MELiSSA

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1. Introduction

Within BELISSIMA, a small-scale MELiSSA loop will be constructed to monitor the behaviour of various microcompounds in this model closed loop system. In TN80.11 till TN80.14, an inventory has been made of possible study items, including genetic stability and axenicity, minerals, hormones, pharmaceutical drugs, other biosafety issues and countermeasures for microcompounds. All these studies cannot be performed within the current contract but the loop design has to be such that it accommodates for all these investigations on the long term.
In this TN, we will summarize the potential study items, analytical methods and their design requirements. The topics will be given a priority ranking and taking into account certain incompatibilities, a logical sequence of experiments and associated integration strategy will be proposed.
In preparation of this TN, the selection of microcompounds, the proposed analytical methods and a research plan were discussed with external experts during a workshop. This TN reflects the outcome of the workshop which are extensively documented in the minutes of the meeting.

2. Inventory of study items and analytical methods

The paragraphs below are a summary of TN80.11 till 80.14.

2.1. Genetic stability (TN80.11)

Various types of interaction of microcompounds with micro-organisms exist that may affect genetic stability. Micro-organisms have been shown to possess binding capacity for hormones. Some synthetic hormones possessed antibacterial action, whereas natural estrogens had no significant effect on growth and androgens promoted or inhibited growth of selected bacteria. Microbial decomposition and transformation of steroid hormones has been reported. Some plants produce phytoestrogens which protect against fungal and bacterial pathogens. Widespread use of antibiotics has increased bacterial resistance against antibiotics and introduces shifts in microbial ecosystems. Horizontal gene transfer will be discussed in 2.8.

Information on the influence of micropollutants on plants is rather limited. However, in some cases plants produce metabolites from particular organic pollutants which have estrogenic activity.

Evidently, both plants and micro-organisms need minerals and trace elements for optimal growth. Both shortages and surpluses may induce stress and inhibit growth.

The stresses to be studied in BELISSIMA include shortages and surpluses of minerals, the presence of hormones or pharmaceutical products, etc. Other types of stresses (e.g. pH, oxidative stress, temperature, etc.) occur as well, but are not the scope of BELISSIMA. They are studied in the framework of MELGEN.

Methods to measure metabolic stability and stress response on MELiSSA organisms have been developed in MELGEN. Flow cytometry turned out to be a fast means to obtain information on physiological status and metabolism in the presence of certain stresses. 2D-proteomics can be used to detect protein modifications at the molecular level in stress conditions. This approach has been initiated for the MELiSSA strains *Rhodospirillum rubrum* and *Arthrospira platensis*. The third technology which has been used before is MALDI-TOF-MS. It can be used to monitor physiological changes in intact cells, under specific conditions.

2.2. Axeny (TN80.11 and TN80.14)

Compartments II to IVa in the MELiSSA loop are axenic. It is important to control their axenicity because contaminants may lead to instabilities and even collapse of the closed loop system. Furthermore, the *Arthrospira* and *Rhodospirillum* biomass may become unsuitable for human consumption. Loss of axenicity may lead to problems of horizontal gene transfer (see 2.8), problems with toxin production (see 2.6) or the presence of pathogens.

MELiSSA bacteria may escape from their intended compartment and cause severe problems in other parts of the loop. Particularly the consortium in compartment I will probably be the major source of contamination and a likely source of human or plant pathogens. Candidate pathogens may become highly virulent after repeated passage through a human host. Plant pathogens may affect food quality.
Contamination of the MELiSSA loop with organisms appearing in the air or surfaces of the space vehicles or organisms from the human body can occur when feed is introduced to or waste removed from the loop, when filters need to be replaced, etc.

Biological contaminants can be bacteria, viruses, protozoa or fungi. Contamination of the MELiSSA cycle with higher organisms is unlikely. The feed entering compartment I will inevitably consist of fecal microbiota of healthy persons and of microbiota residing on fresh vegetables or linked to the rhizosphere. In relation to human or plant diseases, pathogens will appear as well. Human pathogens that spread feco-orally include bacteria, viruses and protozoa. The majority of plant diseases are caused by fungi, and to a lesser extent by bacteria.

Even though no known phages have been described in literature, the MELiSSA organisms should be checked for the presence of phages which could revert to the lytic mode of life under certain conditions and hence present a risk to the closed loop.

Finally, in view of the unknown composition of compartment I, unusual organisms such as nanobacteria, or hitherto unknown species, may occur in the MELiSSA loop.

Methods to check axenicity, vary from classical cultivation on solid media or in broth, microscopic techniques, flow cytometry, to molecular techniques. Flow cytometry seems suitable to detect single cell organisms against a background of *Arthrospira*, but not to distinguish contaminants from *Rhodospirillum rubrum*. Proteomics could be used for the characterization of contaminants in the MELiSSA compartments as well. Molecular tools are suitable mainly for well-known pathogens. They are more difficult to use when the identity of the contaminants is not known.

2.3. Minerals (TN80.12)

A multitude of minerals is essential for biochemical and physiological functions of the MELiSSA organisms. The main aim of BELISSIMA is to study mineral uptake by the biomass in the different compartments, and to detect shortages or excesses. Particularly the slow growing nitrifying bacteria and some microbial communities in compartment I (e.g. acetogens) may be very sensitive to the mineral composition of the media. Release of minerals due to biomass lysis, is possible in compartment I.

In addition to biological processes, other types of transformations may occur as well. Concerning the micro-elements, the multivalent ones have the highest potential for non-biological transformation. The presence of carbonates and phosphates and elevated temperatures may lead to precipitation reactions in the first compartment. High levels of carbonates in compartment IVa may have a similar effect. As a result of complexation reactions, ions may be retained by the membrane filtration unit in compartment I. If metals occur in the feed, they may adsorb on the biomass of compartments II and IVa, affecting its nutritional quality. Monovalent ions are expected to flow through the compartments. However, Na, K and Cl are important for the higher plant compartment, for overall salinity and toxicity and for charge balances.

Analytical methods in the required concentration range are Inductively Coupled Plasma Emission Spectroscopy (ICP), Atomic Absorption Spectrophotometry (AAS), etc. for the cations. For the anions, anion chromatography is a suitable technique. Determinations on solid fractions are preceded by microwave digestion.

2.4. Hormones (TN80.12)

Both hormones of natural origin and the synthetic hormone can be present in the feed to compartment I. Natural female and male sex hormones (estrogens and androgens) as well as the synthetic hormone used in the female anticonceptive pill (i.e. 17α-ethinylestradiol) are most likely to occur. Data on their biodegradability varies for the natural hormones. 17α-Ethinylestradiol is generally considered to be recalcitrant. Except for the parent

compounds, metabolites or conjugated compounds can be present or may appear during degradation or transformation in the MELiSSA loop. Deconjugation may occur as well, although it is not clear to what extent the microbial population in compartment I and the other MELiSSA organisms have the potential to do so.

Bioanalytical techniques screening for total mass balances allow hormone measurement in the required concentration range. Selected assays are available as well, to detect the spectrum of specific hormones. In addition, analytical methods (LC-MS) have been developed to measure concentrations of specific compounds.

Other human hormones are the thyroid and corticosteroid ones. Since their environmental effects have not been well studied and no negative influences have been documented, they seem less relevant for the BELISSIMA study than the sex hormones.

Phytohormones occur naturally in all plant material and may be introduced with the plant material in compartment I. In an open loop, the feed to compartment I will be a source of phytohormones. If they are not degraded in compartments I to III, they will reach compartment IVb, and they can interfere with normal growth of the higher plants. In a closed loop system, the phytohormones produced by compartment IVb itself will be fed to compartment I and may accumulate in the loop.

As yet, we can only speculate on the effect and fate of hormones in the loop, as biological pathways at thermophilic conditions are to a great extent unknown.

2.5. Pharmaceutical drugs (TN80.12)

Major inputs of pharmaceutical drugs and their metabolites will occur through urine and fecal material. The number and type of drugs allowed on a space mission is strictly limited. These include antibiotics, anti-inflammatory agents and aspirin.
Since it will not be possible to study all pharmaceuticals, a selection was made as follows. We first compiled monitoring data for pharmaceuticals on earth and then crossed them with the active substances of pharmaceuticals present in space medical kits. These were further narrowed down to a selection that represented various classes of compounds with various physicochemical properties and biodegradability. The proposed set of substances consisted of the antibiotics sulfamethoxazole (sulfonamide) and ciprofloxacin (fluoroquinolone), the analgesic/anti-inflammatory compound diclofenac, the β-blocker metoprolol and the antidepressant diazepam. During the workshop, the experts suggested to also include the anti-inflammatory compound acetylsalicylic acid (aspirin) to the selection (TN80.12).

Since monitoring data are available, analytical methods for detection of these compounds have been developed. The monitoring of these pharmaca is commonly performed in aqueous media and occurs through automated extraction followed by LC/MS.

Determination of the intermediates of these pharmaca and the fractions adsorbed to the solid matrices present in different MELiSSA compartments, remains a largely uncharted territory.

2.6. Microcompounds introduced by MELiSSA organisms (TN80.11 and TN80.14)

All groups of cyanobacteria are known to produce toxins. Their production by the MELiSSA *Arthrospira* strain should therefore be tested. Similarly, the production of bacteriocins has been described for non-sulphur purple photosynthetic bacteria and may thus be relevant for *Rhodospirillum rubrum*. Bacteriocins are proteinaceous toxins produced by non-pathogenic bacteria to inhibit the growth of similar bacteria.
Fungi, e.g. those found on fresh vegetables, may produce mycotoxins.

Quorum sensing bacteria produce chemical signal molecules, which bacteria use to synchronize certain behaviour. Some signals cause plant pathogens to switch to an infective stage. The presence of some of these pheromone-like substances can be determined.

The only pesticides which could enter the MELiSSA loop would be the ones allowed in biological farming. This is mostly copper or insecticidal or fungicidal extracts from plants.

2.7. Microcompounds introduced by crew (TN80.11)

Lists of personal care products used on space missions are available. However, grey water and tissues - the most probable sources of these products - are not fed into the MELiSSA loop and are not further considered here.

2.8. Non-conventional transmissible agents (TN 80.11 and TN80.14)

Non-conventional contaminants of biological origin can disturb the genetic stability of the MELiSSA compartments and may contribute to horizontal gene transfer. Three mechanisms of horizontal gene transfer have been described:

- Transformation is a process in which bacterial cells incorporate naked DNA from their surroundings. This can be achieved in test tubes and requires the presence of competent cells. This process is likely to occur in the MELiSSA loop, particularly under the extreme conditions of compartment I.
- Phages can incorporate small DNA fragments from a first host and inject them in a second one after infection. This process is called transduction and has been demonstrated in several natural environments.

