MELiSSA

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PRELIMINARY REQUIREMENTS FOR MICRO-COMPOUNDS STUDY

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prepared by/*préparé par* Witters Hilda, De Wever Heleen

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Table Of Contents

1 INTRODUCTION 1

2 MINERALS 2
2.1 Definition 2
2.2 Global objectives with respect to the study of minerals 2
2.3 State of the art 3
2.4 Tentative priority selection of compounds 27
2.5 Identification of technical requirements and critical steps of design of BELISSIMA loop & study protocol 28
2.6 Conclusions 34

3 HORMONES 39
3.1 Definition 39
3.2 Global objectives with respect to the study of hormones 39
3.3 State of the art 39
3.4 Priority selection of compounds 55
3.5 Identification of technical requirements and critical steps of design of BELISSIMA loop & study protocol 56
3.6 Conclusions 63

4 PHARMACEUTICAL DRUGS 64
4.1 Definition 64
4.2 Global objectives with respect to the study of pharmaceutical drugs 65
4.3 State of the art 65
4.4 Priority selection of compounds 78
4.5 Identification of technical requirements and critical steps of design of BELISSIMA loop & study protocol 91
4.6 Conclusions 96

5 REFERENCES 97

1 INTRODUCTION

ESA's Micro-Ecological Life Support System Alternative (MELiSSA) is a closed loop system, which consists of five interconnected compartments with a number of connecting gas and liquid streams among them. In this way, it is a model system for bioregeneration enabling waste recycle. Each compartment is colonised by specific organisms and has a specific contribution to the overall biotransformation process. Up to now, large efforts have been devoted to the study of the six most abundant elements in the loop, C, H, N, O, P and S. The behaviour of the remaining inorganic and organic elements is however as important because they can have a significant impact on the functioning and overall health of organisms involved. Changes of micro-compound levels to sub-optimal levels might cause growth limitations, or even contribute to toxicity problems.
So far, mostly synthetic media with well-defined compositions and rich in trace elements have been used as a feed for the different compartments. In a closed loop this will no longer be the case. Wastewater, organic wastes and possibly also urine of variable composition will enter the first compartment and solutes will be transferred through the entire loop. Consumption, sorption or accumulation and biomagnification of a number of elements or compounds will occur. To what extent this will affect the kinetics and stability of the organisms in the different compartments, has never been studied. Therefore, BELISSIMA wants to study microcompounds in the different compartments of the closed MELiSSA loop.

In the framework of BELISSIMA the term microcompounds refers to:

- minerals or oligo-elements which can be divided in
    - macronutrients and
    - micronutrients or trace elements
- hormones or endocrine active compounds, more specific the human estrogens and androgens, and the synthetic hormones used in the anti-conceptive pill
- pharmaceutical drugs and
- genetic elements.

The current technical note will be devoted to the first 3 groups of microcompounds and a review of literature data and experience derived from former MELiSSA studies will be given. In 3 chapters, the fate and potential impact of minerals, of hormones, and of pharmaceutical drugs on MELiSSA organisms and in the different compartments of the loop will be considered. Next, the most relevant information will be evaluated in order to give recommendations for the technical design of the BELISSIMA loop enabling the study of microcompounds.

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2 MINERALS

2.1 Definition

Mineral elements are used by living organisms and an extensive list of these elements is essential. The traditional criteria for essentiality are that absence or deficiency of the element from the medium or diet produces either functional or structural abnormalities and that the abnormalities are related to, or a consequence of, specific biochemical changes that can eventually be reversed by the presence of the essential element (Bennett, 1993; WHO, 1996). Carbon (C), hydrogen (H) and oxygen (O) are in all organisms referred to as the 'building block'. Other elements, called 'primary elements' or macronutrients are e.g. nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), but may differ between groups of organisms. Macronutrients are those elements that are needed in relatively large quantities, compared to micronutrients or secondary elements. The latter are mostly essential elements, but they are needed in relatively small amounts. Micronutrients are therefore often referred to as trace elements. The following elements belong to this group of micronutrients: manganese (Mn), zinc (Zn), iron (Fe), copper (Cu), cobalt (Co), molybdenum (Mo), nickel (Ni), selenium (Se). Except for inorganic elements, vitamins too belong to the group of trace elements or micronutrients.

2.2 Global objectives with respect to the study of minerals

Few data are available on the consumption, sorption or uptake, biotransformation or biomagnification of micronutrients in individual or coupled compartments. It is a vast and yet unexplored area of study with relevance to estimate the accumulation potential of particular elements and their effect on microorganism growth kinetics and nutritional value.
Through establishment of mass balances for essential macro- and micronutrients within the BELISSIMA loop it is the purpose to identify shortages or accumulation of elements. Changes of element concentrations in biomass and in culture media will be related to growth and functional performance of the populations in each compartment.

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2.3 State of the art

2.3.1 THE ROLE OF MINERALS FOR MELISSA ORGANISMS

2.3.1.1 Micro-organisms

2.3.1.1.1 General

According to Schlegel (1988), the elementary composition of micro-organisms is divided into ten macroelements which are present in all cells and a number of microelements or trace elements. The macroelements are C, H, O, N, S, P, K, Ca, Mg and Fe. Trace elements are Mn, Mo, Zn, Cu, Co, Ni, V, B, Cl, Na, Se, Si and others which are not required by all organisms. Dunn (1985) distinguishes different groups of trace elements, based on their occurrence in organisms (Table 1).

Table 1: Trace element requirements by bacteria and fungi (after Dunn, 1985)

Bacteria/fungi	Trace element
All	Mn, Zn, Fe
Many	Cu, Co, Mo, Ca
Some	Na, Cl, Ni, Se
Few	B, Al, Si, Cr, As, V, Sn, Be, F, Sc, Ti, Ga, Ge, Br, Zr, W, Li, I

Burgess et al. (1999) mention as most important trace elements Mn, Zn, Co, Mo, Ni, Cu, V, B, Fe and I. The role of different trace elements in micro-organisms, including the MELiSSA organisms, is summarized in Table 2. They are

- components of enzymes or cofactors and act in the catalysis of reactions and in maintenance of enzyme structure
- metallic enzyme activators which are not part of the reaction they catalyse
- used in electron transport.

Essential trace elements do have to some extent homeostatic mechanisms involving regulation of absorption and excretion, which enable organisms to adapt to varying nutrient intakes to ensure safe and optimum systemic supply for the performance of essential functions and growth. Micronutrient supply must be sufficient to activate cellular enzymes and to provide general nutrients for growth and metabolism.

Deficiencies will reduce growth rates and can eventually prevent growth. This deficiency can have effects at the level of cell division mechanism with modified cell morphology. The appearance of genetic mutations in the course of long-term cultures has been observed as an effect of ion deficiency. The micro-organisms did adapt their metabolism to the ion-deficient medium, and growth of mutated bacteria was similar to non-mutated organisms (Kern et al., 1994).

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Excess micronutrients may exert toxic effects by interference with physiological pathways. Addition of micronutrients does not guarantee bioavailability. Essential micronutrients, among them especially metals, can be precipitated or adsorbed before bacteria can assimilate them. Even when ions are present in soluble form, deficiencies may occur as a result of antagonistic interactions. Certain non-essential metals, which might get into the system as a contaminant e.g. Cd, could give rise to toxic phenomena with growth inhibition (Tsai et al., 2005).
Unfortunately, interactions are impossible to predict because they are influenced by a number of factors, such as type and concentration of the ion, physico-chemical conditions of the medium (pH, conductivity,..), operating conditions (temperature, retention time,..), etc.

Table 2: Role of trace elements in microbial systems (after Burgess et al., 1999)

Element	Requiring micro-organisms	Role
Fe	Aerobic bacteria	Growth factor
Fe^{3+}	Possibly all	Electron transport Synthesis of catalase, peroxidase
Zn	Bacteria	Metallic enzyme activator Stimulates growth Can exacerbate toxic effect other metals Can inhibit metabolism
Co	Bacteria	Metallic enzyme activator Can inhibit metabolism
Mg	Heterotrophic bacteria	Enzyme activator
Mn	Bacteria	Enzyme activator Can inhibit metabolism
Cu	Bacteria	Enzyme activator Can inhibit metabolism Chelates other substances and reduces their toxicity
Ni	Cyanobacteria, activated sludge	Stimulates certain enzymes Maintenance of biomass May inhibit metabolism
Ca	Bacteria	Plays a role in membrane permeability Requirements and effects vary Interacts with other metals

Micronutrients are often required at doses below 1 mg/l. In addition, they occur as impurities in salts of macronutrients and reach media via contamination of glass vessels and dust particles. Their requirements are therefore difficult to demonstrate. Moreover, they are influenced by organic and hydraulic loading rates, cell growth rate, the nature of the waste and the cell residence time (Burgess et al., 1999).

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2.3.1.1.2 Photosynthetic bacteria, Rhodospirillum rubrum

The major content of minerals in biomass of photosynthetic bacteria is given in Table 3. It is observed that next to N and P, the bivalent ions Mg, Si, and Ca have the highest contribution.

Specific studies on assimilation of mineral elements by *R. rubrum* are reported by Saint-Germes (2000). The increase of biomass in test tubes or flasks as a function of time (42 or 10 days) was evaluated in media, deficient of either Mg, Mn or Fe. Both Mg and Fe appeared to be critical for growth (70% inhibition), while lack of Mn did not interfere with normal growth. In bioreactor experiments (up to 20 days), assimilation of Mg, but also of Mn by the cells was demonstrated. Indeed Mn does play a physiological role in enzymatic systems and DNA synthesis. It is suggested that for cultures deficient of Mn, it can be replaced by Zn which however should be further studied (Saint-Germes, 2000). The studies on assimilation of Zn, Fe and Ni did show less consistent results. Levels of Fe and Ni did increase in the culture medium, while Zn remained similar. Further investigations are required to find out whether it concerns an artefact of the method of measurements (ICP).

Table 3: Content of mineral elements in photosynthetic bacteria (after Kobayashi & Kurata, 1978).

Element	% of dry weight
N	9.75
P	2.49
K	0.21
SiO_2	0.82
Ca	0.87
Na	0.31
Fe	0.13
Mg	5.0
Mn	0.001
Cu	0.0021
Zn	0.11

Gauthey (2001) evaluated the role of EDTA, a chelating agent in the culture media. Trace element solubility can be kept at nominal values in the presence of equimolar levels of EDTA, preventing deficiency of essential minerals. It was demonstrated that *R. rubrum* cells did not affect EDTA degradation, through its use as a carbon source. Moreover growth in the presence or absence of EDTA remained the same. Assimilation studies for Mg, Mn, Fe and Ni demonstrated the same results as in the study of Saint-Germes (2000). The increased levels of Fe and Ni appeared to be the result of corrosion of the stainless steel material of the bioreactor, especially upon acid injection to regulate pH.

In a study by Cabello et al. (2002, TN 52.1), mineral requirements for *R. rubrum* were evaluated through uptake studies of Na, K, Mg and Ca. Experiments consisted of subculturing the organisms in fresh medium, deficient for one of the studied ions, compared to complete medium, and medium deficient for these 4 elements.

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It was shown that both divalent ions (Ca and Mg) were essential for *R. rubrum* cells, as growth inhibition was observed when medium was deficient of one of these elements. Growth of cells was not significantly affected if either Na or K was absent in the culture medium. In continuous culture systems, the consumption of ions was estimated. It was the highest for Na with approximately 20.0 ± 5 mg Na per g cell. On the other hand, consumption of K was very low to negligible. It was suggested that in case Na or K ions are lacking, the cells can use both for the same purposes, thus with no effect on growth. If both ions are present, the uptake of Na seems preferential as highest consumption was measured. Consumption of Mg in continuous culture was also high (10.3 ± 0.5 mg Mg per g cell), and it is essential for growth. Ca-consumption was lower (6.4 ± 1.6 mg Ca per g cell) and of the same order of magnitude as measured for K. Ca remains essential for growth as it was shown that depletion of culture medium resulted in a decreased growth.

The role of mineral elements, such as Ni, Fe and S is demonstrated based on their active site (NiFeS-cluster) in carbon monoxide dehydrogenase (CODH), an enzyme system in *R. rubrum* which in the presence of electron donors reduces CO_2 and COS to CO, H_20 and H_2S (Ensign, 1995; Ensign et al., 1990).

The role of selenium and selenite on cultures of *R. rubrum* under oxic and anoxic conditions were studied by Kessi et al. (1999). In aerated aquatic environment selenium predomininantly will occur in the form of selenite and selenate oxyanions, which are freely available to organisms. High levels of selenite (mM range) might affect cell division as a reduction of cell densities to 15% of the control was seen at 1.5 mM selenite. However the organisms have developed multiple detoxification mechanisms by eg. selenite reduction and consequent deposition inside the cytoplasm or outside the cells. It was also observed that elemental selenium was expelled across the cell wall into the medium (Kessi et al., 1999).

2.3.1.1.3 Nitrifying bacteria, Nitrosomas europaea and Nitrobacter winogradskyi

Limited information is found about the mineral metabolism, uptake or effects due to interfering elements for nitrifying bacteria as those selected in the MELiSSA loop.
Generally, biological nitrogen removal by sequential nitrification and denitrification is a widely applied technology in wastewater treatment facilities. Because of low growth rates of nitrifying bacteria and their extremely high sensitivity to pH, dissolved oxygen concentration, temperature, and toxic chemicals environmental conditions should adequately be monitored. Measurements of N-balance, based on data for nitrite, ammonia,… in growth media, and at influent and effluent will show N-turnover within this compartment. These data on nitrifying activity will be compared with densities and growth performance of the cultures. Below a few examples are given which point to critical factors in the nitrification process.

A constant low-level exposure to metals does not typically affect microbial activity due to biomass acclimation, but shock loads of metals can lead to complete failure of biological processes. Short-term studies have demonstrated that nitrification inhibition generally correlates well with the aqueous free metal cation concentration, as can be described by the free-ion-activity in metal speciation models.

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It is assumed that metal transport across cell membranes occurs rapidly and a pseudo-equilibrium is established between metal species in the bulk solution and on microbial surfaces. The mode of action for metals is mainly through interaction with intracellular functional groups which destroys protein structure and function. However, it has recently been shown that the kinetics of Zn, Ni, and Cd internalization are slow and inhibition by these metals is related to their intracellular fraction. Copper on the other hand did appear to have a unique mode of action involving rapid loss of membrane integrity. Hu *et al.* (2004) investigated the impact of metal biomass association (sorption, internalization) and speciation (total, soluble, sorbed and internalized) on nitrifying activity in both batch and continuous flow reactor conditions (Figure 1).

The discrepancy between the batch and continuous flow reactor observations was the largest for Cu among the four metals tested, presumably due to its unique mode of action, being cell membrane disruption. Appropriate remedial strategies for the sudden discharges of metals in a wastewater treatment are suggested. For metals that partition less to biomass (e.g. Ni), temporary hydraulic retention time control might be beneficial in reducing inhibition. For strongly and rapidly partitioning metals (e.g. Cu), no easy remedial action is available although the addition of strong chelating agents (e.g. EDTA) might be able to extract the sorbed Cu species from the biomass (Hu *et al.,* 2004).

Increases of Cl levels in the operation of nitrifying batch cultures might be critical for nitrification rate (Chen *et al*., 2003). The chloride tolerance level of the fresh nitrifying batch culture was found to be 6,5 g/l Cl (Figure 2). *Nitrobacter* was found to be the only dominant species of nitrite-oxidizers in saline batch cultures when the chloride concentration was kept below 10 g/l. When the chloride concentration was increased from 10 to 18 g/l, the dominant species of ammonia-oxidizers was shifted from non-saline resistant species, such as *Nitrosomonas europaea* lineage and *Nitrosomonas eutropha*, to saline-resistant species such as *Nitrosococcus mobilis* lineage.

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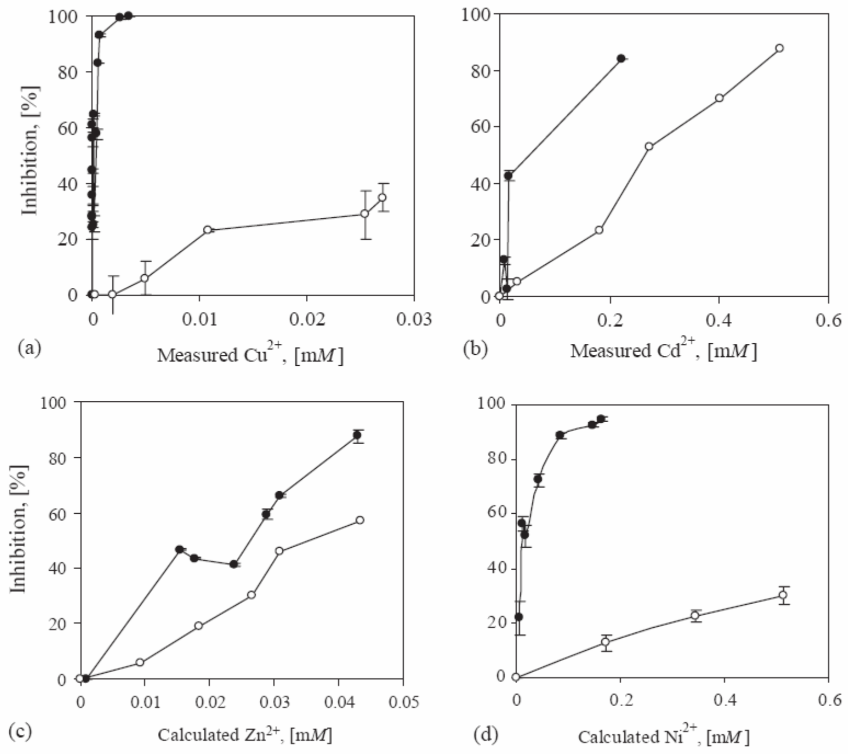

Figure 1: Comparison of nitrifying activity, expressed as ammonium oxidation inhibition as a function of free metal cation concentrations after a 24-hour shock load of (a) Cu, (b) Cd, (c) Zn and (d) Ni to continuous flow reactors (●) and in batch vessels (○). Error bars indicate one standard deviation (after Hu *et al*., 2004).

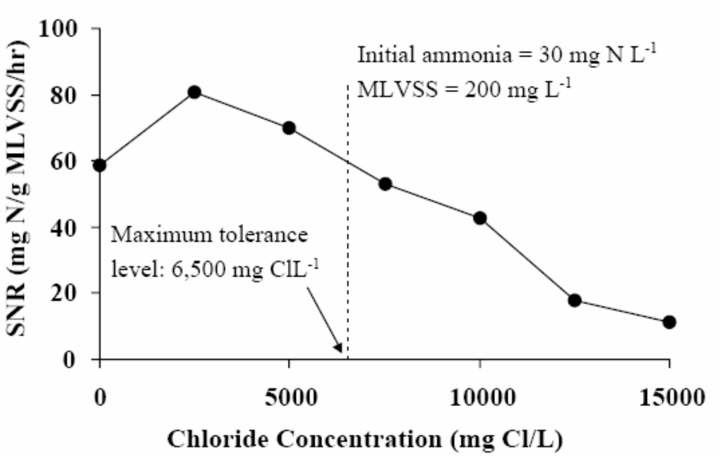

Figure 2: Changes of the activity of the fresh nitrifying batch culture (SNR= specific nitrification rate) at various initial chloride concentrations (after Chen *et al*., 2003).

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The actual consumption of the minerals present in the medium has not yet been studied with the *Nitrobacter/Nitrosomonas* coculture used in the MELiSSA loop. Due to their very slow growth, no significant uptake of minerals is expected and the main biological transformation of inorganic compounds will be the nitrification reaction itself.

2.3.1.1.4 Cyanobacteria, Arthrospira platensis

Arthrospira platensis (also *Spirulina platensis*) is one of the most extensively used microalgae for human and animal nutrition, because of its high protein content at 60-65% on a dry weight basis. This is one of the major reasons for its selection as an organism within the MELISSA loop. However on the other hand, its specific use in waste water sanitation, related to the sorption of heavy metals has been shown by several authors (Mann and Fyfe, 1984; Rangsayatorn *et al.,* 2002, Chojnacka *et al.*, 2004) and should be considered in the context of a closed loop system.

Rangsayathorn *et al.* (2002) demonstrated that *Arthrospira platensis* biomass rapidly sorbs cadmium, with an optimum at pH 7. Maximum sorption capacity was close to 100 mg/g biomass. Inhibitory Cd concentrations were as high as 13 mg/l.

Comparative work on bioaccumulation and selective removal of trace elements by *Spirulina sp.* from industrial waste water has been described (Chojnacka *et al*., 2004). The kinetics of removal of trace elements from refinery effluents do show different patterns as a result of specific bioaccumulation capacity and retention degree for each of the elements. Retention degree ($C_{initial}$-C_{final}/ $C_{initial}$) or effective removal of metals from solution by the organisms (> 50%) was present for Os, Th, W, Zn, Cu, Cd, Hg, Rh, Hf, Pb, Al and Zr. Lower retention degree (~ 20%) was seen for Nb, Sc, Co, Ba and Ni while no changes were seen for Mo, As, Ce, La, Ge, Ti and Cs. Total bioaccumulation capacity was calculated as 7320 µg of cations bound/g dry biomass. Concentration factors in the range of 80 to 4250 were seen and *Spirulina sp*. were shown to be capable of concentrating metal ions in the biomass, even if extremely low levels occur in the medium for a few metals (Os, Rh < 0.0001 µg/l).

Biosorption capacity by *Spirulina sp*. for Pb, Cd and Cr (VI) might be dependent on chemical composition (Hernandez & Olguin, 2002). Cultures were either enriched in protein levels (cultivation in Zarrouk medium), in lipid levels or in polysaccharides . Highest absorption for Pb and Cd were observed at pH 5.0 and pH 4.5 when the cells exhibited the highest polysaccharide content. Cultures with high protein content exhibited the highest adsorption of Cr (VI).

2.3.1.2 Higher plants

2.3.1.2.1 General

About 20 elements which have a role as functional or metabolic nutrient are considered essential for plants (Table 4). For a selected number of elements, their specific role is described in Table 5.

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Table 4: Nutrients essential to plant growth (after Bennett, 1993).

Element	**Chemical symbol**	**Forms taken up by plant**
Macronutrients		
Carbon	C	CO_2
Hydrogen	H	H_2O
Oxygen	O	H_2O, O_2
Nitrogen	N	NH_4^+, NO_3^-
Phosphorus	P	$H_2PO_4^-$, HPO_4^{2-}
Potassium	K	K^+
Calcium	Ca	Ca^{2+}
Magnesium	Mg	Mg^{2+}
Sulfur	S	SO_4^{2-}
Micronutrients		
Iron	Fe	Fe^{2+} , Fe^{3+}
Zinc	Zn	Zn^{2+} , $Zn(OH)_2^0$
Manganese	Mn	Mn^{2+}
Copper	Cu	Cu^{2+}
Boron	B	$B(OH)_3^0$
Molybdenum	Mo	MoO_4^{2-}
Chlorine	Cl	Cl^-
Silicon	Si	$Si(OH)_4^0$
Sodium	Na	Na^+
Cobalt	Co	Co^{2+}
Vanadium	V	V^+

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Table 5: Role of essential mineral elements for higher plants (after Bennett, 1993).

Element	Role
N	Built into amino acid, precursors for protein synthesis and DNA /RNA synthesis Contained in chlorophyll, and structural constituent of cell walls
P	Constituent of enzymes and proteins Role in most metabolic processes, and photosynthetic process Involved in electron transport for oxido-reduction reactions
K	Built up of osmotic cellular potential, regulation of water balance by stomata Counterion for Cl^- and SO_4^{--} Enzyme activator Role in ATP production (energy) and protein synthesis
Ca	Occurs in pectate, component of cell wall Role in cell division and elongation Influence on structural stability and permeability
Mg	Essential element in chlorophyll Cofactor for enzymes, and activates formation of polypeptide chains
S	Constituent of two amino acids, cysteine and methionine, essential in proteins Role in vitamin and hormone synthesis Involved in oxido-reduction reactions
Fe	Essential for synthesis of chlorophyll Involved in N-fixation, photosynthesis, protein syntheses and electron transfer Involved in respiratory enzyme systems , e.g. cytochrome
Zn	Metal component in enzyme systems, acting as electron transfer systems and protein synthesis Built into auxin, best known enzyme regulating plant growth
Mn	Structural component of metalloproteins Enzyme systems
Cu	Involved in enzyme systems Formation of cell wall systems Electron transport and oxidation reactions
B	Membrane transport of sugars Synthesis of cell wall material Cell development and elongation through carbohydrate metabolism, amino acid formation and synthesis of proteins
Mo	Metal component for enzyme systems
Cl	Photophosphorylation reactions for photosynthesis Osmotic regulation, together with K
Si	Structural rigidity of cell wall Enzyme complexes with role in photosynthesis
Na	Osmotic regulator
V	Chlorophyll synthesis Oxidation-reduction reactions
Co	Growth, specific for lower plant organisms Symbiotic N fixation

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Crop performance might be adversely affected by nutrient deficiency as such, or by salinity induced nutritional disorders. These disorders might results from the effect of elevated salt levels on the availability of single nutrient components, an effect through competitive uptake, transport or partitioning within the plants (Grattan & Grieve, 1999).

