M ELiSSA

Technical Note

Memorandum of Understanding 19071/05/NL/CP

TECHNICAL NOTE: 80.14

PRELIMINARY REQUIREMENTS FOR FUTURE BIOSAFE INVESTIGATIONS

prepared by/*préparé par*	W. Schiettecatte, H. De Wever, L. Hendrickx
reference/*réference*	Contract no. 19297/05/NL/SFe
issue/*édition*	1
revision/*révision*	0
date of issue/*date d'édition*	
status/*état*	Final
Document type/*type dedocument*	Technical Note
Distribution/*distribution*	B. Lamaze, H. De Wever, H. Witters, J. Bursens, L. Hendrickx, N. Michel, G. Dussap, F. Godia

CONFIDENTIAL DOCUMENT

A P P R O V A L

Title *titre*	Preliminary requirements for future biosafe investigations	issue *issue* 1	revision 0 *revision*
author *auteur*	W. Schiettecatte, H. De Wever, L. Hendrickx	date *date*	07/03/06
approved by *approuvé by*	Brigitte Lamaze	date *date*	22/05/06

C H A N G E L O G

reason for change /*raison du changement*	issue/*issue*	revision/*revision*	date/*date*

C H A N G E R E C O R D

Issue: 1 Revision: 0

reason for change/*raison du changement*	page(s)/*page(s)*	paragraph(s)/*paragraph(s)*

Table Of Contents

1. Introduction

In the closed loop MELiSSA concept for Advanced Life Support, organic wastes and possibly also urine of variable composition will enter the first compartment of the system and solutes will be transferred through the entire loop. The consumer waste containing fecal material and urine may introduce micropollutants, such as hormones and pharmaceutical drugs and their transformation products and can be a source of pathogenic organisms or their genes associated with virulence. Insights into the behavior and effects of these compounds in natural ecosystems and closed loop systems are largely unknown, but consumption, sorption or accumulation and biomagnification of a number of elements or compounds can be expected to occur.

An infinitely large number of elements and compounds can be studied. To make sure that the small-scale MELiSSA loop which will be constructed in the current BELISSIMA contract, is suited for long-term studies on the behavior and effects of various microcompounds, an overview of potential study items and their effect on the design needs to be made. In this TN, the focus will be on aspects related to biosafety investigations.

2. Definition of Biosafety

The term biosafety implies the protection from the hazards offered by micro-organisms to those who handle them. The term was introduced in the 1970s after reports about the high incidence of laboratory-acquired infections among clinical and medical research laboratory staff.

In Medicine literature biosafety is defined as follows:
'The application of knowledge, techniques and equipment to prevent personal, laboratory and environmental exposure to potentially infectious agents or biohazards. Biosafety defines the containment conditions under which infectious agents can be safely manipulated. The objective of containment is to confine biohazards and to reduce the potential exposure of the laboratory worker, persons outside of the laboratory, and the environment to potentially infectious agents.'

The 'Laboratory Biosafety Manual' of the World Health Organization uses the system of classifying micro-organisms into four groups on the basis of hazards they offer, primarily to the laboratory workers who handle them, and secondarily to the community. This risk group classification is given in Table 1 and is to be used for laboratory work only.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 1. Classification of infective micro-organisms by risk group

Risk Group 1 *(no or low individual and community risk)* A micro-organism that is unlikely to cause human or animal disease. **Risk Group 2** *(moderate individual risk, low community risk)* A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited. **Risk group 3** *(high individual risk, low community risk)* A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available **Risk group 4** *(high individual and community risk)* A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

Biosafety level designations for laboratory facilities are based on a composite of the design features, construction, containment facilities, equipment, practices and operational procedures required for working with agents from the various risk groups.
Laboratory facilities are designated as:

- Basic - Biosafety Level 1;
- Basic - Biosafety Level 2;
- Containment - Biosafety Level 3;
- Maximum containment - Biosafety Level 4.

The assignment of an agent to a biosafety level for laboratory work must be based on a risk assessment. While there are many tools available to assist in the assessment of risk for a given procedure or experiment, the most important components is professional judgement. Several factors have to be considered when classifying agents by risk groups:

- Pathogenicity of the agent and infectious dose;
- Potential outcome of exposure;
- Natural route of infection;
- Other routes of infection, resulting from laboratory manipulations (parenteral, airborne, ingestion);
- Stability of the agent in the environment;
- Concentration of the agent and volume of concentrated material to be manipulated;
- Presence of a suitable host (human or animal);
- Information available from animal studies and reports of laboratory-acquired infections or clinical reports;

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

- Laboratory activity planned (sonication, aerosolization, centrifugation,…);
- Any genetic manipulation of the organism that may extend the host range of the agent or alter the agent's sensitivity to known, effective treatment regimes;
- Local availability of effective prophylaxis or therapeutic interventions.

On the basis of the information ascertained during the risk assessment, a biosafety level can be assigned to the planned work, appropriate personal protective equipment selected, and standard operating procedures (SOPs) developed to ensure the safest possible conduct of work.

The risk assessment procedure described above works well when there is adequate information available. However, there are situations when the information is insufficient.

The above definition of biosafety is mainly related to the handling of consumer waste to be fed to the MELiSSA loop, the handling of waste generated by the MELiSSA loop or to other manipulations related to loop operation, preparation of feed or media, sampling, harvesting of biomass, etc. and the risks associated with them. However, in the framework of BELISSIMA, biosafety should be placed in a wider framework and all risks which the loop can present to human beings should be considered. We therefore propose to consider the following aspects:

- risks associated with the uptake of food or edible biomass produced by the loop
- risks associated with the consumption of drinking water coming from the loop
- risks related to the gas phase, either of the MELiSSA loop or of the space environment in which the astronauts work
- allergies or other phenomena related to contact with water, solids, etc. originating from the loop
- malfunctioning of crucial hardware such as the MELiSSA loop due to the presence of contaminants.

3. Inventory of hazardous elements

A definition of the design requirements for future biosafe investigation starts with an inventory of possible hazardous elements that can enter and/or accumulate in the MELiSSA-loop. This inventory will consist of an overview of micro-organisms and hazardous elements:

- which can enter the loop through the consumer waste, either through the fecal material or the non-edible plant waste
- which are associated with the plants in compartment IVb.

This overview will be completed with knowledge from microbial monitoring during space missions.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

3.1. Fecal material

The fecal material entering the MELiSSA loop will be the major source of contamination with possible hazardous elements. Although tremendous strides have been made, exact knowledge of the ecology of the human intestinal microflora is still poor. This natural ecosystem represents one of the most complex and concentrated groups of micro-organisms in nature. Adding to the complexity is that each individual intestinal ecosystem may have its own distinct characteristics and those characteristics again are not uniform over time.
Information was firstly gathered from available literature on the composition of the intestinal biomass in healthy adults. This was extended with an overview of possible pathogens that can be spread feco-oraly.

3.1.1. Fecal microbiota of healthy humans

The gastro-intestinal tract is a major reservoir of microbiological flora. It is estimated for example that there are some 1 to 2 kg of bacteria in the adult gastro-intestinal tract.

According to statistical analyses of classical culture based studies, bacteria are distributed in 400 to 500 different bacterial species (Demey *et al.,* 1995; Rigottier-Gois *et al*., 2003; Lay *et al*., 2005, Lenoir *et al.,* 2005). The majority (70%) of these species belong to six bacterial genera as given in Table 2.

Table 2. Bacterial genera in human intestines according to culture-based studies

Genera	Gram	Morphology	Spores?
Bacteroides	G^-	Straight, curved and spiral rods	non
Eubacteria	G^+	Irregular rods	non
Clostridia	G^+	Rods	endospores
Ruminococcus	G^+	Cocci	non
Fusobacteria	G^-	Straight rods	non
Bifidobacteria	G^+	Bone-shaped rods	non

Besides these dominant genera, the following minor groups and genera are retrieved: *Enterobacteria*, *Lactobacilli, Streptococci, Veillonella, Enterococci, Bacilli, Micrococci, Staphylococci,* methanogens, sulphate reducing bacteria, anaerobic cocci, ...

The concentrations of some genera are given in Table 3.

Most bacteria from the fecal microbiota are strict anaerobes and thus difficult to culture.
Except from the genus *Clostridium*, the intestinal bacteria are non-spore-forming.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 3. Concentration of intestinal microbiota (Lenoir Wijnkoop and Hopkins, 2003)

Genus	Concentration [Log_{10} bacteria / gram feces]
Bacteroides	10 - 11
Bifidobacteria	10 - 11
Fusobacteria	7 - 10
Eubacteria	7 - 10
Lactobacilli	7 - 9
Streptococci	6 - 8
Clostridia	6 - 10
Enterobacteria	6 - 8
Veillonella	5 - 7
Enterococci	5 - 7

Culture-based studies allow to partially identify the composition of the fecal microbiota. To study such a complex ecosystem, the combination of both culture- and molecular technology-based non-culture techniques are required.
Many bacteria appeared detectable only by the application of molecular technologies such as 16S rRNA gene analysis. Molecular approaches based on the direct study of 16S rRNA genes or using 16S rRNA probe hybridisation have revealed the predominance of four major phylogenetic groups, gathering the above six dominant cultivable genera. Table 4 gives an overview.

Table 4. Phylogenetic groups according to molecular studies (4 dominant and 2 subdominant)

Group	Included genera or species
Bacteroides	*Prevotella*
	Porphyromonas
Clostridium coccoides	*Clostridium*
	Eubacterium
	Ruminococcus
	Butyrivibrio
Clostridium leptum	*Clostridium*
	Eubacterium
	Ruminococcus
	Anaerofilum
	Fusobacterium prausnitzii
Bifidobacterium	
Enterobacteria	*Escherichia coli*
	Atopobium
Coriobacterium	

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Rigottier-Gois *et al.* (2003) compared two 16S rRNA probing methods to characterize the composition of fecal microbiota in 23 healthy persons: a similar set of probes targeting six phylogenetic groups using rRNA dot-blot hybridization, and whole cell fluorescent *in situ* hybridization (FISH) combined with cytometry. These techniques are discussed in greater detail in paragraph 6.2. As seen in Figure 1, the results are not statistically different for *Clostridium coccoides*, *F. prausnitzii*, *Bifidobacterium* and *Enterobacteria*. However, the proportions were significantly different for *Bacteroides*.
The metabolic state of the *Bacteroides* within the colon would explain the discrepancy observed between the rRNA level and the actual cell proportion.