- Plasmid-mediated conjugative transfer is probably most important in confined environments. Especially, conjugative plasmids with a broad host range may present a problem because they are not only able to disseminate the genes they carry, but can also mobilize other plasmids or trigger the release of transposable elements or genes (which can be related to virulence, antibiotic resistance, toxin production). Among the MELiSSA strains *R. rubrum* seems most permeable to plasmid-mediated gene dissemination. Its position just downstream of compartment I may enhance this feature.

Methods to study horizontal gene transfer are available.

With respect to free genetic elements, the uptake of free RNA or proteins by bacteria has not yet been described in literature. As opposed to RNA, free DNA and plasmids may be more stable and cycle through the loop. Their uptake as soluble substances can be achieved in test tubes but requires specific conditions to occur in 'natural' environments. Plasmid-mediated transfer of genes rather than the direct uptake of free plasmids is expected to be much more relevant for further study.

Prions are structurally proteins, but they are highly resistant towards classical inactivation and sterilization methods. According to recent literature, they are common in nature and may have beneficial or detrimental effects (oral communication Pechère). However, the consequences of their abundance are not known and no monitoring methods are currently available. The probability of prion occurrence in the loop is considered to be low. In any case, they have not been linked to pathogenicity.

2.9. Hormones and pharmaceutical drugs countermeasures (TN80.13)

Countermeasures include both physicochemical and biological techniques which may be implemented in the MELiSSA loop when e.g. accumulation of specific microcompounds has been demonstrated. Biological techniques require the availability of micro-organisms capable of their degradation and can be highly specific. Physicochemical techniques such as membrane filtration or sorption processes are typically not selective and will remove all microcompounds with similar properties. Other types of countermeasures may consist of preventive measures, e.g. separate treatment of waste from crew members temporarily taking drugs, avoidance of recalcitrant drugs and hormones, or pretreatment of the feed to the MELiSSA loop, e.g. pretreatment of urine or waste.

3. Priority ranking

3.1. Selected microcompounds

Based on discussions with external experts the following set of microcompounds was selected for further study

- micro-organisms: bacteria, viruses, fungi, protozoa, higher organisms, unusual organisms
- minerals
  - macro-elements: P, S
  - cations: Na, K, Ca, Mg, Mn, Fe, Zn, Cu, Co, Mo, Se, W
  - anions: Cl
- hormones: natural male and female sex hormones, synthetic female hormone, thyroid and corticosteroid hormones, phytohormones
- pharmaceutical drugs
  - antibiotics
    - sulfonamide: sulfamethoxazole
    - fluoroquinolone: ciprofloxacin
  - analgesic and anti-inflammatory agents: acetylsalicylic acid and diclofenac
  - β-blocker: metoprolol
  - antidepressant: diazepam.

The choice of countermeasures has not been finalized. This will largely depend on the results obtained for the various microcompounds in the MELiSSA loop. Current knowledge indicates that the synthetic hormone 17α-ethinylestradiol is recalcitrant, that diclofenac is poorly degradable in aerobic conditions and that ciprofloxacin is mainly removed through sorption. So these may be suitable candidates for further study of countermeasures.

3.2. Prioritization

The list of study items of 3.1 will be ranked according to priority. Two criteria will be used to achieve this. The first one relates to the availability of well-described analytical methods for quantification of the target compounds. The second one estimates the relevance of the study item for BELISSIMA. The different topics were then given a priority score. Priority 1 is the highest priority and is given to topics for which analytical methods are available and which have high relevance for further study. Priority 3 is the lowest score and is assigned to topics for which no method is available or quantification is difficult and which have low relevance for BELISSIMA. Study items with intermediate relevance received a priority 2 score.

Table 1 describes the analytical methods and relevance per topic as well as the final priority scores.

3.2.1.Genetic stability and axenicity

For the study of genetic stability, methods have been tested in MELGEN. Four methods can be used, each providing a different level of detail on changes in the micro-organism concerned. Relevance for BELISSIMA and for MELiSSA in general is high because shifts in the metabolism or physiological status may affect the kinetics of the MELiSSA organisms and the overall performances and therefore control of the loop.

Three MELiSSA compartments are operating under axenic conditions, CII till CIVa. Loss of axenicity may lead to collapse of the compartment. In addition, the potential presence of pathogenic contaminants will affect nutritional quality of the biomass in compartments II and IVa. Methods for axenicity assessment have as yet been tested in MELGEN for *Rhodospirillum* and *Arthrospira*. The main problem consists of detecting contaminants against a high background of the 'indigenous' organism. This has not yet been completely resolved.

When the different classes of micro-organisms are considered, the availability of analytical methods as well as their relevance for BELISSIMA varies largely. Protozoa and higher organisms are generally aerobic and grow optimally at mesophilic temperatures. Hence, they will not survive the conditions in compartment I. Furthermore, they will be retained by the ultrafiltration membranes used for solid-liquid separation in compartment I, since the pore diameters of 50 nm are smaller than the cell dimensions. The same is true for fungi but their relevance for BELISSIMA is somewhat higher because they have the capacity to form spores and may also form toxins. Both types of products will not be retained by the membrane filtration unit. Fungi therefore were given a priority score 2 as opposed to protozoa and higher organisms.
Bacteria and viruses have the highest priority for further study. Both classes will continuously enter the first compartment through the feed. They will mainly be mesophilic species coming from the gastro-intestinal tract or contaminants of the plant material. Although their numbers will strongly be reduced under the pasteurising conditions in compartment I, total elimination is not expected due to the high initial numbers. Bacteria will additionally be retained by the membrane to a large extent, but may still appear in the permeate. Some bacteria may form spores (particularly under extreme environmental conditions) and toxins as well, which make them highly relevant for further study. Compared to bacteria, reduction of viral counts will be much lower, due to their smaller dimensions and to their resistance to adverse environmental conditions.
The class of unusual organisms gets the lowest priority because we very often do not know what we have to look for and no defined analytical methods are available.

Table 1: Priority ranking of microcompound study items in BELISSIMA. Italics refer to general study items, which are further subdivided when relevant. CI: compartment I of the MELiSSA loop.

Study item	analytical method	relevance for BELISSIMA	priority
genetic stability	*flow cytometry* *2D-Proteomics* *Transcriptomics* *MALDI-TOF-MS*	*high because system behaviour and control model rely on it*	*1*
axenicity	*flow cytometry* *proteomics*	*high due to potential impact on food production and quality*	*1*
bacteria	classical culture methods molecular methods	high due to high input in CI and high potential for transfer through loop	1
viruses	first concentration step then cell cultures, molecular methods, immunoassays	high due to high input in CI and high potential for transfer through loop	1
fungi	culture-based methods	intermediate, these aerobic organisms will probably not survive in CI but may form spores and toxins	2
protozoa	first concentration step then immunoassays, microscopy, etc. cysts very difficult to measure	low because they will hardly survive the thermophilic anaerobic conditions in CI and are retained by the ultrafiltration unit	3
higher organisms	various depending on target organism	low, not expected in the loop	3
unusual organisms	mostly not available	low because we do not know what to look for	3
minerals	*ICP* *AAS* *Anion chromatography*	*high in view of mass balances, growth kinetics, potential toxic effects*	*1*
hormones			
human sex hormones	bioanalytical techniques selected bioassays	high because always present in urine	1
other human hormones	methods available	low because no environmental effects documented	3
phytohormones	available for selected compounds	intermediate because presence in feed to CI not known	2
pharmaceutical drugs	*various techniques depending on compound properties*	*high, but not continuously present in feed to CI, only during illness of crew*	*1*
other microcompounds introduced in the MELiSSA loop			
toxins	immunoassays cell cultures	intermediate because toxin production by micro-organisms in MELiSSA loop is not documented	2

Study item	analytical method	relevance for BELISSIMA	priority
pesticides	various techniques depending on compound considered	very low because they are not expected in the feed to CI	3
microcompounds introduced by crew	various methods depending on compounds considered	low because grey water not fed to MELiSSA	3
non-conventional transmissible agents			
free RNA	available	low because not stable	3
free DNA	available	low because competent cells needed for uptake	3
plasmids	available	intermediate: direct uptake by micro-organisms not documented, but conjugation possible	2
proteins	available	low because degraded by proteases	3
prions	not available for water samples	low because no human pathogenicity documented	3

3.2.2.Minerals

Analysis of minerals in the required concentration ranges can be achieved through ICP, AAS or graphite furnace determinations. For anions, anion chromatography is suitable.
Relevance of mineral studies in BELISSIMA is very high. Up to now, mass balances have only been made for C, H, O and N. For the macro-elements P and S, only preliminary data are available. More extensive data are needed to further update the stoichiometry of the reactions in the different compartments and to study mass balances through the loop. For the micro-elements, it is current practice to use synthetic media which have not been optimised for trace element composition. Micro-element utilization and requirements have been studied to some extent in compartment IVa, but more research is needed to detect shortages or surpluses and the stresses they may cause. For these reasons, mineral analysis has the highest priority in BELISSIMA.

3.2.3.Hormones

Of the different groups of hormones, the human sex hormones have the highest relevance for BELISSIMA. Indeed, assuming a mixed crew, the organic waste will always contain the natural male and female hormones. Providing that the anticonceptive pill is going to be used, the synthetic female hormone will be continuously present as well. For other human hormones, no (negative) impact on the ecosystem has been documented. Therefore, their relevance for study in the loop is low. For phytohormones, the situation is intermediate. They may be introduced in the first compartment with the plant material, but there is currently no hard evidence to confirm this.
In terms of analytical methods, a wide range of mechanism-based bioassays and targeted quantification methods is available for human hormones. For the phytohormones, methods have been described for a limited number of compounds.
As a result, human hormones get the highest priority for research, as opposed to other human hormones and phytohormones.

3.2.4.Pharmaceutical drugs

Many research papers report on the environmental concentrations of pharmaceuticals all over the world. This implies that methods have been developed for their analysis. This is also the case for the six pharmaceutical compounds selected in BELISSIMA. All of them appear in medical kits for space missions. During periods of crew illness, these compounds will appear in the loop and their discontinuous presence may lead to stresses on the microbial populations. Antibiotics in particular have bactericidal or bacteriostatic effects. For the other compounds, the effect on the MELiSSA organisms and their degradability in the loop remains to be studied. Pharmaceutical drugs therefore get a priority 1 score.

3.2.5.Other microcompounds

Other microcompounds introduced in the MELiSSA loop, consist of toxins, pesticides and personal care products used by the crew.
Toxins may originate from bacteria and fungi in the loop. At present, toxin production by the MELiSSA organisms is however not documented. Therefore, its relevance for the BELISSIMA study is considered to be intermediate, e.g. when compared to the study of bacteria themselves, and toxin analysis ranks second in terms of priorities.
Pesticides and personal care products have the lowest priority. On the one hand, the input of pesticides is very low since the plant material for compartment I will be purchased from biological agriculture. On the other hand, personal care products typically end up in the grey water stream which is not being directed into the MELiSSA loop.

