogeneity		

	Detail	Requirements	Comment
	Heating	yes	
Measurements	Liquid feed	flow	
	Gas injection	flow	
	Reactor	temperature	
		pH	retractable fitting, if allowed by reactor size
		pressure	
		level	
	Filtrate	flow	
		temperature	
		pressure	

Some of the above system requirements need additional clarifications:

Sterility:

- ✓ Only sterility of the filtrate lines will be required.
- ✓ An ultra-filtration membrane, followed by a dead-end filtration will be used to sterilize the filtrate. The used membrane should be comparable to the one used in the MELiSSA Pilot Plant.
- ✓ Cleaning and sterilization will be done regularly and controlled by the PLC. No filtrate production will occur during these steps, but the process must be adapted to have however a constant filtrate production every day.

Liquid flows:

- ✓ The feeding could be done in a semi-continuous way, by means of a recirculation loop on the influent tank, and an automated valve equipped with a timer, that opens in the direction of the bioreactor at regular intervals of time. The flow rate through the reactor will be in the range of 4-5 liters per day.

Gas-flows:

- ✓ As the pressure in the reactor has to be kept at constant level a passive gas loop control is required, meaning that nitrogen is automatically introduced when the pressure decreases, and some gas is automatically released to the outside of the system when the pressure increases above the set point. A filter should be provided on the gas outlet preventing contamination from the surroundings.
- ✓ Routine follow-up of the gas phase for monitoring of process performance will be complemented with analysis of selected trace elements.
- ✓ The gas outlet will be equipped with a condensation system, to prevent loss of the studied contaminants in the gas phase.

- ✓ The gas production will be measured by means of a gas column similar to the one currently used for compartment I (for the MELiSSA Pilot Plant). This consists of a small tank allowing to calculate gas production rates from pressure and temperature measurements.

The same type of ceramic membranes as in the pilot plant are recommended. This will be the Alpha membrane from Atech with a support of AI_2O_3 and a top layer of titanium oxide. The membrane is a monochannel of 8 mm diameter,

2.1.3.2 Buffering vessels

The inlet vessel should provide sufficient volume to contain the feeding of one week operation. No sterility is required. In order to guarantee absence of oxygen the content must be flushed with N_2. On the other hand, the filtrate vessel has to be kept sterile.

The buffering vessels should be mixed by means of appropriate propellers.

In order to prevent contamination of the content and changing of the composition following requirements should be considered:
- ✓ vessels should be kept gastight;
- ✓ cooling at 4±1 °C is mandatory;
- ✓ illumination must be minimized.

As an alternative to the flow measurement of the inlet and outlet, a weight measurement of the buffering vessels could be foreseen.

2.1.4 OTHER REQUIREMENTS

This part includes requirements concerning control and maintenance.

The online-measurements, as listed in Table 4, are part of a control loop:
- ✓ pH-control: the on-line pH measurement controls the dosing of acid and base. Once the process is in steady-state acidification occurs and only one-way pH correction will be required using base. During start-up acid dosing will however be needed. The deviation around the set-point should be minimal as indicated in Table 2. Possible disturbances can be caused by the changing composition of the influent (with urine in case of BELISSIMA). The control response time should be reduced to 5 minutes. Attention should be paid to the influence of the dosing on the mass-balances studied within BELISSIMA. A too high (5,8) or too low pH (5,1) in the reactor should generate an alarm and stop the feeding of the reactor.
- ✓ Temperature control: as the process requires thermophilic conditions, heating of the reactor will be needed. The heating system will be activated by the on-line temperature measurement. Only a slight deviation around the set-point is allowed as stated in the

process requirements (Table 2). The control response time should be maximal 1 hour. The room temperature and the dosing of cooled influent will determine the required heating.
For the buffering vessels at 4°C a control response time of 0,5 hours is suggested. Besides the room temperature, the addition of new influent in the inlet buffer and the inflow of effluent at 55°C in the outlet vessel will determine the required cooling.

- ✓ Level-control: a constant active volume in the reactor is maintained by means of the flow control of the outlet pump based on the level in the reactor.
- ✓ Pressure-control: will preferably be controlled mechanically by using a pressure regulator. Pressure regulator actions should be recorded and gas losses monitored. The control response time for the bioreactor and the buffering vessels should be less than 5 seconds. Possible disturbances of the pressure inside the bioreactor can be caused by the inlet of influent and outlet of the filtrate, the use of the reactor drain and the nitrogen flush. For the inlet vessel, the opening of the tank and the addition of new liquid waste will cause pressure variations. On the other side, the pressure within the effluent vessel will drop in case of draining the tank.

The design of the waste compartment must take into account maintenance requirements:

- ✓ solutions must be foreseen in order to keep the compartment clean. It is recommended to include a CIP option even though this will be considered as the last option for cleaning to avoid the input of microcompounds;
- ✓ cleaning and sterilization of the sampling ports should be facilitated;
- ✓ ergonomic follow-up procedures will be developed;
- ✓ calibration of probes must be facilitated. For safety of the operators an Intrack connection of the pH probe is recommended.

Finally, some laboratory requirements are listed:

- ✓ Ventilation is recommended to reduce the smell of VFA. Although not meant to escape from the reactor and the buffering vessels, volatilisation of part of the VFA can not be excluded.
- ✓ General laboratory and operation personnel safety equipment must be provided.
- ✓ Especially for the handling of the influent and the drain of the reactor safety protocols should be foreseen.

2.2 Compartment II

2.2.1 GENERAL REQUIREMENTS

Compartment II is a mesophilic photoheterotrophic reactor and will receive in closed loop operation the liquid output of the waste compartment CI. The *Rhodospirillum rubrum* culture will convert the volatile fatty acids (VFA) of low molecular weight.
These specifications are translated in the general requirements for the reactor CII as given in Table 5.

Table 5. General requirements CII

Parameter	Requirement
VFA	maximal conversion
Biomass	maximal production
Oxygen	anaerobic
Temperature	30±0,5 °C
Irradiation	Radial light, but maximal illuminated surface
Light spectrum	350 - 950 nm

2.2.2 PROCESS REQUIREMENTS

The process requirements for optimal conversion by *R. rubrum* are summarized in Table 6.

Table 6. Process requirements CII

Parameter	Requirement	Comment
Oxygen	< 0,5 %	anaerobic
Temperature	30±0,5 °C	
pH	7±0,15	
Suspended solids	0,5 - 2 g/l	
Dilution rate	0,015 - 0,15 h^{-1}	

Some critical issues on the influence of these process parameters on the behaviour of nutrients within the BELISSIMA-study are described in chapter 3.1.