Iron, a micronutrient, and phosphorus, a macronutrient, are essential minerals for both human and plants. Often these elements are abundant in soil, but not in a form that they are readily available for uptake by plants. Except for its role as a limiting factor in plant growth, iron deficiency is one of the leading nutritional human disorders in the world today (http://www.who.int/nut/ida.htm). Fe (III) reduction was thought to be the rate-limiting step for uptake in plants. Grotz and Guerinot (2002) give examples of new strategies with gene manipulation in plants, for isolated components of pathways within the metabolism to enhance nutrient availability.

2.3.1.2.2 Higher plant species in MELiSSA loop: wheat, tomato, potato, soybean, rice, spinach, onion and lettuce.

For a number of selected plant species, mineral content has been measured, but mostly for plants which are grown in soil. Vegetables relevant for the MELiSSA loop were monitored for minor and trace elements, and values are given in Table 6. The order of concentration levels in the vegetables was Ca>Na>K>Mg>Fe. Concentration of these elements generally is higher in plants than in the growth medium, because of their role as major nutrients. For trace elements, no general ranking was possible. Environmental conditions related to availability and plant uptake characteristics highly determine the final content of trace elements in the plant. Especially accumulation of toxic elements such as Cd, Pb and Ni generally mirrors the composition of the growth medium (soil, hydroculture).

Table 6: Minor and trace elements in vegetables cultivated in soil (after Mohamed *et al*., 2003).

Element (mg/kg)	**Tomato**	**Potatoes**	**Lettuce**	**Spinach**	**Onion**	**Soil (subsurface)**
Ca	3372	384	6114	17144	7450	547
Cd	0.77	0.84	1.04	0.77	0.76	0.36
Co	1.29	1.47	0.67	1.41	1.07	10.9
Cu	4.47	0.88	0.9	2.71	2.81	8.8
Fe	60.2	48.2	323.9	166.4	93.6	1720
K	1740	1700	2040	3100	2360	438
Mg	1938	1668	1866	2370	2000	115
Na	2440	2720	22	5480	280	152
Mn	7.39	5.67	20.37	9.9	3.62	34.9
Ni	14.64	10.74	0.7	17.14	18.37	2.26
Pb	2.59	2.81	3.7	9.44	10.29	1.2
Zn	14.4	4.5	42	9.6	17.6	5.9

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Plants in general are very sensitive to heavy metal toxicity, because of interference with essential trace elements (Ross, 1994). An example concerns the toxicity of cadmium which is shown through bioaccumulation and inhibitory effects on biomass productivity of wheat. Cd^{2+} uptake was seen to be at maximum during the initial growth period in seedlings of wheat. Cadmium did show interference with uptake of nutrients, especially Ca, Mg, K, Fe, Zn and Mn from the growth medium. Growth reduction and altered levels of major biochemical components such as chlorophyll, proteins, free amino acids, starch and soluble sugars were observed in response to Cd exposure (Shukla *et al*., 2003).
Bioconcentration of metals in plants should also be considered in terms of food-chain contamination. The most important metals with respect to food safety issues are arsenic, cadmium, mercury, lead and selenium. Therefore, plant breeding conditions and agricultural crop management needs to be critically evaluated in order to minimize plant-human transfer of potential toxic metals (McLaughlin *et al*., 1999; WHO, 2002a; Donma & Donma, 2005).

2.3.1.3 Man

About 50 of the known elements occur in measurable concentrations in the living systems. Four elements (oxygen, carbon, hydrogen, and nitrogen) account for 96% of living matter. In humans and other mammals, 23 elements have known physiological activities. From these elements, 11 can be classified as ‘‘trace elements’’ because of their essentiality and very limited quantity in humans. Out of these 11 trace elements, eight are in the period 4 of the Periodic Table (Figure 3), suggesting an optimal relationship of nuclei size/electron availability of the elements in this period to interact with organic molecules present in biological systems. These trace elements include the transition metals vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), zinc (Zn), and molybdenum (Mo); and the non-metals selenium (Se), fluorine (F), and iodine (I). All of these belong to the category of micronutrients, which are needed by the human body in very small quantities (generally less than 100 mg/day), as opposed to elements considered macronutrients, such as sodium, calcium, magnesium, potassium, chlorine, etc., which are required in larger quantities.

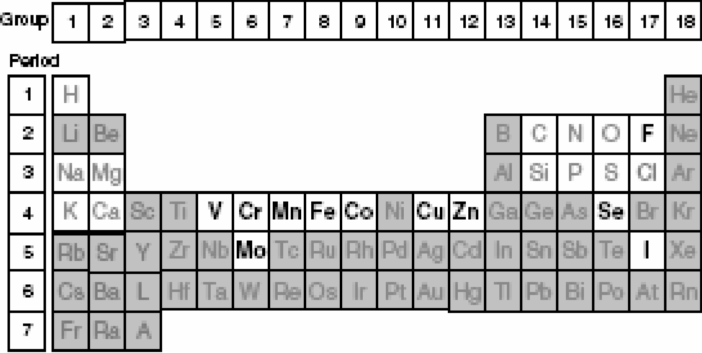

Figure 3: Periodic Table indicating elements essential for humans (white background) and the trace elements (black characters) (after Fraga, 2005).

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Trace elements are essential components of biological structures, but at the same time they can be toxic at concentrations beyond those necessary for their biological functions (Figure 4). Homeostatic mechanisms involve regulation of absorption and excretion and tissue retention, which enable adaptation to varying nutrient intakes. These mechanisms provide for an optimal systemic supply for the performance of essential functions (Table 7) and have been considered to establish values for recommended daily intake (RDA) or values for acceptable range of oral intake (AROI). Values for recommended daily intake have been defined by EU and are given in Table 8.

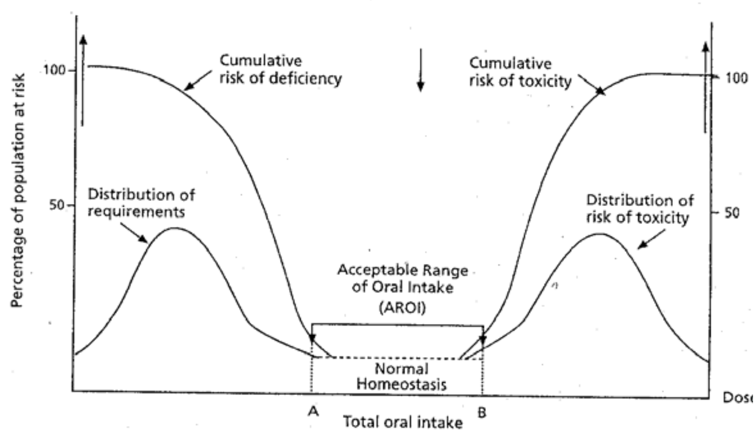

Figure 4: Percentages of population at risk of deficiency and toxicity effects according to oral intake (after WHO, 2002a).

Table 7: Functions, dietary sources, presence, and potential of toxicity for manganese, iron, copper, zinc, and selenium (after Fraga, 2005).

Element	Role
Manganese (Mn)	Associated with bone development, and with amino acid, lipid, and carbohydrate metabolism. Found in different enzymes, e.g. mitochondrial Mn superoxide dismutase, glutamine synthetase, arginase, and activates several hydrolases, transferases and carboxylases. Transported in the body by transferrin and by macroglobulins and albumin Sources of dietary Mn include grain, rice, tea, and nuts. Toxic in excess; in brain it can cause a Parkinson-type syndrome
Iron (Fe)	Found in 4 classes of proteins: Fe-heme proteins; Fe–sulfur enzymes, proteins for Fe storage and transport (transferrin, lactoferrin,..), and other Fe-containing or Fe-activated enzymes (e.g. NADH dehydrogenase, succinate dehydrogenase, ..) Total iron intake ranges from 14.4 to 20.2 mg/day. Serum Fe is about 1.3 mg/L, mostly bound to transferrin. Iron content in an adult man is about 4 g, decreasing to about 3 g in menstruating women. Fe deficiency causes anemia. Sources of heme Fe (15% of consumption) are hemoglobin and myoglobin from animals.

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	Sources of non-heme Fe are cereals, seeds of leguminous plants, fruits, vegetables, and dairy products. One of the most serious forms of Fe overload is acute Fe poisoning. Chronic Fe intoxication occurs frequently associated to genetic and metabolic diseases, repeated blood transfusions, or excessive intake.
Copper (Cu)	Necessary for development of connective tissue, nerve coverings, and bone, participates in both Fe and energy metabolism and as a reductant in the enzymes superoxide dismutase, cytochrome oxidase, … and several other oxidases that reduce molecular oxygen. Transported in the organism by the protein ceruloplasmin. There is about 80 mg of Cu in the adult body (highest in liver and brain) and median intake of Cu ranges between 1.0 and 1.6 mg/day in adults (US data). Sources of dietary Cu are liver and other organ meats, nuts, seeds, dark chocolate, and whole grains. Cu deficiency is rare, but when it occurs leads to anemia, leucopenia and neuropenia, and inclusive osteoporosis in children Excessive dietary Zn can cause Cu deficiency. Chronic Cu toxicity is rare in humans, and mostly associated with liver damage. Acute Cu intoxication leads to gastrointestinal effects characterized by abdominal pain, cramps, nausea, diarrhea, and vomiting.
Zinc (Zn)	Involved in the activity of about 100 enzymes, e.g. RNA polymerase, carbonic anhydrase, .. Present in Zn-fingers associated with DNA. Transported by ceruloplasmin. There are 2–3 g of Zn present in the human body (second to Fe in body content) and about 1 mg/L in plasma. Zn deficiency is common in underdeveloped countries and is mainly associated with malnutrition, affecting the immune system, wound healing, the senses, and impairing DNA synthesis. Support normal growth and development in pregnancy, childhood, and adolescence. Found in red meat and poultry, beans, whole grains, fortified breakfast cereals, and dairy products. Toxicity has been seen in both acute and chronic forms. Intakes of 150–450 mg of Zn per day have been associated with low Cu status, altered Fe function, reduced immune function, and reduced levels of HDL
Selenium (Se)	Incorporated into proteins to make selenoproteins, to be important antioxidant enzymes. Found in glutathione peroxidase, thioredoxins, and selenoprotein P. Obtained from grains, cereals, red meats and seafood. Deficiency is rare in the US but seen in other countries, most notably China, where soil concentration of Se is low. Deficiency may contribute to a form of heart disease, hypothyroidism, and a weakened immune system with higher susceptibility to illnesses caused by other nutritional, biochemical or infectious stresses High blood levels of Se (>1 mg/L) can result in selenosis. Symptoms include gastrointestinal upsets, hair loss, white blotchy nails, garlic breath odor, fatigue, irritability, and mild nerve damage. Se toxicity is rare, the few reported cases being associated with accidental exposure.

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Table 8: European Commission recommended daily intake (EC RDA, 1990) of micronutrients from all sources (after Flynn *et al.*, 2003).

Micronutrient	Amount	EC RDA recommended value
Vitamin A (retionol)	µg	800
Thiamin	mg	1.4
Riboflavin	mg	1.6
Niacin	mg	18
Vitamin B6	mg	2
Total folate	µg	200
Vitamin B12	µg	1
Panthotenic acid	mg	6
Vitamin C	mg	60
Vitamin D	µg	5
Vitamin E	mg	10
Biotin	µg	150
Calcium	mg	800
Copper	mg	1.15
Iodine	µg	130
Iron	mg	14
Magnesium	mg	300
Phosphorus	mg	800
Zinc	mg	15
Selenium	µg	55

Body of evidence exists that significant subgroups of population do have suboptimal nutritional status, as well as intakes below nationally recommended levels for many vitamins, minerals and trace elements. Several literature reviews and reports do give lists of minerals which are essential and in case of deficiency, they do impact human health. Other nutrient elements are also essential but give result to toxic insults or chronic diseases if optimal levels are exceeded (Campbell, 2001). This is illustrated by an epidemiological study on 2000 individuals, living in Western-Canada. Mineral levels were determined by analysis of hair samples (Table 9), and complementary information on health status was derived from a questionnaire. The most reported health related impairment was the occurrence of depression (31%). Lack of nutrients such as selenium and calcium has been shown to negatively impact our mind and emotions. The occurrence of low back pain (24%) and arthritis (20%) can be, at least, partly be attributed to mineral imbalances and especially lack of calcium and magnesium. Cardiovascular diseases were reported in 16 % of the tested population. Chromium, the most deficient mineral, is closely associated with the development of heart diseases, with elevated levels of cholesterol and plaque formation. Adequate chromium levels can result in an increase of high-density lipoprotein (HDL, or “good” cholesterol) and improves insulin utilization.

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Table 9: Percent of 2000 individuals either deficient in 12 essential minerals or showing excess of toxic minerals (after Campbell, 2001).

Essential mineral element	% deficient	Toxic mineral element	% excess
Chromium (Cr)	56	Aluminium (Al)	9.4
Magnesium (Mg)	49	Lead (Pb)	3.0
Zinc (Zn)	47	Cadmium (Cd)	0.8
Calcium (Ca)	46	Arsenic (As)	0.1
Manganese (Mn)	40	Mercury (Hg)	0.1
Selenium (Se)	40		
Potassium (K)	37		
Iron (Fe)	25		
Copper (Cu)	25		
Molybdenum (Mo)	15		
Phosphorus (P)	9		
Sodium (Na)	6		

Both drinking water and plant foods will be the major source of essential micronutrients to man in space. Except for total amounts, bioavailability of micronutrients in food is as important. Quantities of minerals in edible portions of crops are influenced by numerous complex, dynamic and interacting factors, including plant genotype, soil properties, environmental conditions and nutrient interactions. In addition, numerous dietary and host factors interact to affect the bioavailability of mineral nutrients. Micronutrient bioavailability can be improved by either increasing the quantity of substances in food that enhance the absorption and utilization of micronutrients or by decreasing the quantity of dietary antinutrients that inhibit micronutrient absorption (House, 1999). Examples are e.g. iron and zinc. In all mammalian cells, Fe is an integral constituent for hemoglobin and myoglobin, and a component in many enzymes (heme and non-heme Fe enzymes), which are involved in oxidation-reduction reactions. Fe in the body is stored as ferritin and hemosiderin, the amount of which is the determinant for alimentary absorption of Fe. Fe content of the body is well regulated and in general the human body does adapt successfully to a wide range of Fe requirements and intakes by modifying the rate of gastrointestinal absorption. Dietary factors which may depress iron absorption include (1) interactions with mineral elements through similar physico-chemical properties and shared absorptive pathways, (2) proteins from food origin, (3) polyphenolic compounds such as tannins, (4) saponins, (5) lectins, (6) stage of plant maturity , (7) phyate or inositol hexaphosphate and lesser phosphorylated derivates of inositol (House, 1999).

A proposal for nutritional requirements for man in space is given in Table 10 below. Comparison of a few values for minerals with values for recommended daily intake (Table 8) does show similarity.

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Table 10: Nutritional day advice for missions on ISS for a maximum of 360 days (Lane and Schoeller, 1999, after Régis *et al*. 2002).

Nutrient	Units	Requirement
Energy	kilojoules (kilocalories)	WHO equation
Protein	% total energy consumed	12 – 15
Carbohydrate	% total energy consumed	50 – 55
Fat	% total energy consumed	30 – 35
Fluid	ml per MJ consumed or ml/kcal	238-357 or 1.0-1.5 or at least 2l/d
Vitamin A	μg retinol equivalent	1000
Vitamin D	μg	10
Vitamin E	mg α-tocopherol equivalent	20
Vitamin K	μg	80
Vitamin C	mg	100
Vitamin B_{12}	μg	2
Vitamin B_6	mg	2
Thiamin	mg	1.5
Riboflavin	mg	2
Folate	μg	400
Niacin	mg niacin equivalent	20
Biotin	μg	100
Panthotenic acid	mg	5
Calcium	mg	1000 – 1200
Phosphorus	mg	1000-1200 < 1.5 times Ca intake
Magnesium	mg	350
Sodium	mg	1500 – 3500
Potassium	mg	3500
Iron	mg	10
Copper	mg	1.5 – 3
Manganese	mg	2 – 5
Fluoride	mg	4
Zinc	mg	15
Selenium	μg	70
Iodine	μg	150
Chromium	μg	100 – 200
Amino acids (*)		
Histidine	mg/kg body wt	8.0 - 12.0
Isoleucine	mg/kg body wt	10
Leucine	mg/kg body wt	14
Lysine	mg/kg body wt	12
Methionine and Cystine	mg/kg body wt	13
Phenylalanine and Tyrosine	mg/kg body wt	14
Threonine	mg/kg body wt	7
Tryptophan	mg/kg body wt	3.5
Valine	mg/kg body wt	10

(*) Essential Amino Acids (Cenci - McGrody and Stiller, 1997).

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2.3.2 THE FATE OF MINERALS IN DIFFERENT COMPARTMENTS

Mass balances for the macronutrients are available for the different MELiSSA compartments. These are represented by stoichiometric equations derived from the metabolic pathways of the respective organisms. Generally, they have been established in culturing conditions where the trace element requirements have been satisfied by some standard nutrient formulations (Godia *et al.,* 2002; Tikhomirov *et al*., 2003).

2.3.2.1 Compartment I

The feed composition has not yet been determined in terms of minerals and reactor content and permeate samples have only been analysed once. Hence, there is no information available on which minerals are taken up and to what extent. Because the influent is a complex mixture of beet, wheat, straw and faeces with urine, micronutrient demands are most probably covered by the feed.
Following tables do give some idea about content of minerals, including metals as trace elements in several parts of the feed in compartment I. In Table 11, values for metal content in domestic waste based on earth activities with a daily water consumption of 118 l/person are given. Though absolute values are not that relevant, the relative distribution does give an idea about major components. Zinc is one of the most occurring metals present in human faeces, followed by copper, lead, nickel and cadmium. Not only faeces but numerous domestic activities contribute to a significant proportion of metals discharged to sewers in residential areas (Atkins & Hawley, 1978). The distribution of the load of heavy metals is as follows: 48 % for hygiene water, 30 % corrosion of the water distribution system, 16 % faeces and urine, 5 % laundry.

Table 11: Amount of contaminants discharged from human waste through earth activities (after Atkins & Hawley, 1978).

Parameter	Amount	Concentration
	l/day	
Flow	112	
	g/Capita.day	mg/l
BOD	44	393
COD	94	839
Kj-N	10	89
P	1.7	15
SS	55	491
	mg/Capita.day	µg/l
Arsenic	0.55	5
Cadmium	0.14	1
Chromium	0.47	4
Copper	26.38	236
Mercury	0.05	0
Lead	4.19	37
Nickel	0.95	8
Zinc	30.72	274
Total Heavy metals	63.45	567

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Table 12 is an attempt to compile the different media used in the MELiSSA loop and the analyses so far available. In addition, preliminary data on the fecal and plant material and on urine have been included. In bench scale loop tests, inlet medium of compartment I was compared to outlet of compartment I and to artificial media required for compartments II, III and IVa (Creus et al., 2002a; 2002b). Difference between in- and outlet of compartment I might only be partly attributed to biomass production, and differences could additionally result from artefacts of the sampling procedure. Comparison of the compartment I permeate performed during continuous operation of a prototype reactor with the medium composition for downstream compartments either operated alone or coupled, points to potential shortages for Co, Cu, Mg, Mn, Mo and Na. Hence, the effect of limiting concentrations of several essential elements on compartment behaviour is of high interest to this study.

The first compartment is operated at 55°C under anaerobic conditions. Overall, we expect that minerals will be partly included in the micro-organisms biomass as a result of growth. In general, anaerobic treatment systems require trace elements such as iron, cobalt, nickel, zinc, copper, manganese, molybdenum, selenium, tungsten and boron, mainly to sustain the activity of the methanogenic population. Since the anaerobic compartment I of the MELiSSA loop does not include methanogenic activity, nutrient limitations are also less likely to occur. Speece (1996) confirms that trace element bioavailability is assured when the anaerobic process is performing satisfactorily. The degradation efficiencies achieved in the first compartment seem to confirm that no nutrient limitation occurs.

On one occasion, a bioreactor and permeate sample from compartment I have been analyzed at EPAS for micronutrient composition (see Table 13). The comparison of reactor content and permeate learns that some elements are retained in the reactor e.g. Al, Ba, Cd, Ca, Cr, Cu, Zn. This may be due to their complexation in larger molecules which are then physically retained by the membrane. For other elements e.g. Fe, K, Mg, Mn, permeate concentrations are similar or higher than the reactor content, which may be due to release from hardware material. Because the feed has never been analyzed, no information is available on biological incorporation in new biomass.
A second observation is that the permeate composition does not correspond with the synthetic medium fed to compartment II (seeTable 12). Several minerals are present in higher concentrations than needed for compartment II, e.g. Mg, Ca, K, Fe, Ni, Zn. The effect of a surplus on the downstream compartments, and their accumulation in closed loop will have to be evaluated. This is particularly an issue for the metals, though the choice of equipment and the design of the loop will exclude as much as possible any contamination by metals.
Mn, Na and Mo in the permeate (Table 12) are present in lower concentrations than those of the medium Segers and Verstraete which is used for cultivation of *Rhodospirillum rubrum* in compartment II. It will be subject of this research project to evaluate whether these levels of minerals may turn out to be problematic for growth performance and microbiological processes.

In TN 75.2 other compositions for CI effluent are mentioned but since these were determined after filtration, they are not considered to be representative enough.

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Table 12: Oligoelement concentrations in feed, compartment I reactor content and permeate, synthetic media for compartment II, III and IVa and for tests with coupled compartments II to IVa. Urine data were obtained from TN2 of the Microbial Water Treatment contract. Feed data were obtained from various internet sources and have to be considered with caution. Elements indicated in bold may become limiting because they are present in lower concentrations in the permeate of compartment I than in the media used for downstream compartments.

	Feed CI mg/g dw				CI content	urine	effluent CI	feed CII	feed CIII	feed CIV	feed CII-CIV
	wheat straw	lettuce	beet	fecal material	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
anions											
Cl	3.2					1300-5200		991	0.36	625	47
P	0.5-1	0.003-0.2			87	830		205	311	89	198
S	1.7					1170		150	327	211	965
B		< 0.04						0.02		2	0.002
cations											
Al					2.8	0.06	<0.1	/	/	/	/
As					0.019	0.016	<0.02	/	/	/	/
Ba					0.43		0.24	/	/	/	/
Be					<0.015		<0.005	/	/	/	/
Ca	2-5	0.02-0.19	7		250	150-200	190	14	0.2	12	24
Co					0.021		**0.013**	**/**	**/**	**0.04**	**/**
Cd				0.0003	0.014		<0.001	/	/	/	/
Cr				0.0006	5.2	0.007	0.14	/	/	0.04	/
Cu				0.03	2.2	0.067	**<0.01**	0.001	0.001	**0.082**	0.001
Fe		0.005-0.5	0.3		70	0.35	88	4	0.5	2	6
Hg					0.003		<0.001	/	/	/	/
K	14	0.05-0.2	34	8	570	600-1400	550	373	195	513	362
Mg	4	0.01-0.05			50	100-750	**46**	20	5	20	**117**
Mn		< 0.2			1.3	0.07	**1.7**	**3**	/	**2**	**3**
Mo		0.05			0.72		**<0.01**	**0.02**	**93**	**0.07**	**94**

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Table 12 (continued)

	Feed CI mg/g dw wheat straw	lettuce	beet	fecal material	CI content mg/l	urine mg/l	effluent CI mg/l	feed CII mg/l	feed CIII mg/l	feed CIV mg/l	feed CII-CIV mg/l
Na	1	0.09	16		120	1300-2500	**71**	**243**	**449**	**8088**	**251**
Ni				0.002	1.9	0.005	1.1	0.1	/	0.04	/
Pb		0.001		0.0006	0.073	0.02	<0.02	/	/	/	/
Sb					<0.01		<0.03	/	/	/	/
Se					0.01	0.025	<0.01	/	/	/	/
Si					32	6.5	28	/	/	/	/
Sr					0.69		0.58	/	/	/	/
Sn					0.16	0.019	<0.05	/	/	/	/
Ti					0.15		<0.02	/	/	/	/
V					0.021		<0.02	/	/	/	/
W					<0.01		<0.025	/	/	/	/
Zn	0.006			0.3	8.3	0.03	0.32	0.02	0.0009	0.20	0.001

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Table 13: Mineral composition of reactor content and permeate from the first compartment (source: EPAS).

Compound	Concentration in reactor content (µg/L)	Concentration in permeate (µg/L)
Total phosphor P	87 000	
Aluminium Al	2800	<100
Antimony Sb	<10	<25
Arsenic As	19	<20
Barium Ba	430	240
Beryllium Be	<15	<5
Cadmium Cd	14	<1,4
Calcium Ca	250000	190000
Chromium Cr	5200	140
Cobalt Co	21	13
Iron Fe	70000	88000
Potassium K	570000	550000
Copper Cu	2200	<10
Mercury Hg	2.7	0.16
Palladium Pb	73	<20
Magnesium Mg	50000	46000
Manganese Mn	1300	1700
Molybdenum Mo	720	<10
Nickel Ni	1900	1100
Selenium Se	10	<10
Silicon Si	32000	28000
Sodium Na	120000	71000
Strontium Sr	690	580
Tin Sn	160	<50
Titanium Ti	150	<20
Vanadium V	21	<20
Tungsten W	<10	<25
Zinc Zn	8300	320

Apart from biological processes, the presence of carbonates and phosphate in the first compartment may lead to the precipitation of multivalent ions such as Ca, Mg, …. For monovalent ions, a general flowthrough is anticipated.

Based on the above, the multivalent ions are of most interest because consumption and precipitation processes may lead to shortages. On the other hand, some of them appear in higher concentrations in the effluent than required for compartment II and may lead to accumulation.