A final category of microcompounds with potential relevance in terms of biosafety, are the non-conventional transmissible agents. For various reasons, all these agents are considered to have the lowest priority for BELISSIMA. By themselves, these agents do not present a biosafety risk in MELiSSA. Free RNA and proteins are not stable and will be removed through the action of released nucleases and proteases. Free DNA, plasmids and prions are more stable. All these agents may however be involved in horizontal gene transfer and exchange genetic information with host cells. Uptake of free RNA, proteins or plasmids by bacteria is not documented in literature. Uptake of free DNA by cells requires contact with competent bacteria under rather extreme environmental conditions. Plasmids may be exchanged between bacteria through conjugation, e.g. from contaminants to *Rhodospirillum* in compartment II. Prions seem to be ubiquitous in nature, but have not been linked to pathogenicity.
For free DNA, RNA, proteins and plasmids, quantification in aqueous samples is possible. This is not the case for prions.

4. Sampling requirements and incompatibilities

In this paragraph the impact of microcompound studies on sampling in the BELISSIMA loop will be evaluated. Most of the priority 3 compounds were assigned the lowest category because no analytical methods are currently available or because they are expected to occur in very low concentrations or at low probability. They will therefore not be included in this evaluation. Indeed, it can be expected that the sampling volumes required for their analyses will be even higher than for the first priority compounds. Hopefully, new analytical methods will develop in the future which will then allow their study at higher sensitivity and with lower sampling volume requirements.

Table 2 and Table 3 give an overview of sampling volumes, sampling frequency, conditions and incompatibilities for priority 1 and 2 study items respectively. Sampling volumes quickly add up to large volumes, when the target compound is present in low concentrations. Even when tests will be performed at fairly high concentrations at the input (through spiking), the outlet concentrations may be much lower due to sorption, degradation, etc. Since the outlet of one compartment is the inlet for the next one, operation in closed loop will become very difficult at laboratory scale. Tests with coupled compartments II to IVa[1] at UAB were performed at respective reactor volumes of 2 l – 0.5 l – 2.5 l.

Relative reactor volumes for the MELiSSA loop compartments at pilot scale have been determined for a different scenario than the one used in BELISSIMA (namely 20% of the diet of 1 man produced by the MELiSSA loop) but provide an order of magnitude of reactor volumes and associated liquid flows. Estimated reactor volumes were 100 l for compartment I, 50 l for compartment II, 8 l for compartment III and 77 l for compartment IVa (information from UAB). From these numbers it is evident that operation in coupled set-up at laboratory scale does not even allow for samplings of 0.5 l. At pilot scale, the throughput of liquid will be around 10 l/d for compartment I. So even here, samplings of 2 and more litre per day would seriously affect liquid loop closure. When this can be reduced to 2 litre per week, collected over different days, a high degree of loop closure[2] is feasible. In addition, sampling for general reactor performance monitoring should not be forgotten. This amounts to e.g. 300 ml of liquid per week for compartment I.

[1] In coupled compartments the outlet of one compartment is used as inlet for the next one, but the outlet of the last compartment is not recycled to the first one

[2] Closed loop operation means that the outlet of the last compartment is recycled to the first one

Table 2: Sampling requirements for Priority 1 study items. In principle, the volumes stated allow for duplicate analysis.

study item	sample volumes/ frequency	sampling conditions	incompatibilities
genetic stability	flow cytometry: 5 ml 2D-proteomics: 2x50 ml genomics: 2x80 ml total: 265 ml intensive sampling campaign	axenic store at -80°C	only one type of stress to be investigated at a time
axenicity (overall)	flow cytometry: 5 ml proteomics: 2x10 ml genomics: 2x10 ml total: 45 ml intensive sampling campaign	axenic store at -80°C	
bacteria	100 ml, but 1-3 l when low concentration of pathogens weekly or intensive sampling campaign 2 g of biomass per week	axenic glass or plastic bottles store at 4°C if needed	difficult in closed loop at lab-scale for low concentrations
viruses	several l depending on concentrations weekly or intensive sampling campaign 2 g of biomass per week	axenic glass or plastic bottles store at 4°C if needed	difficult in closed loop at lab-scale for low concentrations
minerals	15 ml daily, 80 ml weekly, 0.5 g biomass intensive sampling campaign	axenic homogeneous sampling	
human sex hormones	immediately after spike: samplings at T0-T4-T8-T24-T48-T96-T144 from CI Otherwise: 1 l for all compartments, with frequency 2-1x/week biomass occasionally	store at 4°C in the dark	cannot be combined with bacterial or viral analyses
pharmaceutical drugs	immediately after spike: samplings at T0-T4-T8-T24-T48-T96-T144 from CI Otherwise: 1 l for all compartments, with frequency 2-1x/week biomass occasionally	axenic store at 4°C or freeze	difficult in closed loop at lab scale

Table 3: Sampling requirements for Priority 2 study items.

study item	sample volumes/ frequency	sampling conditions	incompatibilities
fungi	100 ml at high fungi concentrations, otherwise several l once per week	axenic store at 4°C	difficult in closed loop at lab-scale for low concentrations
phytohormones	probably large volumes needed for detection once per week	?	difficult in closed loop at lab-scale
toxins	probably large volumes needed for detection once per week	?	difficult in closed loop at lab-scale
plasmids	25 ml once per week	axenic store at 4°C	

Connecting compartments should occur in two steps. First, the compartments I to IVa should preferably be connected without closing the liquid loop. Second, the loop should actually be closed. As reported in TN80.11 till 80.14, samples are ideally taken from influent and effluent of each compartment. In coupled configuration without loop closure, sample withdrawal in between compartments could be accommodated by gradually reducing the water flow from compartments I to IVa. In a closed loop, this is of course not possible, except when additional water is continuously supplied to close the water balance. Even when the loop is operated under stepwise reduced flow rates, we propose to limit sample volumes to 15% of the total flow at maximum to avoid too strong size and/or dilution effects.

The sampling volume could be reduced by performing tests under 'crashing' conditions. The maximal concentration expected for a particular microcompound during a typical Mars mission could be calculated for specific scenarios. If this concentration were spiked, the system response in a worst case scenario could be evaluated. The relatively high concentrations would allow for lower sample volumes. In addition, studying the different microcompounds separately and sequentially rather than simultaneously, will reduce the overall sampling volumes.

For biomass sampling the situation is more limiting. Obtaining 2 g of biomass sample from a culture of around 1-1.5 g/l as in compartment II and IVa, means withdrawing a volume of 1 to 2 l of mixed liquor. On a total reactor volume of several litres this is unacceptable. In continuous culture and using individual compartments rather than a coupled configuration, the effluent would contain the same concentration of biomass as the reactor, provided that a completely stirred reactor concept is used. This would allow biomass sampling without affecting reactor operation. Other options could be to operate the compartments at maximum biomass concentrations to reduce the overall sampling volume or to collect the total required biomass sample through a combination of small

daily subsamples. Biomass sampling and analysis is probably less critical in compartment I, where accumulating solids have to be wasted on a regular basis to maintain the solids concentration below 5%. However, separation of the biomass from other solid particles will be difficult. In compartment III, frequent biomass sampling is impossible because it is conceived as a fixed bed reactor and the nitrifying bacteria are very slow growing. Sampling through removal of carrier material would disturb the flow patterns and presents a danger of contamination. Analysis should therefore be limited to the suspended biomass appearing in the effluent after backwashing. Or appropriate measures have to be taken to be able to remove carriers in a non-disturbing way through sampling ports at different heights.

Most microcompounds for which intensive sampling campaigns are desirable, require lower sample volumes than the ones with lower sampling frequency (see Table 2). Intensive sampling campaigns should therefore preferably be performed with the individual compartments, rather than with the coupled set-up. Sampling frequency is mostly determined by the dynamics and hydraulics of the system, e.g. the hydraulic retention time. The first approach in BELISSIMA would be to focus on microcompound behaviour during steady state behaviour, and not on transition stages, in particular for those microcompounds which require larger sample volumes. Indeed, steady state operation allows to reduce the frequency of sampling without affecting significance and relevance of the data obtained. The study of transitional behaviour on the other hand, requires intensive sampling. After discussion with MELiSSA partner UBP, it seemed most appropriate to first focus on stoichiometry and mass balances for e.g. uptake of minerals. The study of dynamics is not considered achievable at this stage.
Rationalization of samplings will however be needed. In general, once a coupled set-up is in operation, samplings between compartments will have to be reduced in frequency, compared to sampling of the inlet and outlet of the interconnected compartments. For the genetic stability study, a total analysis would require a maximum volume of 265 ml of mixed liquor for each sampling occasion. In coupled set-up, this volume can be rationalized by reducing the sampling frequency or by selecting the most appropriate analytical tool, rather than applying all of them. This selection of methods could be made through preliminary tests on separately operated compartments or in batch cultures.

To be able to study genetic stability and the effect of stress factors, only one variable can be changed at a time. In this respect, the use of urine in a first step may not be appropriate because it is a complex mixture of minerals and organics, in variable concentrations. A more gradual approach could consist of testing individual hormonal compounds first, and then testing real urine of selected donors. This must somehow be reflected in the research plan. Furthermore, the genetic stability and axenicity studies will preferentially be performed simultaneously with tests on a specific microcompound for maximum efficiency. If this is a problem in terms of sampling volumes, it might be more feasible to only take samples after finalization of e.g. the hormone or drug monitoring test, rather than throughout the test. When steady states have been achieved and effluent

concentrations no longer change, the biomass will have adapted to the stress factor and show the impact on its metabolism. This does however not allow the study of the dynamics of the adaptation process. In any case, the sampling approach will always be chosen in such a way that a useful and relevant data set is obtained.

Sampling conditions are fairly similar for most analyses and no incompatibilities exist. They should be performed under axenic conditions. The samples should be stored at 4°C for limited periods of time. They can be frozen afterwards when needed. Attention should be paid to homogeneous sampling. This is particularly an issue for biomass. Material for sample collection is preferably (precoated) glass in view of sterility, the minimization of adsorption or release of interfering compounds.

5. Critical issues

A number of issues are critical with respect to the study of microcompounds and the study of a closed water loop in MELiSSA. These include:

- Batch or continuous cultures: batch cultures have several advantages. Due to the absence of a steady state, they will result in significant concentration differences between the start and end of the cultivation, which may be easier to detect than concentration differences in inlet and outlet of continuous cultures. Furthermore, sampling could be limited to the beginning and end of the incubation. However, biomass concentrations, environmental conditions and substrate concentrations will vary continuously. Therefore, preference will be given to continuous cultures. Batch cultivations can be considered as a first step or may be used as additional side studies to the main test plan.

- pH correction: currently, pH in the different MELiSSA compartments has been controlled through the use of the following acids or bases
    - HCl
    - H_2SO_4 because it is less corrosive than HCl
    - CO_2 which is sometimes simultaneously used as C-source or may be required for e.g. the degradation of propionate or butyrate in compartment II
    - NaOH.