For the general follow-up of the reactor, sampling of the inlet and outlet will be required, as well as analysis on the biomass itself and the gas-flow. For those parameters to be analysed, Table 7 provides an overview of the proposed frequency and the required volumes. In case the compartments will be coupled, the influent of CII will equal the effluent of CI. The number of analyses can be reduced at that moment.

Table 7. Sampling requirements for general follow-up CII

	Parameter	Influent	Biomass	Effluent	Off-gas
Frequency	TOC	1/batch		3/week	
	VFA	1/batch		3/week	1/week
	NH_4-N	1/batch		3/week	
	NH_3				1/week
	CO_2				1/day*
	D.M.		1/week		
Required minimal volume	TOC	20 ml		20 ml	

	Parameter	Influent	Biomass	Effluent	Off-gas
for single analysis	VFA	2 ml		2 ml	
	NH_4-N	5 ml		5 ml	
	D.M.		20 ml		
Total		**~30 ml /batch**		**~100 ml /week**	

* Could be automated using analyser

As the reactor is a completely stirred system the biomass concentration in the effluent will equal the concentration in the reactor. A sampling port on the reactor however is required. As a biomass sensor is required in the reactor, a single analysis on dry matter per week will be sufficient for control reasons.
There will be CO_2 present in the off-gas in case that mainly acetate is consumed. When the feeding of CII contains butyrate, propionate,… CO_2 will be consumed. Sampling of the off-gas could be automated. A gas analysis system that measures the concentrations in the off-gas of all compartments intermittently could be foreseen.

Details on the sampling of micro-nutrients that will be studied in the BELISSIMA-project are given in paragraph 3.4.2.
Both the influent and effluent tank should be constructed so that the risk for biological contamination is minimized. Sampling should be done in an axenic way. All these issues are addressed in more detail within paragraph 2.2.3.

2.2.3 SYSTEM REQUIREMENTS

This chapter reviews the system hardware requirements. A distinction is made between the reactor itself and the interfaces (buffer vessels and harvesting system).

2.2.3.1 Compartment CII

Table 8 reviews the basic system requirements for CII within MELISSA. Some of the requirements are already adapted to the specific BELISSIMA-study.

Table 8. System requirements CII

	Detail	Requirement	Comment
Operation	Feeding	continuous	
	Automation	intermediate	control described further
	Cleaning	yes	all parts should be easy accessible
	Sterility	yes	
	Gas closure	yes	
	Pressure	100±20 mbar	slight overpressure
	pH-correction	yes	

	Detail	Requirement	Comment
	Gas flow	Ar or He	for anaerobic conditions and overpressure
		CO_2	alternative to create anaerobic conditions and required for butyrate, propionate,… consumption can be used for pH correction as well
Reactor	Active volume	to be defined in TN80.21-23	maximum at low sample volumes CI minimum during intensive sampling C I
	Shape	cilindrical	for optimal mixing and illumination
	Illumination	0 - 300 W/m² variable	light/dark zones (TN 49.2): - cycling between light and dark zones is required; - non illuminated headspace to be minimized.
	Stirring	good radial homogeneity 300 - 400 rpm	mechanical mixing (TN 47.1): proven that less biofilm is formed compared to air-lift system
	Cooling	yes	due to the irradiation of the reactor cooling will be required
Measurements	Liquid feed	flow	or weight of inlet vessel
	Liquid outlet	flow	or weight of outlet vessel
	Gas injection	flow	CO_2 Ar/He injection based on pressure
	Reactor	temperature	on-line
		pH	on-line
		pressure	on-line
		biomass concentration	on-line
		conductivity	on-line
		level	on-line
		redox	on-line to measure possible disturbances due to e.g. presence of oxygen
		light intensity	calibration at start-up
	Off-gas	VFA	

Detail	Requirement	Comment
	CO_2	
	Ammonia	

Some of the system requirements need additional clarification:

Sterility:
- ✓ The equipment should be easily sterilisable. It must be possible to disconnect the reactor from its side-equipment (pumps, filters, buffers,…). However, these parts need to operate in an axenic way and must be sterilisable as well. Manual valves for disconnection should be provided where needed. Smaller parts can be sterilised in an autoclave. For the reactor however, a sterilisation in place is preferable.
- ✓ Sterility filtration should be foreseen on the liquid and gas input of the reactor. The filter must be changeable in an easy and axenic way. Detection of the presure drop over the filter could be used as indication of the fouling.
- ✓ The axis seal of the stirring equipment should guarantee sterile conditions within the reactor.
- ✓ Those on-line measurements that require frequent calibration, e.g. pH probe, should be separable from the reactor in an easy and axenic way.

Gas measurement:
- ✓ As water balances will be studied in BELISSIMA a condenser should be foreseen on the off-gas line. The condensates should be returned to the reactor. As an alternative the humidity of the off-gas can be measured.
- ✓ A multiplex gas analysis system that can measure the off-gas of all compartments intermittently could be foreseen. This could be foreseen for O_2 and CO_2 measurements.

Cooling system:
- ✓ Minimisation of biofilm formation should be aimed for. From previous experience in the MELiSSA-consortium, an external jacket is therefore preferable.
- ✓ Ventilation around reactor to be foreseen.

The general material requirement for the reactor and its equipment and instruments can be summarized as follows:
- ✓ optimal transparency of the illuminated zone;
- ✓ resistant to corrosion;
- ✓ resistant to biofilm formation;
- ✓ resistant to chemical disinfection;
- ✓ resistant to steam sterilisation;
- ✓ heat and pressure proof.

Besides, as BELISSIMA aims to study micro-compounds, inert material must be used. The release of contaminants into the system should be excluded. Both organic components from

sealings, coating of materials,… as well as inorganic contaminants, mainly metals could pose a problem.

2.2.3.2 Interfaces

The interfaces of CII are on the one hand the influent and effluent buffering vessels and on the other hand the solid-liquid separation or biomass harvesting system. All tubing and the sterilisation unit can be seen as interfaces as well and will be briefly discussed later in this document.

The main requirement of the buffering vessels is to avoid biological contamination and maintain sterility of its content. This can be obtained by:
- ✓ keeping the vessels under slight overpressure and keeping them gas tight;
- ✓ cooling at 4±0,5 °C;
- ✓ minimizing illumination;
- ✓ filtration of the inlet.

As an alternative to the flow measurement of the inlet and outlet, a weight measurement of the buffering vessels could be foreseen.

Homogeneity within the vessels is guaranteed by good mixing, e.g. the use of a magnetic stirrer. The material requirements are comparable to those of CII itself.

The biomass harvesting system should fulfill the following requirements:
- ✓ maximal biomass separation and recovery;
- ✓ minimal loss of liquid phase;
- ✓ no use of chemical;
- ✓ minimal influence on the micro-compounds concentration in the liquid phase as the BELISSIMA study aims for optimal nutrient mass balances;
- ✓ probably batch operation as the liquid flow rates of the lab-scale loop will be low.