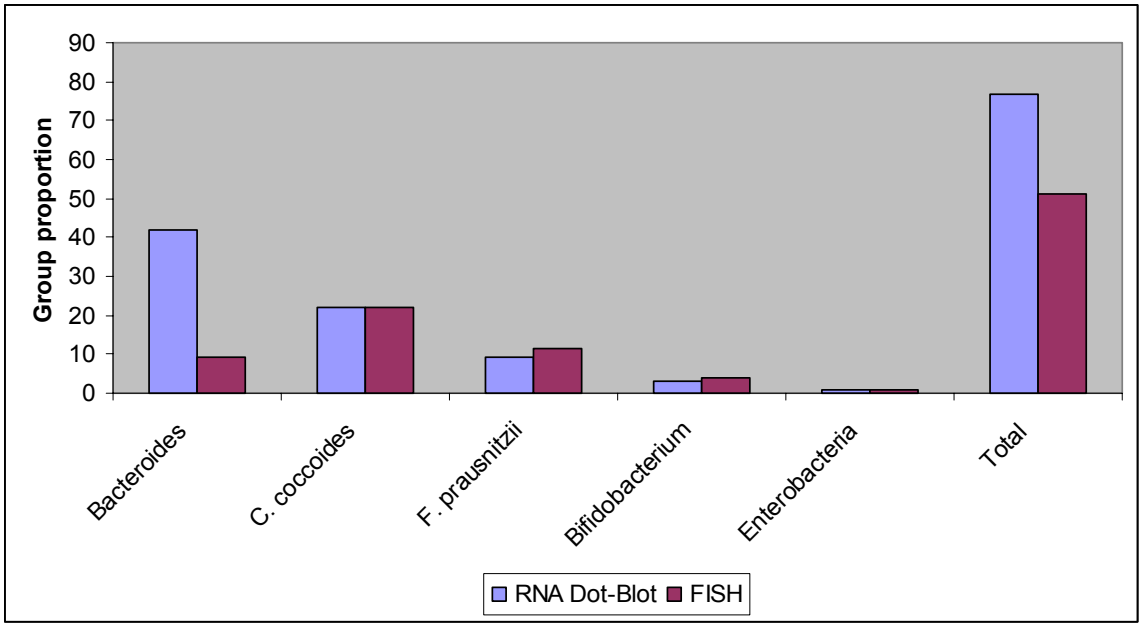

Figure 1. Distribution of the proportion of bacterial groups in human feces assessed by relative rRNA dot-blot hybridisation and by relative cell enumeration by FISH combined with flow cytometry

The optimal temperature for most of the gastro-intestinal bacteria is 36 to 38°C.

Beside the presence of bacteria some Protozoa can be found in the intestine, in the absence of disease, including *Entamoeba coli*, *Endolimax nana* and *Entamoeba dispar* (Hart and Shears, 2004). Heller *et al.* (2003) mention as well the presence of *Candida*, as it can be found in over 80% of human in the feces.

Finally, the gastro-intestinal tract contains enteric viruses, including a variety of bacteriophages, a number of known human viruses and uncharacterised viruses. Bacteriophages can influence food digestion by regulating microbial communities in the human gastro-intestinal tract through lytic and lysogenic replication (Weinbauer, 2003).

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Bacteriophages may also contribute to human health by controlling invading pathogens (Zhang *et al.*, 2006). Phages likely influence the composition of bacterial populations in the intestine through specific predation on microbial hosts. As a particular strain becomes dominant, phages can infect and lyse that host, giving another bacterial strain the opportunity to become abundant (Breitbart *et al*., 2003).
Through lysogenic conversion of resident intestinal bacteria, phages may introduce new phenotypic traits, such as antibiotics resistance and the ability to produce exotoxins. This topic is further elaborated in paragraph 5.2.5.2.

In addition to bacteriophages, the other well-studied human enteric viruses are viral pathogens associated with gastroenteritis. This is described in greater detail in the following paragraph.

Zhang *et al.* (2006) studied the RNA viral community in human feces. The vast majority of over 36000 viral sequences obtained were similar to plant pathogenic RNA viruses. The most abundant one was the plant pathogenic Pepper Mild Mottle Virus (PMMV), which was found in high concentration up to 10^9 virions per gram of dry weight fecal matter and for which dietary origins were suggested. The data demonstrated that PMMV nucleic acids survived standard food processing and that humans might act as a vehicle for transmission of certain plant viruses. In MELiSSA, this implies a potential for food crop disease if viruses present in fecal material are not inactivated throughout the loop.

A final concern is related to the fact that many human viruses may be present in a latent form and that many are still unknown.

3.1.2. Fecal microbiota during illness

A non-exhaustive list of diseases by pathogens that can spread feco-oraly is given in Table 5 (Hart and Shears, 2004). The pathogens cover a large spectrum, from small virusses at one end to protozoa and multicellular parasites at the other. The list contains info on the micro-organism and the disease it causes.

More detailed data on the organisms and their predicted survival rate within the MELiSSA loop and possible risk is provided further on in the TN.

Table 5. Pathogens that can spread faeco-oraly (Hart and Shears, 2004)

Group	Species	Major infection
Viruses		
RNA viruses	*Enteroviruses* (Picornaviridae)	Polio, meningoencephalitis …
	Hepatoviruses (Picornaviridae)	Acute hepatitis

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Group	Species	Major infection
	Astroviruses	Diarrhea, vomiting
	Calicivirus	Diarrhea, vomiting
	Rotavirus	Diarrhea, vomiting
DNA viruses	*Adenovirus*	Upper/lower respiratory tract Pneumonia, diarrhea
Bacteria		
Aerobic G^+ cocci	*Staphylococcus aureus*	Boils, impetigo, wound infections, osteomyelitis, septicemia
	Streptococcus zooepidemicus	Bacteremia
Aerobic G^+ bacilli	*Bacillus cereus*	Food poisoning
	Listeria monocytogenes	Neonatal sepsis
Aerobic G^- bacilli (Enterobacteriaceae)	*Escherichia coli*	Urinary tract, wound infection, septicemia, neonatal meningitis
	Shigella dysenteriae	Bacillary dysenteria
	S. flexneri	
	S. boydii	
	S. sonnei	
	Salmonella typhi	Typhoid and paratyphoid fever, gastroenterititis
	S. paratyphi A	
	S. paratyphi B	
	S. typhimurium	
	Yersinia enterocolitica	Gastroenteritis
	Y. pseudoturberculosis	Mesenteric adenitis
Aerobic G^- bacteria (Vibrios and related species)	*Vibrio cholerae*	Cholera
	V. parahaemolyticus	Food poisoning
	Aeromonas hydrophyla	Gastroenteritis
	Plesiomonas shigelloides	Gastroenteritis
	Campylobacter jejuni	Diarrhea, enterocolitis
Obligate anaerobic G^+ bacteria	*Clostridium perfringens*	Gas gangrene, food poisoning, wound infections
	C. botulinum	Botulism
Parasites		
Protozoa	*Entamoeba histolytica*	Dysenteria
	Giardia intestinalis	Diarrhea
	Cryptosporidium spp	Diarrhea and cramps
Multicellular parasites (Worms)	*Schitosoma spp*	Stomach muscular pain, skin rash and fever

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Group	Species	Major infection
	Ascaris lumbricoides	Usually asymptomatic, except during migration phase: astmatic disease
	Enterobius vermicularis	Irritation of the anal verge

Several of the above mentioned organisms are very unlikely to occur within the fecal material of the astronauts. The risks of diseases e.g. from *Salmonella* and *Shigella* can be reduced significantly if hygienic measures are taken.

The astronauts are exceedingly healthy and screened for several diseases. Examples of unlikely infectious agents include human immunodeficiency virus, tuberculosis and hepatitis B and C. Much more likely are infections from the astronauts' normal microbiological flora. For example, staphylococcal and streptococcal skin infections and urinary tract infections are more likely scenarios. In addition, diseases observed during long-term space missions seemed to be mostly of a gastro-intestinal nature. Some enteric viruses for example can infect the human small intestine cells, causing damage to the epithelial lining and absorptive villi, leading to malabsorption of water and an electrolyte imbalance.

3.2. Non-edible parts of plants

Beside the fecal material entering the MELiSSA-loop, non-edible parts of the plants from the higher plant compartment (CIVb-HPC) are fed to compartment CI as well. It can contain harmless 'native' micro-organisms, e.g. rhizosphere linked, or be infected by pathogens. Furthermore, as the plants are cultivated as hydroponics, micro-organisms can be expected in the hydroponic solution.

Several crops will be cultivated in the HPC: onion, lettuce, rice, soybean, spinach, tomato, wheat, potato, beet.

Only little information can be found on the 'normal microbiota' of vegetables. Information within this paragraph is based on four sources of knowledge:

- phytopathology;
- rhizospere linked microbiota;
- surveys of fresh vegetables, conducted to determine potentially toxigenic and pathogenic micro-organisms;
- literature from microbiological space research.

3.2.1. Phytopathology

Plant diseases may be caused by fungi, bacteria or viruses. For most crops that will be cultivated in the HPC, phytopathogens from these three groups can be found. According to the

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

University of Guelph (MELiSSA partner) fungi are most likely to occur since they cause about 40% of all plant diseases. Bacterial pathogens are fewer than fungi and viruses are the least likely to occur.

Fungi cause the majority of infectious or parasitic plant diseases. These include mildews, rusts, smuts, scabs, leaf spots, cankers, blights, root rots, stem rots, fruit rots, wilts and leaf galls. Typically, fungi do well in moist conditions and they are spread by water or air.