  In BELISSIMA the use of the mineral acids and bases may be a problem when mass balances of Na or Cl have to be closed. Either, provisions have to be taken to adequately measure the dosed amounts of acid or base. Or, alternatives could be considered. Acidification can be achieved through CO_2 or organic acids such as formic acid and citric acid. The use of organic acids may however lead to the presence of undesirable side-products or may affect regular reactor performance.

Application of CO_2 is only feasible with synthetic media when the required amount for acidification can be accurately calculated or under conditions when pH varies only slowly. Alcalinisation could be achieved through the use of ammonia. In any case, a slow pH correction system will be needed to avoid overshoots and overdosing of acids and bases. An additional option consists of unidirectional pH correction, requiring either acid or base but not both. This may be relatively easy for certain compartments (acidification in Compartment I) or on well-defined media, but may become more problematic when connecting compartments or closing the loop. It was finally decided to use mineral acids and bases for pH correction. Some freedom in the pH range may be considered to reduce the introduction of acids and bases, as far as normal reactor operation is not disturbed.

- Memory effects in between tests: it cannot be excluded that memory effects will occur in between tests. Spiking experiments with organic microcompounds in particular may lead to e.g. sorption of these compounds to the biomass, toxic effects or alterations in biomass towards atypical behaviour. The sorbing potential of the microcompounds should be estimated from literature data. In the research plan, non-sorbing compounds should be tested before the ones with a high potential for sorbing, to reduce the memory effects. In case sorption is observed, sufficiently long desorption times will have to provided in between tests. Toxic effects may require replacement of the entire biomass and a new start-up of the compartment concerned. This will be most problematic for compartment III, where the nitrifying biomass is very sensitive to negative impacts. Also for connected compartments or in closed loop, toxic effects should be avoided as much as possible. Batch tests may in this respect be useful for early detection of toxicity, before the actual spiking in the feed to a compartment or the loop is performed. General reactor performance will be taken as an indicator to decide whether corrective measures are needed. Several antibiotics have been selected as pharmaceutical drugs for further study in BELISSIMA. Intrinsically, they will affect microbial metabolism, survival and community composition. After spiking tests, sufficiently long recovery periods will have to be provided, or reinoculation will be necessary. Antibiotic tests should therefore be performed at a later stage in the research plan.

- Axenicity: compartments II to IVa 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. If contamination is detected, the compartment needs to be emptied, sterilized and reinoculated with a back-up culture. Apart from general transfer of micro-organisms in the loop, the introduction of contaminants through various manipulations needs to be taken into account as well. All sampling, addition of media, removal of waste, etc. needs to be performed under axenic conditions. Electrode removal and general maintenance of the loop should be possible without affecting axenicity. The same is true for interfaces, such as centrifugation and (membrane) filtration steps.

- Interfaces: apart from their impact on axenicity, the interfaces may also influence the microcompound concentration in the loop. Filtration techniques may lead to partial removal of compounds due to sorption or retention. Centrifugation may lead to the removal of microcompounds e.g. through sorption to the pellet. Heat sterilization may change medium stability or lead to precipitation. Preference will be given to those techniques that have the least effect on medium composition.

- Introduction of chemicals: The addition of chemicals such as disinfecting agents, anti-foam, membrane cleaning products, etc. introduces undesirable organics in the loop and should be avoided. Specifically for medium sterilization, it is clear that a compromise will have to be sought between influence on medium composition and introduction of chemicals. As long as the effect on medium composition can be evaluated through monitoring of microcompound concentration before and after treatment, sterile filtration of media is preferred.
  Study of the mineral balances may lead to shortages in the loop. These will exert effects on microbial biomass, but will be reversible and normal growth can be restored by returning to a more optimal medium composition. When operating with connected compartments or in closed loop, this may however only be achieved by supplementing minerals.

- General reactor operation: during the tests on microcompound impact, general reactor performance must be monitored. Incomplete elimination of e.g. volatile fatty acids (VFA) in the second compartment or production of nitrite in the third compartment may lead to toxic effects in downstream compartments, which may be related to the presence of VFA or nitrite rather than the microcompound. It is important that the distinction between irregular reactor operation and microcompound effects can be made.

- Closing water balances: the smaller the scale of the BELISSIMA loop, the more difficult it will become to close water balances, which is a prerequisite to close mass balances. All liquid losses through sampling and liquid additions through e.g. pH control need to be quantified. Furthermore, evaporation losses may not be negligible. Attention should therefore be paid to quantification of humidity in the gas phase.

- Closing mass balances: to be able to close mass balances, samples from all relevant environmental compartments are needed, such as gas, liquid and solid phase. In addition, parent compounds and potential metabolites must be monitored. The current status of analytical techniques may however not always allow accurate determination in all environmental compartments or of all metabolites. Analysis of organics associated with biomass turns out to be extremely difficult and inaccurate. For most organic microcompounds, either no or very few metabolites have been described in literature and sophisticated analytical techniques are required for their analysis.

- Closing the liquid loop: in a closed loop configuration, the output of compartment IV (a and/or b) will be used to make up the solid waste suspension feeding into compartment I. It is expected that losses through sampling in the BELISSIMA loop will gradually reduce the liquid output of subsequent compartments. In spite of the fact that urine will be added to compartment I as a source of microcompounds, extra water will probably have to be added to close the water loop. However, in case sampling volumes are negligible compared to the overall liquid flow through the loop and no urine addition occurs (temporarily), the outlet flow of compartment IV may be too high for total recycle. Recirculation of microcompounds is the main aim of BELISSIMA, rather than recycle of the total water volume. This could for instance be achieved by concentrating the microcompounds in the outlet of compartment IV through a reverse osmosis step, by discarding part of the clean water and recycling the concentrated microcompound solution to compartment I. The need for a concentration step will be evaluated case by case .

- Presence of biofilms: microorganisms in biofilms may exhibit different growth rates and kinetics compared to suspended biomass. This implies that the uptake and/or transformation of microcompounds may be different as well. It is therefore important that biomass in the different compartments occurs either in the suspended or the attached form, but not as a combination of both. Compartments I, II and IVa are operated as suspended biomass systems as opposed to compartment III, which contains immobilized nitrifiers. In compartment II and IVa the occurrence of biofilms has been observed under certain conditions. For the microcompound study, operational conditions should be chosen so as to minimize biofilm formation. In this respect, a double-jacketed reactor is preferable for temperature control and mechanical mixing is preferred to an airlift.
  Overall, the occurrence of biofilms in tubing and sampling lines should be avoided because these are perfect environments for the establishment of contaminants and/or pathogens.

- Spiking approach: spiking of selected microcompounds will be performed in a way which approaches real conditions. For natural hormones, continuous spiking will be needed. Pharmaceuticals would be spiked for a period that corresponds with general prescription and administration periods. Volumes and concentrations will be precised in the detailed test protocols later on.

- Back-up strategies: during the tests, the risk of compartment contamination exists or the presence of specific microcompounds may lead to irreversible changes in the biomass behaviour. In such cases, the compartment should be reinoculated with back-up cultures. These should be cultivated under optimal conditions and in quantities that allow a quick restart of the compartment.

- Feed preparation: the feed to compartment I consists of urine, fecal material, toilet paper and lettuce, beet and wheat. The size of the particles in the feed must be minimized to ensure optimal hydrolysis in compartment I and should be below 4 mm to avoid clogging of the membrane filtration unit. The substrates must be collected, treated, stored and fed in a homogeneous mixture. Because of their different properties, the different materials used in the influent cannot be ground by the same technology. Especially wheat straw, which is dry, needs a specific mill grinder and needs to be ground apart from the other fresh materials.

- Operation mode during non-working periods of time: in periods when no microcompound tests are performed, basic reactor performance has to be guaranteed or the system should be operated under such a way that it can quickly return to regular operation conditions.

The choice of hardware materials to avoid release of microcompounds is also critical but will be discussed in the design of the BELISSIMA loop.

Regular points of attention for compartment operation are not discussed here, but in TN80.16 on BELISSIMA loop requirements and the TNs on design of the loop. Extra critical issues for microcompound analysis per compartment are indicated below.

- Compartment I
    - Feed composition: the exact feed composition will depend on the scenarios proposed by ESA. The feed currently consists of wheat straw, beet and lettuce, fecal material, toilet paper and urine. For sizing of the MELiSSA Pilot Plant reactors, the fraction of organic matter from plants was fixed in that the higher plants would provide 20% of the diet of one crew member. Within BELISSIMA, different scenarios could be evaluated including higher percentages of plant material in the feed, maximal urine levels not inhibiting anaerobic biological activity, etc. The toilet paper will be of the unbleached type to avoid inputs of microcompounds (ECOVER) and the plant material will be collected from the same organic supplier (BLIK BVBA) as used by EPAS. Recent literature[3] however indicates that even organic vegetables are not entirely pesticide-free, mainly due to take up of historically used and now banned pesticides from soil. Concentrations were however very low (ppt range) and not harmful. This indicates that determination of background concentrations or effects will always be necessary.

[3] Renner, R. (2006). Organic vegetables are not pesticide-free. Environmental Science and Technology, February 15 2006: 1094-1095

- Distinction between biomass and organic matter: to determine the uptake rates of minerals by biomass and to determine the stoichiometry, it will be necessary to measure the biomass concentration and growth. Due to the continuous influx of hardly degradable organic matter in compartment I, accumulation of inert lignine-like material occurs. Organic matter determinations will include both biomass and accumulated organic matter. Alternative quantification or calculation methods will have to be considered to determine biomass growth.
- Biomass composition: because the biomass is a complex mixed community, it has not yet been completely characterized. This complicates the study of contaminants. Comparison with the feed may however indicate to what extent micro-organisms from the feed establish themselves and to what extent other organisms develop to large numbers.
- Inhibiting effects of urine: so far, urine has never been fed in the MELiSSA loop. On the one hand, a N shortage occurs which can be compensated with urine. In the framework of BELISSIMA, urine is indispensable as a source of hormones, pharmaceuticals or their metabolites. Because chances of degradation of these compounds are highest in the waste degrading compartment I, the point of entry for urine was chosen to be compartment I. On the other hand, a too high N concentration will inhibit the anaerobic conversion processes in compartment I. Unionised NH_3 is the toxic compound, the concentration of which will decrease at lower pH but increases at higher temperature. At pH 6.5 and 55°C, the unionised fraction of NH_3 would be around 1%[4]. There is further evidence that adaptation process can take place and that the acidogens were hardly affected in a concentration range of 4000 – 5700 mg/l NH_4-N as opposed to the methanogens.
- Solids drain: due to the accumulation of hardly degradable plant material and to avoid clogging of the membranes at too high solids concentrations, regular draining of solids is required in compartment I. This will provide a removal mechanism for sorbed microcompounds and will therefore have to be monitored.
- Solids sampling: it will be important to take homogeneous samples of solids.
- Membrane cleaning: compartment I is conceived as a membrane bioreactor. Inevitably, the membranes will have to be cleaned with a certain frequency. The use of chemicals will have to be avoided since these may introduce additional microcompounds in the system. For the pilot plant, ceramic membranes have been selected because these allow thermal cleaning.