TN 37.30 reviewed different biomass harvesting systems and concluded that centrifugation and filtration are the recommended techniques.

2.2.4 OTHER REQUIREMENTS

Besides the general operation and system hardware requirements the BELISSIMA loop should fulfill some additional requirements, including guidelines on control, maintenance and personnel safety.

The on-line measurements were listed in Table 8. Some of these parameters will be part of a control loop:
- ✓ pH-control: the on-line pH measurement controls the dosing of acid and base. The deviation around the entered setpoint should be minimal. Attention must be paid to the

corrosive nature of the used acid and base and their possible influence on the nutrient mass balances. As those mass balances will be studied within the BELISSIMA project, an exact registration of the dosing of acid and base is required. A too high (7,5) or too low pH (6,5) within the reactor CII should generate an alarm and stop the feeding pump.

- ✓ Temperature-control: as the reactor will be illuminated, cooling will be required. During start-up however a short period of low temperature could occur. Heating through the heat exchange system could offer a solution. The cooling circuit will be activated by the on-line T-measurement. The deviation around the given setpoint should be minimal (±0,5 °C). A too high temperature (35°C) should generate an alarm and switch off or minimize the illumination. A too low temperature (25°C) must generate an alarm.
- ✓ Pressure-control: the reactor should work under slight overpressure. The on-line P-measurement controls the injection of gas. A minimal deviation around the setpoint is aimed for (±20 mbar). A security pressure valve should be foreseen.
- ✓ Level-control: a constant level in the reactor should guarantee a constant hydraulic retention time. Two different level setpoints may be required to have a maximal and minimal reactor volume as described in Table 8. The flow rate of the feeding will be set at a fixed rate (see TN80.2). The flow rate of the outlet however will be controlled by the level measurement in the reactor. A too low level in the reactor should generate an alarm and stop the outlet pump. A too high level in the reactor on the other hand should generate an alarm as well and stop the inlet pump.
  The level in the reactor depends on the calculated required active volume and the shape of the reactor.
  The levels in the buffering vessels can be controlled by weight measurement. A low level in the inlet vessel should generate an alarm and stop the inflow pump to the reactor.
- ✓ Illumination: the level of irradiation of the biomass is adjusted by changing the power supply. The relation between the power supply and the light intensity should be determined at start-up, through calibration.

An optimal <u>maintenance</u> of the reactor requires that:

- ✓ pumps, dosing units, gas-injection and interfaces can be disconnected from the reactor in a way that axeny of the other parts remains guaranteed. Manual valves must be foreseen where necessary;
- ✓ all the separate parts of the reactor and interfaces are easily accessible for cleaning. A cleaning in place is preferable;
- ✓ all the separate units can be sterilized when needed. A sterilisation in place is preferable;
- ✓ connections and tubes can be replaced easily;
- ✓ all different parts of the reactor and equipment are easily accessible;
- ✓ the frequency of opening of the reactor should be minimized. Probes that need frequent calibration, e.g. pH-probe, should be separable in an axenic way (intrack);
- ✓ sterility filters on the gas and liquid input should be easily changeable. Parallel spare filters are recommended.

Although the used materials must be chosen in such a way that biomass formation will be minimal, growth of micro-organisms on surfaces in the reactor, the equipment and the tubing

cannot be excluded in the long term. As described above, easy access and facilitated disconnection of all parts, in order to clean or replace them, should be aimed for.

Finally, some laboratory requirements are listed:

- ✓ Sufficient ventilation will be required as 90% of the light power should be removed as heat. The reactor itself will be cooled down by means of the liquid cooling system. However, ventilation will be required to limit room temperature.
- ✓ Ventilation is as well recommended to reduce the smell of VFA. Although not meant to escape from the reactor and the buffering vessels, volatilisation of part of the VFA can not be excluded.
- ✓ Personnel safety equipment must be provided.
- ✓ Axenic operation should be guaranteed.

2.3 Compartment III

2.3.1 GENERAL REQUIREMENTS

The objective of the nitrifying compartment CIII is to oxidize the ammonium ions (NH_4^-) present in the exit stream from CII into nitrate, being the most appropriate nitrogen source for the bacteria in CIVa and the higher plant compartment. This mesophilic reactor consists of a packed-bed reactor with cells of two immobilized bacterial strains (*Nitrosomonas europaea* and *Nitrobacter winogradskyi*). A packed-bed system is required to prevent wash-out as the bacteria are slow growing.
These specifications are translated in the general requirements for CIII as given in Table 9.

Table 9. General requirements CIII

Parameter	Requirement
NH_4^+	maximal oxidation
Oxygen	aerobic
Temperature	30±0,5 °C

2.3.2 PROCESS REQUIREMENTS

The process parameters for optimal conversion of ammonia by the nitrifying bacteria are listed in Table 10.

Table 10. Process requirements CIII

Parameter	Requirement	Comment
Oxygen	> 4 mg/l or 80% saturation	aerobic
Temperature	30±0,5°C	
pH	8,15±0,15	

Parameter	Requirement	Comment
Biomass growth	biofilm on carrier	
Dilution rate	~0,07 h^{-1}	to be specified for BELISSIMA

Some critical issues on the influence of these process parameters on the behaviour of nutrients within the BELISSIMA-study are described within chapter 3.1.

For the general follow-up of the reactor, sampling of the inlet and outlet will be required, as well as sampling on the gas-flow. Analysis on biomass will be more difficult as the biomass is attached to the carrier and axenic operation needs to be guaranteed. Biomass sampling for characterisation can be done on the reactor outlet flow after a backwash procedure. For the parameters to be analysed, Table 11 provides an overview of the proposed frequency and the required volumes. In case the compartments will be coupled, the influent of CIII will equal the effluent of CII. The number of analyses can be reduced at that moment.

Table 11. Sampling requirements for general follow-up CIII

	Parameter	Influent	Effluent	Gas
Frequency	TOC	1/batch	3/week	
	VFA	1/batch	3/week	1/week
	Ntot	1/batch	3/week	
	NH_4-N	1/batch	3/week	
	NH_3			1/week
	NO_2^--N		3/week	
	NO_3^--N		3/week	
	O_2			1/day*
	CO_2			1/day*
Required minimal volume for single analysis	TOC	20 ml	20 ml	
	VFA	2 ml	2 ml	
	Ntot	0,5 ml	0,5 ml	
	NH_4-N	5 ml	5 ml	
	NO_2^--N		2 ml	
	NO_3^--N		1 ml	
Total	**Total**	**~30 ml /batch**	**~90 ml /week**	

* Could be automated using analyser

The above volumes are the absolute minimum for a single analysis. If the effluent contains some biomass, additional volume will be needed as filtration before analysis is required.