MELiSSA partner University of Guelph provided us with the following overview of potential fungal pathogens on the three main MELiSSA candidate crops (Clark and Waters, 2005). For beet, *Aphanomyces cochlioides* can cause Aphanomyces root rot which is a type of black root rot and may cause damping-off. *Pythium spp.* are responsible for Pythium root rot, damping-off and blackleg. Finally, *Rhizoctonia cerealis* causes damping-off, Rhizoctonia root and crown rot and black leg. On lettuce, *Pythium tracheiphilum* causes Pythium stunt, *Botryotinia fuckeliana (Botrytis cinerea)* grey mold rot and *Rhizoctonia solani* bottom rot. For wheat, *Fusarium spp*. have been described to cause head, seed or seedling blight, foot and root rot and *Pythium spp*. are responsible for damping-off and browning root rot. Other fungal pathogens are *Blumeria graminis, Erysiphe communis* and powdery mildew.

Further info was obtained from the University of Arizona and Florida respectively (http://ag.arizona.edu/PLP/plpext/index.html, http://edis.ifas.ufl.edu/CV273).
Pythium spp. can infect the roots of lettuce and tomato in hydroponic production systems (= Pythium root rot). Introduction of these fungi into the nutrient film can result in dramatic disease development. Affected plants develop a wilt symptom, that lengthens with each passing day until plant death.

Botrytis blight, caused by the fungus *Botrytis cinerea*, occurs at humidity levels above 90%. Spores are easily spread through the hydroponic solution. Tomato and lettuce are sensitive to this disease.

Several hundred types of bacteria cause plant disease. The most common types of bacterial disease are soft rots, leaf spots or blotches, blights, stem rots or cankers, wilts and galls. Many of the bacteria that cause rots in ripening fruits or vegetables are secondary invaders, gaining entrance through the wounds caused by other diseases or pests. Since bacteria can be spread by air or water, they will easily spread through hydroponic solution. Most disease-causing bacteria are quickly killed by high temperatures and dry conditions.

Table 6 lists examples of bacteria plant diseases related to the MELiSSA crops.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 6. Phytopathogenic bacteria
(1: http://www.ipm.ucdavis.edu/PMG/crops-agriculture.html, 2: Clark and Waters (2005)

Crop	Bacteria	Disease	Ref
Beet	*Agrobacterium tumefaciens*	crown gall	2
	Erwinia carotovora ssp. carotovora	soft rot	
	Pseudomonas marginalis pv. *marginalis*	soft rot	
Lettuce	*Erwinia carotovora ssp. carotovora*	head rot (slime rot)	2
	Pseudomonas marginalis pv. *marginalis*	marginal leaf blight	
Tomato	*Clavibacter michiganensis*	canker	1
Wheat	*Xanthomonas campestris*	Leaf streak	1
	Xanthomonas translucens pv. translucens	bacterial streak black chaff	2
	Pseudomonas spp.	stem melanosis basal blume rot bacterial leaf blight	2
	Erwinia rhapontici	pink seed	2
Potato	*Erwinia carotovora*	Blackleg	1
Onion	*Pseudomonas gladioli*	Soft rot	1
	Enterobacter cloacae		

Although plant viruses are the least likely to occur, they can cause a great deal of damage to crops. Virus infected plants can display a wide range of different symptoms. The whole plant can be stunted (dwarf-like) and infected leaves can show abnormal colorations like mosaics and yellowing. Very often infected leaves have a rolling or curly appearance and also the fruits can display various symptoms dependent on the virus. The yield can be severely reduced by a virus infection.

Table 7 provides a limited list of examples of virus plant diseases related to the MELiSSA crops, as compiled from the websites http://www.dias.kvl.dk/plantvirology/taxonomy.html, http://image.fs.uidaho.edu/vide/genindex.htm, http://www.dias.kvl.dk/plantvirology/links.html. As can be seen from the list, single stranded RNA viruses are the most common viruses of plants. Small DNA viruses are registered as well. Large DNA viruses have not been identified from plants. More details on the expected survival rate of viruses within the MELiSSA loop will be given in 4.

The most simple way of transmission of a virus is through vegetative propagation. More than 50% of the plant viruses can be transmitted by animal or microbial vectors. When cultivating in hydroponics, spread of viruses can of course easily occur through the hydroponic solution.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Clark and Waters (2005) also mention that viruses rarely transmit through seeds and are most often spread by insects or nematodes.

Table 7. Phytopathogenic viruses

Family	Species	ss/ds	DNA/RNA	envelope	size
Geminiviridae	Beet curly top virus	ss	DNA	no	20 nm
	Tomato pseudo curly top virus				
Reoviridae	Rice ragged stunt virus	ds	RNA	no	70 nm
Rhabdoviridae	Lettuce necrotic yellows virus	ss	RNA	yes	50-200
	Potato yellow dwarf virus				nm
Bunyaviridae	Tomato spotted wilt virus	ss	RNA	yes	80 nm
Potyviridae	Potato virus Y	ss	RNA	no	20-800
	Wheat streak mosaic virus				nm
Sequiviridae	Rice tungro spherical virus	ss	RNA	no	30 nm
Closteroviriodae	Beet yellows virus	ss	RNA	no	10-20
	Lettuce infectious yellows virus	ss	RNA		nm
Caulimoviridae	Soybean chlorotic mottle virus	ds	DNA	no	50 nm

Besides viruses, bacteria and fungi, insects can cause crop damage. Two well known examples are whitefly and aphids.

Whitefly adults are tiny yellowish insects with white wings that can occur on beets (information from University of Guelph). They are found mostly on the undersides of leaves. Whiteflies will feed and deposit eggs on sugar beets. In high populations, whiteflies can damage sugar beet by sucking sap from plants and causing stunting and wilting. The sweet potato whitefly is a vector of lettuce infectious yellows virus, an extremely destructive virus of sugar beet.

Aphids damage potatoes primarily by spreading plant diseases. Occasionally, aphids become so abundant that their feeding weakens and stunts the plants. Potato leafroll virus is mostly spread by green peach aphids. Plants grown from infected seed potatoes will not produce marketable potatoes. An infected Russet Burbank potato often has phloem net necrosis, a brown discoloration inside the potato that reduces quality. The brown discoloration is most intense at the stem end but may extend well into the tuber. Other viruses spread by aphids include cucumber mosaic and alfalfa mosaic (calico).

3.2.2. Rhizosphere linked microbiota

The rhizosphere, or area surrounding plant roots, is a region of enhanced microbial activity because roots release a variety of compounds. The rhizosphere consists of microbial communities that may be attached to root cells, embedded in the root mucilage, or not

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

physically connected to the root at all. The turbulence and rapid flow in a hydroponic system should ensure that microbes are uniformly distributed throughout the solution.

The roots of many plants are closely associated with micro-organisms. Due to the excretion of organic nutrients by the plant roots, growth of bacteria is promoted in the rhizosphere. In return, bacteria may carry out processes which are useful to plants, such as nitrogen fixation and decomposition of almost insoluble salts (Schlegel, 1988).
Apart from bacteria, associations with fungi in mycorrhiza are also found. A large number of fungi can penetrate plant roots and stimulate their growth through the production of auxins. The advantages for the fungus are the easy access to assimilation products of the plants and for the plant the highly effective absorption of mineral salts such as phosphate and fixed nitrogen.
As mentioned before, the association of plants with nitrogen-fixing bacteria is a good example of a symbiotic interaction. Nitrogen fixing bacteria belong to various bacterial genera (e.g. *Rhizobium)* and are typically strictly aerobic. In MELiSSA's HPC hydroponic N-containing solutions are currently being used. In a closed loop system, nitrate-rich effluents from compartment III will be fed to compartment IV. Hence, the chance that nitrogen-fixing bacteria will be present in high numbers is low. Furthermore, they are generally strictly aerobic and will most probably be eliminated during passage through compartment I.

No literature is know to us ascribing human pathogenicity to rhizosphere linked microbiota.

3.2.3. Surveys of fresh and minimally processed vegetables

Commodities can be easily contaminated with variety of microbes, including moulds and yeasts, during cultivation, harvest, storage and at the consumers' hands. Tournas (2005) screened fresh and minimally processed vegetables from local supermarkets in Washington DC area on presence of yeast and moulds. The most commonly found moulds were *Cladosporium*, *Alternaria* and *Penicillum.* Less common was *Geotrichum*. Yeast was found in 100% of lettuce, 62,5% of tomato samples and 66,7% of green onion samples. The author noticed as well that *Penicillium* growth increased during refrigeration. They could produce mycotoxins.

Croci *et al*. (2002) detected the highest quantity of Hepatitis A Virus within fresh lettuce. It has the most favorable conditions for viral persistence, probably because of the size and the wrinkled texture of its leaves. Washing apparently does not guarantee the elimination of the virus.

Goularte *et al.* (2004) state that the micro-organisms are on the surface region of the vegetables although internal tissues can eventually show viable micro-organisms as well, depending on harvesting and processing techniques. Treatments such as blanching, pH controls (3,5-4,4), traditional antimicrobials, high pressure and ozone have potential for immediate use. Other techniques like irradiation, electric pulses and natural antimicrobials are still under research.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

The abundant use of antibiotics in human therapy has resulted in the emergence of large numbers of antibiotic-resistant bacteria. Research on the survival of (potentially) pathogenic bacteria in manured soil, their transfer to crop roots and the presence of antibiotic resistance genes led to the conclusion that antibiotic resistance has the potential to spread in the environment through the survival of original manure-derived bacteria as well as through the transfer of plasmid-mediated antibiotic resistance genes from these bacteria to soil-borne and plant-associated species (Cools, 2001). Although in MELiSSA the bacteria present in fecal material follow a different route, no soil is present and no direct spreading of manure on plants occurs, the risk exists of similar mechanisms for spread of antibiotic resistance.