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2.3.2.2 Compartment II

The synthetic medium used for cultivating *Rhodospirillum rubrum* is the medium of Segers and Verstraete. This contains micronutrients required for its growth (Table 14). Compartment II is operated at 30°C under anaerobic conditions. It is conceived as a photobioreactor and aims at maximal production of biomass. It is however not clear whether the proposed medium is the most optimal for these test conditions within the BELISSIMA loop.

Table 14: Composition of medium Segers and Verstraete (Suhaimi et al., 1987)

Compound	g/l
NH_4Cl	0.76
Na_2SO_4	0.54
EDTA	0.02
$MnCl_2.4H_2O$	0.01
CH_3COOH	2.5
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.05
KH_2PO_4	0.49
K_2HPO_4	0.52
$FeSO_4.7H_2O$	0.02
$NaHCO_3$	0.25
MOPS	21
Trace elements	1 ml/l

Trace elements	g/l
$NiSO_4.6H_2O$	0.5
$ZnSO_4.7H_2O$	0.1
$CuSO_4.5H_2O$	0.005
H_3BO_3	0.1
$Na_2MoO_4.2H_2O$	0.05
Vitamin solution	
Biotin	0.015

Like for compartment I, inorganic compounds can be consumed because they are taken up by growing biomass (Saint-Germes, 2000). In batch cultures, Mg and Fe deficiency reduced growth at 70%. Mn deficiency did not exert a negative effect on growth. A follow-up on trace element concentration indicated that

- Mg and Mn were consumed
- Zn requirements were low since no concentration changes occurred
- Ni and Fe concentrations increased.

Gauthey (2001) observed that the latter results were related to the release of Ni and Fe from the construction material of the reactor. Na uptake has been described as well and interactive effects between Na and K seem to take place.

Due to the presence of carbonates, precipitation of multivalent ions may occur. For monovalent ions, no processes other than uptake in biomass are expected. If metals appear in the effluent of compartment I, they may sorb on the biomass and may affect its nutritional quality.

In conclusion, both multivalent and monovalent ions are of interest for compartment II.

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2.3.2.3 *Compartment III*

As opposed to compartments II and IVa, the main aim of the nitrifying compartment is to obtain nitrifying activity, rather than biomass growth. It is operated at 30°C and it is the first aerobic compartment in the MELiSSA loop. The medium supplied to compartment III is adapted from Wijffels (1994) with the composition given in Table 15.
The actual consumption of the minerals present in the medium has however not yet been studied with the *Nitrobacter/Nitrosomonas* coculture used in the MELiSSA loop. Due to their very slow growth, no significant uptake of minerals is expected and the main biological transformation of inorganic compounds will be the nitrification reaction itself.

Table 15: Composition of nitrifiers medium (Wijffels, 1994).

Compound	**g/l**
$(NH_4)_2SO_4$	1.32
$MgSO_4.7H_2O$	0.052
$CaCl_2.2H_2O$	7.4E-4
KH_2PO_4	0.68
Na_2HPO_4	0.71
$FeSO_4.7H_2O$	2.5E-3
$NaHCO_3$	0.8
$ZnSO_4.7H_2O$	4.3E-6
$CuSO_4.5H_2O$	4.0E-6
$(NH_4)_6Mo_7O_{27}.4H_2O$	0.177

Because nitrifiers are slow growing organisms, they are very sensitive to inhibitory effects and optimal medium composition is crucial to maintain their activity. Sato *et a*l. (1988) observed an increased Cu toxicity towards *N. europeae* at increasing ammonia concentrations. The 50% inhibition concentrations decreased from 0.5 to 0.01 mg/l Cu^{2+} at total ammonia concentrations of 3 and 23 mg N/l respectively.

No precipitation of minerals is expected. However, because compartment III is the first aerobic one, some oxidation reactions (e.g. iron) may occur which eventually result in precipitates.

In conclusion, compartment III is an ideal compartment to evaluate the inhibitory effect of inorganic compounds which accumulated or were released in upstream compartments. No major changes in mineral composition of the incoming medium are anticipated here except for the nitrification reaction itself. Performance of the cultures and N-balances will be evaluated and data can be used as a measure of any unwanted effects through toxicity or accumulation trace elements.

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2.3.2.4 Compartment IVa

Compartment IV consists of an aerobic photobioreactor operating at 36°C. *Arthrospira platensis* is typically grown in Zarrouk medium (Table 16), which has a high pH and contains a high salt concentration.

Table 16: Composition of Zarrouk medium (Zarrouk, 1996)

Compound	g/l
$NaNO_3$	2.5
$NaHCO_3$	13.6
K_2SO_4	1
NaCl	1
$MgSO_4.7H_2O$	0.2
$CaCl_2.H_2O$	0.04
$FeSO_4.7H_2O$	0.01
EDTA-Na_2	0.08
K_2HPO_4	0.5
Na_2CO_3	7.6
Solution A5	4 ml/l
Solution B6	4 ml/l
pH	9.5-11

Solution A5	g/l
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
MoO_3	0.015

Solution B6	g/l
$KCr(SO_4)_2.12H_2O$	0.096
$NiSO_4.7H_2O$	0.048
$(NO_3)_2Co.6H_2O$	0.049
$Na_2MoO_4.2H_2O$	0.018

Uptake rates by growing biomass have been studied to some extent. Cogne *et al*. (2002) concluded from batch and continuous cultures that

- B, Mo, V, Cr, Ni, Co, W and Ti are not required
- Zn, Fe, Mn and Mg are fixed in the biomass.

The suggested minimum medium for growth contains 0.23 mg/l $MnCl_2.4H_2O$, 0.11 mg/l $ZnSO_4.7H_2O$ and 0.03 mg/l $CuSO_4.5H_2O$. Chloride concentrations should exceed 32 mg/l. In bench scale loop tests, Creus *et al*. (2002b) mention that the K/Na ratio should not exceed 5 in order to prevent growth inhibition in *A. platensis*. Li *et al.* (2003) demonstrated that sodium selenite concentrations below 400 mg/l stimulated growth of *A. platensis*. The Se content of the biomass increased with the selenite concentration in the medium. At higher concentrations, toxic effects were apparent. Like *R. rubrum*, *A. platensis* could reduce selenite to elemental Se, which is less toxic.

Non-biological elimination is expected as well. Because the medium contains high levels of carbonate, bicarbonate, .. precipitation reactions may occur. Sorption of metals (those which are essential micronutrients) on the biomass may affect its nutritional quality.

For compartment IVa some information is already available on microcompound uptake. Both multi- and monovalent ions seem to be of interest.

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2.3.2.5 Compartment IVb

Composition of the nutrient solution in the higher plant compartment (HPC) is derived from the ESA annual report, 2005 (personal communication G. Waters, see Table 17).

Table 17: Composition of hydroponic solution HPC.

Component	Concentration (mM)
Ca^{2+}	3.62
Mg^{2+}	1.0
Mg^{2+}	1.0
Mn^{2+}	0.005
Zn^{2+}	0.035
Cu^{2+}	0.0008
Fe^{3+} (as Fe-DTPA)	0.025
B^{3+}	0.02
Mo^{6+}	0.0005
K^{+}	5.0
Na^{+}	0.008
NH_4^{+}-N	4.0
$P0_4^{3-}$	1.5
$N0_3^{2-}$-N	11.75
$S0_4^{2-}$	2.0
pH	5.5 (with NaHCO3)

No information is yet available on changes of mineral balance for the HPC compartment.

2.4 Tentative priority selection of compounds

It has been shown in the foregoing overview that a multitude of trace elements or micronutrients are essential for organisms in the loop. Given the fact that multivalent ions were identified as interesting in the analysis of individual compartments (especially I-IV) and that some of them may be transformed in organic compounds which show tendency to bioaccumulate, these are potential candidates for analysis in this study. The cationic elements of major interest due to their physiological role in biochemical and cellular processes in micro-organisms, plants, and humans will be Fe, Co, Cu, Mg, Mn, Mo, Zn and Ca. Na and K are major elements, occurring in each of the compartments, and important as an indicator of increased salt levels. As is demonstrated in literature, this might also be critical to normal functioning of the system. With respect to salt levels, the counterions Cl^- and SO_4^{2-} should be monitored in the loop to estimate mass balances. In addition to being a counterion, S does also play an important physiological role as an element in several amino acids, and thus protein structures.

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Additionally, P is an essential element in the structure of nucleic acids present in all organisms. Therefore, both S and P as major anions should be considered for their fate in the BELISSIMA loop in relation to their role in essential functions for all organisms involved.

2.5 Identification of technical requirements and critical steps of design of BELISSIMA loop & study protocol

2.5.1 EXPERIMENTAL SET-UP

The media for growth and normal functioning of organisms in the MELiSSA loop are defined (see above). The fate of minerals will be evaluated by sampling influent and effluent and selected samples of the biomass in each compartment. Estimates of requirement of trace elements can also be determined from the composition of the cells, taken into account the growth rate and the turn over of fresh medium.

The experimental procedure will be set-up stepwise (individual compartments, or off-line batch experiments) in order to prevent irreversible effects in coupled compartments of the BELISSIMA loop. Because compartment I has never been coupled to compartment II, the effects of compartment I effluent on the micro-organisms and behavior of compartments II to IVa are not yet fully understood. Therefore we propose to characterize the fate of micronutrients in compartment I and the subsystem with compartments II to IVa separately and in parallel. After analytical methods have been validated, the accuracy of the measurements and number of repeated measurements will be taken into account to get reproducible results and allow statistical analysis. This approach will be described in the validation step and will be based on preliminary experiments. This information is part of the experimental design being reported in future TN.

The cation elements of major interest are Fe, Co, Cu, Mg, Mn, Mo, Zn, Ca, K and Na. Methods for sample treatment and analytical determination will be optimized primarily for adequate results for these elements. However as ICP analysis is proposed for those elements where detection limit is not critical, simultaneous analyses of about 15 compounds is possible. These include also some metals (Cd, Zn, Al, ..) which might result from contamination, which should be warranted for with respect to the design of the BELISSIMA loop.

Anion elements to be measured will be S, P and Cl. ICP can also be used to screen for total levels of S and P. In order to screen for total levels of Cl, colorimetric test methods can be used (e.g. Merck Spectroquant). In these methods total levels are measured.

It is foreseen that during the experimental set-up at least weekly samples from different flows (influent/effluent/biomass) of individual compartments, or coupled compartments are needed to characterise the fate of mineral changes (bioaccumulation and shortages). Subsequent sampling for a longer time period will allow to map dynamic changes in the system.

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The protocol for sampling of compartments and data collection will be discussed with UBP, responsible for the modelling work, in order to provide them with the minimal required data (which elements, frequency) for their models (Régis *et al*., 2002; draft TN 79.1).

Stepwise changes in trace element concentrations will not be aimed for because concentration increases may lead to medium instability e.g. precipitation reactions. Instead, changes in flow are preferred taken into account not to add any significant stress to the micro-organism populations. Due to a lack of operational experience, particular attention needs to be paid to the future liquid transfer between compartment I and II. The composition of compartment I effluent will be compared with the synthetic feed used up to now for compartment II to IVa. With regard to routine operation, the mineral content of feed to compartment I will extensively be studied for overall mineral content in order to get an idea about the variability of the composition.
If process limitations will be observed during tests with individual compartments, particular trace elements may have to be added in between the 2 compartments. However, this will be reduced to the minimum. After a while, transfer of medium without trace element addition will have to be evaluated as well.
The experimental design will be set up in a way to get a minimum number of accurate data to perform statistical analysis in order to detect significant differences in time, and between compartments. Routine statistical methods (Student T-test, Anova, Dunnet test) will be used.

2.5.2 SAMPLE PROCESSING

The different types of samples are inlet media (feed), outlet media, and biomass. They might be of different quality: solid samples, or liquid samples with different turbidity. Samples should be stored adequately. Samples which can be analysed on the same day of sampling will be stored in the refrigerator and in the dark. However, in most occasions, samples need to be stored for longer periods prior to analysis. In that case, liquid samples will be filtered and stored in a freezer at -20°C. Biomass samples to be analysed will be centrifugated , and supernatant and pellet samples will be stored separately at -20°C. Technical design should be as such that at least weekly 15 ml of influent and effluent samples can be taken in axenic conditions. Occasionally, samples of biomass will be taken to check for mineral content of organisms. Based on data for mineral content and biomass density, given by Creus *et al.* (2002a, TN52.6), it is estimated that solid samples of approximately 0.5 g will be required (thus on average 1 l samples taken into account the density of 1.5 g/l *R. rubrum*, or *S. platensis* of 1 g/l) for determination of trace element concentrations. Care must be taken with respect to minerals in biomass, needed for the calculation of requirements, because excess of micronutrients can be adsorbed onto the cell walls. Therefore determination of concentration in biomass ash could exceed the actual required (= assimilated internally) amount of mineral elements. Biomass samples should adequately be rinsed prior to processing for analysis. Adequate rinsing procedures will be optimised through evaluation of a sequence of 3-4 rinsing steps with physiological saline (isotonic conditions), and measuring the mineral content in the sequence of rinsing solutions.

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Specific saline solutions with required osmotic potential should be chosen in order to allow measurements of the background of any of the target mineral elements. Sample treatment methods (e.g. total digestion, acid extraction, ..) of biomass, or suspended particulate matter in general will be evaluated for each type of sample according to pre-defined performance characteristics. In general, depending on the purpose and the method of the analyses in the sample, storage conditions and sample treatment procedures will be defined and available through standard operating procedures (SOP). During phase I of the project, preliminary tests on batch cultures will be performed in order to provide adequate methods for sample treatment and subsequent analysis of liquid media and biomass subsamples.

2.5.3 ANALYTICAL METHODS

A number of critical aspects related to analyses of micronutrients have already been defined (see 2.2 and 2.3). Presence of interfering mineral elements at high levels (salts), effect of pH or chelating component in medium samples, or the complex matrix can modify the analytical result. While the total content of macronutrients can be reasonably high (up to mg/l range), the level of micronutrients and free ions of metals is likely to be in the lower concentration range (µg/l).

Therefore taken into account the method of choice (ICP, AAS,…), the matrix of the sample, methods will be validated according to current VITO procedures (Zwijzen *et al.*, 2003). The method of choice will depend on the element and concentration range to be expected. A number of methods as specified in Table 18 and Table 19 can be used. Specific quality criteria, such as reproducibility and sensitivity will be determined for each of the target elements and matrices during the course of the study.

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Table 18: performance characteristics for 4 analytical methods of choice for mineral analysis (after www.perkinelmer.com)

Table 2. Comparison of the Various Atomic Spectroscopy Techniques

Criterion	Flame AA	GFAA	ICP-OES	ICP-MS
Detection limits	high ppb	sub ppb	sub ppb-ppm	sub ppt
Analytical capability	single element	single element	multielement	multielement
Sample throughput	~3-10 sec/ element/sample	~2-3 min/ element/sample	~1-5 min/sample	~1-4 min/sample
Dynamic range	mid ppm range	low ppm range	high ppm range	mid ppm range
Precision				
Short-term	0.1-1%	0.5-5%	0.1-2%	~0.5-2%
Long-term	1-2% (double-beam)	1-10%	~1-5%	< 4% (4 hours)
Interferences				
Spectral	few	very few	some	few
Chemical	many	many	very few	some
Physical	some	very few	some	some
Dissolved solids handling	up to 5%	up to 10%	up to 20%	< 0.2%
Elements applicable to	> 60	> 50	> 70	> 80
Sample volume required	4-8 mL/min	~0.2-1 mL	~1-2 mL/min	~0.02-2 mL/min
Semi-quantitative analysis	no	no	yes	yes
Isotopic analysis	no	no	no	yes
Ease-of-use	very easy	more difficult	easy	more difficult
Method development	easy, cookbooks	fairly easy, cookbooks	fairly easy	more difficult
Unattended operation	no, flammable gas	yes	yes	yes
Initial costs	low	med	high	very high
Operating costs	low	high	med	high
Cost per sample (overall)	low	med	low	med

In most conditions, micronutrient uptake from media and in the organisms is followed by inductively coupled plasma emission spectroscopy (ICP) or for lower detection levels by this technique in combination with mass spectrometry or by atomic absorption spectrophotometry (AAS). Under ideal conditions, detection limits for ICP typically vary between 0.68 and 77 µg/l for Mg and Se respectively. For graphite furnace AAS, these are typically reduced to between 0.004 and 0.15 µg/l for Mg and Co respectively (see Table 19). For both techniques, this is far below the concentrations to be expected. In complex media, the detection limit will obviously change but they will still be in the required range for this study.

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Table 19: Detection limits for various analytical techniques for most mineral determination (theoretical values for optimal conditions).

	ICP-OES	ICP-MS	Flame-AAS	Graphite Furnace AAS
	µg/l	µg/l	µg/l	µg/l
Na	40,83	0,0003	0,3	0,005
K	1731	0,0002	3	0,005
Ca	3,42	0,0002	1,5	0,01
Mg	0,68	0,0003	0,15	0,004
Mn	1,38	0,00007	1,5	0,005
B	-	0,003	1000	20
Fe	8,65	0,0003	5	0,06
Zn	2,19	0,0003	1,5	0,02
Cu	9,46	0,0002	1,5	0,014
Co	4,97	0,0009	9	0,15
Ni	-	0,0004	6	0,07
Mo	-	0,001	45	0,03
Se	76,76	0,0007	100	0,05
P	47,10	0,1	75000	130
S	10	30	-	-

To determine total levels of anions, and eventually some of the speciation forms (see Table 20) several techniques are available which are listed in the table below. In general it is estimated that up to 50 ml is required for analysis of these elements. The method of choice will depend on the levels of ions to be expected. For the analysis of a single anion, one should collect at least 5 ml to apply one of these methods.

Table 20: Range of measurement for various methods to determine anions in aquatic samples.

	Colorimetric test kits	Automated flow analysis	Ion chromatography
	mg/l	mg/l	mg/l
Cl	1-250	10-150	0.1-50
PO_4^{3-}	0.03-300	0.01-1 (as -P)	0.1-20
SO_4^{2-}	5-500	10-200	0.1-100
SO_3^{2-}	1-20		0.1-100
S_2^-	0.02-1.5		
N	0.5-150		
NO_3^-	0.4-100	2-10 (as –N)	0.1-50
NO_2^-	0.03-2.3	0.25-1.0 (as –N)	0.05-20

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2.5.4 TECHNICAL REQUIREMENTS FOR LOOP DESIGN

A critical issue, as appeared from former studies might be the materials to be used, which could lead to release of contaminants into the system. Both organic components from sealings, coating of materials,…as well as inorganic contaminants, mainly metals could pose a problem. Metals can either compete for mineral uptake, and if levels do accumulate they can reach toxic levels for MELiSSA organisms. The construction material should ideally be an inert material. Moreover, as pH control is performed by addition of a concentrated acid solution, the sites of dosing the acid should be protected for interference or effects by acid corrosion.

Application of acid & base solution (normally HCl/NaOH) should take into account the enrichment of specific minerals such as Na. Depending on the mass balances of measured minerals, other solutions for pH correction could be considered. To avoid interference with the concentration level of several minerals, unidirectional pH adjustment using either HCl or NaOH may be preferred. It should anyway be limited as the total conductivity, or specific salt levels can increase up to sub-optimal levels for organisms. Technical design should be as such that at least weekly 65 ml samples of influent, effluent and reactor content can be taken to analysis the anion (50 ml) and cation (15 ml) concentrations. However, if specific elements (e.g. metal contaminants) need to be measured at levels close to detection limits with other methods than ICP, then an additional 15 ml sample should be taken. For the collection of data for modelling work of mass balance for selected mineral elements (Na, K, Cl, Mg, Fe and Ca), as required by Prof. Gilles (UBP) daily samples should be taken. In that case, a volume of 15 ml will be required. These 15 ml samples will simultaneously be used for daily measurements of conductivity. At a 2-week period, samples of biomass will be taken to check for mineral content of organisms. Based on data for mineral content and biomass density, given by Creus *et al*. (2002a, TN52.6), it is estimated that solid samples of approximately 0.5 g will be required, corresponding to 1 l at maximum.

Related to the content of the compartments, and the composition of the influent and effluent, either biomass or suspended solids might be present. Equipment for sample collection and access to the reactor compartments should be designed in a way that allows to take a homogenous sample. The homogeneity of samples to be collected is critical based on the likelihood that precipitates of mineral salts can be present, or adsorption phenomena can take place, which might lead to non-representative samples.

Continuous cultures are preferred compared to batch culturing because they will allow to study mass balances under steady state conditions and constant concentration levels of the medium constituents.

This is summarized in Table 21.

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2.6 Conclusions

So far, few data are available on the consumption, sorption or uptake, biotransformation or biomagnification of micronutrients in individual or coupled compartments. This remains a vast and yet unexplored area of study with relevance to estimate the accumulation potential of particular elements and their effect on microorganism kinetics and nutrional value.
The direct coupling of compartments I and II has not yet been attempted within the MELiSSA consortium. Given the complex composition of the feed to the first compartment and the limited knowledge on the compartment I permeate, its impact on the other organisms of the loop is expected to be very high. Based on preliminary data, the composition of compartment I effluent may lead to shortages in downstream compartments for some minerals. The elements of interest seem to be Fe, Co, Cu, Mg, Mn, Mo, Zn, Ca and Na and K for cations. Given the fact that multivalent ions were identified as interesting in the analysis of individual compartments and that some of them may be transformed in organic compounds which show tendency to bioaccumulate, these are potential candidates for analysis in this study. Analytical techniques in the required concentration ranges are available. Anions to be considered are P, S and Cl, based on their presence as counterions in salt balances, and mostly because of their essential physiological role and constituent in e.g enzyme systems.

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Table 21: Design requirements for minerals study.

	Compartment I	Compartment II	Compartment III	Compartment IV
operation mode: batch < > continuous	continuous	continuous	continuous	continuous
culture medium: as is, optimal nutrient concentrations, limitations, ..	Several conditions: - As is - Increased flow - nutrient levels to be optimised related to performance of culture	sSeveral conditions: - As is - Increased flow - Medium with nutrients to be optimised related to performance (batch tests)	Several conditions: - As is - Increased flow - Nutrients content eventually modified for bether performance (batch test)	Several conditions: - As is - Increased flow - Nutrient to be improved based on results batch test
sterile cultivation/operation: yes/no	No specific requirements	axenic	axenic	axenic
choice acid/base: - only acid/only base/both - which acid/base	HCl/NaOH (to be changed if levels of Cl & Na: salt become critical for organisms!!)	HCl/NaOH (to be changed if levels of Cl & Na: salt become critical!!)	HCl/NaOH (to be changed if levels of Cl & Na: salt become critical!!)	HCl/NaOH (to be changed if levels of Cl & Na: salt become critical for organisms!!)
samples: influent, effluent, biomass (solids), supernatant, at different reactor heights or locations, gas phase	I, E, B, S ->homogenous	I, E, B, S ->homogenous	I, E, B, S ->homogenous	I, E, B, S ->homogenous

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	Compartment I	**Compartment II**	**Compartment III**	**Compartment IV**
sampling conditions : - axenic - continuous versus grab samples	Grab, consider human safety of handling sample (infectious)	grab	grab	grab
sample volume : indicate maximum volume	See below Liquid phase: 80 ml Biomass: 0.5g (1 liter)	See below Liquid phase: 80 ml Biomass: 0.5g (1 liter)	See below Liquid phase: 80 ml Biomass: 0.5g (1 liter)	See below Liquid phase: 80 ml Biomass: 0.5g (1 liter)
sampling frequency: - daily, weekly, .. - intensive sampling campaign - over which period	Liquid phase: - Daily: 15 ml - Weekly: 80 ml Biomass: each 2 weeks	Liquid phase: - Daily: 15 ml - Weekly: 80 ml Biomass: each 2 weeks	Liquid phase: - Daily: 15 ml - Weekly: 80 ml Biomass: each 2 weeks	Liquid phase: - Daily: 15 ml - Weekly: 80 ml Biomass: each 2 weeks
sample collection: - in effluent tank or separate collection - through sampling ports - protect from light - temperature	Effluent tank Not critical Not critical As is (not exceeding temp of compartment)	Effluent tank Not critical Not critical As is (not exceeding temp of compartment)	Effluent tank Not critical Not critical As is (not exceeding temp of compartment)	Effluent tank Not critical Not critical As is (not exceeding temp of compartment)

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	Compartment I	**Compartment II**	**Compartment III**	**Compartment IV**
sample treatment: - centrifugation, filtration, mixing, concentration, .. - sterilization	Likely filtration for influent/effluent & supernatant (should be tested for eventual loss, otherwise centrifugation) Reactor samples to be sterilised Biomass samples centrifuged and separate frozen samples for pellet and supernatant	Likely filtration for influent/effluent & supernatant (should be tested for eventual loss, otherwise centrifugation) Reactor samples to be sterilised Biomass samples centrifuged and separate frozen samples for pellet and supernatant	Likely filtration for influent/effluent & supernatant (should be tested for eventual loss, otherwise centrifugation) Reactor samples to be sterilised Biomass samples centrifuged and separate frozen samples for pellet and supernatant	Likely filtration for influent/effluent & supernatant (should be tested for eventual loss, otherwise centrifugation) Reactor samples to be sterilised Biomass samples centrifuged and separate frozen samples for pellet and supernatant
sample preservation:T, protect from light, max. period	*4°C, dark (max 3 days)→ to be verified as it is function of sample type and treatment condition* *-20°C (1 month)*	*4°C, dark (max.3 days)→ to be verified as it is function of sample type and treatment condition* *-20°C (1 month)*	*4°C, dark (max.3 days)→ to be verified as it is function of sample type and treatment condition* *-20°C (1 month)*	*4°C, dark (max. 3 days)→ to be verified as it is function of sample type and treatment condition* *-20°C (1 month)*

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	Compartment I	Compartment II	Compartment III	Compartment IV
required measurements/instrumentation - as for normal reactor operation: yes/no - on-line measurements – which	Yes	Yes	Yes	Yes
required control: pH, T, ..	pH, Temp	pH, Temp	pH, Temp	pH, Temp
material selection	Omit metal parts, sensitive for corrosion by acid Choice of materials should omit any release of organic or inorganic contaminant	Omit metal parts, sensitive for corrosion by acid Choice of materials should omit any release of organic or inorganic contaminant	Omit metal parts, sensitive for corrosion by acid Choice of materials should omit any release of organic or inorganic contaminant	Omit metal parts, sensitive for corrosion by acid Choice of materials should omit any release of organic or inorganic contaminant

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3 HORMONES

3.1 Definition

The hormones or endocrine active compounds, which will be evaluated are the human estrogens and androgens, and the synthetic hormones used in the female anti-conceptive pill (Greenspan & Gardner, 2001). These groups of hormones are most likely to occur in urine, and belong to the feed in compartment I. Except for the parent compounds, metabolites or conjugated compounds can occur in urine, and may appear during degradation processes in compartment I. In faeces too, hormones and derivates can occur but their amounts are only a fraction compared to the urine content. All these compounds from human origin are likely to occur as input to the BELISSIMA loop and will be considered. The estimates for levels of hormones will be mainly derived from data in urine, as only for this most accurate data have been reported in literature.