Besides the possibility of characterising the biomass after backwash sampling, sampling ports should be foreseen at different levels on the reactor. As contamination of the slow growing nitrifying community must be prevented, these ports should be easily sterilisable. Sampling through these ports could be done by means of a syringe through a septum.

As an alternative to momentary gas sampling and O_2 and CO_2 analysis, a continuous gas analyser checking intermittently the in- and outlet gas phases of all compartments can be used.

Details on the sampling of micro-nutrients that will be studied in the BELISSIMA-project are given in paragraph 3.4.2.
Both the influent and effluent tank should be constructed so that the risk for biological contamination is minimized. Sampling should be done in an axenic way. These issues are addressed in more detail within paragraph 2.3.3.

2.3.3 SYSTEM REQUIREMENTS

In this paragraph the system hardware requirements are reviewed. A distinction is made between the reactor itself and the interfaces.

2.3.3.1 Compartment CIII

Table 12 summarizes the basic system requirements for CIII of the MELiSSA-loop. Some of the parameters are adjusted to meet the requirements for the specific BELISSIMA-project.

Table 12. System requirements CIII

	Detail	Requirement	Comment
Operation	Feeding	continuous	batch during start-up
	Recirculation	5:1	recirculation/feed ratio
	Backwash	yes	every 4 to 6 months according to TN 47.2; but to be related to the pressure drop over the reactor
	Sterility	yes	
	Gas closure	yes	gas release out of reactor due to aeration
	Pressure	100±20 mbar	slight overpressure
	pH-correction	yes	
	Gas flow	constant	to guarantee constant mixing
		air + x% O_2	oxygen supplied when requirements higher than provided with air, but total gas flow will be kept constant
Reactor	Size	to be defined in TN80.21-23	max. at low sample volumes CI and CII min. during intensive sampling CI and CII To be determined for BELISSIMA

	Detail	Requirement	Comment
	Shape	cilindrical	for optimal mixing
	Fixed bed	carrier support	biostyr, glass beads or other
	Stirring	magnetic stirrer 300 rpm	to provide an optimal mixing between inlet and recirculation stream
	Temperature-control	yes	for cooling and heating
Measurements	Liquid feed	flow	or weight of inlet vessel
	Recirculation	flow	
	Gas injection	flow	
	Reactor	temperature	on-line
		pH	on-line
		oxygen	on-line
		level	on-line
		pressure	on-line
	Off-gas	CO_2 - O_2	
		Ammonia	

Some of the system requirements need additional clarification:

Liquid flows:

- ✓ Although the reactor will be inoculated with the two strains of bacteria, no nitrification is to be expected at start-up. Batchwise operation at start-up is recommended until the nitrification process starts. During normal operation a continuous inflow will be required.
- ✓ Recirculation of the liquid phase over the reactor will be required to increase ammonia conversion. The ratio will be fixed by the design. A backwash of the reactor should be possible to prevent clogging of the reactor medium (TN 47.2).
- ✓ If part of the carrier is removed, the flow patterns wil change.
- ✓ The use of a magnetic stirrer is prefered for optimal and sterile mixing of the recirculation and inlet stream.

Sterility:

- ✓ The reactor without the carrier should be sterilisable before start-up. If expanded polystyrene beads are used (Biostyr) no steam sterilisation is allowed. Glass beads on the other hand can be sterilised.
- ✓ It must be possible to disconnect the reactor from its side-equipment (pumps, filters, buffering vessels,…). All parts need to operate in an axenic way and should, as a

consequence, be sterilisable as well. Manual valves for proper disconnection should be provided. Larger parts can be sterilised in-line. Smaller parts are preferably sterilised in an autoclave.

- ✓ Sterility filtration should be foreseen on the liquid and gas input of the reactor.
- ✓ Those on-line measurements that require frequent calibration, e.g. pH probe, should be separable from the reactor in an easy and axenic way (Intrack).

Gas flows:

- ✓ The gas flow rate into the reactor should be constant to prevent changes in the mixing pattern of he reactor. However, changes in biomass concentration and the loading of the reactor make the oxygen demand variable. As a result, the oxygen concentration in the inlet gas flow should be adjustable. This can be achieved if pure gases (O_2, CO_2 (and N_2)) are used instead of air.
- ✓ As water balances will be studied in BELISSIMA a condenser should be foreseen on the off-gas line. The condensates should be recycled to the reactor.
- ✓ A multiplex gas analysis system that can measure the off-gas of all compartments intermittently could be foreseen.

The general material requirements for the reactor and its equipment can be summarized as follows:

- ✓ the possibility of visual observation is recommended. However to prevent microbial growth on the inside of the reactor the illumination should be minimized, e.g. by covering the reactor with insulation material;
- ✓ resistant to corrosion;
- ✓ resistant to biofilm formation;
- ✓ resistant to chemical disinfection;
- ✓ resistant to steam sterilisation. Except for the carrier material if polystyrene beads are used;
- ✓ heat and pressure proof.

Besides, as BELISSIMA aims to study micro-compounds, inert material must be used. The release of contaminants into the system should be excluded. Both organic components from sealings, coating of materials,… as well as inorganic contaminants, mainly metals could pose a problem.

2.3.3.2 Interfaces

The interfaces of CIII are on one hand the influent and effluent buffering vessels and on the other hand the solid-liquid separation.

The main requirement of the buffering vessels is to avoid biological contamination or changes of its content. This could be obtained by:

- ✓ keeping the vessels under slight overpressure and keeping them gas tight;
- ✓ cooling at 4°C;
- ✓ minimizing illumination.

As an alternative to the flow measurement of the inlet and outlet, a weight measurement of the buffering vessels could be foreseen.

Homogeneity within the vessels is guaranteed by good mixing. The axis seal of the stirring equipment should guarantee axenic operation. The use of a magnetic stirrer could offer a solution. The material requirements are comparable to those of CIII itself.

The solid-liquid separation system should be based on the recovery of clean liquid. As biomass is immobilized in the reactor no significant concentrations are expected in the output. However, low concentrations of detached cells will have to be removed. The selected technology should overcome the fact that nitrifying bacteria are difficult to sediment and to centrifuge (TN 72.6).
The separation system should fulfill as well the following criteria:

- ✓ minimal loss of liquid phase;
- ✓ no use of chemicals;
- ✓ minimal influence on the micro-compounds concentration in the liquid phase as the BELISSIMA study aims for optimal nutrient mass balances;
- ✓ probably batch operation as the liquid flow rates of the lab-scale loop will be low.

2.3.4 OTHER REQUIREMENTS

Besides the general operation and system requirements CIII and its side-equipment should fulfill some additional requirements, including control, maintenance and personnel safety.