3.2.4. Plant microbial communities in space

Plants grown in space have been found to be more susceptible to pathogens due to stress (Clark and Waters, 2005). On the other hand, they are exposed to fewer pathogens. To our knowledge, no information on phytopathogens determined on plants during space missions is available. However it may be anticipated that sterilization actions and the reduced number of bacterial species in a confined environment may present possibilities for opportunistic pathogens to establish themselves.

The following information is reported for plant microbial communities in closed systems, as for life support. Roberts *et al*. (2004) indicated that rhizosphere communities associated with potatoes in hydroponic nutrient delivery systems initially showed a high variability, but became increasingly more predictable over time. This was even more the case for tests with dwarf wheat. They concluded that inoculation with known mixtures of microbial isolates is of limited value. It is better to use stable communities which have already established on older plants. Tirranen (2001) reports that the microbial community on vegetables grown in polyculture is more diverse and stable than the community of a monoculture of wheat.

3.3. Presence and production of toxins

Mycotoxins are secondary metabolites of certain fungi. They are toxic to higher animals and humans. Aflatoxins are best known, due to the outbreak of Turkey X disease in the UK (1960). They are produced by several strain of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Since the late 1980s the aflatoxins are classified by the WHO as Group 1 carcinogens.
Aflatoxin B_1 is generally the most common and the most toxic in terms of both acute and chronic toxicity. The standard for Aflatoxin B1 according to European legislation is 2µg/kg.
There are several food commodities in which aflatoxins have been reported, e.g. soybeans, wheat and rice (Moss, 2002; Pittet, 1998).
Although the highest levels of aflatoxins are undoubtedly associated with post-harvest spoilage of food commodities stored under inappropriate conditions of water activity and temperature, the aflatoxigenic fungi have more complex ecologies. The spores of *Aspergillus* can germinate on plants under stress conditions, like drought. In this case aflatoxins may be produced in the plant tissue during growth (Moss, 2002).

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Beside Aflatoxin, several other mycotoxins are defined. A large family of mycotoxins of geberal concern is the trichothecens, which are mainly produced by moulds of the genus *Fusarium*. *Fusarium species* produce also several other mycotoxins: zearalenone, fumonisin. *Aspergillus* and *Penicillium* species can produce ochratoxin A.

As research on bovine milk indicates the presence of mycotoxines, it can be concluded that the microflora in the rumen only partly eliminates these chemicals. Aflatoxin M_1 will survive pasteurisation and has been reported in UHT milk.

Table 8 gives an overview of acute LD_{50} values for a selection of microbial toxins.

Table 8. Microbial toxins and their toxicity

Toxin	Producing organism	LD_{50} [mg/kg]
Aflatoxin B1	*Aspergillus flavus*	5,5 (male rat)
T-2 toxin	*Fusarium sporotrichioides*	5,2 (rat)
Sporidesmin	*Pithomyces chara*	1 (lamb)
Verrucarin A	*Myrothecium spp.*	0,87 (rat)
Aeroginosin	*Microcystis aeruginosa*	0,05 (mouse)
Botulinum toxin	*Clostridium botulinum*	10^{-6} (mouse)

As can be seen from the list, the botulinum toxin from the bacteria *Clostridium* is by far the most toxic, when compared to the mycotoxins. In addition, it is the only bacterial toxin.
Botulinum toxin causes, on ingestion of the food, paralysis of the nervous system and is lethal. This toxin is heat labile and can be easily inactivated by boiling for 15 minutes. Whether it will also be eliminated during a 10-day retention at 55°C in compartment I, is unsure.

Many plant pathogenic fungi and bacteria are known to produce toxic metabolites which contribute to symptom development in the infested plant. These so-called *phytotoxins* can be divided into i. host-selective toxins, which are produced only by a few fungal species (e.g. *Alternaria*, *Cochliobolus*) and ii. non-selective toxins, which are synthesized by a lot of fungi and bacteria and cause damage not only to the host plant but also to other plant species that are not normally attacked by the pathogen in nature.
The phytotoxin fusicoccin, for example, which is produced by *Fusicocum amygdali* induces stomata opening by stimulating K^+-uptake into the guard cells leading to uncontrolled transpiration and finally wilt (Heiser *et al*., 1998).

3.4. Genetic elements

Genetic material can be transferred from one bacterial cell to another following several mechanisms. This is addressed in the MELiSSA TN80.11 - Preliminary requirements for genetic stability and axenity study (Hendrickx *et al.* 2006). Plasmids and bacteriophages play

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

important roles in the transfer of pathogenic determinants. These topics are respectively discussed in paragraph 5.2.5.1and 5.2.5.2.

Special attention should be paid to the spread of genetic material encoding for the resistance to antibiotics. The widespread use of antibiotics in animal husbandry for instance has triggered the concern of antibiotic resistance spread from animals to humans. A direct link can be found within the food chain. A less documented risk however is through manure disposal on fields. This way resistance to antibiotics, which is often plasmid-mediated, can be spread to the soil bacterial community. Crops grown on these fields can easily be infected with those antibiotic resistant bacteria. (Sengelov *et al*., 2003, Cools *et al*., 2001)

3.5. Prions

Prions are simple proteins that are much smaller than viruses. They are unique since they lack genome. The known prion diseases are fatal. Since the immune system does not recognize prions as foreign, no natural protection develops.
Prions were first described in mammals as protein infectious particles, hypothesized to be the causative agent of transmissible spongiform encephalopathies (TSEs). The fatal human diseases include Creutzfeld Jacob disease, kuru, Gerstmann-Straussler-Scheinker disease, fatal familial insomnia, and possibly Alpers disease.
The infectious agent is hypothesized to be a self-propagating pathological isoform of the host-encoded prion protein PrP. This is the most widely accepted 'protein only' theory. However, the final proof of this theory will require the engineering *in vitro* of a synthetic infectious protein capable of propagating a prion *in vivo* (Soto and Castilla, 2004).

The term prion is however no longer confined to the infectious agent of TSEs but is used for any protein that adopts a self-sustaining conformational state. In the budding yeast *Saccharomyces cerevisiae*, prions were discovered as heritable epigenetic elements. The altered protein structure, or prion state, produces a change in phenotype that is perpetuated by self-propagation of a protein structure without a change in genotype (True, 2006).

Under natural conditions, the most likely way that the infectious agent could enter the environment is through the decay of infected animal carcasses (with the accumulation of prion in nervous system and lymphoid tissues through the disease), excreta from infected animals, or infected placenta remaining in the ground after whelping. The US Environmental Protection Agency however states that at present there has been little evidence of prion-contaminated manure.
Narang *et al.* (2005) discuss the fact that the expression of the host-encoded glycoprotein PrP is the highest in brain tissue. It can also be detected at low levels in peripheral tissue. However it is unclear whether a significant amount of PrP is released into body fluids and excreted into urine.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

3.6. Microbial monitoring during space missions

Several publications are available on the presence of micro-organisms in spacecrafts and long-term space habitats supporting human exploration. Generally, no exotic micro-organisms are found, but rather these genera which originate from the astronauts themselves. Indeed, the largest microbial reservoir in a space vehicle is the intestinal flora of the crew. It has been noted that the number of bacterial species isolated from the intestinal tract is strongly reduced in confined environments. Particularly some species which are beneficial to human health, decrease sharply. Such shifts may change the health status of the crew and increases the risk of opportunistic infections (Taylor *et al.,* 2005, cited by Pechère, 2005). Several monitoring studies indicate the presence of a wide range of micro-organisms.

An extensive database of environmental parameters has been provided for short-term (<20days) space flight during more than 100 missions aboard the Space Shuttle. The NASA MIR Program provided similar data for long-duration missions. Recently, information from microbial surveys on board the International Space station ISS was published as well.
A continuous environmental monitoring during prolonged exploitation of an orbital station or any moon or planetary base is of major importance to ensure the cosmonaut's health and the integrity of the spatial hardware.

Data were reviewed by Pierson (2001) and Novikova (2004, 2005). The focus in most studies lay on the presence of bacteria and fungi. No publications were found on e.g. the presence of viruses in space vehicles. Interestingly, the major bacterial and fungal species found in the Space Shuttle are similar to those encountered in MIR.

During several **Space Shuttle flights**, air contamination was monitored. Bacterial levels tended to increase modestly during flight, whereas fungal levels were usually low and remained low or even decreased further throughout the mission, probably because of low humidity (generally <50%) and lack of continuous sources of fungi. The most common bacterial genera recovered from the air were *Staphylococcus*, *Micrococcus* and *Bacillus*. With the exception of *Bacillus*, most bacterial species cultured were commonly associated with humans. *Aspergillus*, *Penicillium* and *Cladosporium* were the fungal genera most frequently collected from the air.
Surfaces were analyzed as well. Pre-flight values for bacteria and fungi were respectively 300 and less than 100 cfu/100cm². The same genera as in the air samples were dominant.
The bacterial level in the potable water after treatment was very low, *Burkholderia cepacia* being the most commonly cultured bacterial species.
The microbiological profile of the astronauts themselves, after space flights, was typical of healthy individuals, and no significant changes in microbiota have been detected.
Pierson *et al*. (1996) investigated *Staphylococcus aureus* epidemiology during space flights. They discovered that the *S. aureus* fingerprint was unique for each crew member, and that each individual usually carried only one strain. In one case, transfer between crew members was noted.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

The **space station MIR** has been in operation for nearly 15 years, giving a good opportunity to gain knowledge of long-term microbial buildup, selection and adaptation processes.
Air sampling revealed levels of bacteria from approximately 200 to 425 cfu/m³ of air. The mean of the fungal levels ranged from 175 to 325 cfu/m³. The bacterial and fungal genera most frequently cultured from MIR air were respectively: *Staphylococcus*, *Bacillus* and *Corynebacterium* on the one hand and *Penicillium*, *Aspergillus* and *Cladosporium* on the other hand. *Aspergillus flavus* was recovered from approximately 50% of the samples. A significant number of microbial species in the air were opportunistic pathogens including *Staphylococcus aureus, S. capitis, S. haemoliticus, Flavobacterium meningosepticum, Escherichia coli, Serratia marcescens, Streptococcus sp., Bacillus cereus.*
Mean bacterial levels on surfaces were about 2700 cfu/100cm². Fungal levels were 500 cfu/100 cm² or less. *Staphylococcus*, *Bacillus* and *Micrococcus* were the most frequently isolated bacteria and *Penicillium*, *Candida* and *Aspergillus* the most frequently isolated fungal genera.
Humidity condensate was reclaimed through a water processor system, that included a terminal heat step. Bacterial levels in this water source were very low.