3.2 Global objectives with respect to the study of hormones

Hormones of natural origin, but also synthetic hormones can be present in the feed of compartment I. First, it will be important to perform an extensive characterization of compartment I, especially with respect to the effluent composition in terms of endocrine activity. Metabolisation, degradation, sorption or accumulation and biomagnification of these compounds can occur. Their fate throughout each of the compartments of the loop will be estimated. Furthermore it is questioned to what extent these endocrine active compounds will affect the kinetics and stability of the organisms in the different compartments, and hence the overall reactor performance.

3.3 State of the art

3.3.1 HUMAN HEALTH AND ENVIRONMENTAL CONCERN FOR ENDOCRINE DISRUPTION

There is growing concern about harmful effects to human health and wildlife by the occurrence of antropogenic compounds with hormone disrupting potential, named ‘xeno-oestrogens’ and pollution by natural and synthetic hormones (Colborn & Clement, 1992; Colborn *et al*., 1996, Vieira da Silva, 2004; WHO 2002b). Indeed, within the EU, the problem of endocrine disruption has been recognized and action plans have been communicated through the EU strategy paper (EU Com, 2001). Within extended lists of compounds to be assessed for their estrogenic potency and risk as part of future research and policy (e.g. industrial compounds to be banned, or limit values to be defined) natural and synthetic hormones too are on one of the priority lists.

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The occurrence of endocrine disrupting compounds in the aquatic environment has been extensively studied in the past few years. Inventories of hormone distribution and national monitoring programmes (Blok & Wösten, 2000; Witters *et al.,* 2000; Witters *et al.,* 2002; Witters *et al.,* 2003; Vethaak *et al.,* 2002) have pointed to the likelihood of elevated levels of these hormones and xeno-oestrogenic chemicals in the rivers, in effluents of waste water treatment plants, in ground water wells and in drinking water resources. Especially hormones discharged through effluents from municipal waste water treatment plants and natural hormones from agricultural activity appear to pose a significant threat to the environment (Johnson *et al*., 2000; Ternes *et* al., 1999a; Ternes *et al.,* 1999b; Van den Belt *et al.,* 2002; Van den Belt *et al.,* 2003; Lange *et al.,* 2002). Based on some recent studies which model the release of hormones, present in human waste and livestock waste, it appears that there is still a lack of data on the environmental fate of steroidal hormones. The concern remains high, as a few studies have shown that there is no complete degradation at treatment plants (Hanselman *et al.,* 2003; Johnson & Williams, 2004). Compared to other industrial chemicals with hormone-like properties (e.g. xeno-estrogenic compounds), these natural and synthetic hormones are of particular importance for environmental risk assessment because they have the highest potency. At extremely low concentrations (range ng/l) estrogenic natural and synthetic hormones have shown biological hazardous effects to aquatic organisms. Harmful effects such as sex reversal, vitellogenin induction, and failure of normal reproduction in natural fish populations and other wildlife organisms have been demonstrated (Vos *et al*., 2000; Van den Belt *et al.,* 2002; Van den Belt *et al.,* 2003). In addition, there is concern for human health effects through transfer of xeno-oestrogens and androgens in food and drinking water (WHO, 2002b; Vieira da Silva, 2004).

The occurrence of hormones in food and water and their potential effects for human health is highly relevant, and should be considered in the context of the BELISSIMA loop. Major risks are dependent upon final exposure conditions (concentration in e.g. drinking water resources or in food). Within the BELISSIMA loop, the potential degradation of the natural and synthetic hormones, the mobility or transfer between compartments and the bioconcentration and uptake in organisms (as part of the food chain) will contribute to the final exposure concentration. Though it is initially not the purpose to consider any contribution of phytohormones, we anyway should take into account that components in plants might have hormonal activity which can be measured in assays for total biological activity. This contribution can be assessed through analyses for specific fractions of plant material. However it is expected that the activity of natural & synthetic hormones in human excretion as will be present in the BELISSIMA loop is much higher compared to any phytohormone, if at all present.

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3.3.2 THE ENDOCRINE SYSTEM

The endocrine system is one of at least three important integrating and regulatory systems in humans and other animals. The other two are the nervous and immune systems. Hormones influence important regulatory, developmental, growth, and homeostatic mechanisms, such as reproductive structure and function, maintenance of normal levels of glucose and ions in blood, control of general body metabolism, blood pressure, and other glandular, muscle, and nervous system functions. Some of the major endocrine glands include the pituitary, thyroid, pancreas, adrenal, and the male and female gonads (testes and ovaries). These secretory products of endocrine glands travel in the blood in very small concentrations and bind to specific cell sites called receptors in distant target tissues and organs, where they exert their specific functions.

Steroid hormones are naturally occurring molecules based on a fused hydrocarbon ring structure. The tetracyclic ring structure is numbered and labeled as shown in Figure 5. Substituents occurring at specific sites within the molecule allow classification of steroids into three main chemical categories, based on the androstane, pregnane or oestrane ring structures, demonstrated in Figure 6. However, steroidal hormones are more often classified by their biological activities into androgens, oestrogens, progestogens, corticosteroids and mineralocorticoids. Within this project only oestrogens and androgens will be considered as they are the major female, respectively male sex hormones.

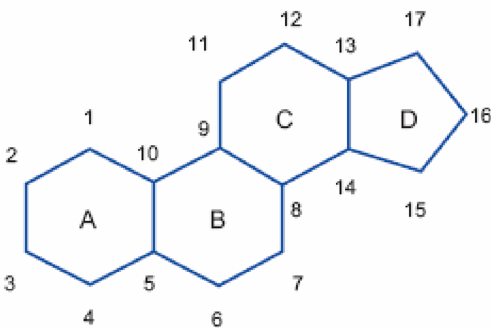

Figure 5: Tetracyclic steroid ring structure (after Andrew, 2001).

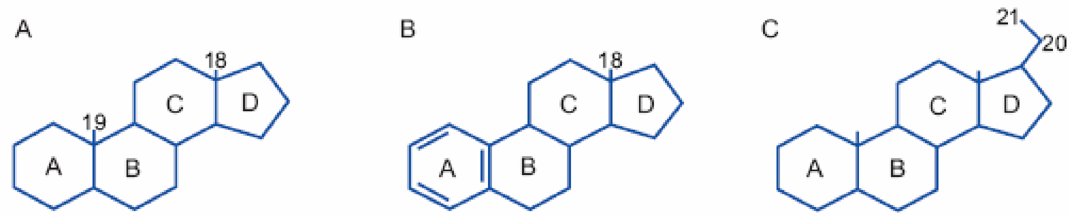

Figure 6: Structures of (A) androstane, (B) oestrane and (C) pregnane ring systems (after Andrew, 2001).

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The male and female sex hormones are the 'messenger substances' which control the balance between sex-related processes in the body, and their regulation is under control of the hypothalamic-pituitary-gonadal axis. They occur either as free molecules, but more often they are bound to transport proteins. Via the bloodstream these hormones do reach the target cells, those that contain receptor proteins, which are able to bind the hormone so that they can elicit a particular effect. The way in which that effect is triggered varies from one type of cell or hormone to another. Sex hormones e.g. bind to receptors that are located in the cell nucleus (Figure 7). Within the nucleus the hormone receptor complex binds to a specific site on the DNA, the so called hormone-responsive element or HRE, whereupon transcription of one or more genes into messenger RNA takes place. RNA therefore contains the code that is subsequently translated into specific proteins, as a result of which the cell will perform a particular function. The specific activity to target organs may change by the occurrence of various reversible hormonal conversions in the body (Anonymous, 1999; You, 2004).

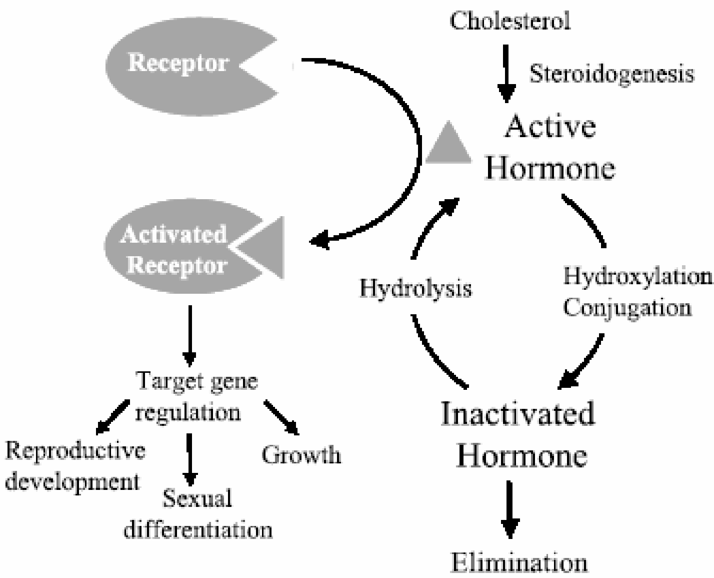

Figure 7: Steroid hormones, maintained through a dynamic balance between steroidogenesis and steroid inactivation to ensure physiological functions, mediated by activation of ligand-specific steroid hormone receptors (after You, 2004).

Biosynthesis of hormones takes place from cholesterol via the conversion by certain enzymes (P450-system) in gonadal tissues, the adrenal gland or a few other organs and is summarized in Figure 8. All of these conversions leave the skeletal structure of the steroid intact, but a number of substituted groups might change. Methyl groups can be disconnected, -OH groups converted to =O groups and vice versa. This gives rise to a large number of extremely similar molecules, of which only a limited number are hormonally active.

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Steroids are hydrophobic substances and thus difficult to be excreted by the kidneys. Therefore mainly the liver is involved in processes of metabolisation of steroid hormones (e.g. oxidation, hydrolysis, methylation, ..) before they are conjugated with glucuronic acid, phosphates or sulphates. Steroids can be enzymatically inactivated by steroid hydroxylases of the CYP family and by conjugations with glucuronide and sulfate. Hydroxylation and conjugation increase the hydrophilicity and tendency for excretion. Protein binding of steroid species may hinder their elimination. Several nuclear receptors, including the glucocorticoid receptor, the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the proxisomal proliferator activated receptors (PPAR), are know transcriptional regulators for some of the steroid biotransformation enzymes (You, 2004). The conversion of steroid hormones makes them more water soluble and facilitates the excretion through urine and faeces. The overall pathway of hormones in the human body is summarized in Figure 9.

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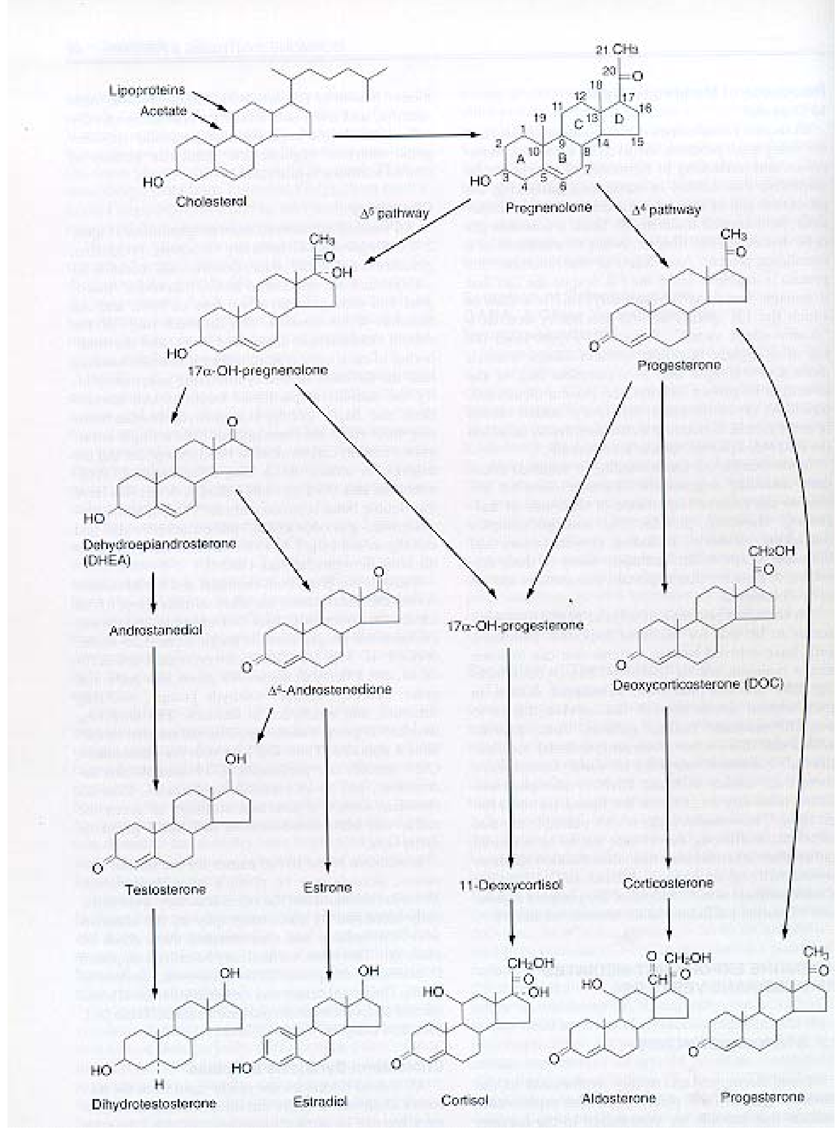

Figure 8: Pathways and structure of steroid hormones (after Greenspan & Gardner, 2001).

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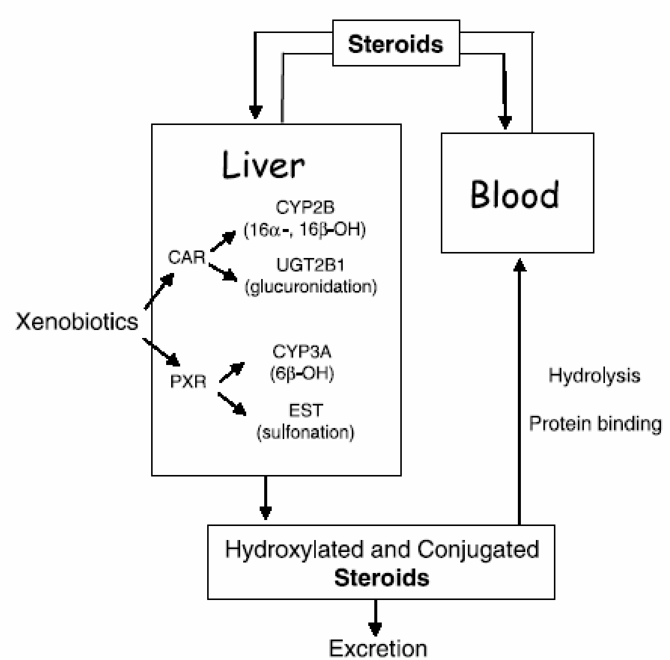

Figure 9: Steroids and pathways for metabolisation and excretion (after You, 2004).

3.3.3 NATURAL ESTROGENS AND THEIR METABOLISM

The most important representative of the natural estrogens is 17β-estradiol (E2). In addition, there are less potent oestrogens, such as estrone (E1) and oestriol (E3). These hormones are in general produced by theca and granulosa cells, which surround the maturing oocytes in female ovaries. Structures of major estrogens is shown in Table 22.

The half-life of E2 in the human blood is about three hours. Its removal is accomplished by irreversible conversion into metabolites that may be passed into urine or bile. There are multiple pathways that convert E2 to products that have widely different biological activities (Lord *et al.,* 2002). Oxidation to form hydroxyderivatives is a principal route of endogenous steroid metabolism (Figure 10). Isoenzymes of the cytochrome P450 (CYP) class can insert hydroxyl groups at the 2-, 4-, or 16- positions of E1. The iso-enzyme that catalyzes 2-hydroxylation of E2 (CYP1A1) is an inducible enzyme. A separate enzyme, CYP1B1, catalyzes 16α-hydroxylation. After estrone hydroxylation, the various poly-hydroxy derivatives are conjugated with glucuronate or sulfate, or methylation occurs prior to excretion in urine. The catechol-O-methyl transferase (COMT) enzymes that catalyze the methylation reactions require S-adenosyl methionine. A portion of conjugated and unconjugated steroids also passes into bile, some of which may be reabsorbed via enterohepatic circulation. The principal hydroxylation products are 2-hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 4-hydroxyestrone (4-OHE1), 4-hydroxyestradiol (4-OHE2), and 16α-hydroxyestrone (16α-OHE1).

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Table 22: Major compounds of natural estrogens (after Vieira da Silva, 2004).

Natural Hormone	Chemical formula	Molecular formula	Molecular weight
Estrone	$C_{18}H_{22}O_2$		270.4 g/mol
Estradiol	$C_{18}H_{24}O_2$		272.4 g/mol
Estriol	$C_{18}H_{24}O_3$		288.4 g/mol

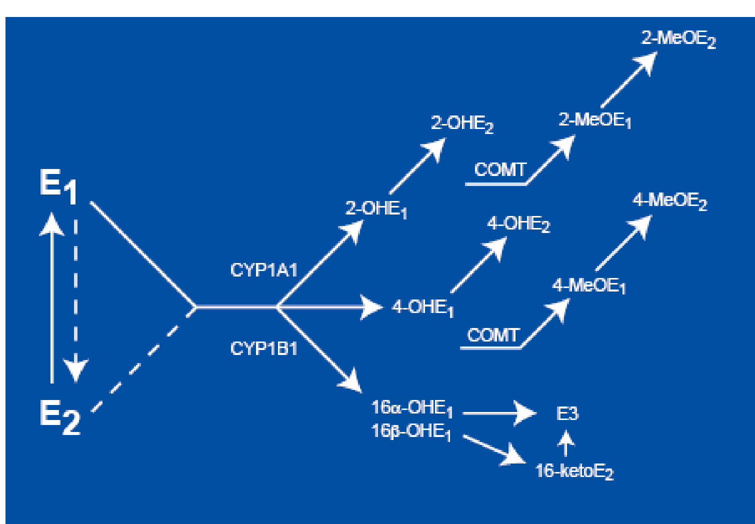

Figure 10: Metabolism of estradiol and estrogen (after Lord et al., 2002)

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Thus final urinary excretion in mammals, including humans consists mainly of metabolites of estradiol and estrone. 17 β-estradiol oxidizes into estrone, which can be converted to estriol via 16 α-hydroxy estrone. Moreover estrogens can be excreted in various forms, like 17 β-hydroxy estrone, 16-ketoestrone or 16-epiestriol. 17 β-estradiol is mainly present in human urine as 17 β-estradiol-3-glucurone, estrone-3 sulphate and estriol, while estriol itself is mainly excreted in the form of estriol-16-glucurone.

The majority of excreted steroids in the faeces are in the unconjugated form (Macdonald *et al.*, 1983; Johnson & Williams, 2004). The important role of intestinal metabolism of estrogens is experimentally shown. Conjugated estrogens, both sulfate and glucuronide, excreted from the bile are largely deconjugated by the natural intestinal flora prior to excretion. Significant deconjugation of sulfate forms occurs in the gut, indicating the presence of steroid desulfating bacteria. Strictly anaerobic desulfating bacterial strains have been isolated from human faeces, which were capable of cleaving estrone-3-sulphate and estradiol-3-sulphate.

Levels of excretion of hormones can differ according to age and gender. Especially with regard to estrogen excretion, levels can highly fluctuate from 20 µg/day (children) up to 250 µg/day (non pregnant women). During pregnancy the estrogen hormone levels strongly increase and may amount up to 30000 µg/day at the end of pregnancy. Within a 9 months-period, the average daily excretion is assumed to be 10000 µg/day. With specific data of urinary excretion of estrogens for each population group, and demographic data on proportion of sex and age classes, and birth rates within a population, an estimation of average daily excretion of estrogens/head of population can be made. A summary of available literature data (minimum-maximum values) on human excretion of estrogens has been made by Witters *et al.* (2002) and is given in Table 23.

Table 23:Natural estrogens in human urine as derived from several literature reports (Witters *et al.*, 2002).

Population group	Daily estrogen excretion (µg/day)		% of Belgian population	Average daily excretion (µg/day)	
	Min.	Max.		Min.	Max.
Children (0-15 yrs)	nd	20	17.8		
Male (15-64 yrs)	7	85	33.1		
Female (15-64 yrs)	16.3	250	31.9		
Pregnant female	6859	30000	0.83		
Eldery (> 64 yrs)	nd	28	16.5		
Population head				72.3	365.1

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Based on a mean composition of crew in space consisting of 3 man and 3 women, an average range of daily excretion of estrogens in urine per population head will be between 12 and 182 µg total estrogens (Table 24). The latter does not consider the use of anti-conceptive pill. This estimated range can be taken into account to assess the content of estrogens which will be put into compartment I, as soon as is decided upon the amount of urine to be put into the feed.

Table 24: Estimates of excretion of natural estrogens in human urine in space, based on proposed composition of crew (ESA) and numbers from Table 23.

Population group	Daily estrogen excretion (µg/day)		Crew in space (# persons)	Range daily excretion (µg/day)	
	Min.	Max.		Min.	Max.
Children (0-15 yrs)	nd	20	0		
Male (15-64 yrs)	7	85	3	21	340
Female (15-64 yrs)	16.3	250	3	48.9	750
Pregnant female	6859	30000	0		
Eldery (> 64 yrs)	nd	28	0		
Total crew			6	69.9	1090
Population head (average)				11.7	181.7

3.3.4 SYNTHETIC FEMALE HORMONES

Except for the natural hormones, the synthetic hormone 17 α-ethynylestradiol (EE2), the most common and persistent hormone present in the contraceptive pill, is relevant for this study (Table 25). The daily dose of women using the contraceptive pill is calculated to be approximately 35 µg. Literature data report urinary excretion between 26% and 90 % of this daily dose. These highly variable numbers are the results of individual biological variation and inaccuracy of methods to be used.
On the ISS medical Checklist, the birth control pill contains a combination of synthetic hormones, which is norethindrone and ethinylestradiol (Ortho-Novum).

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Table 25: Examples of synthetic estrogens which are used in the oral contraceptive pill (after Vieira da Silva, 2004).

Synthetic Hormone	Chemical formula	Molecular formula	Molecular weight
Ethynylestradiol (Estinyl) 17α-ethinylestradiol	$C_{20}H_{24}O_2$		296.4 g/mol
Mestranol 3-methyl ether	$C_{21}H_{26}O_2$		310.4 g/mol

The pathway of fate and excretion of the synthetic hormone, 17α-ethinylestradiol in the female body is illustrated in Figure 11. Except for total amounts of excreted EE2 in urine and faeces, which are in the range of µg/day, it is also clear that not only the parent compound is present, but several conjugate forms. EE2 does also give result to glucuronide forms or sulfated conjugates, similar to the metabolites of the natural hormones. It is a challenge to detect and quantify each of these forms in a total spectrum by analytical techniques.