[4] Speece R.E. (1996. Anaerobic biotechnology for industrial wastewaters. Archae Press, Nashville, TN

- Compartment II
    - Light/dark zones: external conditions such as the light transfer and ratio between dark and illuminated zone may affect biomass composition and synthesis of storage products and hence uptake of minerals or removal of microcompounds.
    - Color of CI effluent: in connected compartments, the permeate of CI will be fed to CII. The CI effluent has a brownish color and this may affect the light absorption in CII.
    - PHB: upon cell lysis, PHB may be released into the medium. This can lead to biofilm formation and hence to non-representative biomass sampling. Biofilms will also reduce the light intensity towards the bulk liquid of CII.

- Compartment III
    - Biomass sampling and quantification: the nitrifiers grow as biofilms on the carrier material. Removal of carrier for biomass investigation should be possible in a way that does not affect the flow patterns in the reactor.
    - Sterilization: the Biostyr carrier material is not heat sterilizable. In combination with the slow growth rate of the nitrifiers, this makes the compartment highly susceptible to contamination. Therefore, the compartment should have the flexibility to use another carrier material e.g. glass beads as well.

- Compartment IVa
    - EPS production: the presence of EPS may affect microcompound removal through sorption. Therefore, operational conditions have to be chosen to ensure a constant EPS production when studying organic microcompounds.
    - Illumination: external conditions such as the light transfer may affect biomass composition and synthesis of storage products and hence uptake of minerals or removal of microcompounds. As opposed to *Rhodospirillum rubrum*, *Arthrospira platensis* does not require an alternation between light and dark zones. However, to obtain maximal efficiencies, dark zones due to sensors and other equipment should be minimized.

- Compartment IVb
    - Associated microflora: no studies have been performed yet to characterize and identify the microbial populations present on the higher plants or in the hydroponic solutions. In case CIVb will be included in the loop, this will be important information to determine what constitutes the natural microflora and when contaminants occur.

6. Research plan

6.1. Logical sequence of tests

Based on discussion during the microworkshop the following series of tests is proposed. Minerals will be tested first because their effects on cell growth and cell condition are expected to be less pronounced than for organics. Furthermore, negative effects due to e.g. mineral shortage are typically reversible and can easily be remediated in the loop. In a next step organic microcompounds will be investigated. Hormones always occur in fecal material and urine and are therefore the first priority. Pharmaceuticals are often dosed for limited periods of time and will therefore be tested next.

- Minerals: within the minerals study the logical sequence of tests would be to first use synthetic media for each compartment. These may not have optimal compositions for the micro-organism concerned, but they would constitute a good reference situation since most tests so far have been performed with them.
    - For compartment I, the feed composition currently consists of wheat, beet and lettuce, fecal material and toilet paper. These make up respectively 25.8, 25.8, 25.8, 14 and 8.6% on dry weight basis and are added as a 2% suspension in water. Within BELISSIMA the relative amounts may change depending on the (mission) scenario to be studied. Urine will be added as well.
    - The synthetic medium for compartment II is the medium of Segers and Verstraete.
    - The synthetic medium for compartment III is from Wijfels.
    - The medium for compartment IVa is Zarrouk medium.

  Attention should be paid to the original composition of the media. In e.g. the medium of Segers and Verstraete, the absence of particular amino-acids may lead to biofilm formation.

  After the tests with the synthetic media mentioned above, a simulated compartment I effluent will be used. This will be a synthetic medium with a composition similar to real compartment I effluent, and will be determined through an extensive characterization of compartment I effluent. Characterization studies are currently planned on the compartment I pilot at EPAS and can be complemented with additional characterization in BELISSIMA.

  The final step will be to use real effluent of compartment I and to transfer the real effluent of one compartment to the next one.

  For each condition, selected minerals will be added in various concentrations as long as these do not lead to precipitation or other medium changes, or at various flow rates, to simulate conditions of shortage or excess.

- Hormones: since urine contains both natural hormones, minerals and other compounds at once, it will be very difficult to relate effects to specific microcompounds. Therefore, we suggest the following test sequence:
    - Measurement of background endocrine activity in the feed to compartment I, in the absence of urine.
    - Tests with pure natural male hormones: the male hormones will be tested before the female ones because their natural concentration levels are less variable.
    - Tests with pure natural female hormones.
    - Tests with pure synthetic female hormone: this compound is expected the most recalcitrant one.
    - Tests with real urine from male donors.
    - Tests with real urine from female donors not using the contraceptive pill: donations will be from healthy women to avoid the presence of pharmaceuticals and metabolites and preferably not during the menstruation period. Again, tests with real urine of male donors may have to be considered as well.
    - Tests with real urine from healthy women taking the contraceptive pill.
    - In between the tests intermediate periods without hormone or urine dosing will be provided to allow the system to recover.

- Pharmaceuticals: these organics have various physicochemical characteristics. We propose to first use polar compounds, because their tendency to adsorb to biomass will be low and the risk of memory effects and replacement of biomass will be lower. In a second step, apolar organics will be tested.

- Axenicity: axenicity control can be performed in parallel with all the other tests. The intermediate periods where no spiking is taking place, can be used as reference situation.

- Genetic stability: the first stresses to be investigated in BELISSIMA are mineral shortages or surpluses, the presence of hormones or pharmaceuticals. These tests are planned as described above. The genetic stability investigation can be performed in parallel with the study of the fate of these compounds.

- Countermeasures: this study will largely depend on the monitoring results obtained for endocrine active compounds and pharmaceuticals. At present, we estimate that the first tests will be performed with synthetic media with a composition similar to the one observed in the BELISSIMA loop. At a later stage, real water samples will be taken from the loop directly. The test scale is basically laboratory-scale but since the type of technology is not known yet, it is difficult to estimate which amount of water will be needed.

6.2. Integration approach

Compartment I is the biodegrading compartment and is therefore expected to have the highest potential for removal or degradation of organic microcompounds. So this will be the point of entry within BELISSIMA for urine or for the spiking of hormones or pharmaceuticals. Because compartment I has never been coupled to the downstream compartments of MELiSSA, we propose to first test it thoroughly before coupling it to compartment II.

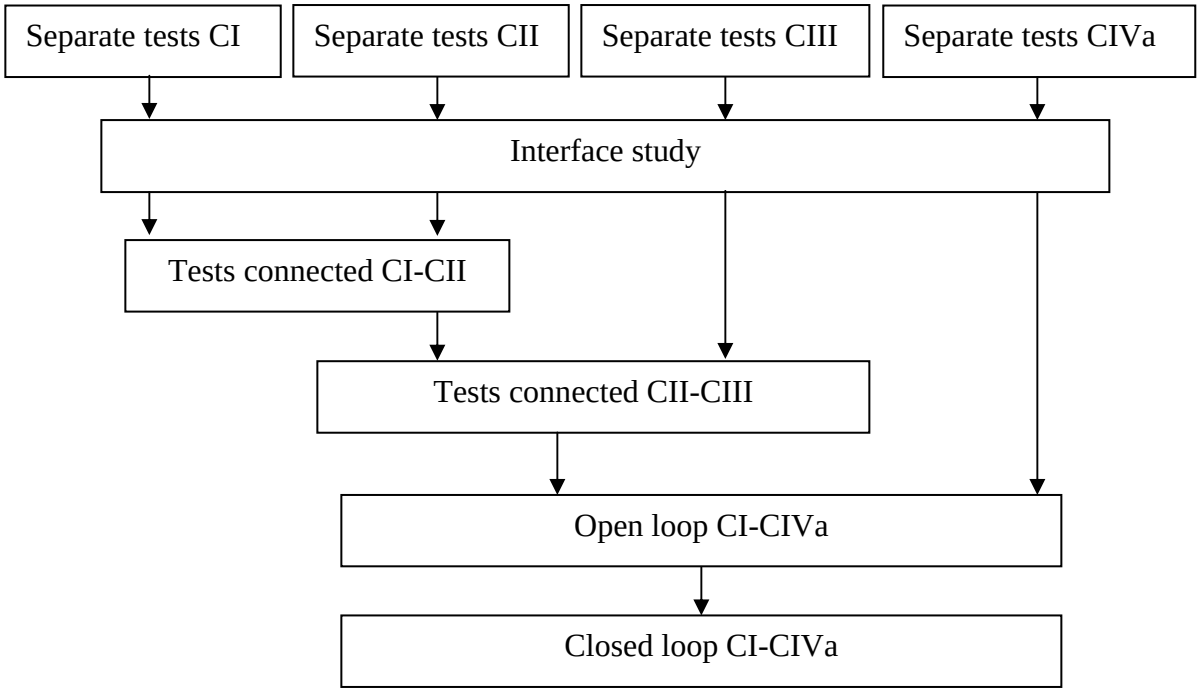


Figure 1: Integration sequence for microcompound study

Coupling of compartments will in any case be a stepwise process. First, all compartments will be tested as separate units. This is an advantage in terms of available sampling volumes. It avoids quick contamination by upstream compartments by chemical or biological agents and is easier when it comes to the replacement of biomass. Second, compartment II will be coupled to compartment I. Third, compartment III will be coupled to I and II. Fourth, this connected set-up will be linked to compartment IVa in an open loop. The final step will consist of loop closure.

6.3. Proposed test plan

Based on the above we propose a separate test plan for compartment I (Figure 2) and for compartments II to IVa (Figure 3). This is based on the current knowledge of the MELiSSA compartments and associated processes. However, at the time of definition of

detailed test plans and protocols and their performance, progressive adaptation of the overall test plan might occur.

Because the feed and content of compartment I is not well characterized, we first plan an extensive characterization in terms or minerals and hormone composition (Figure 2).
Then, the fate of micronutrients will be studied as they appear in the feed at various flow rates.
Thirdly, the effect of endocrine active compounds will be studied. The sequence of tests consists of the study of background endocrine activity, the effect of the pure natural male hormones, the pure natural female hormones and the synthetic female hormone EE2-norethindrone. Subsequently, real urine from healthy donors will be used. As mentioned before, the point of entry for urine in the loop will be the first compartment. The study of parent compounds and metabolites in compartment I effluent, will allow us to determine the relevant inputs for the downstream compartments.
Fourthly, the fate of pharmaceuticals will be monitored through the consecutive spiking of polar and non-polar target compounds.
In between all tests, sufficiently long intermediate non-spiking periods need to be planned to allow for system recovery to normal operation.