Ott *et al.* (2004) published results of microbial analysis on humidity condensate that had accumulate behind panels aboard MIR. As these floating masses of liquid come into contact with the astronauts and the engineering systems, they have the potential to affect both crew health and systems performance. A wide variety of organisms were isolated including *Escherichia coli*, *Serratia marcescens* and a presumed *Legionella* species. In addition, microscopic analysis indicated the presence of protozoa, dust mites and spirochetes.

The **International Space Station (ISS)** is an orbital living and working environment actually under construction by the United states in collaboration with Russia, 11 nations of the European Space Agency, Canada, Japan and Brazil. The projected lifetime of the ISS after completion is approximately 10 years. Over the past few years monitoring of air, water and surfaces has been done within ISS.
Concentrations of airborne bacteria and fungi were lower than 710 and 44 cfu/m³, respectively. *Staphylococcus* sp. was by far the most dominant airborne bacterial genus, whereas *Aspergillus* sp. and *Penicillium* sp. dominated the fungal population (Novikova *et al*., 2006).
The bacterial concentrations in surface samples fluctuated from 25 to $4{,}3.10^4$ cfu/100cm². *Staphylococcus* sp. dominated in all of these samples. The number of fungi varied between 25 and $3{,}0.10^5$ cfu/100cm², with *Aspergillus* sp. and *Cladosporium* sp. as the most dominant genera.
The viable counts in potable water did not exceed 100 cfu/ml. *Sphingomonas* sp. and *Methylobacterium* sp. were identified as the dominant genera.
Molecular analysis demonstrated the presence of nucleic acids belonging to various (opportunistic) pathogens and strains involved in the biodegradation of structural material.

Table 9 states acceptability limits for bacteria and fungi, as will be used for the ISS.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 9. Bacterial and fungal limits for air, surfaces and water (Pierson, 2001)

	Bacteria	Fungi
Air	1000 cfu/m³	100 cfu/m³
Surfaces	10 000 cfu/100cm²	100 cfu/100cm²
Water	100 cfu/100ml (heterotrophic)	

The microbes determined in the atmosphere during space missions may enter the MELiSSA loop through airlocks, waste or biomass elimination systems and filters which are present in the system. Particularly during filter replacement, a high risk of contamination exists (Pechere, 2005).

4. Associated risks

We will discuss in this chapter risks associated with the uptake of food, the consumption of drinking water and contact with the gas phase, contact with water and waste and risks of hardware or MELiSSA compartment malfunctioning.

4.1. Uptake of food or edible biomass

On earth, food safety is determined by the presence or absence of food-related pathogens or the degree at which pathogens occur (Van Campenhout, 2004). Two types of food pathogens are considered, those which cause food infections and those which produce toxins and hence cause food intoxications. Food infections and intoxications differ in the way they cause disease.
Food infections originate from the presence of living pathogens on food products, the associated uptake by the consumer and their establishment in the intestinal tract and disturbance of the regular intestinal microflora. Disease only occurs when the minimal infectious dose has been reached. This is dependent on the virulence or pathogenicity of the micro-organism, the physical condition of the host and the food matrix. Food infections typically cause (gastro)-enteritis which are either due to the adherence of the microbial cells to the gut or due to the production of toxins by the microbes in the gut. In case of food intoxications, microbially produced toxins rather than the microbes themselves are ingested with the food. Both bacteria and fungi can produce toxins during growth in the food. If the minimal toxic dose is reached, biological reactions will take place in the body of the consumer leading to disease.

The five most important bacteria causing food infections are *Campylobacter*, *Salmonella*, *Staphylococcus aureus, Clostridium perfringens* and *Bacillus cereus*. Food intoxications are mostly caused by *Bacillus cereus, Staphylococcus aureus* and *Clostridium botulinum.* They spread fecal-orally and can hence enter the MELiSSA loop through the fecal material (see

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 5). If not eliminated or retained through the MELiSSA cycle, they can appear on the edible biomass produced.
Important Protozoa with respect to food infections are *Cryptosporidium*, *Giardia* and *Entamoeba*, which also spread faeco-orally.

Conditions for storage and conservation of the food produced in the MELiSSA loop, and for handling and preparation of the food, will further determine whether or not the potentially present pathogens will increase or decrease in number, whether they can produce toxins, and whether the minimal infectious or toxic dose will be reached.

On MIR, most diseases seemed to be gastro-intestinal infections although no documents are publicly available to confirm this. On the early Apollo missions, gastroenteritis was among the most common infectious diseases. Once the Crew Health Stabilization Program was implemented, fewer infectious diseases occurred and these were mainly skin infections and gingivitis (Pierson, 1992).

4.2. Consumption of drinking water

The risks associated with consumption of contaminated drinking water are largely the same as those related to uptake of food. Indeed, mostly fecal-orally transmitted pathogens are concerned. These are either bacteria, protozoa, viruses, cyanobacteria, or higher organisms, which may cause diarrhea, fever, enteritis, etc.

Analysis of drinking water aims largely at the presence of *Escherichia coli*. This bacterium is a normal inhabitant of the human intestine and is harmless (except for 4 subtypes which can cause diarrhea and the subtype O157:H7). Hence, its presence in drinking water is not dangerous. However, it is used as an indicator of fecal contamination of the water.
Demonstration of *E. coli* in water samples indicates contamination with intestinal content and bacteria, among which could be pathogens. 'Good indicator organisms' should fulfil the following criteria: universally present in large numbers in all human and animal feces, simple to detect, persistent in water, and their removal is related to the removal of actual pathogens. In current legislation, the indicator organisms used are coliforms, *E. coli*, fecal streptococci and *Clostridium perfringens* which should all be absent in 100 ml water samples. A multibarrier approach during drinking water treatment should ensure that appropriate desinfection is achieved. In fact, in several occasions viruses or protozoa caused an outbreak of disease even when no indicator organisms had been detected. This shows that the concept is not 100% reliable particularly with respect to other groups of organisms. Hence, other indicator organisms are being looked for or additional norms for specific pathogens rather than indicator organisms are being considered. Over the years, a number of organisms have been suggested as tentative alternative indicators for the coliforms. These include fecal streptococci, *Pseudomonas aeruginosa*, *Bacteroides* spp., *Candida albicans* (Gleeson and Gray, 1997).

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

4.3. Contact with gas phase

Both fungi, bacteria and viruses spread through water and air. Fungi, responsible for plant diseases, have for instance evolved specifically to be dispersed as air-borne particles (spores). Plant viruses tend to be dispersed by insect vectors and direct air-borne spread is unusual. Plant pathogenic bacteria, such as *Erwinia carotovora* can be transported by aerosol particles such as water droplets or other air-borne particles.
Another pathogen which spreads through aerosols is *Legionella*. This organism can proliferate in warm water distribution and air conditioning systems. Its optimal growth temperature is between 20 and 46°C. At higher temperatures, it only survives for a couple of minutes. Hence, compartment I will eliminate all *Legionella* cells.
(Opportunistic) pathogens which are resistant to desiccation, will survive in aerosols. Several mechanisms exist through which bacteria can protect themselves against adverse environmental conditions. These include the production of spores (e.g. *Bacillus, Clostridium*) and the presence of a characteristic thicker cell wall (e.g. *Mycobacterium*) or of slime sheaths (e.g. *Shigella dysenteriae*). Compared to slime sheaths, capsules are more tightly bound to the cells (e.g. *Streptococcus* and *Bacillus*) and due to their hydrophilic nature protect bacteria against desiccation by preventing water loss.
Finally, toxins may be spread through the air as well, e.g. in aerosols or associated with particles. The presence of toxins, dust mites, spores, etc. may induce allergies.

In general, manipulations with airlocks, filtration systems and waste removal systems of the MELiSSA loop present a risk to the crew due to potential contact with the gas phase and inhalation thereof.

Studies on MIR indicated that the gas phase in the station contained opportunistic pathogens such as *Staphylococcus aureus, Flavobacterium meningosepticum, Serratia marcescens*, etc. (Novikova, 2004). In MIR's operational lifetime bacterial contamination of the air remained stable and did not exceed the limit in 95% of the samples. Whether they were present under sufficiently high number to infect the astronauts as a result of inhalation is not clear. Also on earth, dose-response effects of bioaerosols are not really understood.

4.4. Contact with water and waste

Contact with water and waste which is contaminated with pathogens may present a risk to the crew provided that the pathogens exert their effect through inhalation of air or aerosols, or contact with the skin. In compartment I for instance, organic material will regularly have to be introduced and waste removal removed. Because this compartment is operated under a slight overpressure, opening of the airlock presents a theoretical risk of contamination to the crew. In addition, several filtration systems are available in the loop. Filter changes may present a risk because they are contaminated with e.g. the bacteria they retained.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Bauer *et al.* (2002) state that raw wastewater is a potential carrier of pathogenic micro-organisms and may pose a health risk when pathogenic micro-organisms become aerosolized during aeration. They measured the concentration of cultivable bacteria and fungi in the emitted aerosols at two different types of wastewater treatment plants. Up to 17 000 cfu/m³ of mesophilic bacteria were measured and 2100 cfu/m³ of bacteria associated with certain waterborne virulence factors. The concentration of mesophilic fungi remained below 2000 cfu/m³. Compared to this, the concentrations at a fixed bed treatment were 3 to 5 times lower for bacteria and even 10 times lower for fungi.

Furthermore, contact with micro-organisms may lead to allergies. This has e.g. been described for spores of moulds such as *Aspergillus* and *Penicillium* sp. but is not considered to present a real biosafety risk. Fungal diasporas from plants may induce infections of respiratory ducts and the gastrointestinal tract both through direct contact and through the air. *Candida* yeasts can infect the crew through contact (Tirranen, 2001).