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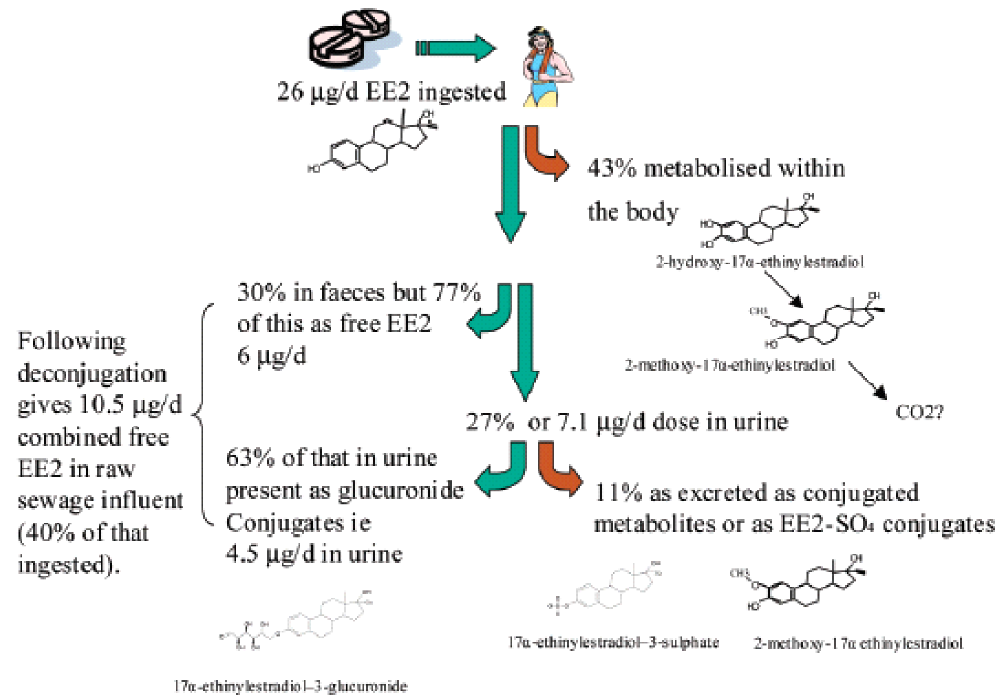

Figure 11: Fate and excretion of 17α-ethinylestradiol (after Johnson & Williams, 2004).

3.3.5 NATURAL ANDROGENS AND THEIR METABOLISM

As far as the androgens are concerned, this group of C19 steroids comprises principally testosterone and its steroid A-ring saturated metabolites 5α-dihydrotestosterone (DHT) and the isomeric androstane-3,17-diols. In addition, androstenedione (AD) and dihydro-epiandrosterone (DHEA) are classified as androgens, although they are less potent (Shimada *et al.,* 2001). They are produced by the Leydig cells, present in the interstitial tissue of the testis. But the adrenal gland can also be an important source of androgen production, especially in women.

It is generally accepted that the androgenic potency of testosterone occurs as a result of its very active conversion to DHT in target tissues such as the prostate. In many other tissues too (testes, adrenals, ovaries, cardiac muscle, salivary glands,..), there is extensive breakdown of testosterone (T). It is converted to AD and next to 17-oxosteroids or 17-ketosteroids such as androsterone (A), epiandrosterone (epiA) and etiocholanolone (ET). Further metabolisation of T and AD through hydroxylation at C-6, C-7 and C-16 is also described.

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The principal urinary 17-ketosteroids occur in urine as a mixture of glucuronides and sulfates (Shimada *et al.,* 2001; Rittmaster, 1993). Glucuronide and sulfate conjugates can be formed at the 17-carbon position of DHT, the 3-carbon position of androsterone, and at either the 3-or 17-carbon position of androstanediol. Conjugation probably serves the dual role of removing the steroids from the pool of potentially active androgens and enhancing their urinary excretion (Figure 12). The majority of 17-ketosteroids in the urine however, are formed from the metabolism of adrenal steroids, such as cortisol and cortisone (Greenspan and Gardner, 2001).

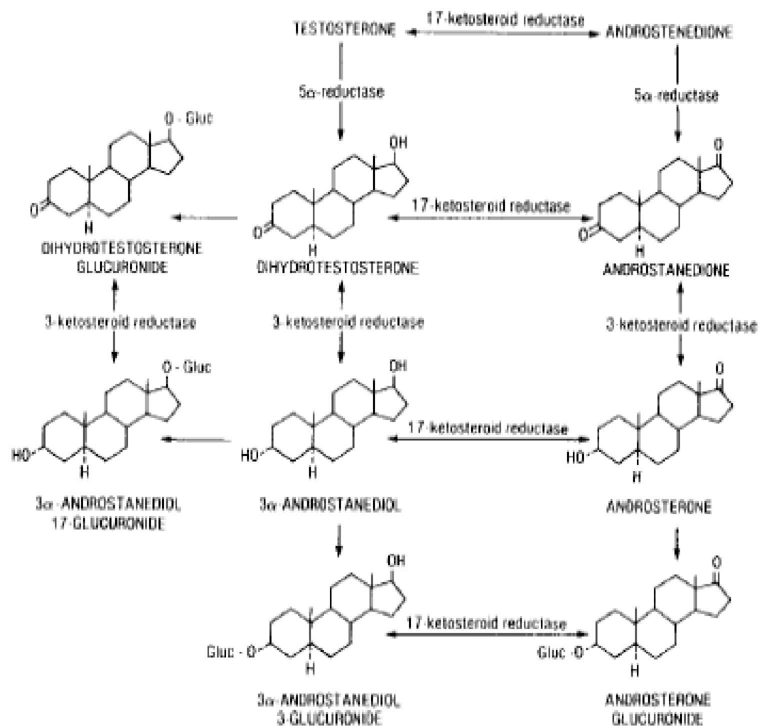

Figure 12: Pathways of androgen glucuronide formation from DHT. Androgen sulfates are formed from similar metabolic pathways (after Rittmaster, 1993).

With regard to levels of excretion of androgen hormones, less data were found. Ditmer (1961) reported total androgen levels up to 100 µg/ml in daily urinary excretion by male. These numbers are rather high compared to a more recent paper where basal levels of urinary excretion of male hormones, including several metabolite forms, range from 0.028 µg/ml for testosterone which is low due to its conversion into several metabolites up to 1.9 µg/ml for androsterone being much higher as one of the major ketosteroids, the endproduct of conversion of male hormones (Wang *et al*., 2005). The latter data, which were obtained by GC-analysis, are likely more accurate. These values are also in line with reference values for steroid profile in urine of male and female (ARUP, 2006; Weykamp et al; 1989).

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Details of reference values were given by Weykamp et al. (1989) as a result of an extended study on urine samples from 288 healthy volunteers, with triplicate samples for analysis by capillary gas chromatography by 24 institutes in The Netherlands and in Belgium. Targetcompounds which were analysed were urinary excretion products of androgens, progestagens and corticosteroids (Weykamp, et al. 1989) and these were reported for 6 separate age classes. In the next table, a selection of reported values is presented (Table 26).

Table 26: urine reference values for men and women in 3 age categories (selected from Weykamp et al. 1989)

µmol/day	Men			Female		
	3m-12y	13y-50y	51y-70y	3m-12y	13y-50y	51y-70y
Androsterone (A)	0.2-4.5	1.5-14.1	2.3-11.6	0.4-3.7	1.9-11.8	0.6-5.5
Etiocholanolone (E)	0.2-5.0	1.2-15.8	2.5-12.7	0.4-2.4	1.7_10.3	1.2-6.1
Dehydroepi-androsterone (D)	0.0-0.9	0.0-2.8	0.0-3.0	0.0-1.1	0.1-2.0	0.2-0.9
11-Ketoandro-sterone (11-KA)	0.1-1.6	0.2-2.8	0.1-2.4	0.1-0.9	0.2-1.9	0.3-1.9
11-Ketoetiocho-lanolone (11-KE)	0.1-1.4	0.2-2.7	0.4-2.1	0.1-0.8	0.3-1.8	0.3-1.6
11-Hydroxyandro-sterone (11-HA)	0.1-3.5	0.9-7.5	2.0-7.5	0.3-2.2	0.8-4.8	1.4-4.1
11-Hydroxyetiocho-lanolone (11-HE)	0.1-1.8	0.2-3.4	0.5-2.4	0.1-1.2	0.3-2.7	0.5-2.6

The amount of 17-ketosteroids (mg/day) in urine is primarily the result of adrenal androgen production (2/3) and second for 1/3 from the testes. This amount is orders of magnitude higher than the testosterone level in male and female urine . Total values given by ARUP (2006) are used to estimate levels at the input of Belissima CI (Table 27).

Table 27: Estimates of excretion of natural androgens in human urine in space, based on proposed composition of crew (ESA) and numbers from ARUP (2006).

Population group	Daily excretion ketosteroids (mg/day)		Daily excretion testosteron (mg/day)		Crew in space (# persons)	Range daily excretion androgens (mg/day)	
	Min.	Max.	Min.	Max.		Min.	Max.
Male (17-50 yrs)	5.3	17.6	0.025	0.125	3	16.0	53.2
Female (17-50 yrs)	4.4	14.2	0.005	0.034	3	13.2	42.7
Total crew						29.2	95.9
Population head (average)						4.9	16.0

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Levels to be expected in human waste (urine/faeces) are within measurement ranges of current analytical equipment (GC-MS and /or LC-MS). With respect to the bioassays to be used, detection will depend on the potency of each of separate metabolites which occur in the system but an overall assessment of biological activity is feasible as the concentrations are high compared to the estrogen levels.

3.3.6 THE FATE OF HORMONES IN DIFFERENT COMPARTMENTS

3.3.6.1 General microbial degradation processes

For literature information on bacterial transformations on hormones, we refer to TN80.13.

3.3.6.2 Expected transformations in the MELiSSA loop

Since hormones leave the human body primarily via urine, a complication to study their effect in the MELiSSA loop exists in the fact that the MELiSSA consortium has not yet decided on the point of entry for urine in the loop. Still the consortium is aware that urine is crucial to close the nitrogen mass balances in the loop. Because the first compartment is the main one in which biodegradation processes take place, it would presumably be best suited to remove or reduce the hormone concentration (in urine). Based on a preliminary simulation performed by ESA, this study will use urine addition to compartment I as a starting point. For 6 crew members (3 females – 3 males) a daily urine volume is estimated to be between 3.6 liter and 12.0 liter (derived from terrestrial data, Weykamp et al. 1989). For further assessment of the fate of hormones (and pharmaceuticals) on Belissima design and sampling requirements, a scenario with input of total urine volume in CI is taken into account. It needs to be noted though that feeding urine to compartment I releases high ammonia concentrations that may inhibit the anaerobic transformation processes at concentrations above 3 g/l.

3.3.6.2.1 Compartment I (anaerobic, 55°C)

Literature data on hormone elimination in anaerobic conditions is limited because endocrine active compounds are associated with municipal wastewater which is typically treated in aerobic purification systems in terrestrial environments. Work by Holbrook *et al*. (2002) is in this respect interesting. They evaluated the mass balance of estrogen activity (by use of yeast estrogen receptor test) in liquid and solid phases of pilot and full-scale waste water treatment facilities. They demonstrated that 5 to 10% of the estrogenic activity of the influent became associated with the biosolids, and between 26 (aerobic) to 43% (anaerobic) appeared in the treated liquid effluent. Except for the estrogenic activity which is not biodegradable and will appear in effluents, the digestion process of biosolids appeared to be a significant sink for estrogenic compounds (Holbrook *et al*., 2002). Similar observations were made by Braga *et al*. (2005).

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In a primary sewage treatment plant (STP), removal of estrogens was mainly due to sorption to the solids. In an advanced STP with activated sludge reactors (anoxic & aerobic zones) 85 to 96% of natural estrogens were removed, while EE2, the synthetic hormone appeared resistant to biological treatment and undetectable levels were explained as the result of sorption. Johnson & Williams (2004) suggest that sulfate conjugates of estrogenic compounds are likely to pass sewage treatment works, as the principal organisms responsible for desulfating activity are obligate anaerobes. It should be investigated in the MELiSSA loop whether the populations in compartment I will be stressed, or whether they can cope with the accumulation of steroid compounds, or whether the present species are able to contribute to desulfating activity. A few other papers on anaerobic conditions indicate that steroid compounds will remain almost unchanged. Ying *et al.* (2003) evaluated the sorption and degradation of 5 endocrine disrupting compounds, including E2 and EE2, in sediment and groundwater from an aquifer. The estrogens did show medium affinity for the aquifer material (sorption coefficients K_f of 24.2 for EE2 and 90.9 for E2). In anaerobic conditions, no degradation was observed for EE2 while E2 degraded very slowly within 70 days in native groundwater (Ying *et al*., 2003). Similarly Ivashechkin *et al*. (2003) indicate that endocrine disrupting chemicals sorb to sludge and that no degradation of these compounds is expected during anaerobic digestion. These data point to high variability, or even discrepancies which might be related to the inaccuracy of methods used to measure hormone levels in complex matrices, and to differences in performance conditions of the different WWTP and their set-up.

Hormones are in general excreted in a conjugated form. The potency of the bacteria present in C1 to deconjugate them with glucuronidase and sulfatase enzymes is not known. Literature data indicate that hardly any biodegradation of steroid compounds is likely to occur in anaerobic conditions. Although no data are available for thermophilic conditions, we anticipate that biodegradation processes will be insignificant because the polycyclic structure of hormones make them rather recalcitrant. Limited sorption will probably occur. Chemical transformations at the elevated temperatures are not known and have not yet been described.
Physical retention of hormones by the ultrafiltration membranes of compartment I is not expected because they are too small to be retained.

3.3.6.3 Compartments II to IVa

In case no transformations occur in the first compartment, hormones may enter into the downstream compartments. These are not active biodegrading compartments but are rather meant to produce edible biomass or perform nitrogen transformations.
If no transformations took place in compartment I with its highly diverse microbial community, hormone elimination is unlikely to occur in compartment II which operates under anaerobic conditions as well. Non-biological transformations may include sorption onto biomass and photochemical reactions.
The following two compartments are aerobic. Ternes *et al.* (1999b) demonstrated that bacteria present in activated sludge (aerobic), were able to dissociate the glucuronides and sulphate groups from excreted conjugated oestrogens.

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Effluents from municipal waste water treatment plants were shown to mainly contain the biological active, unconjugated oestrogens such as 17β-estradiol, estrone, 16α-hydroxy estrone and 17α-ethynylestradiol. Further degradation occurs principally via bacteria. Under aerobic conditions, estradiol is converted to estrone and degradation of 70-95% within one week has been established in bacterial cultures. The latter is comparable to the fate of natural hormones in waste water treatment plants (Tabak *et al*., 1970; Ternes *et al*. 1999a, 1999b). For ethynylestradiol the situation is even worse because it has been shown to be more persistent to bacterial degradation. A recent study by Johnson & Williams (2004) demonstrated the use of a model to estimate the levels of major estrogens at a STP influent and effluent for estradiol (E2), estrone (E1) and (EE2). Calculated values obtained by the model were compared with measured values at 6 Italian and 1 British STP and appeared to be in agreement. Measured mean values in the effluents ranged for respectively E1, E2 and EE2 between 4.1-44.6 ng/l, between 0.7-2.4 ng/l and between 0.35-7 ng/l. These are rather low concentrations, but which still remain detectable provided that adequate sample treatment methods (e.g. solid phase extraction) are used prior to analysis (Witters *et al.,* 2000; Vieira da Silva, 2004; Nielen *et al.* , 2004). Most chemical analytical and bio-analytical techniques (e.g. receptor binding tests: see below) do allow to detect natural and synthetic estrogens in the ng/l range.
Whether the MELiSSA bacteria of compartments III and IVa are capable of the same transformations under the conditions used in the loop, is not documented. Taking into account the slow growing biomass in compartment III, its sorption capacity will be insignificant. For compartment IVa sorption on the biomass and photochemical transformations may occur.

When hormone transformations do occur in compartment I, metabolites will enter the loop. In that case, similar observations hold as for the parent compounds.

3.4 Priority selection of compounds

The main source of hormones will result from human urine, planned to be added in compartment I as the point of entry in the BELISSIMA loop. The majority of hormones in urine will be natural male and female sex hormones, excreted by man in space. As a guidance compound of synthetic hormones, 17α-ethinylestradiol is proposed because of (1) its general use as a constituent in the anti-conceptive pill (likely also in space) and (2) its known environmental concern because of its persistence, as shown in terrestrial studies at sites of municipal WWTP. Both parent compounds, and metabolised compounds which are likely to show biological effects through interaction at the hormone receptor systems will be considered.

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3.5 Identification of technical requirements and critical steps of design of BELISSIMA loop & study protocol

3.5.1 EXPERIMENTAL SET-UP

The effect of endocrine active compounds will be studied in the BELISSIMA loop.
An extensive characterization of the compartment I effluent, with and without urine application, will be performed in terms of endocrine activity. This will allow to estimate the background and to determine the presence of phytoestrogens from the plant material or other sources (e.g. contaminants, see below) as opposed to estrogens from human origin.
In order to get some standardization with respect to the input of urine into compartament I, urine will be collected from healthy individuals. This means, individuals which do not use any pharmaceutical drug or synthetic hormone. Moreover , a questionnaire should be completed by human donators in order to get some idea about other sources of contaminants through the use of food (such as pesticides) or through their daily activity (eg. occupational exposure).
As mentioned before, the point of entry for urine in the loop will be the first compartment. The behavior of male hormones will be evaluated first. Urine and fecal material donations will be from healthy male persons to avoid the presence of pharmaceutical drugs and their metabolites. Next, the effect of female hormones will be studied through donations of healthy women. We will first attempt to collect female waste containing only natural hormones, as it is expected that these are less resistant to biodegradation. In a final step, urine and faeces will be used of female using the contraceptive pill (composition preferably EE2-norethindrone, similar to use in space), as the latter is expected to be most retained in the system.
Since no spiking experiments are performed but real urine is fed to compartment I, we propose to run the hormone monitoring tests simultaneously with the monitoring of oligo-elements. Each test condition should last at least 5 residence times to achieve equilibrium. Due to the long residence time in the first compartment, this means at least 50 days for each condition in that compartment.
Hormone monitoring is scheduled in compartments II to IVa only when the fate of hormones has been evaluated in compartment I and the residual concentration or metabolites in its effluent are known to be ≥ 0.1 ng/l in the liquid phase. At these levels, chronic effects might be relevant and suitable tests for the downstream compartments can be proposed provided that at these low levels, at least 1 liter of sample volume can be taken. Based on numbers given in Table 24 where the minimum input in CI (volume: eg. 100 liter), based on total recovery of urine of 6 crew members, will be ± 70 µg estrogens, a 7000-fold reduction of hormone levels should occur to exclude further needs to monitor for these specific groups of hormones. As the input levels of androgens is higher, the likelihood that these compounds remain above the cut-off level is very likely. In that case, for any of the hormones to be studies, hormone monitoring in further compartments is recommended.

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3.5.2 SAMPLE PROCESSING

Freshly collected samples should be stored in a refrigerator. In order to get representative measurements for the conditions in the BELISSIMA loop, samples will be treated by solid phase extraction (SPE) methods, as soon as possible after collection (within less than 24 hours). Sample extracts, or even SPE columns which retained the target components can be stored in the dark, at 4°C for a longer time period (max. 2 weeks).

Sample treatment methods to analyze complex matrices will be selected and optimized. The current procedure used at VITO will be evaluated for its performance for the matrix of the different compartments, especially CI, of the Belissima loop. An overview of different pretreatment methods for subsequent analysis of steroids is given by Gomes *et al*. (2003) and illustrated in Figure 13. Available SPE techniques (solid phase extraction) for surface water analyses which are currently in use at VITO in combination with biological screening techniques (Witters *et al*., 2000) will accordingly be modified in order to get optimal recovery of selected compounds. The matrix of Belissima compartments will be spiked with known concentrations of hormones (estrogens/androgens) and extractions with concentration procedures will be evaluated for % of recovery of spiked compounds by eventually modifying filtration steps, changing/adding other solvents, time of extraction, volumes of solvents, types of solid phase such as Lichrolut, SDB, C4-C8-C18, ENV+ or combinations of these. Especially with regard to low volume sampling and levels of detection, extraction methods will be optimized. In order to identify the content of endogenous hormones in e.g. urine samples (if applied to any of the compartments) and samples of compartments, both conjugated and unconjugated fractions will be determined by application of enzymatic steps with glucuronidase/arylsulfatase (Nielen *et al.,* 2004; Bovee *et al.,* 2005). In this way, metabolites are again converted into their parent compounds, which have the highest potency in the biological detection systems.

In order to follow up hormone levels in the BELISSIMA loop, it is estimated that a sampling regime of twice a week would be recommended. However this frequency is only feasible if low volumes are required (eg. 75-500ml) which is dependent upon the concentration of the target compound.
However as concentration of hormones (and drugs, see next chapter); the sampling volume should be higher (up to 1 liter) in order to detect the compounds after sample treatment (extraction and concentration) down to levels in the ng/l range. Therefore, the samples to be taken should consist of 75 to 1000 ml effluent volume, depending on behavior in the compartment where levels can range from extremely low (ng/l), if degradation/adsorption processes occur, up to high values (µg/l). For cases where the 1 liter sample should be taken, the frequency for practical reasons (design) should be once a week.

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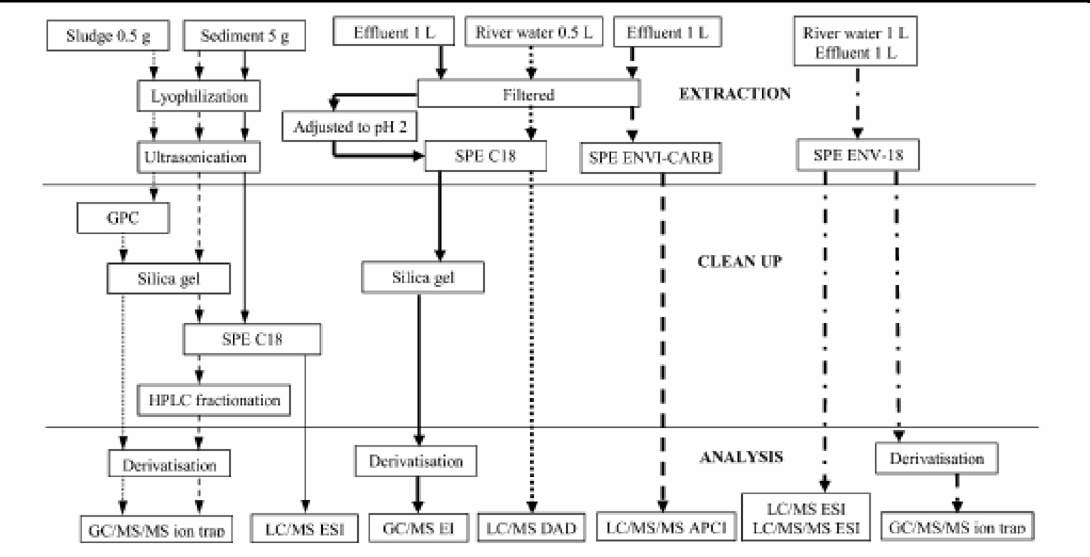

Figure 13: Several methods of sample treatment for analysis of steroids in solid and aqueous samples (after Gomes *et al.*, 2003).

3.5.3 ANALYTICAL METHODS

3.5.3.1 General approach

Optimization and validation of measurement techniques (both chemical and bio-analytical) will consist of getting concentration/response or concentration/signal relationships for each of the selected compounds, detection limits, range of quantification, … for parent compounds and relevant metabolites. Methods include cellular estrogen-receptor tests (MVLN, YES and YAR) and HPLC for fractionation and GC-MS and LC-MS. Levels down to 'ng/l' can be detected, which is well above the range expected. Other techniques for steroid measurements involve ELISA (enzyme linked immunosorbent assay), EIA (enzyme immunoassay) and RIA (radio immuno assay) methods. These methods make use of specific antibodies for target compounds to be analyzed and the detection system can be based on either fluorescence, absorbance or radioactivity for RIA tests. The working range of these methods is approximately 0.05 to 5 µg/l.
Each of these methods consist of dedicated target compound analysis. However within either biological matrices (e.g. urine) or environmental matrices (waste water, feed in MELiSSA loop) steroids might exist in a metabolized or conjugated form and several hormones might occur in the same sample. A bio-analytical approach which makes use of mechanism-based bioassays is an approach which allows an integrated screening of micro-compounds present, which act through the same biological mechanism (e.g. genotoxicity, estrogenic activity, ..). This approach does offer advantages in cases were due to the complex matrix, or due to presence of many unknown compounds, such as metabolites at low levels, the current analytical techniques do face limitations.

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Bioassays in that case will assess the total amount of toxicological relevant compounds, which can be used to estimate mass balances. If requested, in samples which give positive signal, further bio-assay directed fractionation or TIE[1]-procedure is performed and the complex matrix is simplified allowing better performance of analytical techniques to identify the target compounds (Gomes *et al.*, 2003, Figure 14). This approach is e.g. applied for estrogenic compounds with the yeast assay in urine samples or waste water samples (Desbrow *et al.* 1999; Nielen *et al.*, 2004; Labadi & Budzinski, 2005).