The tests in compartments II are scheduled as shown in Figure 3. The fate of selected micronutrients will be studied in the individual compartment first. If the micronutrients exert stresses on the biomass, this will most probably be a reversible phenomenon that can be counteracted by returning to the original mineral concentration levels. Preference will be given to variations in flow rates rather than in mineral concentration levels in the feed, because the latter could lead to precipitation reactions or other media instabilities. The following sequence of tests is proposed:

1. Mineral studies will be performed in the currently used and probably non-optimal synthetic medium.
2. A simulated compartment I effluent will be tested as feed. This implies that the compartment I effluent characterization has been completed.
3. The real compartment I effluent will be fed to compartment II. At this point, compartment II is physically coupled to compartment I.

In general, there will be a time delay between the tests on compartment II on the one hand and II and IVa on the other hand, because the latter will be assembled at a later stage. For compartments III and IVa, tests with synthetic media (as described in step 1 for compartment II above) will probably start in parallel with step 3 of compartment II. When mineral studies on the currently used synthetic media are finalized for compartments III and IVa, they will stepwise be coupled to the previous compartments. This implies that the investigation on minerals will be less exhaustive in compartments III and IVa than in compartment II. Furthermore, once the coupling is achieved, the test programme for compartment I will completely determine the one for the downstream compartments.

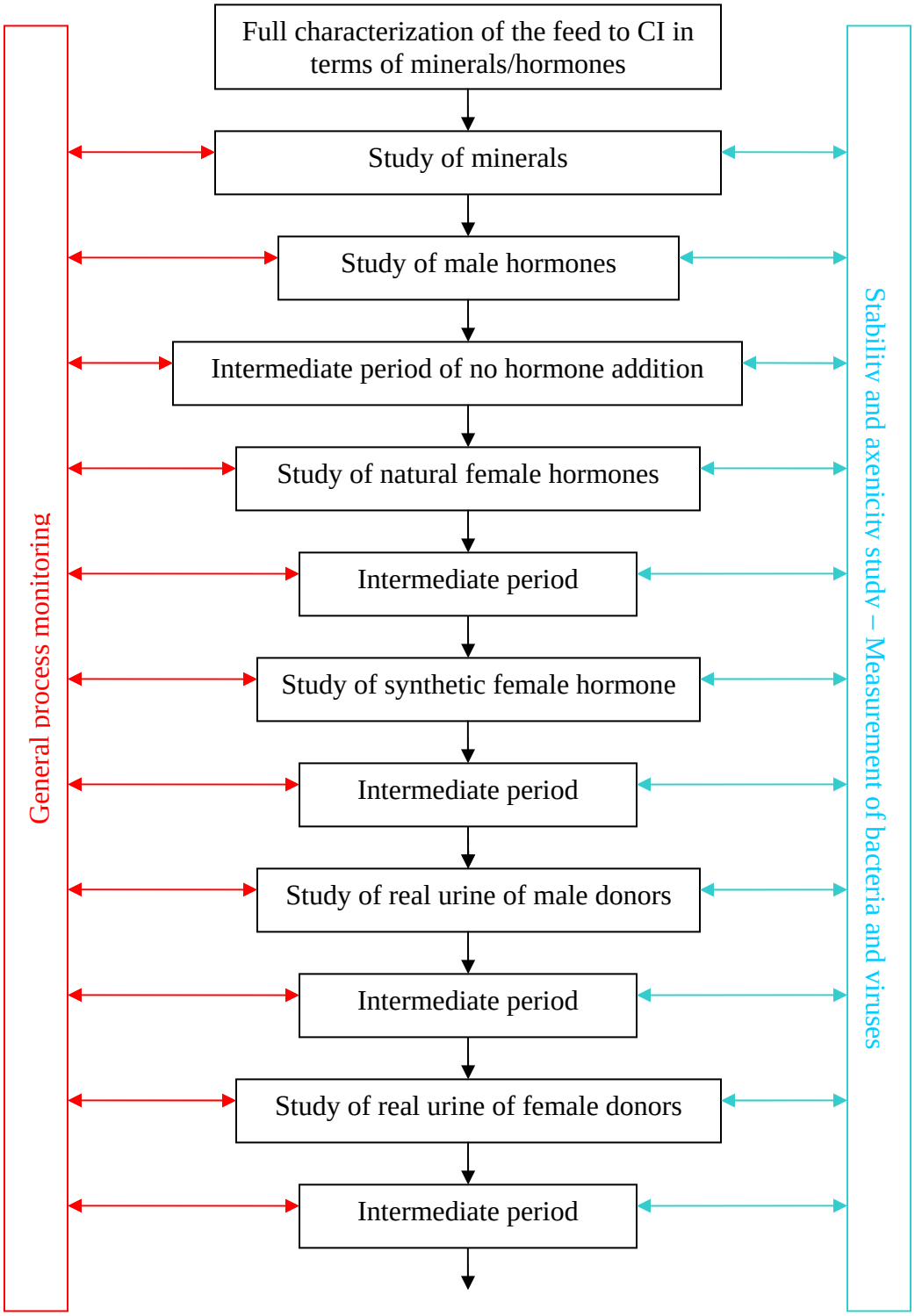


Figure 2: Proposed test plan for compartment I in BELISSIMA.

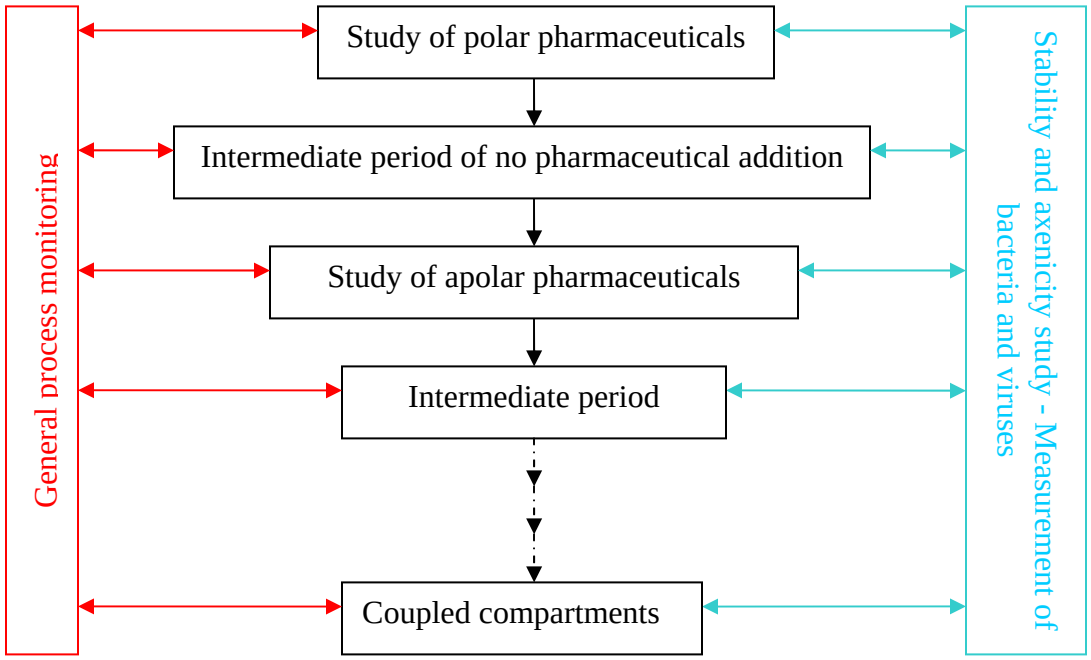


Figure 2 (continued)

Based on a preliminary estimation of start-up time and timing for the various microcompound studies, the connection of compartment III to I and II will immediately receive effluent from compartment I which contains spiked male hormones. If further mineral studies in the absence of hormones are of interest, male hormone spiking in compartment I will need to be postponed. The early coupling of compartments has however the advantage that compounds or metabolites formed in one compartment can immediately be introduced in the next one, eliminating the need for spiking.

During all tests described above samples will be collected for axenicity evaluation. For the measurement of bacteria and viruses, samples will be taken in particular during intermediate periods where sampling frequency for the other studies is probably lower.

All tests will be performed under steady state concentrations, this is after approximately 5 hydraulic retention times. For all test conditions, either with separate or coupled compartments, general reactor performance will be monitored through regular samplings and measurements of gas, liquid and biomass.

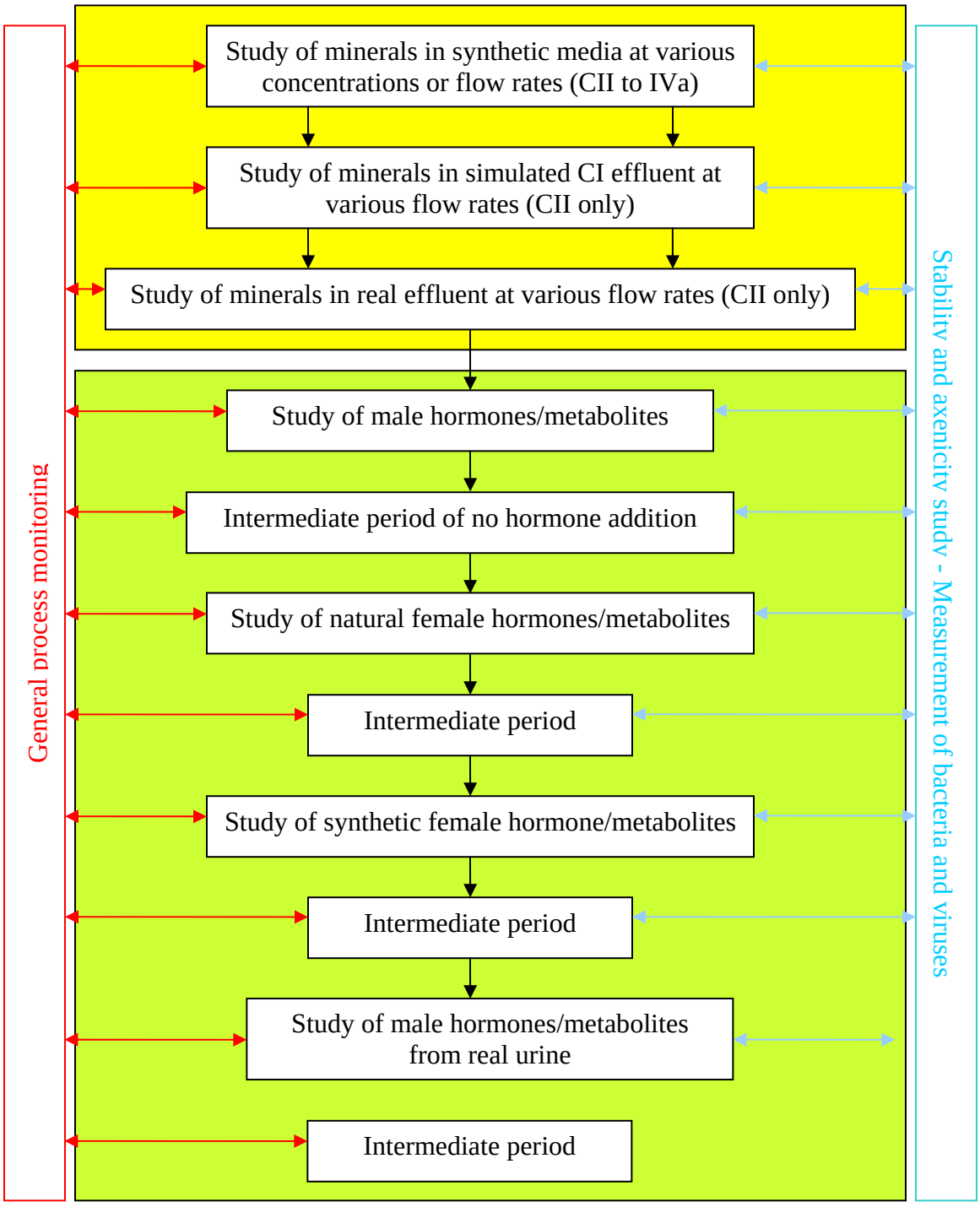


Figure 3: Proposed test plan for compartments II to IVa in BELISSIMA. The yellow part refers to tests in separate compartments which are more extensive in compartment II than in III and IVa, the green part to connected compartments I to IVa.