4.5. Malfunctioning of MELiSSA compartments and hardware

4.5.1. Effects of MELiSSA loop contamination

On the one hand, operation of the different MELiSSA compartments will depend on the presence of the desired microbial population. Changes in microbial population may lead to malfunctioning of the compartments and reductions in efficiency. Even when not related to the presence of pathogenic organisms, this indirectly poses risks to air, food and water production for the crew. Sources of contamination may be

- inoculated micro-organisms from upstream compartments
- organisms or elements originating from the fecal and plant material entering compartment I not being retained by the filtration unit, such as viruses, nanobacteria, prions, etc.
- organisms present in the atmosphere and entering the system through manipulations of airlocks, replacement of filters, removal of waste.

Particularly compartment III is highly susceptible to contamination due to the low growth rates of the nitrifying organisms.

As phytopathogens will cause a reduction of the crop yield in the HPC, they cause in a sense a risk for the crew as well.

Roberts *et al*. (2004) confirm that functional or compositional changes in the microbial communities in bioreactors and life support systems can lead to reduced yield in food production systems, the failure of waste recycling systems, the outbreak of disease among the crew or changes in efficiency of the digestive system of the crew.
Temporal changes in community composition of closed systems depend on the availability of new species and their performance (selection) in the system. Since the introduction of new

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

species will be limited due to sterilization protocols prior to launch, mutation and recombination will be the main mechanisms producing new genotypes. Whether the latter can establish themselves in existing communities will depend on the fitness of the organism to the existing conditions. Both populations with very low mutation rates and hypermutable ones are typically present at very low proportions except when they have a selective advantage (e.g. pathogens).
Roberts *et al.* (2004) further state that simple communities developed in closed systems are inherently unstable. They are likely to fail in terms of system function and are very susceptible to invasion by organisms from other systems or organisms generated by mutation. Although controversy exists on this topic, community stability is generally higher at higher species diversity. The authors were able to prove this for rhizosphere communities of dwarf wheat in hydroponic solution. Further evidence was presented that community richness may decline over time leading to more predictable successional dynamics and community composition.

Under stress conditions, the risk of hypermutation exists. This is a (temporary) stage in which bacteria generate multiple mutations, which may be related to an increase in the rate of polymerase errors (which may or may not be triggered by template damage) and/or through abrogation of mechanisms such as proofreading and mismatch correction. In bacteria, there are numerous examples of transient mutator states, often occurring as a consequence of stress. They may be targeted to certain regions of the DNA, for example by transcription or by recombination. The initial errors are made by various DNA polymerases. They may be genuinely spontaneous polymerase errors or they may be triggered by damage to the template strand, for example as a result of stress.

4.5.2. Biocorrosion

Proper hardware functioning may be jeopardized by biocorrosion. All surfaces under natural and artificial conditions, except for extremely clean rooms, are covered with micro-organisms. Biofilms consist of micro-organisms embedded in an organic matrix of biopolymers (extracellular polysaccharides) which are produced by the micro-organisms under natural conditions. Under humid conditions or when immersed in an aqueous environment, all materials, including metals, inorganic minerals and organic polymers are susceptible to the formation of microbial biofilms. The micro- or biofouling is a forerunner for substantial corrosion and/or deterioration of the underlying material.
The organization and mechanisms of micro-organisms within a biofilm are material and organism specific. It depends on the surface properties and the ambient environmental conditions. Important factors affecting the rate of biodegradation include material composition (molecular weight, chemical composition,…), the indigenous microflora and environmental conditions. Environmental conditions determine the dominant groups within the biofilm: aerobic micro-organisms when oxygen is involved, anaerobic consortia in the absence of free or bound oxygen. Both types play an important role in the degradation of materials. However, sulfate reducing bacteria under anoxic conditions play a major role.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Beech and Sunner (2004) and Gu (2003) reviewed the current understanding of biocorrosion. Knowledge on the exact biodegradation processes of metal surfaces is still limited. The attack on metal occurs as a result of the activity of diverse microbial species. These processes include the consumption of oxygen, production of acids, sulfides and enzymes that promote the establishment of chemical gradients at the metal surface. Extracellular polymeric substances (EPS) seem to play an important role in the process of cell attachment to metal surfaces. The EPS consist of lipids, polysaccharides, proteins and nucleic acids. One of the important properties of EPS is their ability to complex with metal ions.
Polymers are as well potential substrates for heterotrophic micro-organisms, including bacteria and fungi. Generally, an increase in molecular weight (MW) results in a decline of polymer degradability. High MW results in a sharp decrease in solubility making them unfavorable for microbial attack. Nevertheless, even highly resistant polymers like polyethylene and polypropylene are subject to biocorrosion. Novikova (2004) notes that the majority of fungal species on structural materials in MIR are well-known potential degraders of polymers and can be expected to cause damage to structural materials, malfunctioning and failure.
Metallic surfaces are frequently coated with polymeric materials to provide a physical barrier to prevent direct contact between the metal and the external environment. Protection of polymers and specific coatings can be achieved to some extent through surface engineering and control of the physical, chemical and biological environments, so that the material surfaces can be as inert as possible. Applications of biocides has been widely used, but the development of resistant bacteria becomes a serious problem. As a control measure, lowering humidity is a very effective means to slow down the growth of micro-organisms on surfaces in enclosed environments.
Novikova (2004) concludes that bacteriofungal associations reside on materials of space interiors. In particular the technophylic fungi may cause biointerference with hardware functioning, degradation of various materials or provoke corrosion of metals.

Gu *et al.* (1998) investigated the formation of microbial biofilms on surfaces of a wide range of materials being considered as candidates for use on the International Space Station. The materials included a fibre-reinforced polymeric composite, an adhesive sealant, a polyimide insulation foam, teflon cable insulation, titanium and an aliphatic polyurethane coating. They were exposed to a natural mixed population of bacteria under controlled conditions of temperature and relative humidity. Biofilms formed on the surfaces of the materials in a wide range of temperatures and humidity. The biofilm population was dominated by *Pseudomonas aeruginosa*, an opportunistic pathogen, *Ochrobactrum anthrop*, *Alcaligenes denitrificans*, *Xanthomonas maltophila* and *Vibrio harveyi*. However, a significant decline in bacterial numbers on material surfaces was observed when the relative humidity was lowered to 45%. It appeared to be a more effective measure than lowering the temperature from 30 to 22°C.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

5. Relevance of study items in melissa loop

Based on the above inventory a broad range of micro-organisms can enter the MELiSSA loop through the input of feces and non-edible plant residues. Attention should be paid to those elements and organisms that are likely to pass the different compartments. When accumulation within the loop and infection of the HPC occurs, a serious danger of infection of the crew can occur. Appropriate measures concerning risk reduction should be taken.

5.1. MELiSSA compartments

The MELiSSA loop (Figure 2) consists of several compartments, all having a dedicated function. The different process conditions and the intermediate separation techniques are expected to eliminate the major part of the contaminants entering the loop. In this paragraph, we will try to evaluate the survival of the different micro-organisms and -elements entering the circuit.

Compartment CI is the 'liquefying waste compartment'. It is an anaerobically operated bioreactor connected with a filtration unit. Table 10 lists some process conditions (TN 71.9.4).

Table 10. Operating conditions of the bioreactor

Process parameter	Value	unit
pH	5 - 5,5	
Temperature	55	°C
Hydraulic Retention Time	10	days
Type membranes	ceramic	
Cut-off	50	nm

Operation at 55°C was chosen to achieve a pasteurization effect. The membrane filtration system was selected to at least retain bacteria and produce a bacteria-free effluent.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