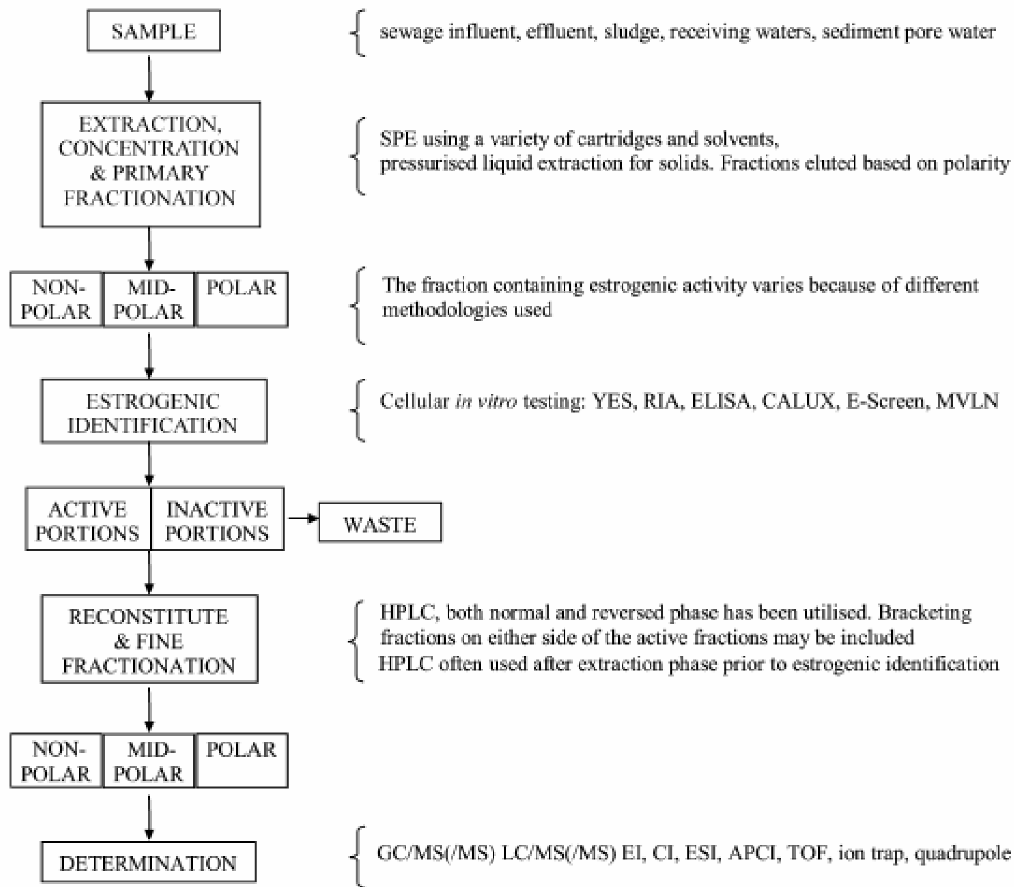

Figure 14: TIE approach to determine compounds responsible for estrogenic effects (after Gomes et al., 2003).

Thus, the use of *in vitro* biological tests to assess the presence of hormones, consisting of parent compounds and metobolites with different endocrine activity, can offer advantages. In this project, for evaluation of mass balances in complex media (especially CI), receiving human waste with metabolites of sex hormones, the endocrine activity should be assessed (Andersen *et al.,* 1999).

[1] TIE= toxicity identification and evaluation

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Sex hormones exert their specific activity through interference at the receptor, either estrogen or androgen receptor.
An extended list of bioassays has been developed in the past years, but none of them has yet been validated. Currently both in the US, Japan, and EU organizations involved in test strategy (OECD, ICCVAM, ECVAM) are in the phase of evaluation of *in vivo* and *in vitro* tests which can be used to evaluate endocrine disrupting properties of compounds. VITO is involved in an EU-research project (REPROTECT, 2004-2009) for the prevalidation of a few estrogen and androgen receptor based tests using mammalian cell lines. Either human or yeast cells have been genetically engineered to be used as *in vitro* screening tools to detect steroid hormones which bind to the estrogen or androgen receptor.
This type of biological test systems indeed, has internationally been recognized by OECD and EDSTAC as a tool for high throughput screening of compounds with potential hormone disrupting activity (Tattersfield *et al*., 1997; IEH, 1998). Both for detection of estrogen and androgen-active compounds, tests for hormone receptor binding through transcriptional activation of a reporter system have been developed in yeast and mammalian cells. The principle of most commonly used assays for environmental screening of hormones is described below.

3.5.3.2 Hormone receptor transactivation and reporter system in yeast cells

The DNA sequence of the human estrogen receptor is stably integrated into the yeast genome, which also contains expression plasmids carrying estrogen-responsive sequences controlling expression of the reporter gene lac-Z. Upon binding an active ligand, the estrogen-occupied receptor modulates gene transcription, and the reporter gene lac-Z is expressed producing the enzyme β-galactosidase, which is secreted into the medium. It then metabolizes the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), from normally yellow into a red product, which can be measured by absorbance at 540 nm. This assay (Routledge and Sumpter, 1996) is referred to as the YES-assay or yeast estrogen screen, and a similar assay has been developed to screen for androgenic activity (YAR-screen) (Purvis *et al*., 1991). The test principle is shown in Figure 15. A similar yeast estrogen bioassay, based on the expression of green fluorescent protein (GFP), has been developed and validated by Bovee *et al.* (2005). The assay has been applied for detection of natural and synthetic estrogens in calf urine (Bovee *et al.,* 2005). This GFP-yeast assay from RIKILT has been compared at VITO with the YES-screen from Brunel University and both assays demonstrated similar sensitivity (Thijs, 2003). The advantage of the RIKILT assay might be the short response time (< 24 hours) and its demonstrated applicability for urine samples.

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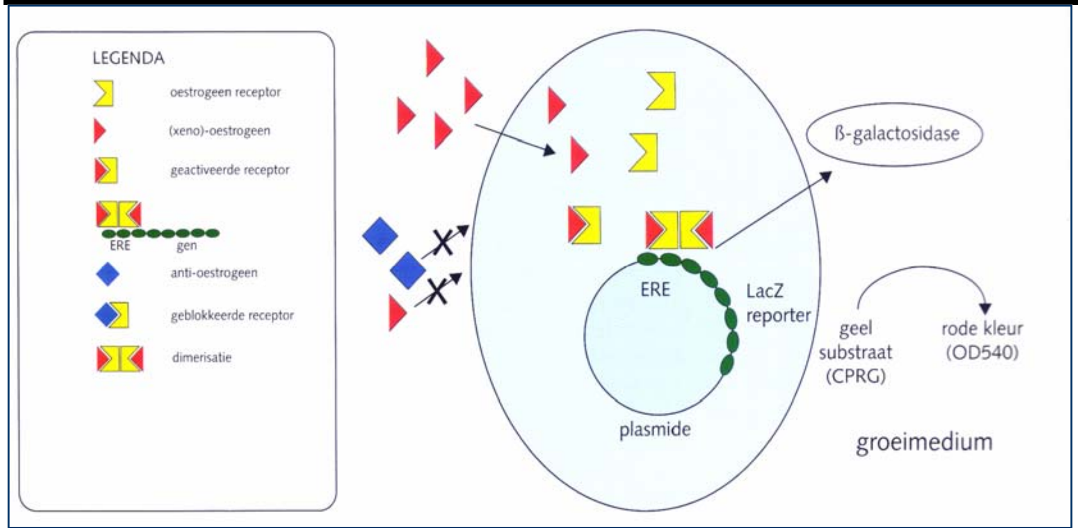

Figure 15: Test principle of the Yeast Estrogen Screen (YES)-test for detection of estrogenic compounds (after Vethaak *et al*., 2002).

3.5.3.3 Hormone transactivation and reporter system in mammalian cancer cells

The MVLN-cell line was developed at INSERM (France), and it consists of human breast cancer cells (MCF-7 cells) which were stably transfected with a pVit-tk-Luc plasmide (Pons *et al.,* 1990). The transfected cells contain an estrogen response element (ERE) which is coupled to a luciferase reporter gene. Binding of compounds to the estrogen receptor leads to gene transcription of the luciferase reporter gene and the production of the luciferase enzyme. After addition of the Luciferase Assay Reagens, the light production is measured with a luminometer as a measure for the receptor binding of the tested compound. A similar biological assay which makes use of prostate cells (PC-3) has been developed in order to detect the presence of androgenic compounds through activation of a luciferase reporter gene (Térouanne *et al*., 2000). This assay, called PALM-test does allow detection of androgenic compounds down to levels of 0.01 nM.

3.5.3.4 Performance of bioassays

Comparison of the MVLN-assay and the YES-assay has demonstrated higher sensitivity (detection limit up to 50 times better) for the MVLN, with higher human relevance, while the YES assay is more robust and easy for routine use (Witters *et al*., 2000; Witters *et al.,* 2003; Van den Belt *et al*., 2004). In Figure 16, performance characteristics (working range, lowest detectable levels) for 17β-estradiol (E2), estrone (E1) and 17α-ethinylestradiol (EE2) are illustrated. It is clear that these assays easily do allow to detect levels of hormones in urine samples, even if diluted (µg/l range).

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However, as it has been demonstrated that concentrations down to ng/l still can exert biological effects, with effects of EE2 even more potent than these of the natural estrogens, detection limits down to ng/l range will indeed be required. Moreover, in this case sample treatment through extraction and concentration might be required.

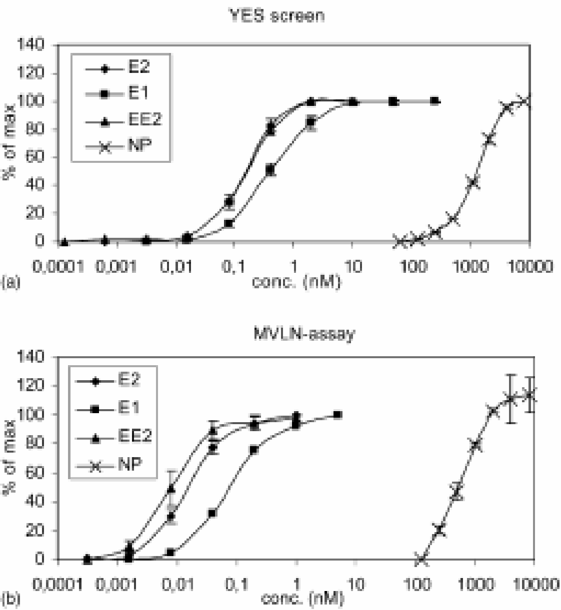

Figure 16: Comparison of dose-response curves of Er-activated yeast and mammalian cellular assay to detect estrogenic compounds (Van den Belt *et al*., 2004).

3.5.4 TECHNICAL REQUIREMENTS FOR LOOP DESIGN

The major concern related to measurements of hormones and other organic compounds in the BELISSIMA loop will be to exclude sources of contaminants from synthetic origin. It has been demonstrated that constituents in plastic material, in sealings or coatings of material might have hormone disrupting properties. More specific components such as bisphenol A and several phtalates are generally recognized for their estrogen- or androgen like activities.
In addition, certain cleaning agents containing surfactants of the group of alkylphenols, do occur in the aquatic environment and were also shown to be detected by hormone receptor tests, and cause hormone disrupting effects in organisms (Van den Belt *et al*., 2004; Vethaak *et al*., 2002). The use of detergents should carefully be selected, or tested prior to use, or even be omitted if possible. Proper hardware material should be selected which avoids the release of endocrine disrupting compounds.

For an overview of design requirements we refer to the chapter on pharmaceutical drugs (Table 36).

3.6 Conclusions

As yet, we can only speculate on the effect of hormones on the MELiSSA loop. Since urine is the main source of hormones, its point of entry in the MELiSSA loop is of crucial importance. For this study, urine will be fed to compartment I without any pretreatment. Proposed bio-analytical techniques to screen for total mass balances will allow hormone measurement in the required concentration ranges. Selected assays will allow to detect, eventually after adequate sample treatment (deconjugation steps, solid phase extraction, ..) the spectrum of natural male hormones, and natural and synthetic female hormones which are likely to occur in space. For detailed chemical analysis of biological active samples, a spectrum of compounds present will be obtained and based on the use of standards for parent compounds, major peaks will be identified.

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4 PHARMACEUTICAL DRUGS

4.1 Definition

A drug or pharmaceutical compound is a chemical agent which can affect living processes, it concerns mainly small molecules which affect cellular processes. Most of these are xenobiotics (Gr. *xenos* - stranger), chemicals that are not synthesized by the body, but introduced into it from outside. Pharmacology (Gr. *pharmakon* - a drug or poison, *logos* - word or discourse) is the science dealing with actions of drugs on the body (pharmacodynamics) and the fate of drugs in the body (pharmacokinetics). In order for a drug to work, it must enter the body and somehow be distributed in such a way that it gets to its site of action. In most cases the site of action is a macromolecular "receptor" located in the target tissue. Most drug effects are temporary, because the body has systems for drug detoxification and elimination.

Until the 1990s, relatively little consideration was given to the likely fate, occurrence or effects of pharmaceuticals in the environment following normal use and emission through human waste to the environment. Common drugs such as paracetamol or aspirin are sold in quantities comparable to high production volume (HPV) materials—close to or exceeding 1000 tons/annum in EU countries such as the UK or Germany. Total use of human prescription drugs in these countries will even exceed this amount (Webb, 2001). Drugs are also inherently biologically active and often extremely potent. They are often resistant to biodegradation as metabolic stability is necessary to pharmacological action. Certain pharmaceuticals (or their metabolites) are also highly water soluble. When combined with a lack of biodegradation, removal during wastewater treatment will be limited for such compounds. These compounds will consequently enter the aquatic environment and it is currently questioned to what extent a risk to human health and the ecosystem is apparent.

With respect to the BELISSIMA loop, the release of pharmaceuticals compounds (and PPCP in general) is relevant. It is expected that major input will occur through human faeces and urine, being part of the feed of compartment I. It should be considered whether these pharmaceuticals present in human waste, do pose a potential risk to the functioning of the BELISSIMA system. It should be investigated whether either through cumulative chronic toxicity, or specific mechanistic effects related to the potent pharmacological mechanism of compounds, potential hazards due to release of pharmaceuticals might be present.

The literature study on human metabolism and effect evaluation of pharmaceutical drugs will be made only for a selection of pharmaceutical drugs which are most relevant for manned space missions. A list of priority compounds for the group of antibiotics has been proposed by Vieira da Silva (2005).

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Other groups of compounds will be selected later on taking into account this literature review. Technical requirements of the loop and analytical tools to follow up these components will be discussed.

4.2 Global objectives with respect to the study of pharmaceutical drugs

The number and type of pharmaceutical drugs allowed on a space mission is strictly limited and regulated. On the International Space Station for example, 99 different drugs are available. These include antibiotics, anti-inflammatory agents, aspirin, and components of a burns and wounds kit (Anonymous, 2000). Furthermore, lists are available of the antibiotics which are allowed by the Russian and American Space Agencies. All these drugs or their metabolites may enter the MELiSSA loop via urine or fecal material and may accumulate in the loop or exert stress on the microorganisms. So far, their behavior and effects have not yet been studied within the MELiSSA framework. A review paper by Vieira da Silva (2005) on biological degradation of antibiotics confirms the need to identify the microbial population in order to assess their role and the way they cope with antibiotics. It should be considered whether these different classes of pharmaceuticals present in human waste, do pose a potential risk to the overall functioning of the BELISSIMA system, and specific populations. It should be investigated whether either through cumulative chronic toxicity, or specific mechanistic effects related to the potent pharmacological mechanism of compounds, potential hazards due to release of pharmaceuticals might be present.

4.3 State of the art

4.3.1 HUMAN HEALTH AND ECOLOGICAL RISKS RELATED TO THE ENVIRONMENTAL DISTRIBUTION OF PHARMACEUTICALS

Generally, drugs are absorbed by the organism after intake and are subject to metabolic reactions. However, a significant fraction of the original substances leave human or animals organisms unmetabolized via urine or faeces Therefore they are next emitted into raw sewage, sewage sludge or manure, and can be distributed into the environment. For example in human faeces, several antibiotics still can be detected during 6 days of regular application. Furthermore if parent compounds are metabolized, some of these excreted metabolites can even be transformed back to the original active drug through microbiological activity in the environment. Recent monitoring studies in influents and effluents of WWTP, in rivers, in groundwaters and soils have detected low levels of a wide range of pharmaceuticals, including hormones, steroids, antibiotics and parasiticides. (Hirsch *et al*, 1999; Ternes 2001; Sacher *et al*.,2001; , 2003; Schrap *et al*., 2003; or review by Fent *et al*., 2006). It seems very difficult to make general predictions with regard to the release of pharmaceuticals into the environment.

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The form of application, the pathway of excretion, including parent compounds and metabolites, the physico-chemical properties of the drugs, as well as the type of treatment at the WWTP do all contribute to highly variable concentrations of compounds at the influents of WWTP, with removal efficiencies in the range of 0 to 100% (**Table 28**).

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Table 28: Influent and effluent concentrations and removal efficiency in sewage treatment plants (different equipment, different countries, sampling in different seasons) (after Fent *et al.*, 2006).

Compound	Influent concentration (µg/L)	Effluent concentration (µg/L)	Maximal removal (%)	Reference
Analgesics and antiinflammatory drugs				
Acetylsalicylic acid	3.2	0.6	81	Ternes et al. (1999)
Salicylic acid	57	0.05	99	Metcalfe et al. (2003a)[a]
	330	3.6		Carballa et al. (2004)
Dextropropoxyphene	0.03	0.06	0	Roberts and Thomas (2005)[a]
Diclofenac	3.0	2.5	17	Heberer (2002)
	n.r.	n.r.	69	Ternes (1998)[b]
	0.33–0.49	n.r.	75 (10–75)	Andreozzi et al. (2003a)[c]
	[5]	[1.5]	53–74	Strenn et al. (2004)[a]
	1.3	n.r.		Metcalfe et al. (2003a)[a]
	0.47–1.9	0.31–0.93		Buser et al. (1998b)
	2.8	1.9	23 ± 30	Quintana et al. (2005)[b]
	0.4–1.9	0.4–1.9	0	Tauxe-Wuersch et al. (2005)[c]
	0.35 ± 0.1	0.17–0.35	9–60	Lindqvist et al. (2005)[c]
	1.0	0.29	71	Roberts and Thomas (2005)[a]
Ibuprofen	3		96	Buser et al. (1999)
	38.7	4	>90	Metcalfe et al. (2003a)[a]
	9.5–14.7	0.01–0.02	99	Thomas and Foster (2004)
	[0.54]	[0.08–0.28]	22–75 99 (52–99)	Andreozzi et al. (2003a)[c]
	[1.5]	[0.01]	12–86	Strenn et al. (2004)[a]
	2.6–5.7	0.9–2.1	60–70	Carballa et al. (2004)[a]
	5.7	0.18	97 ± 4	Quintana et al. (2005)[b]
	28.0	3.0	98	Roberts and Thomas (2005)[a]
	2–3	0.6–0.8	53–79	Tauxe-Wuersch et al. (2005)[c]
	13.1 ± 4	0–3.8	78–100	Lindqvist et al. (2005)[c]
Ketoprofen	0.41–0.52	0.008–0.023	98	Thomas and Foster (2004)
	[0.55]	[0.18–0.3]	48–69	Stumpf et al. (1999)[b]
	5.7	n.r.		Metcalfe et al. (2003a)[a]
	0.47	0.18	62 ± 21	Quintana et al. (2005)[b]
	0.25–0.43	0.15–0.24	8–53	Tauxe-Wuersch et al. (2005)[c]
	2.0 ± 0.6	0–1.25	51–100	Lindqvist et al. (2005)[c]
Mefenamic acid	1.6–3.2	0.8–2.3	2–50	Tauxe-Wuersch et al. (2005)[c]
	0.20	0.34	0	Roberts and Thomas (2005)[a]
Naproxen			66	Ternes (1998)[b]
	40.7	12.5	40–100	Metcalfe et al. (2003a)
	10.3–12.8	n.d.-0.023	100	Thomas and Foster (2004)
	[0.6]	[0.1–0.54]	15–78	Stumpf et al. (1999)[b]
			93 (42–93)	Andreozzi et al. (2003a)[c]
	1.8–4.6	0.8–2.6	40–55	Carballa et al. (2004)[a]
	0.95	0.27	71 ± 18	Quintana et al. (2005)[b]
	4.9 ± 1.7	0.15–1.9	55–98	Lindqvist et al. (2005)[c]
Paracetamol	6.9	0	100	Roberts and Thomas (2005)[a]
β-Blocker				
Metoprolol	n.r.	n.r.	83	Ternes (1998)[b]
	n.r.	n.r.	10 (0–10)	Andreozzi et al. (2003a)[c]

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Table 28 (continued)

Compound	Influent concentration (μg/L)	Effluent concentration (μg/L)	Maximal removal (%)	Reference
Propranolol	n.r.	n.r.	96	Ternes (1998)[b]
	70	304	0	Roberts and Thomas (2005)[a]
Atenolol	n.r.	n.r.	<10 (0–10)	Andreozzi et al. (2003a)[c]
Blood lipid lowering agents				
Bezafibrate	[1.18]	[0.6–0.84]	27–50	Stumpf et al. (1999)[b]
	n.r.	n.r.	83	Ternes (1998)[b]
	[5]	[0.01]	10–97	Strenn et al. (2004)[a]
	0.6	0.2		Metcalfe et al. (2003a)[a]
	2.6	0.24	91 ± 4	Quintana et al. (2005)[b]
	0.42 ± 0.3	0–0.85	15–100	Lindqvist et al. (2005)[c]
Gemfibrozil	n.r.	n.r.	69	Ternes (1998)[b]
	[0.3]	[0.18–0.28]	16–46	Stumpf et al. (1999)[b]
	n.r.	n.r.	75 (10–75)	Andreozzi et al. (2003a)[c]
	0.7	1.3	n.r.	Metcalfe et al. (2003a)[a]
Fenofibric acid	[0.44]	[0.22–0.4]	6–45	Stumpf et al. (1999)[b]
	n.r.	n.r.	64	Ternes (1998)[b]
Clofibric acid	n.r.	n.r.	6–50	Stumpf et al. (1996)
	[1]	[0.68–0.88]	15–34	Stumpf et al. (1999)[b]
	n.r.	n.r.	51	Ternes (1998)[b]
	0.15–0.25	0.15–0.25	0	Tauxe-Wuersch et al. (2005)[c]
	0.34	0	91	Roberts and Thomas (2005)[a]
Neuroactive compounds				
Carbamazepine	n.r.	n.r.	7–8	Ternes (1998)[b]
	0.7	0.7	<50	Metcalfe et al. (2003a)[a]
	n.r.	n.r.	8	Heberer (2002)
	[1.5]	n.r.	4	Clara et al. (2004)[a]
	n.r.	[1.5]	53 (0–53)	Andreozzi et al. (2003a)[c]
Diazepam	0.59–1.18	0.1–0.66	93	Van Der Hoeven (2004)
Various				
Ethinylestradiol	0.003	0.0004	85	Baronti et al. (2000)
Clotrimazole	0.031	0.14	55	Roberts and Thomas (2005)[a]
Ifosfamide	0.007–0.029	0.010–0.043	0	Kümmerer et al. (1997)[a]
Tamoxifen	0.15	0.20	0	Roberts and Thomas (2005)[a]
X-ray contrast media	0.18–7.5	0.14–8.1	0	Ternes and Hirsch (2000)[b]

Data estimated from graphical data are in square brackets. n.r.: not reported.
[a] Median concentrations or percent.
[b] Average concentrations or percent.
[c] Maximal concentrations or percent.

Additionally, specific data provided by Ternes (2001) for measurements of drugs in effluents of WWTP, do demonstrate a significant number of drugs with levels above limit of quantitation. The latter is the case for most groups of compounds with different therapeutic and chemical structure (Table 29). The reported concentrations in surface waters are generally low and usually less then 1 μg/l (Table 30). Compounds which belong to the group of lipid regulators were consistently present in surface waters.

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Furthermore, an anti-epilepticum, carbamazepine, a few β-blokkers (propanolol, metaprolol), several analgetics (diclofenac, ibuproben, naproxen,..), and a few antibiotic compounds, sulfametaxozol and a metabolite of erythromicin were measured. Their occurrence in the aquatic environment does point to their ability to pass degradation at the WWTP and their persistence in the aquatic environment. These findings have raised questions about environmental abundance and persistence of veterinary and human medicines in general, and consequently concern is raised about their impact on organisms in the environment and on human health.

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Table 29: Concentrations of pharmaceuticals in municipal German STP effluents, results from 1996 to 1998 (after Ternes, 2001).

Analyte	LOQ (µg/l)	Number STPs	n > LOQ	Median (µg/l)	90-percentile (µg/l)	Maximum (µg/l)
Lipid regulator						
Bezafibrate	0.25	49	48	2.2	3.4	4.6
Gemfibrozil	0.050	49	39	0.40	0.84	1.5
Clofibric acid (metabolite)	0.050	49	47	0.36	0.72	1.6
Fenofibric acid (metabolite)	0.050	49	41	0.38	0.68	1.2
Antiphlogistics						
Diclofenac	0.050	49	49	0.81	1.6	2.1
Ibuprofen	0.050	49	42	0.37	1.2	3.4
Indomethacin	0.050	49	49	0.27	0.40	0.60
Naproxen	0.050	10	10	0.30	0.42	0.52
Ketoprofen	0.050	49	37	0.20	0.25	0.38
Phenazon	0.10	30	28	0.16	0.30	0.41
ASA	0.10	49	22	0.22	0.32	1.5
Salicylic acid (metabolite)	0.050	36	9	< LOQ	0.063	0.14
Betablocker						
Metoprolol	0.025	29	29	0.73	1.3	2.2
Propranolol	0.025	29	28	0.17	0.23	0.29
Betaxolol	0.025	29	17	0.057	0.10	0.19
Bisoprolol	0.025	29	17	0.057	0.13	0.37
β_2-Sympathomimetics						
Terbutalin	0.050	29	11	< LOQ	0.087	0.12
Salbutamol	0.050	29	10	< LOQ	0.072	0.17
Psychiatric drug						
Diazepam	0.030	20	8	< LOQ	0.03	0.04
Antiepileptic						
Carbamazepine	0.050	30	30	2.1	3.7	6.3
Antibiotics						
Clarithromycin	0.020	8	8	0.14	0.24	0.26
Roxithromycin	0.020	10	10	0.68	0.80	1.00
Chloramphenicol	0.020	10	1	< LOQ	< LOQ	0.56
Sulfamethoxazol	0.020	10	10	0.40	0.90	2.00
Trimethoprim	0.020	10	9	0.32	0.62	0.66
Dehydrato-erythromycin (metabolite)	0.020	10	10	2.50	5.10	6.00
X-ray contrast media						
Iopamidol	0.010	25	21	0.66	8.0	15
Iopromide	0.010	24	23	0.75	4.4	11
Diatrizoate	0.010	25	22	0.08	1.5	8.7
Iomeprol	0.010	12	10	0.37	2.8	3.8
Estrogens						
Estrone	0.001	38	20	0.001	0.021	0.070
17β-Estradiol	0.001	38	13	< LOQ	0.002	0.003
17β-Estradiol-17-valerate	0.004	38	0	< LOQ	< LOQ	< LOQ
17α-Ethinylestradiol	0.001	38	9	< LOQ	0.001	0.015
16α-Hydroxyestrone	0.001	15	11	0.001	0.004	0.005

LOQ: limit of quantification. STP: sewage treatment plant effluents (identical with the number of investigated STPs).