The on-line measurements were listed in Table 12. Some of these parameters are part of a control loop:

- ✓ pH-control: nitrification will decrease pH. The aeration flow through the reactor however increases the pH again as it strips CO_2 out of the liquid phase (TN 37.510). The on-line pH measurement should control the dosing of acid and base. Attention must be paid to the corrosive nature of the used acid and base and their possible influence on nutrient and carbon mass balances. As those mass balances will be studied within the BELISSIMA project, an exact registration of the dosing of acid and base is required. A too high (8,3) or too low (8,0) pH within the reactor CIII should generate an alarm and stop the feeding pump. A minimal deviation around the pH setpoint is required (±0,15).
- ✓ Temperature-control: the reactor should be connected to a thermostatic bath to guarantee a constant temperature in the reactor. The deviation around the temperature setpoint should be minimal. A too high (35°C) or too low temperature (25°C) in the reactor should generate an alarm.
- ✓ Level-control: a constant level in the reactor is required. The inlet pump will work at a constant, calibrated flow rate. The outlet pump should work at an equal flow rate. However, as the flows will not be perfectly identical, the level-control should generate alarms whenever a too high or too low level is obtained in the reactor. The high level should switch off the inlet pump, whereas a low level should stop the outlet pump.

An optimal maintenance of the reactor requires that:

- ✓ pumps, dosing units, gas-injection and interfaces can be disconnected from the reactor in a way that axeny of the other parts remains guaranteed. Manual valves must be foreseen where necessary;
- ✓ all the separate parts of the reactor and interfaces are easily accessible for cleaning. A cleaning in place is preferable;
- ✓ all the separate units can be sterilized when needed. A sterilisation in place is preferable. Smaller parts will be sterilised in an autoclave;
- ✓ connections and tubes can be replaced easily;
- ✓ all different parts of the reactor and equipment are easily accessible;
- ✓ the frequency of opening of the reactor should be minimized and restricted during test runs to essential samplings or operational problem solving. Probes that need frequent calibration, e.g. pH-probe, should be separable in an axenic way (intrack);
- ✓ the carrier should be removable from the reactor and replaceable when necessary;
- ✓ sterility filters on the gas and liquid input should be easily changeable. Parallel spare filters are recommended.

Although the used materials must be chosen in such a way that biomass formation will be minimal, growth of micro-organisms on surfaces in the reactor, the equipment and the tubing cannot be excluded in the long term. As described above, easy access and facilitated disconnection of all parts, in order to clean or replace them, should be aimed for.

Finally, some laboratory requirements are listed:

- ✓ Personnel safety equipment must be provided.
- ✓ Axenic operation and handling.

2.4 Compartment IVa

2.4.1 GENERAL REQUIREMENTS

The main task of the mesophilic photosynthetic compartment CIVa within the MELiSSA loop is the fixation of CO_2, concomitant with the generation of the edible mico-algae *Arthrospira platensis*. This cyanobacterium presents a high nutritional value. Besides the production of biomass, this compartment aims for a maximal production of oxygen as well. The liquid flow, rich in nitrates from CIII, will provide the minerals and nitrogen source to CIVa.
These specifications result in the general requirements for the reactor CIVa as listed in Table 13.

Table 13. General requirements CIVa

Parameter	Requirement
NO_3^-	maximal removal

Parameter	Requirement
Biomass	maximal production
Oxygen	maximal production
Temperature	36±0,5 °C
Irradiation	maximal illuminated surface zone
Light spectrum	400-700 nm

2.4.2 PROCESS REQUIREMENTS

The process requirements for optimal photosynthesis by *Arthrospira platensis* are summarized in Table 14.

Table 14. Process requirements CIVa

Parameter	Requirement	Comment
CO_2	to be injected	not in case synthetic media contains additional carbonates
Temperature	36±0,5 °C	
pH	9,5	constant productivity between pH 8 and 10
Suspended solids	0,5 - 2 g/l	
Dilution rate	0,005 - 0,05 h^{-1}	to be specified for BELISSIMA

Some critical issues on the influence of these process parameters on the behaviour of nutrients within the BELISSIMA-study are described within chapter 3.1.

For the general follow-up of the reactor, sampling of the inlet and outlet will be required, as well as analysis on the biomass itself and the gas-flow. For those parameters to be analysed, Table 15 provides an overview of the proposed frequency and the required volumes. In case the compartments will be coupled the influent of CIVa will equal the effluent of CIII. The number of analyses can be reduced at that moment.

Table 15. Sampling requirements for general follow-up CIVa

	Parameter	Influent	Biomass	Effluent	Gas
Frequency	TOC	1/batch		3/week	
	NH_4-N	1/batch		1/week	
	NO_3^--N	1/batch		3/week	
	NO_2^--N	1/batch		3/week	
	O_2				1/week*
	CO_2				1/week*
	D.M.		3/week		

	Parameter	Influent	Biomass	Effluent	Gas
Required minimal volume for single analysis	TOC	20 ml		20 ml	
	NH_4-N	5 ml		5 ml	
	NO_3^--N	1 ml		1 ml	
	NO_2^--N	2 ml		2 ml	
	D.M.		**20 ml**		
Total		**~30 ml /batch**		**~100 ml /week**	

* Could be automated using analyser

As the reactor is a completely stirred system the biomass concentration in the effluent will equal the concentration in the reactor. As a result no sampling within the reactor itself will be required.
Details on the sampling of micro-nutrients that will be studied in the BELISSIMA-project are given in paragraph 3.4.2.
Both the influent and effluent tank should be constructed so that the risk for biological contamination is minimized. Sampling should be done in an axenic way. All these issues are addressed to in more detail within paragraph 2.4.3.2.

2.4.3 SYSTEM REQUIREMENTS

This chapter reviews the system hardware requirements. A distinction is made between the reactor itself and the interfaces (buffer vessels and harvesting system).

2.4.3.1 Compartment CIVa

The basic MELiSSA system requirements for reactor CIVa are listed in Table 16. Some of the parameters are already adjusted for the specific BELISSIMA-study.

Table 16. System requirements CIVa

	Detail	Requirement	Comment
Operation	Feeding	continuous	
	Automation	intermediate	control described further
	Sterility	yes	
	Gas closure	yes	gas release out of reactor due to aeration
	Pressure	100±20 mbar	slight overpressure
	pH-correction	yes	
	Gas flow	air + x% CO_2	in case of closed loop operation. For tests with Zarrouk medium sufficient bicarbonates are available in the medium.