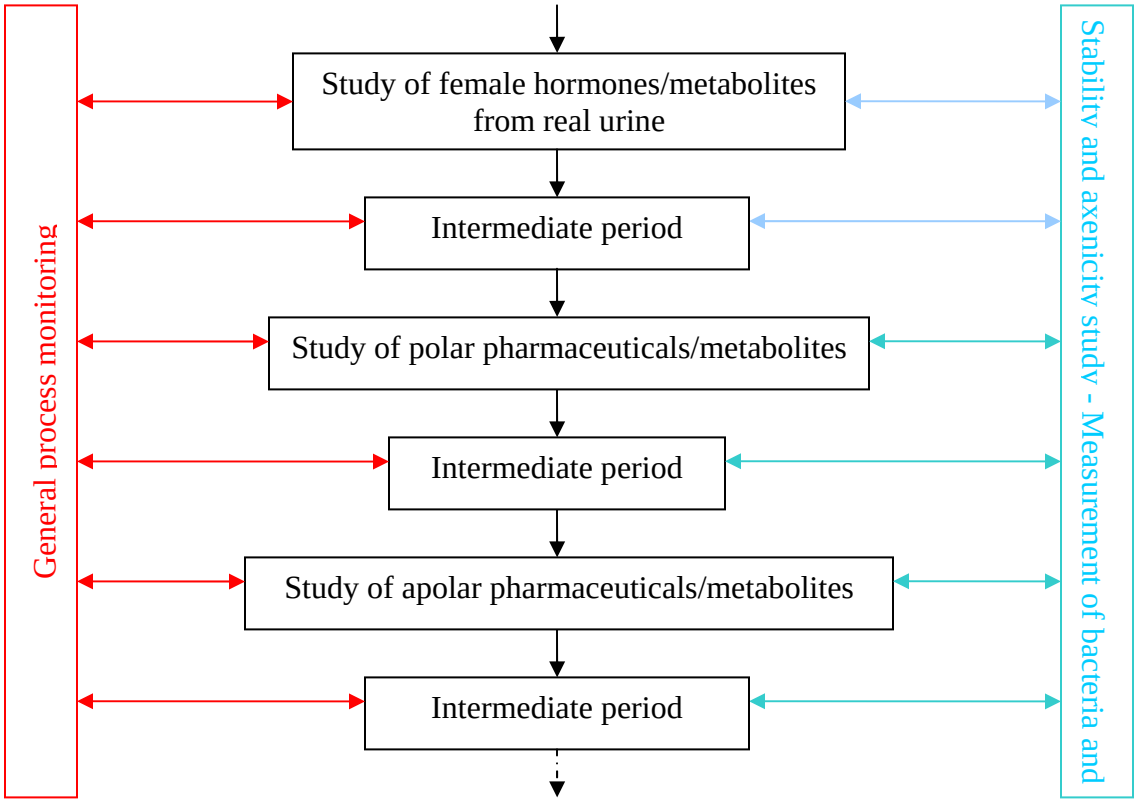


Figure 3 (continued)

The resulting test plan is detailed for compartment I first, then for the individual compartments II to IVa and finally for the coupled compartments and closed loop.

6.3.1.Compartment I individually

The tests related to compartment I will start with an extensive analysis of the feed. This implies the analysis of its composition in terms of minerals and its background in endocrine activity and pharmaceuticals. To make sure that the analysis are statistically relevant we propose to perform analyses on at least 5 samples. Two types of feed will be considered: one with fecal material of healthy male donors, and one with fecal material of healthy female donors. The amounts of toilet paper and wheat, beet and lettuce will be as defined in the mission scenario for BELISSIMA. No urine will be added for this phase.

Compartment I will be started up with an inoculum from the corresponding unit in the Pilot Plant because this material will be best adapted. First, the inoculum will be cultivated to the final volume of the bioreactor without starting the membrane filtration unit. Once the active volume is reached at a dry matter concentration of around 20 g/l and

proper conversion rates are achieved, the membrane filtration unit will be started up. When the system has reached equilibrium, general reactor performance will be evaluated and compared to previous results obtained with compartment I. However, we will have to take into account that the mission scenario will be different compared to the one which has been used so far in MELiSSA and this may change the performance results. Once stable hydrolysis and VFA production has been achieved, the actual microcompound tests will be initiated. Taking into account that the hydraulic retention time (HRT) in the first compartment is close to 10 days, we estimate that start up will take at least 2 months. Stable performances achieved here, will be used as reference situation.

First, the fate of minerals will be investigated under continuous operation mode and in test periods, which will each last 5 hydraulic retention times to ensure that equilibrium conditions have been reached. No urine will be added to the feed. The target minerals were selected in TN80.12 and are given below

- anions: sulphate, phosphate and chloride
- cations: Na, K, Ca, Cu, Mg, Mn, Zn, Fe, Co, Mo.

Minerals will be determined on influent, effluent and biomass samples taking according to the details given in TN80.12 and uptake or release quantified. The main aim of the tests is to determine the stoichiometry rather than the dynamics. For each test period, at least 8 sampling occasions will be scheduled.
At a HRT of 10 days, each test will last at least 50 days. To rationalize the overall period of mineral testing, we propose to work at 2 different flow rates. These will be the normal flow corresponding to a 10 d HRT and e.g. a 2 times lower flow. Lower flows are preferred to higher ones to make sure that the microbial consortium can hydrolyze the feed at the same efficiency. However, if the first test indicates a shortage for a specific mineral, higher loads and flows may be more appropriate.

Changes in hydraulic conditions may exert stresses on the microbial biomass, so an intermediate period will be provided at normal operating conditions. Since it is assumed that potential stresses due to mineral shortages or surpluses are reversible, this is expected to be sufficient to return to the performance in the reference situation ± 10%. All further tests will be performed at a HRT of 10 days.

Second, the study of hormones will be initiated. It was agreed before (TN80.12) that the target compounds are the male hormones (androgens), the natural female hormones (estrogens) and the synthetic female hormone (17α-ethinylestradiol).
Because the urine matrix may confound interpretation of the effects and fate of hormones, the hormone compounds will first be spiked to the feed without urine. They will be purchased as pure compounds. The order of testing is chosen based on the concentration levels expected and the complexity of the response. First the androgens will be spiked, then the natural female hormones and finally the synthetic female hormone. For men, the daily excretion varies between 7 and 85 µg/d, for women between

16 and 250 µg/d. Excretion of synthetic hormone have been calculated to be between 9 and 32 µg/d (see TN80.12). Because we do not know the extent of removal of hormones in the first compartment, we propose to perform the tests at the highest expected dosis. This will increase the chance of measuring differences in endocrine activity between inlet and outlet. Each test will last 5 HRTs, i.e. 50 to 100 days, and per test at least 8 sampling occasions will be planned. In between tests, intermediate periods without hormone spiking will be provided to recover reference situation performances ± 10% and to wash out remaining spikes or to desorb fractions attached to the biomass. In the intermediate periods the background endocrine activity will have to be monitored as well. In case no removal through adsorption is observed and no effects are measured on the microbial biomass or reactor performance, then intermediate periods are not required.
In a next step, real urine will be used. The first group of donors will be healthy male persons, the second group healthy females not using the anticonceptive pill and the third group healthy females using the anticonceptive pill. The main hormones present are respectively androgens, estrogens and estrogens + 17α-ethinylestradiol. This implies that the effect of 17α-ethinylestradiol will have to be evaluated in an indirect way, as the difference in response between the two last conditions. Moreover, it is not necessary to provide an intermediate test period between the latter two conditions because the estrogens will remain present anyway. To have some kind of standardization, urine from a number of people will be pooled. As was the case for the previous study items, each test will last at least 5 HRT and will include at least 8 sampling occasions. Unless specified otherwise, intermediate periods are provided to return to the reference situation ± 10%, if this is necessary.

Third, the study of pharmaceuticals is scheduled. The selected compounds are

- the sulfonamide antibiotic sulfamethoxazole
- the fluoroquinoline antibiotic ciprofloxacin
- analgesic and anti-inflammatory compounds: diclofenac and aspirin
- the β-blocker metoprolol
- the antidepressant diazepam.

A logical sequence is to use a polar compound before an apolar because the former has a lower tendency to sorb on the biomass and to cause memory effects. Ciprofloxacin would be the most and diclofenac the least polar compound of the selection. However, the use of antibiotics has a high potential to affect microbial community composition through the elimination of the bacterial fraction susceptible to it. In that case, aspirin is the most polar non-antibiotic compound, followed by metoprolol and diazepam. Finally, one compound may induce the enzymes for degradation of a structurally similar compound. The current selection of pharmaceuticals seems to be diverse enough at first sight. Due to the fact that little is known about the degradation pathways, it is not possible to anticipate interferences. Therefore we propose the following order of tests: aspirin, metoprolol, diazepam, ciprofloxacin, sulfamethoxazole, diclofenac. All tests will last at least 5 HRT until stable measurements are obtained. A minimum of 8 sampling occasions will be

provided. Intermediate periods are thought to be necessary to remove sorbed compounds and dilute remaining pharmaceuticals. However, when effects on reactor performance remain lower than a 10% deviation from the reference situation, they can be cancelled.
In view of time restraints, the number of pharmaceuticals tested may be reduced to 2 or 3.

Even then, the total timeframe for the tests amounts to 3 years (see also Figure 4), not considering any events or failures or serious effects warranting reinoculation.

At all test stages, regular reactor operation will be monitored and biomass samples will be collected for the axenicity and genetic stability study. Furthermore, batch tests may complement the work on microcompounds to elucidate removal mechanisms or e.g. to expand the concentration ranges tested.