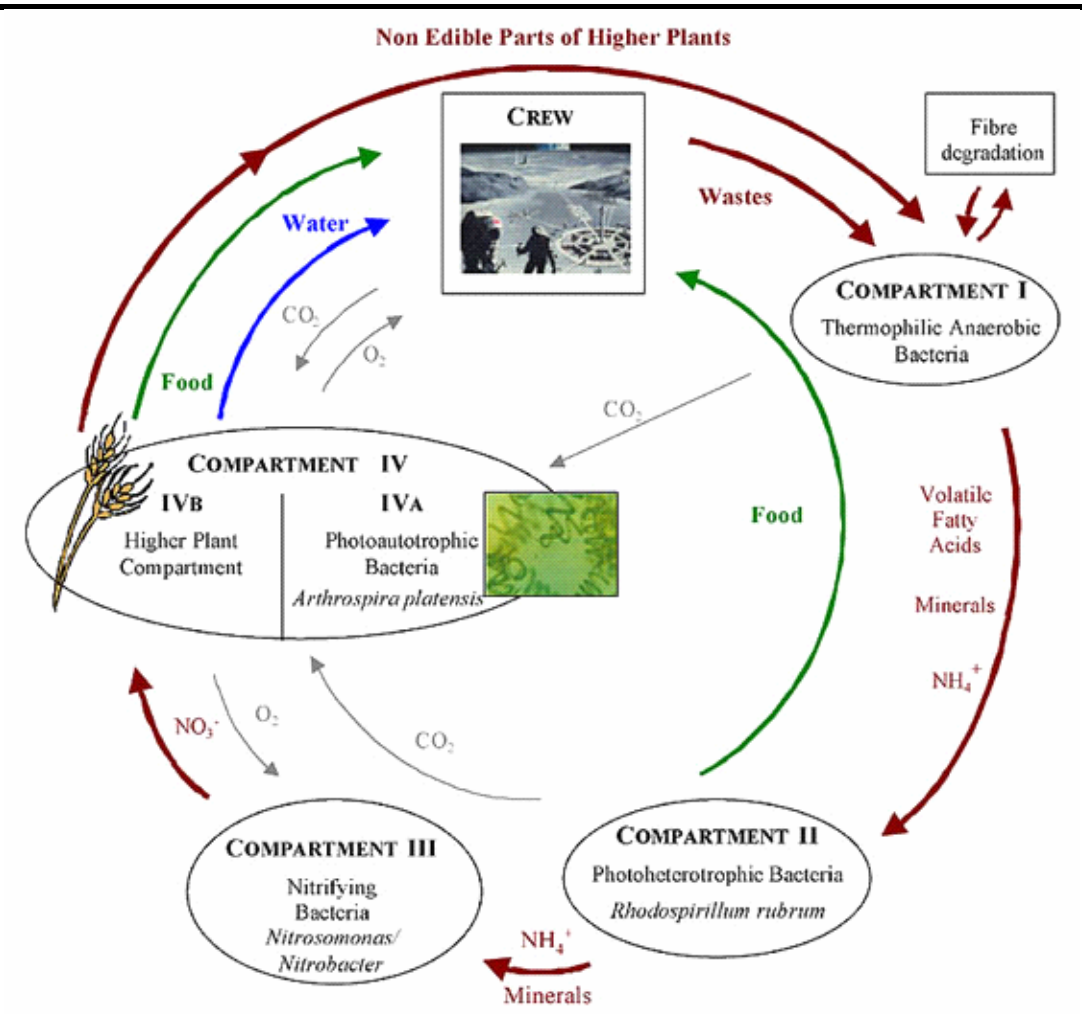

Figure 2. MELiSSA loop

Compartment CII is a photoheterotrophic reactor, responsible for the elimination of the Volatile Fatty Acids (VFA) coming from CI. The bacteria *Rhodospirillum rubrum* is kept in pure culture. The excess biomass is harvested and can be used as nutrition for the crew, however at low concentrations.

Within the nitrifying compartment CIII, the ammonia is converted into nitrate, being the most favorable source of nitrogen for the higher plants and *Arthrospira platensis*. A mixed culture of *Nitrosomonas* and *Nitrobacter* is maintained within a fixed bed reactor. The organisms are very slow growing and the carrier material to which they are attached cannot be sterilized by heat.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

The fourth compartment is split into CIVa and CIVb. The first one is the photoautotrophic cyanobacteria (algae) part colonized by *Arthrospira platensis*. The second is the higher plant compartment where several crops are cultivated. The harvest of both compartments is used as food for the crew.

5.2. Behavior of organisms/elements in the MELiSSA loop

As described within the previous chapter a broad range of organisms can enter the MELiSSA loop through the feed. This paragraph highlights the different elements and organisms, gives general information on the life cycle and tries to predict the survival rate within the loop. Because elimination and behavior is expected to be similar for different micro-organisms of one class, the discussion will be performed per class of organisms, rather than for each of them individually.

Prioritization of risks performed in the Biosafety consulting contract which kicked off in March 2005, indicated that microbiological risks leading to acute toxic risks are the most important to consider. This was explained by the fact that potentially pathogenic microbes present a risk to the crew in case of contamination and that microbiological developments may disorganize the MELiSSA loop functioning. Infection risks to the crew were listed as:

- Acute respiratory infections
- Injuries and traumas leading to infections
- Opportunistic infections from commensal flora due to a reduction of bacterial species in a confined environment.

Chemical and genetic elements were considered to be less critical.

5.2.1. Viruses

The overview in chapter 3 clearly stated that viruses can occur in the feed. Feces, as well as plant residues could contain viruses. These organisms differ from others in the sense that they only contain one type of nucleic acid: DNA or RNA, covered by a protein coat. This nucleocapsid can either be naked, or enclosed by a membrane (lipid envelope).
Furthermore, viruses are unable to reproduce outside living cells. The necessary reproduction within the host usually leads to the death of the host cell.

Table 11 summarizes the viruses and provides additional information on the type of nucleic acid, structure of the organism, presence of an envelope and size.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 11. Viruses occurring in the MELiSSA feed

Virus	DNA/RNA	Enveloped	Structure	Average size
Feces				
Enteroviruses	RNA	no env.	eicosahedral	30 nm
Hepatoviruses	RNA	no env.	eicosahedral	30 nm
Astroviruses	RNA	no env.	eicosahedral	30 nm
Calicivirus	RNA	no env.	eicosahedral	40 nm
Rotavirus	RNA	no env.	eicosahedral	70 nm
Adenovirus	DNA	no env.	eicosahedral	80 nm
Phytopathogens				
Curtovirus	DNA	no env.	twin eicosahedral	20 nm
Oryzavirus	RNA	no env.	eicosahedral	70 nm
Cytorhabdovirus	RNA	enveloped	bacilloform	200 nm length 50 nm wide
Tospovirus	RNA	enveloped	sphere	80 nm
Potyvirus	RNA	no env.	filamenteous	800 nm length 20 nm wide
Waikavirus	RNA	no env.	eicosahedral	30 nm
Closterovirus	RNA	no env.	flexuous filaments	10 -20 nm wide
SbCMV virus	DNA	no env.	bacilloform or eicosahedral	50 nm

As can be seen from Table 11 several types of viruses occur. Most of them have RNA as nucleic acid and are non-enveloped. Non-enveloped viruses are known to survive longer in the inanimate environment (Hart and Shears, 2004). Loss of the lipid envelope is associated with a loss of infectivity. They can be inactivated by ether or detergents.

Huyard *et al.* (2000) measured a 4 log reduction of *Enterovirus* (RNA - non-enveloped) after a thermophilic acidenogenic reactor at 55°C. The hydraulic retention time was 2 days. Although a substantial reduction in virus counts may be observed at 55°C, the cut-off of the CI filtration unit (50 nm), is insufficient to guarantee complete virus elimination in the permeate. However, it is interesting to note that elimination of particles smaller than the membrane cut-off may be higher than expected due to the presence of a cake layer on the membranes which may decrease the actual pore diameter. In any case, this cake layer is only built up after some period of operation and its effect will not play during initial start up.

Thus, despite the elevated temperature and the post filtration unit it can be expected that viruses will pass through to CII. The mild conditions within the compartments CII till CIV give no guarantee on further elimination of the viruses. As a result, a certain risk of (re)-

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

infection of the food producing compartment CIV with (phyto)pathogenic viruses exists. Hence, consumption of the crops and algae could lead to illness.

As mentioned in the inventory, bacteriophages can be present in almost every species of bacteria and play an important role in the transfer of pathogenic determinants (Dobrindt and Hacker, 2001). In terms of elimination, they presumably behave in a similar way as the other viruses or are eliminated with their bacterial host.

5.2.2. Bacteria

Within the waste compartment CI an important reduction of several bacteria can be expected. The elevated temperature (55°C) will cause a high elimination rate of the mesophilic intestinal bacteria whose optimal growth temperature is around 37°C. As the size of bacteria lies within the range of 0,5 to 5 µm, the filtration unit will in addition remove the possible remaining bacteria. The same can be expected for the removal of bacteria linked to plants: phytopathogens, rhizospere linked bacteria, … since they are not adapted to elevated temperatures. Indeed, the higher plant compartment is typically operated at 20 to 22°C.

In case some bacteria appear in the permeate or some bacteria are released in the downstream compartments, few barriers are present to avoid their transfer to the food producing compartments. The University of Guelph has for instance strong indications that nitrifiers occur and grow in the hydroponic solution of compartment IVb. Upon MELiSSA loop closure, we must take into account that some nitrifying organisms will be sloughed off of the biofilm in compartment III and will be transported in solution to compartment IVb where they can grow on the ammonium in the hydroponic solution. Similarly, other bacteria which have entered the system after compartment I or which passed through the membrane filtration unit, will transfer through the loop and may adhere to the crops or edible biomass. In addition, when biofilms occur in the loop, these may provide an excellent environment for opportunistic pathogens to establish and maintain themselves. Since biofilms are very difficult to remove, their formation should be avoided in the first place.

Bacteria present an extra risk due to possible production of toxins and the formation of spores under certain conditions.

There are two general kinds of *bacterial toxins*. Exotoxins are produced during the growth phase of certain kinds of bacteria and are liberated into the medium or tissue. Exotoxins are protein-like and their reactions are specific. For example, *Clostridium botulinum* produces an exotoxin of unusual potency which affects only neurological tissue. Other well-known examples of exotoxins are tetanus toxin, Shiga toxin, and diphtheria toxin.
The term endotoxin is usually interchangeable with the term pyrogen. The effects produced by endotoxins on the host are systemic such as fever and general body reactions, rather than strictly neurological effects, as is the case with most exotoxins. Endotoxins are found in the gram-negative bacteria mostly, and are obtained subsequent to the death and autolysis of the

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

cells. The endotoxins are extracted from and associated with the cell structure (cell wall). The best-studied endotoxin is lipopolysaccharide (LPS), a major compound of the outer membranes of Gram-negative bacteria. Endotoxines are thus ubiquitous pathogenic molecules. Good examples of pyrogen producing bacteria are *S. typhosa*, *E. coli*, and *Ps. aeruginosa*.

Some bacteria can form spores. These are rod-shaped, gram-positive bacteria and can be found within the aerobic genus *Bacillus* and the anaerobic genus *Clostridium.* Sporulation is a complex process. The cytoplasmic membrane divides off a portion of the cell protoplast, containing part of the nuclear material. The spore protoplast is gradually enveloped by the cytoplasmic membrane of the mother cell. Both membranes take part in the synthesis of the spore wall. The spores are liberated upon autolysis of the vegetative cell. Spores are not an obligate part of the life cycle of bacteria. Spores are most often formed under unfavorable environmental conditions, e.g. when nutrients are exhausted or when unfavorable metabolic products accumulate. Due to low water content and the impermeability of the spore envelope to many chemicals, they are highly resistant to extreme environmental conditions such as heat. Some spores are resistant to boiling water for several hours.
Spore-forming genera and spores themselves can be present in fecal material and may enter the MELiSSA loop. The conditions in the first compartment may further induce spore formation and due to their high resistance, the spores may persist.

Both toxins and spores can be smaller than bacterial cells and will not be retained by the ultrafiltration membrane of compartment I. In addition, the conditions in the first compartment are probably not sufficiently harsh to eliminate them, but may for instance initiate spore germination and return to the vegetative form of the cells. Hence, it is assumed that spores and toxins may present a risk in the loop.