	Detail	Requirement	Comment
Reactor	Size	to be defined in TN80.21-23	maximum at low sample volumes CI-CIII minimum during intensive sampling C I-CIII
	Shape	cylindrical	for optimal mixing and illumination
	Diameter	small	to avoid dark zones in centre dark zones at headspace should be minimal as well
	Illumination	0 - 300 W/m²	excess light becomes inhibitory
		variable	avoid dark surfaces and zones
	Stirring	good radial homogeneity	mechanical stirrer: easy to control
		max. 300 rpm	mixing with gas flow could be possible as well
	Cooling	yes	due to the irradiation of the reactor cooling is required
Measurements	Liquid feed	flow	or weight of inlet vessel
	Gas injection	flow	
	Reactor	temperature	on-line
		pH	on-line
		pressure	on-line
		biomass concentration	on-line
		oxygen	on-line
		light intensity	calibration
	Off-gas	CO_2 - O_2	on-line

Some of the system requirements need additional clarification:

Sterility:

- ✓ The equipment should be easily sterilisable. It must be possible to disconnect the reactor from its side-equipment (pumps, filters, buffers,…). However, these parts need to operate in an axenic way and must be sterilisable as well. Manual valves for disconnection should be provided where needed. Smaller parts will be sterilised in an autoclave.

- ✓ Sterility filtration should be foreseen on the liquid and gas input of the reactor. The filter must be changeable in an easy and axenic way. Detection of the pressure drop over the filter could be used as indication of the fouling.
- ✓ The axis seal of the stirring equipment should guarantee sterile conditions within the reactor.
- ✓ Those on-line measurements that require frequent calibration, e.g. pH probe, should be separable from the reactor in an easy and axenic way.

Gas measurement:

- ✓ As water balances will be studied in BELISSIMA a condenser should be foreseen on the off-gas line. The condensates should be recycled to the bioreactor..
- ✓ A multiplex gas analysis system that can measure the off-gas of all compartments intermittently could be foreseen.

Cooling system:

- ✓ Minimisation of biofilm formation should be aimed for. An external jacket could offer a solution.
- ✓ Ventilation around reactor to be foreseen.

The general material requirement for the reactor and its equipment can be summarized as follows:

- ✓ optimal transparency of the illuminated zone;
- ✓ resistant to corrosion;
- ✓ resistant to biofilm formation;
- ✓ resistant to chemical disinfection;
- ✓ resistant to steam sterilisation;
- ✓ heat and pressure proof.

Besides, as BELISSIMA aims to study micro-compounds, inert material must be used. The release of contaminants into the system should be excluded. Both organic components from sealings, coating of materials,… as well as inorganic contaminants, mainly metals could pose a problem.

2.4.3.2 Interfaces

The interfaces of CIVa are on one hand the influent and effluent buffering vessels and on the other hand the solid-liquid separation or biomass harvesting system.

The main requirement of the buffering vessels is to avoid biological contamination or other changes of its content. This can be obtained by:

- ✓ keeping the vessels under slight overpressure and keeping them gas tight;
- ✓ cooling at 4°C;
- ✓ minimizing illumination.

As an alternative to the flow measurement of the inlet and outlet, a weight measurement of the buffering vessels could be foreseen.

Homogeneity within the vessels is guaranteed by good mixing. The use of a magnetic stirrer could offer a solution. The material requirements are comparable to those of CIVa itself.

The biomass harvesting system should fulfill the following requirements:
- ✓ maximal biomass separation and recovery;
- ✓ ensure the integrity of the cells. Cell breakage and shear stress release EPS into the liquid phase and increase the viscosity of the liquid. Centrifugation appears difficult as fresh *Arthrospira platensis* contains vacuoles and floats (TN 62.5);
- ✓ minimal loss of liquid phase. Recuperation of liquid between 75 and 90% (TN 72.6);
- ✓ minor or no use of chemical;
- ✓ minimal maintenance;
- ✓ minimal influence on the micro-compounds concentration in the liquid phase as the BELISSIMA study aims for optimal nutrient mass balances;
- ✓ probably batch operation as the liquid flow rates of the lab-scale loop will be low.

A discontinuous separation will be preferable as the flow rate of the reactor outlet of the lab-scale plant will be small. *Arthrospira platensis* can be stored at 4°C for 48 hours before degradation occurs (TN 72.6).

2.4.4 OTHER REQUIREMENTS

Besides the general operation and system hardware requirements the BELISSIMA loop should fulfill some additional requirements, including guidelines on control, maintenance and personnel safety.

The on-line measurements were listed in Table 16. Some of these parameters will be part of a control loop:
- ✓ pH-control: the on-line pH measurement controls the dosing of acid and base. Attention must be paid to the corrosive nature of the used acid and base and their possible influence on the nutrient mass balances. As those mass balances will be studied within the BELISSIMA project, an exact registration of the dosing of acid and base is required. A too high (10) or too low pH (8) within the reactor CIVa should generate an alarm and stop the feeding pump.
- ✓ Temperature-control: as the reactor will be illuminated, cooling will be required. The cooling circuit will be activated by the on-line T-measurement. The deviation around the given setpoint should be minimal. A too high temperature (38°C) should generate an alarm and switch of the illumination. A too low temperature (34°C) must generate an alarm.
- ✓ Pressure-control: the reactor should work under slight overpressure. Air is injected. A pressure control valve should be foreseen.
- ✓ Level-control: a constant level in the reactor should guarantee a constant hydraulic retention time. Two different level setpoints may be required to have a maximal and

minimal reactor volume as described in Table 8. The flow rate of the feeding will be set at constant level. The flow rate of the outlet however will be controlled by the level measurement in the reactor. A too low level in the reactor should generate an alarm and stop the outlet pump. A too high level in the reactor on the other hand should generate an alarm as well and stop the inlet pump.
The levels in the buffering vessels can be controlled by weight measurement. A low level in the inlet vessel should generate an alarm and stop the inflow pump to the reactor.

- ✓ Illumination: the level of irradiation of the biomass is adjusted by changing the power supply. The relation between the power supply and the light intensity should be determined at start-up through calibration.

An optimal maintenance of the reactor requires that:

- ✓ pumps, dosing units, gas-injection and interfaces can be disconnected from the reactor in a way that axeny of the other parts remains guaranteed. Manual valves must be foreseen where necessary;
- ✓ all the separate parts of the reactor and interfaces are easily accessible for cleaning. A cleaning in place is preferable;
- ✓ all the separate units can be sterilized when needed. A sterilisation in place is preferable;
- ✓ connections and tubes can be replaced easily;
- ✓ all different parts of the reactor and equipment are easily accessible;
- ✓ the frequency of opening of the reactor should be minimized. Probes that need frequent calibration, e.g. pH-probe, should be separable in an axenic way (intrack);
- ✓ sterility filters on the gas and liquid input should be easily changeable. Parallel spare filters are recommended.