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Table 30: Concentrations of pharmaceuticals in German rivers and streams, results from 1996 to 1998 (after Ternes, 2001).

Analyte	LOQ (µg/l)	Number STPs	n > LOQ	Median (µg/l)	90-percentile (µg/l)	Maximum (µg/l)
Lipid regulator						
Bezafibrate	0.025	43/22	39	0.35	1.2	3.1
Gemfibrozil	0.010	43/22	28	0.052	0.19	0.51
Clofibric acid (metabolite)	0.010	43/22	35	0.066	0.21	0.55
Fenofibric acid (metabolite)	0.010	43/22	26	0.045	0.17	0.28
Antiphlogistics						
Diclofenac	0.010	43/22	43	0.15	0.80	1.20
Ibuprofen	0.010	43/22	35	0.07	0.28	0.53
Indometacin	0.010	43/22	35	0.04	0.17	0.20
Naproxen	0.010	20/20	20	0.07	0.15	0.39
Ketoprofen	0.010	43/22	5	< LOQ	0.12	0.12
Phenazon	0.020	26/20	21	0.024	0.15	0.95
ASA	0.020	43/22	17	< LOQ	0.16	0.34
Salicylic acid (metabolite)	0.010	35/19	24	0.025	0.13	4.1
Betablocker						
Metoprolol	0.010	45/23	38	0.045	1.2	2.2
Propranolol	0.010	45/23	26	0.012	0.44	0.59
Betaxolol	0.010	45/23	1	< LOQ	< LOQ	0.028
Bisoprolol	0.010	45/23	19	< LOQ	0.19	2.9
β_2-Sympathomimetics						
Terbutalin	0.010	45/23	0	< LOQ	< LOQ	< LOQ
Salbutamol	0.010	45/23	2	< LOQ	< LOQ	0.035
Psychiatric drug						
Diazepam	0.030	30/20	0	< LOQ	< LOQ	< LOQ
Antiepileptic						
Carbamazepine	0.030	26/20	24	0.25	0.82	1.1
Antibiotics						
Clarithromycin	0.020	33/22	7	< LOQ	0.15	0.26
Roxithromycin	0.020	52/40	23	< LOQ	0.20	0.56
Chloramphenicol	0.020	52/40	4	< LOQ	< LOQ	0.06
Sulfamethoxazol	0.020	52/40	26	0.03	0.14	0.48
Trimethoprim	0.020	52/40	10	< LOQ	0.09	0.20
Dehydrato-erythromycin (metabolite)	0.020	52/40	31	0.15	0.63	1.7
X-ray contrast media						
Iopamidol	0.010	25/25	24	0.49	1.6	2.8
Iopromide	0.010	25/25	22	0.10	0.55	0.91
Diatrizoate	0.010	25/25	23	0.23	6.4	ca. 100
Iomeprol	0.010	12/12	12	0.10	0.47	0.89
Estrogens						
Estrone	0.0005	15/15	3	< LOQ	0.001	0.0016
17β-Estradiol	0.0005	15/15	0	< LOQ	< LOQ	< LOQ
17β-Estradiol-17-valerate	0.002	15/15	0	< LOQ	< LOQ	< LOQ
17α-Ethinylestradiol	0.0005	15/15	0	< LOQ	< LOQ	< LOQ
16α-Hydroxyestrone	0.0005	15/15	0	< LOQ	< LOQ	< LOQ

LOQ: limit of quantification.

In the past few years, a number of studies have been performed on the environmental fate and health impact of pharmaceuticals (Kümmerer, 2001). With respect to human health, comparison of measured concentrations in water (such as in Table 30) with therapeutic dose information, drinking water limits and health advisories indicates that the concentrations of therapeutic compounds in surface waters are well below levels that would be of concern to human health (Webb, 2001). It therefore seems that indirect exposure to pharmaceuticals through the water supply is unlikely to pose a risk to humans.

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However, risks through other routes of exposure, such as uptake from soils into crops and biomagnification through the food chain have yet to be quantified and cannot be ruled out.
The impact on environmental health is more difficult to assess. Phenomena with respect to chronic toxicity to organisms are expected on the one hand, and on the other hand antibiotic resistance might be a key issue. Environmental impact studies investigate the potential negative effects on fish, daphnids, algae, bacteria, earthworms, plants and dung invertebrates. These data provide a reasonable body of evidence for likelihood of effects, but there are questions about the real-world value of these studies. Indeed, these data mostly are based on standard ecotoxicity tests, which are often short-lived and focus predominantly on mortality as the endpoint. Moreover, aquatic tests tend to focus on the water compartment and do not take into account pharmaceuticals residing in sediments. In general, the effects observed in these studies occur at much higher concentrations than those that are measured in the environment. This is illustrated (Figure 17) with comparative data for selected pharmaceuticals in a review by Fent *et al.* (2006).

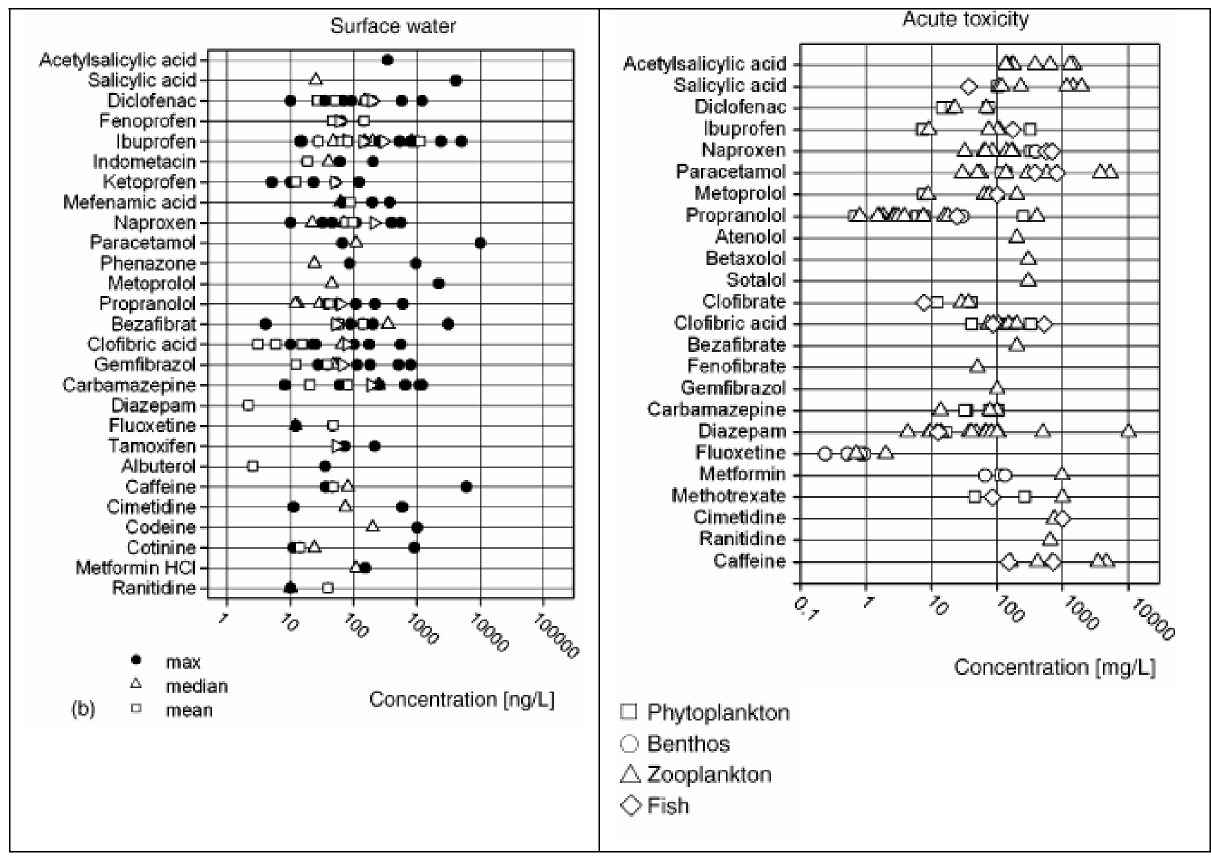

Figure 17: Range of concentrations in surface waters (ng/l) compared to measured acute effects on aquatic organisms (after Fent *et al.*, 2006)

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Therapeutically active substances can have more subtle, chronic effects on organisms in the environment, such as growth, fertility or behavior. Pharmaceutical compounds are designed either to be highly active and interact with receptors in humans and animals or to be toxic for many infectious organisms, including bacteria, fungi and parasites. But this does not mean that they affect only these living forms. Many lower animals have receptor systems similar to humans and animals used in agriculture. Furthermore, many groups of organisms that affect human and animal health, which are targeted by pharmaceuticals, have a crucial role in the functioning of ecosystems. A wide range of subtle impacts has been reported so far (Table 31), including effects on oocytes and testicular maturation, impacts on insect physiology and behavior, effects on dung decomposition, inhibition or stimulation of growth in aquatic plant and algae species, and the development of antibacterial resistance in soil microbes. Equally, antibiotics from human and veterinary use have an effect on soil microbes and algae. Macrocyclic lactones can affect invertebrate larvae in dung at fairly low concentrations. Earthworms appear sensitive to the parasiticides used in veterinary medicine and plants may be sensitive to many antibiotics. Despite the fact that these studies do not include effects for specific BELISSIMA organisms, some of the listed effects in Table 31 should be considered of potential concern for these organisms as they are dealing with similar, often conserved, mechanisms of biochemical and physiological receptor related functions.

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Table 31: Reported subtle effects of pharmaceutical compounds on aquatic and terrestrial organisms (after Boxall, 2004).

Substance(s)	Medicine class	Reported effect	Reference
Fenfluramine	Anorexic	Enhances release of serotonin (5-HT) in crayfish which in turn triggers the release of ovary-simulating hormone resulting in larger oocytes with enhances amounts of vitellin In fiddler crabs, stimulates the production of gonad-stimulating hormone accelerating testicular maturation	Daughton & Ternes, 1999
17a-Ethinylestradiol	Synthetic steroid	Endocrine-disrupting effects on fish, reptiles and invertebrates	Young *et al*, 2002
Methyltestosterone	Synthetic steroid	Impersex, reduced fecundity, oogenesis, spermatogenesis in snails	Schulte-Oehlmann *et al*, 2004
Avermectins	Parasiticide	Adults insects: loss of water balance, disruption of feeding and reduced fat accumulation, delayed ovarian development, decreased fecundity and impaired mating Juvenile insects: delayed development, reduced growth rates, development of physical abnormalities, impairment of pupariation or emergence and a loss of developmental symmetry	Floate *et al*, 2005
Tetracyclines, macrolides and streptomycin	Antibacterials	Antibacterial resistance measured in soil bacteria obtained from sites treated with pig slurry	Sengelov *et al*, 2003
Cypermethrin	Ectoparasiticide	Impact on dung decomposition	Sommer & Bibby, 2002
Fenbendazole	Parasiticide	Impact on dung decomposition	Sommer & Bibby, 2002
Tylosin	Antibacterial	Impacts on the structure of soil microbial communities	Westergaard *et al*, 2003
Erythromycin	Antibacterial	Inhibition of growth cyanobacteria and aquatic plants	Pomati *et al*, 2004
Tetracycline	Antibacterial	Inhibition of growth cyanobacteria and aquatic plants	Pomati *et al*, 2004
Ibuprofen	Anti-inflammatory	Stimulation of growth of cyanobacteria and inhibition of growth of aquatic plants	Pomati *et al*, 2004
Fenofibrate	Lipid regulator	Inhibition of basal EROD activity in cultures of rainbow trout hepatocytes	Laville *et al*, 2004
Carbamazepine	Analgesic	Inhibition of basal EROD activity in cultures of rainbow trout hepatocytes Inhibition of emergence of *Chironomus riparius*	Laville *et al*, 2004; Nentwig *et al*, 2004
Diclofenac	Analgesic	Inhibition of basal EROD activity in cultures of rainbow trout hepatocytes	Laville *et al*, 2004
Propanolol	Beta blocker	Weak EROD inducer in cultures of rainbow trout hepatocytes	Laville *et al*, 2004
Sulphamethazole	Antibacterial	Inhibition of basal EROD activity in cultures of rainbow trout hepatocytes	Laville *et al*, 2004
Clofibrate	Lipid regulator	Inhibition of basal EROD activity in cultures of rainbow trout hepatocytes	Laville *et al*, 2004
Diazepam	Antianxiety drug	Inhibition in the ability of dissected polyps from the cnidarian *Hydra Vulgaris* to regenerate a hypostome, tentacles and a foot	Pascoe *et al*, 2003
Digoxin	Cardiac glycoside	Inhibition in the ability of dissected polyps from the cnidarian *Hydra Vulgaris* to regenerate a hypostome, tentacles and a foot	Pascoe *et al*, 2003
Amlodipine	Calcium channel blocker	Inhibition in the ability of dissected polyps from the cnidarian *Hydra Vulgaris* to regenerate a hypostome, tentacles and a foot	Pascoe *et al*, 2003

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4.3.2 THE USE OF PHARMACEUTICALS AND HUMAN METABOLISM

In order for a drug to work, it must enter the body and somehow be distributed in such a way that it gets to its site of action. In most cases the site of action is a macromolecular "receptor" located in the target tissue. Most drug effects are temporary, because the body has systems for drug detoxification and elimination. The pharmacokinetic process, including eventual metabolisation of compounds, is illustrated in a general scheme (Figure 18) and described below in a number of steps.

1. A pharmaceutical drug may enter the body in a variety of ways: an oral liquid, pill, or capsule; as an inhaled vapor or aerosol; absorbed through intact skin or a mucous membrane; injected into muscle, subcutaneous tissue, spinal fluid, or directly into the bloodstream. The physical properties of the drug and the specific way it is prepared greatly influence the speed and the magnitude of absorption.
2. If the drug is given orally and swallowed, it must be absorbed from the gastrointestinal tract into the portal circulation. If it is absorbed from the skin, mouth, lungs or muscle it will go directly into the systemic circulation. If drug is injected directly into the bloodstream (e.g. intravenous injection), 100% of it is available for distribution to tissues. The latter will not be the case for other modes of administration. For example, a drug which is absorbed via the portal circulation must first pass through the liver which is the primary site of drug metabolism (biotransformation). Some of the drug may therefore be metabolized before it ever reaches the systemic blood. In this case, "first-pass" metabolism reduces the bioavailability to less than 100%.
3. Once the drug is in the bloodstream a portion of it may exist as free drug, dissolved in plasma water. Some drug will be reversibly taken up by red cells and some will be reversibly bound to plasma proteins. For many drugs, the bound forms can account for 95-98% of the total. This is important because it is the free drug which traverses cell membranes and produces the therapeutic effect.
4. The unbound drug may then follow its concentration gradient and distribute into peripheral tissues. In some cases, the tissue contains the target site and in others the tissue is not affected by the drug. Sites of non-specific binding act as further reservoirs for the drug. This total volume of distribution determines the equilibrium concentration of drug after a specified dose.
5. Tissue-bound drugs eventually reenter the bloodstream where they perfuse the liver and kidneys. The liver plays the crucial role in the production of metabolites. It metabolizes most drugs into inactive or less active compounds which are more readily excreted. These metabolites and some of the parent compound may be excreted in the bile and eventually may pass out of the body in the faeces. Alternatively, some of the drug may be reabsorbed again, further down the gastrointestinal tract (the so-called enterohepatic cycle). Any biotransformed drug which is not excreted in bile passes back into the systemic circulation.

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6. Parent drug and metabolites in the bloodstream may then be excreted: most are filtered by the kidney, where a portion undergoes reabsorption, and the remainder is excreted in the urine. Some drugs are actively secreted into the renal tubule. Another route of excretion is the lung (e.g. alcohol and the anesthetic gases). Smaller amounts of drug are eliminated in the sweat, tears and breast milk.

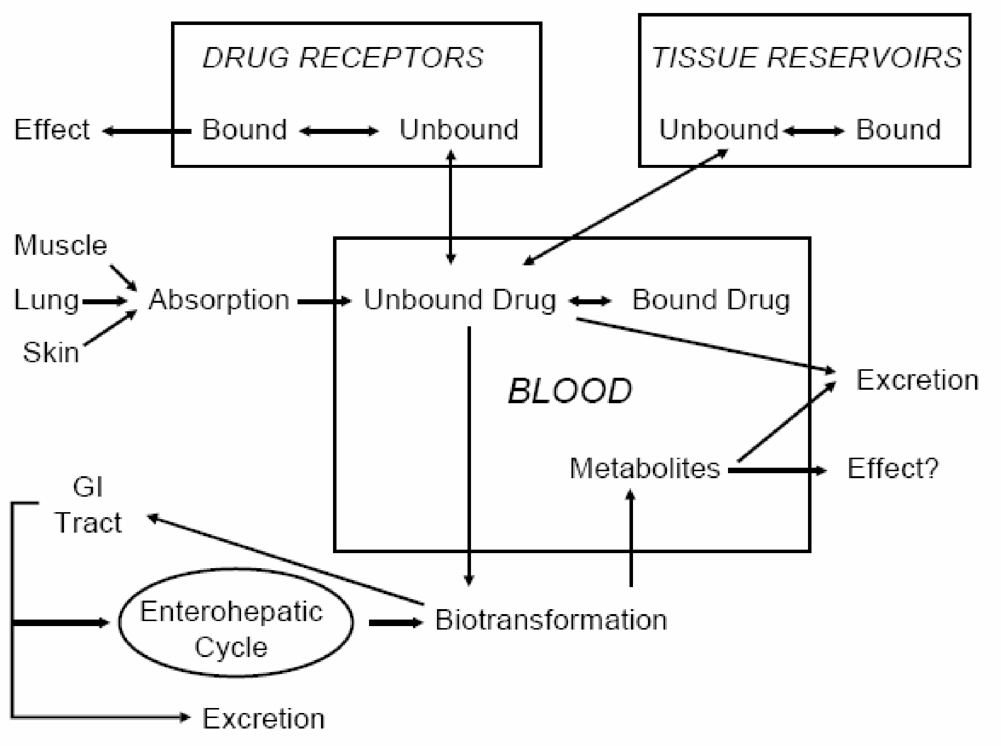

Figure 18: Pathway of pharmaceutical drug in human body (after Rosow C., lecture)

The desirable and undesirable effects of a drug arising from its concentrations at the sites of action are usually related either to the amount administered (dose) or to the resulting blood concentrations, which are affected by its absorption, distribution, metabolism and/or excretion. Elimination of a drug or its metabolites occurs either by metabolism, usually by the liver, or by excretion, usually by the kidneys and liver. Hepatic elimination occurs primarily by the cytochrome P450 family of enzymes located in the hepatic endoplasmic reticulum but may also occur by non-P450 enzyme systems, such as N-acetyl and glucuronosyl transferases. P450 enzyme systems located in gut mucosa can also significantly affect the amount of drug absorbed into the systemic circulation. Many factors can alter hepatic and intestinal drug metabolism, including the presence or absence of disease and/or concomitant medications.

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The pharmacokinetic behavior of drugs in the human body directly influences the potential environmental contamination. A drug which is only excreted as metabolites should in general not be found in its parent form in sewage and the environment. Thus, for those compounds it makes more sense to monitor the stable excreted principal metabolites. However pharmacokinetic data show that human excretion rates of unchanged drugs sometimes even exceed 50%. Additionally, excreted metabolites formed by conjugation with glucuronic acid or other polar compounds are likely to be cleaved by microorganisms into the unchanged pharmaceuticals, and hence the environmental concentration will increase.

Extended information on drug characteristics and related research for specific target compounds and metabolites can be found at the website of the US FOOD and Drug administration (FDA), section CDER (Center for drug evaluation and research: http://www.fda.gov/cder/). Additionally an inventory of more than 1500 pharmaceuticals with description of their use and pharmacokinetic pathways can be consulted at http://www.rxlist.com/. These information sources will be used for further estimation of potential metabolites, in relation to selected parent compounds as subject of this study.

4.3.3 THE FATE OF PHARMACEUTICALS IN DIFFERENT COMPARTMENTS

Compartment I

As for other organic compounds, the potential for biological/chemical transformations of pharmaceutical drugs under anaerobic conditions at elevated temperature is unknown. Biodegradation will strongly depend on the molecular structure of the drug considered. Sorption effects may turn out to be more significant, in particular for apolar compounds. Through regular draining of the accumulating solids in compartment I, some degree of removal from the loop will be achieved.

Retention by the ultrafiltration membrane will be limited because the molecular weight of the substances concerned is too low.

Compartments II

No biological transformations are expected to occur in compartment II if they did not occur in compartment I. Sorption on the biomass seems a more likely removal mechanism and may be problematic for food quality. Photochemical reactions may take place as well.

Compartments III

No biological transformations are expected but the biomass will probably be very sensitive to negative influences and stresses.

Due to low biomass concentrations and limited growth, no sorption is expected. Some chemical transformations may occur because this compartment is the first contact with oxygen.

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Compartments IVa

Similar observations hold as for compartment II. Metabolites formed in previous compartments may sorb on the biomass. Due to the production of extracellular polymers (EPS), this effect may be more pronounced than in compartment II. Photochemical, chemical and biological transformations are largely unknown. The presence of drugs and/or metabolites may alter the EPS production pattern.

The effect of pharmaceutical drugs in the MELiSSA loop is not known. Sorption phenomena may turn out to be important removal mechanisms. The availability of analytical methods will depend on the compounds studied.

4.4 Priority selection of compounds

Numerous pharmaceutical drugs of very different therapeutic groups (antibiotics, analgesic agents, cardiovascular drugs, anti-inflammatory agents,..) are likely to be used in space. These drugs are listed in reports by CHECS (including Soyuz onboard pharmacy and service module medical kits), with the comparison of the US medical kit. A literature review by Vieira da Silva (2005) does discuss the extended group of several types of antibiotics. As a starting point, it has been decided to evaluate space literature on the use of pharmaceutical drugs in relation to available terrestrial data on the environmental occurrence of such drugs. A selection is proposed with guidance compounds for selected therapeutic groups with varying physico-chemical properties.

4.4.1 PROCEDURE OF SELECTION AND LIST OF COMPOUNDS

It would be impossible to describe the metabolism of all pharmaceuticals within the framework of BELISSIMA, even when the list is limited to those compounds which are allowed on space mission. Therefore, we selected a number of compounds for intensive study. The selection procedure was as follows:

- Pharmaceutical drugs can be divided in antibiotics, analgesic and anti-inflammatory agents, lipid regulators, β-blockers, antidepressants, anti-epileptics, cytostatics and contrast media. Because of the crew's physical condition, lipid regulators, ati-epileptics and cytostatics are not expected to be used. Furthermore, the use of contrast media is linked to hospitals rather than to a space mission and can be eliminated as well. This leaves 4 classes of pharmaceuticals of which the antibiotics can be further subdivided according to their molecular structure into macrolides, sulfamides, β-lactams, tetracyclines and fluoroquinolones, mainly.
- Literature data on terrestrial monitoring of these pharmaceutical classes were compiled, including monitoring data of wastewater treatment plant effluents, surface water and drinking water. The concentrations measured were divided in several subclasses. The availability of monitoring data indicates that analytical methods for the compounds exist and the concentration ranges give an indication of the persistence or removal in wastewater treatment or in the environment.

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- Compounds which were found in terrestrial environments were crosschecked with pharmaceuticals allowed on space missions. Lists of the latter were available for the Russian medical kit (Soyuz) and US medical kit for ISS. Compounds occurring in the terrestrial environment and used in space, were retained.
- Data were collected for the selected compounds to ensure that they represented a range in biodegradability and in physicochemical properties.
- For these compounds human metabolism is reported in this TN and bacterial metabolism in TN80.13.

Table 32 shows monitoring data in effluents of sewage treatment plants, surface waters and drinking water for a number of parent compounds and metabolites. It appears that concentrations in effluents are always below 10.000 µg/l except for salicylic acid. β-lactams are always below detection limit, most probably because the parent compound is easily degraded. Concentrations in surface water are similar or lower than the ones in the wastewater treatment plant effluents. Drinking water data are limited but several compounds are present above detection limit, indicating that they pass all treatment steps. Their presence does however not pose a threat to human health.

The compounds of Table 32 were then crosschecked with pharmaceuticals used in space, as shown in Table 33. From this table, it becomes clear that the sulfonamide antibiotic sulfamethoxazole and the fluoroquinoline antibiotic ciprofloxacin are used both by the Russians and Americans and are hence relevant for further study. For the group of analgesic and anti-inflammatory compounds, none are present in both medical kits. Taking into account toxicological data, we propose to study diclofenac (used by the US). The only β-blocker and antidepressant allowed on ISS are metoprolol and diazepam.
This selection of pharmaceutical drugs was discussed with external experts during a microworkshop. The experts agreed with the selection but suggested to add acetylsalicylic acid (aspirin).