6.3.2.Compartment II individually

Compartment II will be inoculated with a preculture of *Rhodospirillum rubrum*. Through gradual increase of the light intensity and the flow rates to a predetermined value, the biomass will be increased towards the desired equilibrium concentration. Culturing will occur in continuous mode. The medium will be as defined by Segers and Verstraete. Although this medium may not have the optimal mineral composition, all past MELiSSA experience is based on it. Therefore, we need this condition as a reference. HRT is typically between 1 and 5 days. The minimum time to achieve steady state conditions will therefore be between 5 and 25 days. Once VFA removal rates and biomass production have been reached which are comparable to previous tests performed under the same conditions, this will be considered as the reference situation.

After start-up, the mineral study will be initiated at the design flow rate. Attention will be paid to the fact that light intensity, C, N and P availability etc. are not rate limiting. It is assumed that equilibrium conditions will be achieved after 5 HRT. Influent, effluent and biomass samples will be taken at regular time intervals for mineral analysis, quantification of uptake and/or release and stoichiometry determination of growth kinetics. The minimum number of sample occasions will be 8. Subsequently, flow rates will be decreased down to 2 different levels. Lower flow rates are preferred compared to higher ones because the former will reduce the total amount of minerals supplied and may indicate shortages. If reactor performances are suboptimal under these conditions, some intermediate phase must be provided to restore the normal operating conditions and transformation rates.
After the synthetic medium of Segers and Verstraete, a simulated version of compartment I effluent will be tested. This will mimick the mineral composition of the compartment I effluent under the reference situation. Again, mineral uptake and/or release will be determined at the same three flow levels as tested before and under the same conditions as mentioned before.
In a final step, the real effluent of compartment I will be fed to compartment II. This may require some manipulations to sterilize it. In separate tests, the effect of e.g. sterile

filtration on mineral composition will be verified. Due to the color of compartment I effluent, the illumination may have to be adjusted. As for the previous tests, the mineral study will be performed at three flow rates. This phase is the actual coupling of compartment II to I. Form then on, all tests in compartment II will be determined by the test plan of compartment I since this operates at a higher retention time. For instance, the hormone investigations will be started directly with real effluent from compartment I and will contain either the non-degraded or non-transformed parent compounds or th emetabolites.

At all test stages, regular reactor operation will be monitored and biomass samples will be collected for the axenicity and genetic stability study. Furthermore, batch tests may complement the work on microcompounds to elucidate removal mechanisms or e.g. to expand the concentration ranges tested.

When severe stresses on the MELiSSA organisms are detected or contaminations occur, compartments may need to be reinoculated. Stresses due to mineral imbalances are expected to be reversible and will most probably not warrant replacement of the biomass. Contaminations may particularly become problematic once real compartment I effluent is being used. In any case, backup cultures will be needed to allow for a quick restart of the reactor.

6.3.3.Compartments III and IVa individually

Although the design of compartment III will allow both the use of biostyr carriers and glass beads, the carrier material in compartment III will first be glass beads to avoid sterilization problems. Following the start-up procedures developed by UAB, the coculture of *Nitrosomonas europeae* and *Nitrobacter winogradskyi* will be grown on the carriers, first under batch conditions with internal recycle, later on in continuous mode. The synthetic medium will be the one described by Wijfels. Once nitrification rates have been reached which are comparable to previous tests performed under the same conditions, this will be considered as the reference situation.
For compartment IVa, the start-up procedure will be similar to the one described for compartment II, using *Arthrospira* as the inoculum and the synthetic medium defined by Zarrouk. Reference performance will refer to biomass production rates and nitrate removal comparable to other tests performed in the MELiSSA consortium.
Although the synthetic media may not have the optimal mineral composition, all past MELiSSA experience is based on it. Therefore, we need this condition as a reference.
The minimum time to achieve steady state conditions will be 5 HRT.

The first series of tests with the individual compartments will be performed using the synthetic media at three flow rates. The maximum flow is the design flow. The 2 others will be lower. These tests will most probably be finalized in the same time period as the mineral investigations with real compartment I effluent in compartment II. Therefore, coupling of compartment III to the 2 previous ones is possible.

6.3.4.Coupled compartments

Compartment III will be coupled to compartment I and II once the mineral tests for both have been finalized. At that time, compartment I will receive synthetic male hormones, which will be passed on to compartment II and III.

Coupling of compartments implies an extensive interphase study to evaluate the effect of specific technologies on effluent composition and this should result in a trade-off of technologies. Coupling will be performed stepwise: first II to I, then III to the 2 previous ones, then IVa. Once the compartments are coupled, the test plan in compartment I determines what happens in the rest of the loop because it operates at the highest HRT and because some compounds may be transformed or (partially) degraded here..
All studies for the coupled compartments will be performed at the design flow rates. First, the compartment I effluent obtained after spiking with the pure hormones will be used as feed to the coupled set-up, and afterwards the effluent obtained during real urine tests. The order of hormone and pharmaceutical drugs studies is also valid for compartments II to IVa.

Intermediate periods will be foreseen if severe diversions from regular reactor operation are being observed.

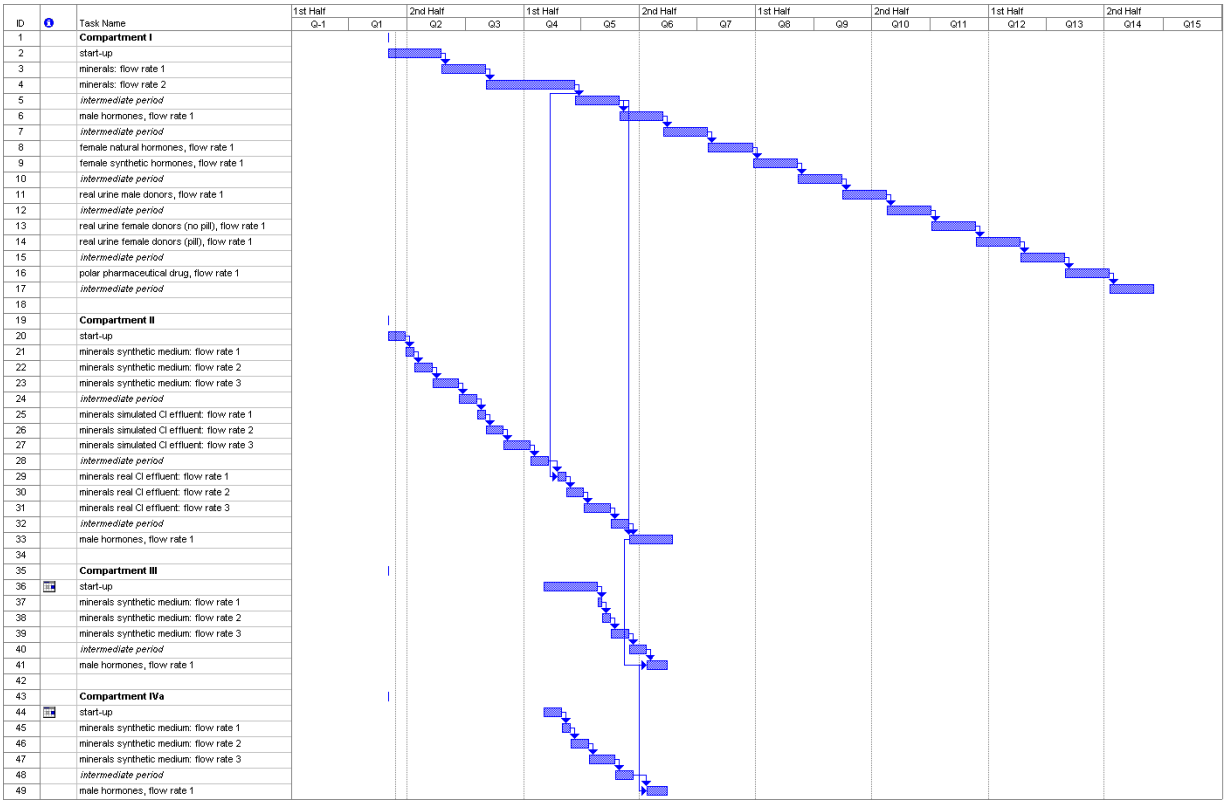
6.3.5.Countermeasures

It is not clear yet which type of countermeasures will be relevant for BELISSIMA. The test plan will largely depend on the outcome of the various microcompounds studies.

6.3.6.Tentative test planning

Figure 4 shows the relative timing of the various microcompound studies as described in detail before. Around 8 months of operation, compartment II will be coupled to compartment I.

Construction of compartments III and IVa will be finalized 8 months after start-up of compartments I and II. After the mineral investigations with synthetic media at various flow rates it will be possible to couple them to compartments III and IVa after about 12 months of operation for compartment I and II and 4 months of operation for III and IVa.



ID	Task Name
1	**Compartment I**
2	start-up
3	minerals: flow rate 1
4	minerals: flow rate 2
5	*intermediate period*
6	male hormones, flow rate 1
7	*intermediate period*
8	female natural hormones, flow rate 1
9	female synthetic hormones, flow rate 1
10	*intermediate period*
11	real urine male donors, flow rate 1
12	*intermediate period*
13	real urine female donors (no pill), flow rate 1
14	real urine female donors (pill), flow rate 1
15	*intermediate period*
16	polar pharmaceutical drug, flow rate 1
17	*intermediate period*
18	
19	**Compartment II**
20	start-up
21	minerals synthetic medium: flow rate 1
22	minerals synthetic medium: flow rate 2
23	minerals synthetic medium: flow rate 3
24	*intermediate period*
25	minerals simulated CI effluent: flow rate 1
26	minerals simulated CI effluent: flow rate 2
27	minerals simulated CI effluent: flow rate 3
28	*intermediate period*
29	minerals real CI effluent: flow rate 1
30	minerals real CI effluent: flow rate 2
31	minerals real CI effluent: flow rate 3
32	*intermediate period*
33	male hormones, flow rate 1
34	
35	**Compartment III**
36	start-up
37	minerals synthetic medium: flow rate 1
38	minerals synthetic medium: flow rate 2
39	minerals synthetic medium: flow rate 3
40	*intermediate period*
41	male hormones, flow rate 1
42	
43	**Compartment IVa**
44	start-up
45	minerals synthetic medium: flow rate 1
46	minerals synthetic medium: flow rate 2
47	minerals synthetic medium: flow rate 3
48	*intermediate period*
49	male hormones, flow rate 1

Figure 4: Tentative planning of the microcompound tests in the different MELiSSA compartments.

6.3.7.Concluding remarks

At all test stages, regular reactor operation will be monitored and biomass samples will be collected for the axenicity and genetic stability study. Furthermore, batch tests may complement the work on microcompounds to elucidate removal mechanisms or e.g. to expand the concentration ranges tested.

During and in between tests, time will have to be provided to perform maintenance activities, calibrations, etc.

7. References

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