Although the used materials must be chosen in such a way that biomass formation will be minimal, growth of micro-organisms on surfaces in the reactor, the equipment and the tubing cannot be excluded in the long term. As described above, easy access and facilitated disconnection of all parts, in order to clean or replace them, should be aimed for.

Finally, some laboratory requirements are listed:

- ✓ Personnel safety equipment must be provided.
- ✓ Axenic handling must be possible.

2.5 Overall requirements

In addition to the specific requirements given for separate compartment some overall issues will be discussed in this paragraph, including power supply, steam supply, automation…

The power supply required will be 400 VAC + N + PE 50Hz. An isolation transformer should be foreseen. The analog input/output signals should be a 4-20 mA signal, the digital signals a 24V or TTL.

A steam line for sterilisation purpose should be foreseen. All gases, as mentioned in previous compartment descriptions, should be available: Ar, N_2, CO_2, O_2 … Pressurized air must be provided for aeration and valve operation. A drain for liquid waste will be needed.

The automation and operation control should fulfil the following requirements:

- ✓ automation is needed to minimize the operator work and to ensure a sufficient level of control of the processes. However, full automation is out of scope within the BELISSIMA project;
- ✓ clear visual observation is aimed for;
- ✓ standardisation of the used symbols, color indications, tags and labeling… is suggested.

Concerning countermeasures, sufficient valves and connections to the different reactors must be foreseen to ensure a potential future coupling of additional technologies. Extra ports in the reactors could be required as well for possible future on-line measuring probes, different from the ones mentioned in previous paragraphs.

Table 17 reviews the suppliers and references preferentially used within all MELiSSA projects.

Table 17. Harmonized hardware MELiSSA

HARDWARE TYPE	SUPPLIERS/REFERENCE
Programmable Logic Controller	Schneider/Quantum
Electrical connectors	Phoenix
Electrical cupboards	Rittal
Flow controllers	MKS
Lamps	OSRAM 12V, 20W BAB 38°
Port	Ingold
Tubing connections	Swagelok
pH-probe	Mettler-Toledo
O2-probe	Mettler-Toledo
Electrical fuses, circuit breakers…	Hager

Requirements concerning safety have been discussed partly in previous chapters. In summary the overall needs are the following:

- ✓ axenic operation should be possible;
- ✓ sufficient ventilation will be required to remove vapours and heat;
- ✓ the different work spaces should be kept under overpressure to minimize contamination of CII to IVa from outside or under underpressure to avoid spread of pathogenic organisms from CI.
- ✓ the possibility of H_2S release from CI should be addressed.

2.6 Compartment operation

An optimal reactor operation is aimed for. Variations in compartment performance, which are not due to lacks of or accumulation of certain micro-compounds, should be avoided.

As described in TN 80.15, it is proposed to work with separate compartments in a first stage and with a coupled set-up afterwards. This allows intensive sampling to determine removal efficiencies per compartment. Only those compounds which appear interesting from these tests will be further studied in coupled set-up and finally in closed loop.

Operation in closed loop will limit the sample volumes. There will be a conflict between closed loop operation and intensive sampling as the outlet of one compartment will be used as the feed for the following one. As a result, sampling in closed loop operation should be reduced in order to minimize a too strong size or dilution effect.

The residence time, or dilution rate, within the different compartments has been described for all four reactors in previous paragraphs (process requirements). The dilution rate and the sampling volumes will be evaluated to calculate the active reactor volumes. It is currently envisaged that the compartments CII, CIII and CIVa may operate at 2 different liquid levels during different test periods. A high liquid level can be maintained in case sampling from the previous reactors is minimal. Most of the effluent in such a situation can be used as feed to the next compartment. A low level will however be needed in case more sample volume is required. It is crucial however, that once the active volume is fixed, it remains stable and operation occurs at fixed dilution rates.

BELISSIMA mainly aims for the closure and follow-up of the liquid loop. Water losses through drains, interphases, sampling,… must be minimized. However, as the off-gas and solid drains will contain water and contaminants as well and sampling of gas phases and biomass may be needed to close mass balances, a careful characterisation of those streams will be needed:

- ✓ condensers or humidity measurements must be placed on the off-gas streams;
- ✓ the condensates should be analysed to determine possible losses of contaminants;
- ✓ possible losses of volatile contaminants should be monitored as well: ammonia, VFA,…

3 SPECIFIC REQUIREMENTS FOR MICROCOMPOUND STUDY

3.1 Critical issues

In addition to the requirements listed above, some overall points of attention, related to the specific BELISSIMA objectives, are listed in this chapter.

Concerning pH correction, it should be taken into account that the addition of mineral acids and bases may cause a problem when nutrient mass balances have to be closed. Nevertheless, because the use of organic chemicals can lead to undesirable side-effects, preference is given to mineral acids and bases. In that case provisions have to be taken to adequately measure the dosed amounts.

Beside the dosing of pH-correcting agents the addition of other chemicals should be minimal as well. Products such as disinfecting agents, antifoam and membrane cleaning products are to be excluded from normal operation.

3.2 Definition of feed to compartment I

Compartment I will be fed with a mix of wastes (Table 18) grinded and diluted with water.

Table 18. Composition of the feed to CI

Material		Comment
Non edible parts of crops	lettuce beet wheat straw	
Toilet paper		non-bleached
Human fecal material		
Urine		

The selection of the crops was agreed upon within the MELiSSA consortium.
The composition of the CI influent in the MELiSSA pilot plant is presented in Table 19.

Table 19. Composition and load of the CI influent in MELiSSA

Material	Amount DW (g/d)	Percentage (%)
Lettuce	54	25.8
Beet	54	25.8
Wheat Straw	54	25.8
Toilet paper	18	8.6
Total plants and paper	***180***	***86%***
Faecal material	***30***	***14%***
Total amount of material	**210 DW g/d**	**100%**

Urine will be added to the feed for the BELISSIMA study and a different mission scenario will be used, implying changes in the influent composition and load. The mission scenario is the following:

- ✓ 40% of food of 6 crew members daily diet;
- ✓ production of 6 equivalent men of oxygen per day;
- ✓ production of 6 equivalent men of drinking water per day;
- ✓ recycle 6 equivalent men of faecal material, urine and CO_2;
- ✓ if N from urine is too high the overall load should be undersized or the percentage of urine in the overall load lowered.

The equivalent composition and load are presented in Table 20. The amounts of waste produced are based on calculations for men. The total Dry Matter (DM) for this scenario is 2538 g/d.