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Table 32: Monitoring data of pharmaceuticals per class (ng/l). Compounds in italics are metabolites of pharmaceuticals. DL: detection limit. References: a: Snyder *et al*. (2005), b: Vieira da Silva (2005), c: GRWC, d: Ternes *et al*. (2004), e: SVW report, f: Fent *et al*. (2006), g: Aquafin (2002), h: Kummerer (2001), x: Derksen *et al*. (2001), y: Luna *et al*. (2005), z: Hekster and Mons (2004).

		effluents						surface water					drinking water
Antibiotics		> 10000	> 1000	> 100	> 10	> DL	< DL	> 1000	> 100	> 10	> DL	< DL	> DL
macrolide	clarithromycin			x, y, z, h					x, h		z		
	erythromycin		h	x, y, c	z			x, h			z, a		
	erythromycin-H2O		*x*	*z*				*x*	*c*		*z, a*		*a*
	roxythromycin		x, h	y	z, d		d		x, c, h		z		
sulfonamide	sulfamethoxazole		x, h	y, c, e	z, b				x, c, h	a, e	z		z, a, c
	trimethoprim			x, b, c, h	z, b				x, c, h		z, a		
	sulfamethazine						x		c				
β-lactam	cloxacillin						x					x	
	dicloxacillin						x					x	
	methicillin						x					x	
	nafcillin						x					x	
	oxacillin						x					x	
	penicillin						x					x	
tetracycline	doxycycline											x	
	oxytetracycline			c	z		x				z	x	
	tetracycline				z		x	x			z	x	
fluoroquinolone	ciprofloxacin			c	b								
	lomefloxacin			b									
	norfloxacin				b								
	ofloxacin			b									
other	chloramphenicol			x, h	z					x, h	z		

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Table 32 **(continued)**

	effluents						surface water					drinking water
	> 10000	> 1000	> 100	> 10	> DL	< DL	> 1000	> 100	> 10	> DL	< DL	> DL
analgesic + anti-inflammatory												
acetylsalicylic acid		x, h	c, f, h		z			x, f	z	h		x, z, c
salicylic acid	*x*	*f*		*f*			*x, f*					
detropropoxyphene							x					
diclofenac		x, b, d, f, h	y, z, b, c, d, f, h				x, f	c, f, h	h	z		x, c, e
fenoprofen			x, b, c	z, b					x, f	z		
gentisic acid			*x*				*x*					
o-hydroxyhippuric acid		*x*									*x*	
ibuprofen		x, y, d, f, h	z, b, c, d, e, f, h	d, f			f	x, c, f	e, h	z, a		x, z, a, c
ibuprofen-OH		x					x					
ibuprofen-COOH			x						x			
indometacine			x	z				x, f	f	z		
ketoprofen		b, f	x, y, b, f	z, f				f	x, f	z, f		
methotrexate											x	
naproxen		b, f	x, y, z, c, e, f	f				x, f	e, f	z, a		a
paracetamol		x	c		z	f		f		z	x	z, c, e
phenazone			x, b, c, h	z, b	b	b	h	x, f	c	z		c
propyphenazone			x	z			x			z		

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Table 32 **(continued)**

β-blockers										
betaxolol		x, c, f, h		b			x, h			
bisoprolol	x	c, h	z		x, h	c		z		
carazolol		x				x				
metoprolol	x, h	z, b, c			x, f, h	c		z		e
nadolol		c	x					x		
propranolol		x, c, f, h	z, b		f	x, c, f, h		z		e
timolol			x				x			
antidepressants										
diazepam		f	z, c	x, d				z	x	x, c, e

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Table 33: Pharmaceuticals from Table 32 crosschecked for presence in the Russian or US medical kit for ISS. Compounds in yellow are selected for further study.

		Russian medical kit	US medical kit
antibiotics			
macrolide	clarithromycin		
	erythromycin	x	
	erythromycin-H2O		
	roxythromycin		
sulfonamide	sulfamethoxazole	x	x
	trimethoprim		x
	sulfamethazine		
β-lactam	cloxacillin		
	dicloxacillin		
	methicillin		
	nafcillin		
	oxacillin	x	
	penicillin	x	
tetracycline	doxycycline	x	
	oxytetracycline		
	tetracycline	x	
aminoglycoside			
fluoroquinolone	ciprofloxacin	x	x
	lomefloxacin		
	norfloxacin		
	ofloxacin	x	
other	chloramphenicol	x	
analgesic + anti-inflammatory			
	acetylsalicylic acid	x	
	salicylic acid		
	detropropoxyphene		
	diclofenac	x	
	fenoprofen		
	gentisic acid		
	o-hydroxyhippuric acid		
	ibuprofen		x
	ibuprofen-OH		
	ibuprofen-COOH		
	indometacine		
	ketoprofen		
	methotrexate		
	naproxen		
	paracetamol		
	phenazone		
	propyphenazone		

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Table 33 **(continued)**

β-blockers	betaxolol bisoprolol carazolol metoprolol nadolol propranolol timolol		x
antidepressants	diazepam		x

For the final selection of pharmaceuticals, physicochemical data were collected. Although a literature search was performed to collect data on biodegradation under aerobic and anaerobic conditions for all compounds, only the data for the selected compounds are reported in Table 34.

Table 34: Physicochemical and removal data for selected pharmaceuticals. *: observed under thermophilic conditions, ND: no data found, K_{ow}: octanol-water partition coefficient.

compound	removal (%)		MW	pKa	log K_{ow}
	aerobic	anaerobic			
sulfamethoxazole	0-25	85-95*	253	/	0.89
ciprofloxacin	70 (sorption)	ND	331	6.09	0.28
acetylsalicylic acid	75-80	ND	180	3.49	1.19
diclofenac	0-75	unclear	318	4.15	4.51
metoprolol	50-83	ND	267	9.6	1.88
diazepam	93	unclear	285	3.4	2.82

Table 34 shows that the selected compounds are recalcitrant to biodegradable under aerobic conditions and that data on anaerobic degradation, particularly in thermophilic conditions, are scarce or undecisive. Molecular weights vary little. Some compounds have acidic properties and are more hydrophobic in nature (higher log K_{ow}). The variation in physicochemical properties is considered sufficient to lead to variable behavior in the loop.
In agreement with the opinion of the external experts this selection of pharmaceuticals will be studied in BELISSIMA. At a later stage digestive medication and sleeping pill constituents may be included, if they are considered relevant.

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4.4.2 HUMAN USE AND METABOLISM OF SELECTED PHARMACEUTICALS

4.4.2.1 Antibiotic compound, sulfamethoxazole

Sulfamethoxazole is benzenesulfonamide, 4-amino-*N*-(5-methyl-3-isoxazolyl). It is an almost white, odorless, tasteless compound with a molecular weight of 253.28, the molecular formula $C_{10}H_{11}N_3O_3S$, and the following structural formula:

This antibiotic compound is often combined with other chemicals (e.g. trimethoprim, ..) in pharmaceutical drugs.

Sulfamethoxazole is rapidly absorbed following oral administration. It can occur in the blood as unbound, protein-bound, and metabolized forms. The metabolism of the sulfamethoxazole occurs predominately by N4-acetylation, although the glucuronide conjugate has also been identified. The free forms of sulfamethoxazole are considered to be the therapeutically active forms. Approximately 70% sulfamethoxazole will be bound to plasma proteins. Peak blood concentrations of sulfamethoxazole occur 1 to 4 hours after oral administration. The mean serum half-lives of sulfamethoxazole is 10 hours. Detectable amounts of sulfamethoxazole are present in the blood 24 hours after drug administration. During administration of 800 mg of sulfamethoxazole bid, the mean steady-state plasma concentration of sulfamethoxazole were 57.4 µg/ml and 68.0 µg/ml, respectively. These steady-state concentrations were achieved after three days of administration.
Excretion of sulfamethoxazole is primarily by the kidneys through both glomerular filtration and tubular secretion. Urine concentrations of sulfamethoxazole can be considerably higher than the concentrations in the blood. The average percentage of the dose recovered in urine from 0 to 72 hours after a single oral dose is 84.5% for total sulfonamide. Thirty percent of the total sulfonamide is excreted as free sulfamethoxazole, with the remaining as N4-acetylated metabolite.

4.4.2.2 Antibiotic compound, ciprofloxacin

Ciprofloxacin is a main constituent in CIPRO® Tablets and CIPRO Oral Suspension. CIPRO® (ciprofloxacin hydrochloride) Tablets and CIPRO (ciprofloxacin*) Oral Suspension are synthetic broad spectrum antimicrobial agents for oral administration.

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Ciprofloxacin hydrochloride, USP, a fluoroquinolone, is the monohydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid. It is a faintly yellowish to light yellow crystalline substance with a molecular weight of 385.8. Its empirical formula is $C_{17}H_{18}FN_3O_3$•HCl•H_2O and its chemical structure is as follows:

The serum course of ciprofloxacin could best be described by an open three-compartment model. High volumes of distribution (exceeding 200 liters/100 kg) suggested effective diffusions in the extravascular space. The terminal half-life of ciprofloxacin ranged between 3 and 4 h. High total and renal clearances suggested additional elimination pathways, such as tubular secretion, metabolism, or biliary excretion. After oral administration, absorption was sufficient, and the absolute bioavailability varied between 0.77 and 0.63. Maximal serum concentrations were attained 0.5 to 1 h after dosing; the higher dosage tended towards a delay in absorption. The proportion of the relative amount of metabolites to the total amount of drug excreted in urine increased from 29.7% after intravenous administration to 42.7% after oral dosing, indicating a first-pass effect of the liver. Ciprofloxacin concentrations with a bioassay were 3 to 27% higher than with high-pressure liquid chromatography, which may indicate the presence of biologically active metabolites (Hoffken *et al*., 1985).
Proportions of cumulative renal excretion of the administered dose of the parent drug up to 36 h have been shown to be as high as 43.1% for ciprofloxacin XR.

4.4.2.3 Anti-inflammatory and analgesic compound, diclofenac

Diclofenac sodium is a white to slightly yellow crystalline powder. It is freely soluble in methanol, soluble in ethanol, sparingly soluble in water, slightly soluble in acetone, and partially insoluble in ether. The chemical name for diclofenac sodium is: Sodium [o-(2,6-dichloranilino) phenyl] acetate. Diclofenac sodium has a molecular weight of 318.13. The CAS number is CAS-15307-79-6. The structural formula is represented below:

Diclofenac sodium is an active ingredient in several pharmaceutical drugs to be used as a nonsteroidal anti-inflammatory drug which relieves pain and reduces inflammation (swelling). It is used to treat headaches, muscle aches, dental pain, and athletic injuries. It is commonly used to treat the pain, swelling and stiffness associated with arthritis.

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4.4.2.4 Analgesic compound: acetylsalicylic acid

Acetylsalicylic acid is a colorless or white crystalline powder or granules; odorless or almost odorless with a slight acid taste. It is soluble in water. It is used as an analgesic for the treatment of mild to moderate pain, as an anti-inflammatory agent for the treatment of soft tissue and joint inflammation, and as an antipyretic drug. In low doses salicylate is used for the prevention of thrombosis.
Ingested aspirin is mainly absorbed as such, but some does enter into the systemic circulation as salicylic acid because of hydrolysis by esterases in the gastro-intestinal mucosa and liver. Aspirin can only be detected in the body for a short time as it quickly is hydrolysed. The half-life of aspirin is about 30 minutes. Plasma concentrations are low and rarely exceed 20 µg/ml (at normal therapeutic dose). The major urinary metabolites of aspirin include salicyluronic acid, salicyl-O-glucuronide, salicyl ester glucuronide and free salicylic acid.

4.4.2.5 Cardiovascular compound: β-blocker metropolol

This cardiovascular drug can be administered in different forms, including a multitude of controlled release pellets which contain metoprolol in the succinate form. The chemical name is 1-(isopropylamino)-3-[p-(2-methoxyethyl) phenoxy]-2-propanol succinate (2:1) (salt). Metoprolol succinate is a white crystalline powder with a molecular weight of 652.8. It is freely soluble in water and its structural formula is shown below:

Metoprolol is a beta1-selective (cardioselective) adrenergic receptor blocking agent. This preferential effect is not absolute, however, and at higher plasma concentrations, metoprolol also inhibits beta 2-adrenoreceptors, chiefly located in the bronchial and vascular musculature.
Clinical pharmacology studies have confirmed the beta-blocking activity of metoprolol in man, as shown by (1) reduction in heart rate and cardiac output at rest and upon exercise, (2) reduction of systolic blood pressure upon exercise, (3) inhibition of isoproterenol-induced tachycardia, and (4) reduction of reflex orthostatic tachycardia.

Pharmokinetic studies have demonstrated that in man, absorption of metoprolol is rapid and complete. Plasma levels following oral administration of conventional metoprolol tablets, however, approximate 50% of levels following intravenous administration, indicating about 50% first-pass metabolism. Plasma levels achieved are highly variable after oral administration. Only a small fraction of the drug (about 12%) is bound to human serum albumin.

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Metoprolol is a racemic mixture of R- and S- enantiomers, and is primarily metabolized by CYP2D6. When administered orally, it exhibits stereoselective metabolism that is dependent on oxidation phenotype. Elimination is mainly by biotransformation in the liver, and the plasma half-life ranges from approximately 3 to 7 hours. Less than 5% of an oral dose of metoprolol is recovered unchanged in the urine; the rest is excreted by the kidneys as metabolites that appear to have no beta-blocking activity. Following intravenous administration of metoprolol, the urinary recovery of unchanged drug is approximately 10%.

4.4.2.6 Anti-depressant, diazepam

Diazepam (or Valium) is a benzodiazepine derivative. The chemical name of diazepam is 7-chloro-1, 3-dihydro-1-methyl-5-phenyl-2H-1, 4-benzodiazepin-2-one. It is a colorless to light yellow crystalline compound, insoluble in water. The empirical formula is $C_{16}H_{13}C_{1}N_{2}O$ and the molecular weight is 284.75. The structural formula is as follows:

Valium is available for oral administration as tablets containing 2 mg, 5 mg or 10 mg diazepam. In addition to the active ingredient diazepam, each tablet contains the following inactive ingredients: anhydrous lactose, corn starch, pregelatinized.
Diazepam is used to treat anxiety, acute alcohol withdrawal, and seizures. It is also used to relieve muscle spasms and to provide sedation before medical procedures. This medication belongs to a class of drugs called benzodiazepines which act on the brain and nerves (central nervous system) to produce a calming effect. It works by enhancing the effects of a certain natural chemical in the body (GABA).
Specific data on the fate in human body and excretion in urine were not found.

4.4.3 ANALYTICAL METHODS FOR SELECTED PHARMACEUTICALS

Several analytical methods have been published concerning the determination of pharmaceuticals in biological samples such as serum, blood or urine within the µg/l range. The detection methods are mainly high performance liquid chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS) or GC/FID, and occasionally GC/MS/MS. Hernandez *et al*. (1999) provide a method for analysis of classes of antibiotics in biological samples by capillary electrophoresis (CE). These methods used sample volumes of 10 ml and less. In order to obtain quantification limits within the lower ng/l range for selected target drugs in the aqueous environmental matrices, advanced solid phase materials combined with specific derivatization procedures, as well as techniques such as GC/MS and LC-electrospray tandem MS (LC-ES /MS /MS) are essential (Ternes, 2001).

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Due to the continuous improvement of the analytical techniques, more than 50 pharmaceuticals and personal care products (PPCPs) have been detected during the last years in different environmental samples. Many of these samples were taken from wastewater, but also from surface or groundwaters which contain extremely low levels (Ternes, 2001; Lindberg et al., 2005; Batt & Aga, 2005; Lin *et al*., 2005). In Table 35 a summary is given for measurement methods for selected pharmaceutical compounds with corresponding data on sample pretreatment and performance characteristics of the method. This summary does illustrate that sample volumes up to 1 liter are required to detect selected pharmaceuticals in the ng/l range. In all the cases, and dependent upon the analytical technique to be used, sample treatment, mostly SPE is required. This is quite similar to the scheme proposed in Figure 13 by Gomes *et al* (2003) for the analysis of steroids.

Table 35: Overview of methods for the analysis of pharmaceutical drugs in environmental samples

Pharmaceutical compound	Sample type - volume	Sample treatment method	Analysis method	Performance characteristics of method	Reference
Diclofenac	WWTP* -200 ml	SPE (RP-C18/EN) with clean-up & derivatisation rec.: 50-89%	GC-MS	LOQ=50 ng/l	Ternes, 2001
	Surface water* - 1L			LOQ=5 ng/l	
	Drinking water* - 1 L		GC-MS/MS	LOQ=1 ng/l	
Diclofenac	Ground water-surface water-WWTP effluent (200-50ml)	SPE (RP-C18/ENVI; PS-DVB or Oasis HLB) % rec: 54-81		LOQ=2 ng/l	Lin *et al*., 2005

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Table 35 **(continued)**

Pharmaceutical compound	Sample type - volume	Sample treatment method	Analysis method	Performance characteristics of method	Reference
Metoprolol	Surface* & drinking water* - 1L	SPE (RP-C18/EN) with clean-up & derivatisation % rec: 93-98	GC-MS	LOQ=5 ng/l	Ternes, 2001
	Surface* & drinking water*- 1L	SPE % rec: 71-98	LC-MS/MS	LOQ=5 ng/l	
	WWTP* -200 ml	SPE	LC-MS/MS	LOQ=50 ng/l	
Diazepam	Surface* & drinking water* - 1L	SPE (C18) with clean-up & derivatisation % rec: 102	GC-MS	LOQ=20 ng/l	Ternes, 2001
	WWTP* -200 ml			LOQ=100 ng/l	
Sulfamethoxazole	Surface water*- 100 ml	Lyophilisation % rec: 81	LC-ES/ MS/MS	LOQ=50 ng/l	Ternes, 2001
	Surface water*- 1L	SPE (lichrolut C18/EN) % rec: 75		LOQ=5 ng/l	
Sulfamethoxazole	Sewage water-1L	SPE (ENV+) % rec : 101	LC-MS/MS	LOQ= 80 ng/l	Lindberg *et al.* 2005
	Sludge-2g	Extraction by ultrasonication & centrifugation % rec: 71	LC-MS/MS	LOQ=1.1 mg/kg	
Sulfamethoxazole	Surface water*- 1L	SPE (oasis-HLB or C18, several approaches) % rec: 97-107	LC-MS/MS	LOD= 83 ng/l LOQ= 270 ng/l	Batt & Aga, 2005
Ciprofloxacin	Sewage water-1L	SPE (ENV+) % rec : 98	LC-MS/MS	LOQ= 6 ng/l	Lindberg *et al.* 2005
	Sludge-2g	Extraction by ultrasonication & centrifugation % rec: 50	LC-MS/MS	LOQ=0.1 mg/kg	

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Table 35 **(continued)**

Pharmaceutical compound	Sample type - volume	Sample treatment method	Analysis method	Performance characteristics of method	Reference
Ciprofloxacin	Surface water*- 1L	SPE (oasis-HLB or C18, several approaches) % rec: 107-112	LC-MS/MS	LOD= 30 ng/l LOQ= 100 ng/l	Batt & Aga, 2005
Acetylsalisylic acid	Surface water & STP effluent	SPE No derivatization	GC-MS	LOD= 100 ng/l	Ternes, 1998

* spiked

4.5 Identification of technical requirements and critical steps of design of BELISSIMA loop & study protocol

4.5.1 EXPERIMENTAL SET-UP

Though study of the fate of pharmaceuticals is not planned for the immediate future, it is suggested that spiking experiments with a polar and an apolar drug for individual compartments could be set up. The test plan should still be discussed and will be function of available methods.

4.5.2 SAMPLE PROCESSING

With respect to sample collection it is estimated that 1 liter will be required in order to follow up the levels down to detection limits (ng/l) in the loop if normal urine is added into compartment I.

With respect to spiking experiments with pharmaceutical drugs, concentrations of target compounds could be elevated (range mg-µg/l) in relation to levels to be expected in urine. In the latter case, at least during a time course of sampling within the first hours after spiking, less sample volume (50-200 ml) might be enough.

Several methods for analysis are available and accordingly appropriate sample treatment methods (often SPE, either or not with clean-up) can be applied preferentially immediately after sample collection. In any case, samples should be collected in a way that they are representative and homogenous with respect to composition. They should be stored in the dark at 4°C prior to extraction. Sample extracts will be stored for a longer period until analysis.

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4.5.3 DESIGN REQUIREMENTS

Design requirements are similar for the study of hormones and pharmaceutical drugs and are summarized in Table 36.

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Table 36: Design requirements for hormone and pharmaceutical drug study.

	Compartment I	Compartment II	Compartment III	Compartment IV
operation mode: batch < > continuous	continuous	continuous	continuous	continuous
culture medium: as is, optimal nutrient concentrations, limitations, ..	As is, or later being optimal	As is, or later being optimal	As is, or later being optimal	- As is, or later being optimal
sterile cultivation/operation: yes/no	No specific requirements	No specific requirements	No specific requirements	No specific requirements
choice acid/base: - only acid/only base/both - which acid/base	HCl/NaOH	HCl/NaOH	HCl/NaOH	HCl/NaOH
samples: influent, effluent, biomass (solids), supernatant, at different reactor heights or locations, gas phase	I, E, B, S ->homogenous	I, E, B, S ->homogenous	I, E, B, S ->homogenous	I, E, B, S ->homogenous
sampling conditions : - axenic - continuous versus grab samples	Grab, Glass ware bottles, pre-cleaned	Grab, axenic, Glass ware bottles, pre-cleaned	Grab, axenic, Glass ware bottles, pre-cleaned	Grab, axenic , Glass ware bottles, pre-cleaned
sample volume : indicate maximum volume	See below Liquid phase: 75-1000 ml Biomass & solids: 0.5 g	See below Liquid phase: up to 1000 ml Biomass & solids: 0.5 g	See below Liquid phase: up to 1000 ml Biomass & solids: 0.5 g	See below Liquid phase: up to 1000 ml Biomass & solids: 0.5 g

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	Compartment I	Compartment II	Compartment III	Compartment IV
sampling frequency: - daily, weekly, .. - intensive sampling campaign - over which period	Liquid phase: - Immediate after urine application or spike: time sequence T0-T4-T8-T24-T48-T96-T144 followed by - 75-1000ml, with frequency 2-1x/week Biomass: occassionally,	Liquid phase: - 1x/Week: 1000ml Biomass: occassionally	Liquid phase: - 1x/Week: 1000ml Biomass: occassionally	Liquid phase: - 1x/Week: 1000ml Biomass: occassionally
sample collection: - in effluent tank or separate collection - through sampling ports - protect from light - temperature	 Effluent tank Not critical Not critical As is (not exceeding temp of compartment)	 Effluent tank Not critical Not critical As is (not exceeding temp of compartment)	 Effluent tank Not critical Not critical As is (not exceeding temp of compartment)	 Effluent tank Not critical Not critical As is (not exceeding temp of compartment)
sample treatment: - centrifugation, filtration, mixing, concentration, .. - sterilization	As is, stored in dark at 4°C, for immediate transfer to lab for SPE	As is, stored in dark at 4°C, for immediate transfer to lab for SPE	As is, stored in dark at 4°C, for immediate transfer to lab for SPE	As is, stored in dark at 4°C, for immediate transfer to lab for SPE

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	Compartment I	**Compartment II**	**Compartment III**	**Compartment IV**
sample preservation:T, protect from light, max. period	*4°C, dark < 8 hours, unless if sterilised than < 24 hours*	*4°C, dark < 8 hours, unless if sterilised than < 24 hours*	*4°C, dark < 8 hours, unless if sterilised than < 24 hours*	*4°C, dark < 8 hours, unless if sterilised than < 24 hours*
required measurements/instrumentation - as for normal reactor operation: yes/no - on-line measurements – which	Yes	Yes	Yes	Yes
required control: pH, T, ..	pH, Temp	pH, Temp	pH, Temp	pH, Temp
material selection	Omit use of synthetic material as plastic, sealings or glue & silicone which are in contact with reactor content. Omit plastic labware for manipulation of samples unless contact time is negligible (should be tested for contamination)	Omit use of synthetic material as plastic, sealings or glue & silicone which are in contact with reactor content. Omit plastic labware for manipulation of samples unless contact time is negligible (should be tested for contamination)	Omit use of synthetic material as plastic, sealings or glue & silicone which are in contact with reactor content. Omit plastic labware for manipulation of samples unless contact time is negligible (should be tested for contamination)	Omit use of synthetic material as plastic, sealings or glue & silicone which are in contact with reactor content. Omit plastic labware for manipulation of samples unless contact time is negligible (should be tested for contamination)

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4.6 Conclusions

We have demonstrated that the occurrence of pharmaceutical drugs in the BELISSIMA loop is highly relevant and that their fate and behaviour should be investigated in detail. For the multitude of drugs which might be used in space, a selection is proposed based on terrestrial data of occurrence and degradation, their different therapeutic use and chemical characteristics. Such compounds might interfere with biological organisms at very low levels, as they are especially designed to have a biological effect (therapeutic), and specific mode of action at low concentrations (ng/l). Moreover, as is the case for endocrine compounds, sites of action eg. receptors, or biochemical/fysiological mechanisms which are the target for drugs are often highly conserved.

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