Table 20. Composition and load of the CI influent in BELISSIMA

Material	Production / consumption of one man per day	Production / consumption of the 6 men crew	DM	Fresh load
Wastes		Production of 6 equivalent man		
- Fecal material	30 g DW/d	180 g DW/d	33%	545 g/d
- Urine	51 g DW/ d in 1,5 L/d	306 g DW/d in 9 liters	3,4%	9 l/d
- Toilet paper	18 g DW/d	108 g DW/d	100%	108 g/d
Vegetables		40 % of food of 6 crew members daily diet		
- Lettuce	270 g DW/d	648 g DW/d	5%	13 kg/d (~52 crops)
- Red beet	270 g DW/d	648 g DW/d	8%	8,1 kg/d (~34 crops)
- Wheat straw	270 g DW/d	648 g DW/d	100%	648 g/d

In the MELiSSA CI, a total DM concentration of 21 g/L in the influent is used. It is advised to use the same concentration in BELISSIMA. With higher concentrations, the mixing and circulation by pumps of the influent is difficult because of the presence of plants particles. The proposed mission scenario implies the feeding of about 2.5 kg/d of total dry matter. To use the same concentration, this means that the flow of influent fed should be of about 120 L/d (including the 9 L of urine daily produced). In practice, BELISSIMA will be representative of this scenario, though not at full scale. Currently, downscaling to a daily flow of about 5 l/d is envisaged, or a scaling factor of about 24.

The substrate contains particulate material. The size of the particles must be minimised to ensure an efficient hydrolysis of the substrate. The membranes that will be used are tubular membranes and have an internal diameter of 8 mm. A particles size smaller than 2 mm is the objective to avoid clogging of the inlet of the membranes. Particles are present under different shapes that result from the initial composition of the different wastes and the grinding method used. Grinded wheat can for instance mostly present fibrous particles. Therefore the size criteria must be applied to the maximum length of one particle.

The substrate must be collected, treated, stored and fed in a homogeneous mixture in order to guarantee the influent quality. Because of their difference of properties, the different materials used in the influent cannot be grinded by the same technology. Especially wheat straw, which is dry, needs a specific mill grinder and needs to be grinded apart from the other fresh materials. These fresh materials can possibly be grinded with water. Usually, finer particles can be obtained using smaller lab-scale techniques. These techniques have the disadvantage to be time-consuming. On the other hand, bigger-scale techniques allow some automation and treat bigger amounts of material. They therefore consume less time, but the particles size is often bigger. A compromise therefore needs to be made, depending on the amount of substrate that will need to be prepared, which is directly related to the bioreactor size.

3.3 Definition of strains

Table 21 summarizes the strains to be used within the different compartments

Table 21. Overview of strains within the different compartments

Compartment	Strains	Details
CI	*Mixture of bacteria*	fermentative bacteria acidogenic bacteria acetogenic bacteria
CII	*Rhodospirillum rubrum*	ATCC 25903
CIII	*Nitrosomonas europaea* *Nitrobacter winogradskyi*	ATCC 19718 ATCC 25391
CIVa	*Arthrospira platensis*	PCC 8005

In case of calamities and loss or die-off of biomass from a certain compartment a back-up culture should be available. If needed, the compartment must be emptied, sterilized and reinoculated with the back-up culture. Culture conditions of the back-up culture should be as close as possible to the operational conditions in the actual BELISSIMA loop. For CII and CIVa, a back-up culture at 1/10 of the BELISSIMA bioreactor volume is considered sufficient. For the slow growing nitrifiers a larger back-up culture will reduce the start-up times.

3.4 Sampling conditions

3.4.1 AXENY

Compartments CII to CIVa are operated under axenic conditions. Part of the research plan will be devoted to the study of axenicity and the transfer of micro-organisms through the loop. The introduction of micro-organisms in the loop through various manipulations should therefore be minimized.
Specific remarks per compartment on sterility were given in previous paragraphs.

3.4.2 FREQUENCY AND SAMPLE VOLUMES

Within the BELISSIMA-study the behaviour of several micro-compounds will be monitored throughout the reactor. Details on sampling were given already in previous technical notes TN80.11 till TN80.15. Table 22 reviews the frequency and sampling volumes as stated in TN80.11, paragraph 4.5, TN80.12, paragraph 2.6 and 4.6, and TN80.14, paragraph 6.11. Only those study items that were given a priority ranking 1 or 2 in TN80.15 are listed.
For all four described compartments, these volumes must be added to the given volumes for general follow-up of compartment performance.

Table 22. Review of sampling volumes of specific BELISSIMA analysis

Study item	Analyse	Sample volume	Frequency
Genetic stability	flow cytometry	5 ml	intensive
	2D-proteomics	2 x 50 ml	
	genomics	2 x 80 ml	
Axenicity	flow cytometry	5 ml	intensive
	proteomics	2 x 10 ml	
	genomics	2 x 10 ml	
Bacteria	classic culture or molecular	100 ml	weekly or intensive
Viruses	first concentration, then: cell cultures, molecular methods, immunoassays	few liters	weekly or intensive

Study item	Analyse	Sample volume	Frequency
Fungi	culture based methods	100 ml	weekly
Minerals	ICP or AAS or Anion Chromatography	15 ml 80 ml 0,5 g biomass	daily weekly once per 2 weeks
Human sex hormones	bioanalytical techniques selected bioassays	75 ml - 1 l for CI 1 l for all other compartments 0,5 g biomass	immediately after urine addition at T0-T4-T8-T24-T48-T96-T144 (CI), later on 2-1x/week occasionally
Phytohormones	depends on compound	large volumes	weekly
Pharmaceutical drugs	depends on compound	75 ml - 1 l 0,5 g biomass	immediately after urine addition at T0-T4-T8-T24-T48-T96-T144, later on 2-1x/week occasionally
Toxins	immunoassays cell cultures	large volumes	weekly
Plasmids		25 ml	weekly

3.4.3 CONSERVATION AND TRANSPORT

As extensively described in the technical notes 80.11 till 80.15, the method of conservation of the samples depends on the type of analysis.
Axenic conservation will be required for all parameters. Samples that have to be stored before genetic analysis should be frozen at -80°C. Most other samples can be stored at 4°C before use. Samples should be protected from light and should not be stored longer than 8-24 h.

3.5 Protocols

A distinction should be made between continuous operation and follow-up during the test phases on the one hand and the intermediate periods on the other hand. During the

intermediate phase the major objective will be to maintain normal operation conditions or to restore the compartment to normal operating conditions.

Protocols will vary depending on whether the system is in start-up, under normal operating conditions, in maintenance or whether an emergency occurs. Start-up should guarantee an optimal growth of inocula and transition towards normal reactor operating conditions through control of microbial growth, adaptations in light intensity, increasing reactor loads, etc.. Maintenance includes protocols for replacement and calibration of instruments, sterilisation frequencies, manual operations, access to the compartments, etc.

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