Except for the microbial community in compartment I, none of the MELiSSA organisms is associated with pathogenicity for humans.

5.2.3. Fungi

Fungi generally grow best at acidic pH. However because they are most often strictly aerobic, they will most probably not survive the anaerobic conditions and elevated temperatures in CI. Likewise, moulds associated with plants will not survive the thermophilic conditions in compartment I.

As for bacteria, the problem of spores and mycotoxins requires special attention. It can be anticipated that these elements will hardly be eliminated in the loop and may accumulate.

5.2.4. Protozoa and parasites

Protozoa and parasites are higher multicellular organisms and have larger dimensions than bacterial cells. Furthermore, they will typically have mesophilic properties when related to

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

fecal or plant material. Hence, the thermophilic, acidic conditions in compartment I are expected to be harmful to them. Combined with the fact that retention by the ultrafiltration membrane will be even higher than for bacteria, the risk of their appearance downstream compartment I seems to be negligible.

Protozoa and parasites may spread through eggs and cysts. These typically have dimensions of between 10 and 50 µm and will also be retained by an ultrafiltration unit with pore diameters of 50 nm.

5.2.5. Plasmids, bacteriophages, non-conventional transmissible agents

5.2.5.1. Plasmids

In the case of gene transfer via conjugation, the following categories of genes that are associated to mobile genetic elements require special attention:

- genes involved in human pathogenesis (new virulence can be provided to the bacterial hosts)
- genes for resistance to antibiotics and drugs (dissemination of the latter genes in confined environments could affect the therapeutic possibilities in the case of pathogenic outbreaks),
- genes associated with resistance against disinfectants,
- genes associated with biofilm formation or associated EPS production (see also 4.5), which may lead to biocorrosion.

The potential presence of these genes on plasmids implies that chances of horizontal transfer to other bacteria in the microbial community are high and that genetic information which may jeopardize crew safety can spread quickly to a wide range of recipient bacteria, including the MELiSSA organisms.

Uptake of free RNA or proteins by bacteria has not yet been described in literature. Hence, the spread of information through this mechanism can be eliminated. Uptake of foreign DNA from dead cells by living bacterial cells does occur and is called transformation. This process may become important when mesophilic intestinal bacteria are killed under the conditions in compartment I and their DNA is released. These DNA pieces are fairly stable and since they will not be retained by the membrane filtration unit, they can spread throughout the MELiSSA loop.

Interbacterial DNA exchanges are facilitated by a number of shuttle vectors, including plasmids and transposons. These vectors will resist the conditions in compartment I (Pechère, 2005) and may assist in the spread of pathogenic capabilities related to the uptake of foreign DNA.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

5.2.5.2. Bacteriophages

Phages play an important role in the transfer of pathogenic determinants through transduction (Dobrindt and Hacker, 2001). Recent work in the past few years has shown that cholera toxins, shiga toxins, diphteria toxins, and the botulinum toxins all reside on phages, and the recent completion of the genome sequence of two pathogenic *E. coli O157* strains revealed the presence of > 20 prophages in their chromosome (Ohnishi *et al*., 2001), encoding a variety of virulence-related proteins such as Shiga toxins (Stx), zinc/copper-type superoxide dismutases (SOD), and Bor proteins and many Lom homologs (implicated in host serum resistance and cell adhesion, respectively).

5.2.5.3. Prions

Prions are best known for their responsibility for some fatal human diseases, including Creutzfeld Jacob disease, kuru, Gerstmann-Straussler-Scheinker disease, fatal familial insomnia, and possibly Alpers disease. Although they are structurally proteins, prions are extremely resistant towards classical inactivation and sterilization methods. They are heat resistant, requiring exposure to 134°C for 18 minutes for inactivation. They are highly resistant to common sterilants and other chemical agents and to extremes of pH and to ultra-violet or ionizing irradiation (Doblhoff-Dier and Collins, 2001). Prions will easily survive compartment I conditions and are in principle not retained by the filtration unit. If prions are present in the feed to compartment I, they will most probably colonize the whole MELiSSA loop. Indeed, due to the fact that they are clearly more resistant than other proteins, their inactivation or degradation by protease enzymes e.g. from lysed cells is probably highly unlikely.
Pechère (2005) states that prions are common in nature but that the detrimental or beneficial consequences of their ubiquity are unknown. However, no human intestinal prions have been associated with human diseases. They should therefore be placed very low on the hazard list.

5.2.5.4. Nanobacteria

Although some controversy exists, nanobacteria are considered a new form of life rather than bacteria. They have a cell wall but have much smaller dimensions than regular bacteria. They are thought to appear on space travel and have been said to be an infectious risk to astronauts. For more information we refer to TN80.11. As mentioned in this TN, nanobacteria and other unusual organisms will potentially exist in the MELiSSA loop, particularly because of the unknown composition of compartment I.

5.2.5.5. Free RNA/DNA

Free RNA is known to have a very short lifetime. It will most probably be degraded in compartment I and will not circulate in the loop. Free viral RNA for instance will easily be degraded, due to presence of bacterial endonucleases.
Free DNA is assumed to be more stable and DNA fragments can be expected to colonize the whole loop. However, the presence of endonucleases originating from lysed cells (particularly

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

in compartment I) would dramatically lower the concentration of free 'naked' DNA in the medium or culture. Hence, the chances for uptake of free DNA in bacterial cells is expected to be improbable.

5.2.5.6. Behavior in MELiSSA loop

In summary, all the elements overviewed may be associated with the plant and fecal material entering the MELiSSA loop. They are substantially smaller than the membrane filtration cut-off in compartment I. Their elimination at elevated temperatures and under anaerobic conditions (as in compartment I) and under the conditions of the downstream compartments is unknown. As a consequence, they will populate the whole MELiSSA loop. Although their relationship to human disease is most often limited, the risk of their transmission through the loop requires further investigation.

6. Design requirements

For all the different study items, described in the previous chapter, attention is paid to state-of-the-art analysis, sampling methods, requirements for sample handling, … These boundary conditions should lead to specifications and requirements for the MELiSSA-loop design.

6.1. Introduction

The methods for microbial examination of water, as described in the Standard Methods (1997), are mainly focused on detection and enumeration of indicator organisms, rather than of specific pathogens. The coliform group density has been accepted as a major criterion of sanitary water quality. Fecal streptococci and enterococci are also indicator organisms for fecal polution. The 'indicator system' misses or fails to indicate disease-causing viruses and bacteria, such as Hepatitis A virus, Adenoviruses, *Legionella* or protozoa.
For decades, the main method for microbiological determination has been based on culture methods and microscopic research. During the last five to ten years however, a change towards molecular techniques, to determine the specific pathogens, is noticed.

Based on the inventory in previous paragraphs a more detailed determination of specific pathogens will be required compared to the general detection of indicator organisms. Molecular techniques as PCR and FISH will be needed when biosafe investigation is intended. Techniques will be discussed in greater detail, including references, in the following paragraphs. As these techniques are relatively new and in continuous development, definite standardizing remains to be done. Most of the techniques were originally developed and successfully applied for detecting pathogens in clinical samples. However, their application to environmental microbiology is still in its infancy.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Köster *et al.* (2003) state that the traditional cultivation techniques are usually sensitive but the identification is often not as reliable as might be desired. Methods based on molecular biology tend to be sensitive and yield reliable identification.

After some general guidelines and procedures, more specific analysis methods for bacteria, viruses, prions and toxins are described separately.

6.2. Analytical methods

There is no single method to collect, process and analyse a water sample for all pathogenic micro-organisms of interest. From a literature overview it became evident that detecting pathogens in water is often problematic because (1) pathogens are very dilute, (2) established protocols for sample collection, concentration and identification are not sensitive enough to detect dilute agents, and (3) the different methods are not comparable to each other. Some of the difficulties include the physical differences between the major pathogen groups such as viruses, bacteria and protozoa, and the efficient concentration of large water volume samples for the detection of low target concentrations of certain pathogens (Straub and Chandler, 2003). In general it can be stated that most microbiological procedures consist of concentration/enrichment, detection and quantification.

6.2.1. Sampling

Section 9060 of the Standard Methods (1997) describes the collection, preservation and storage of microbiological samples for culture methods.
Sampling should be done in nonreactive borosilicate glass or plastic bottles that have been cleansed and rinsed carefully. In order to avoid sample contamination, aseptic techniques should be used. Sample ports should be flushed to ensure representative content. The sample volume should at least be 100ml.
The microbial analysis of water samples should be started as soon as possible after collection to avoid unpredictable changes in the population. For the analysis of coliform bacteria e.g. the holding time should not exceed 30h. Samples should be stored below 10°C pending analysis.
The above mentioned volume of 100ml applies to classic bacterial culture methods. Detection of viruses implies a pre-concentration phase, requiring more sample. This is described more in detail in the paragraph on analysis of viruses.

6.2.2. Microscopy

The smallest object visible by light microscopes is 200-300nm in diameter. Living, unstained micro-organisms can be visualized using either dark-field of phase-contrast microscopy.
As the wavelength of electrons is much shorter than that of light the conventional electron microscope can resolve objects as close together as 0,5nm.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

6.2.3. Flow cytometry

Flow cytometry is a technology in which a variety of measurements can be made on particles, cells, bacteria and other objects suspended in a liquid. In a flow cytometer, particles are made to flow one at a time through a light beam (laser beam) in a sensing region of a flow chamber. They are characterised by light scattering based on their size, shape and density and also on the dyes that are used either independently or bound to specific antibodies or oligonucleotides that endow a fluorescent phenotype onto components of interest. As a particle flows through the beam, both light scattered by the particle and fluorescence light from the labelled particle is collected either by a photomultiplier or photodiode in combination with light splitters and filters. This makes it possible to make multiple simultaneous measurements on a particle.
A particularly valuable aspect of flow cytometry is its capability of rapid analysis: the assay itself can be completed within three to five minutes. This is likely to be one of the key devices for the routine multiple monitoring of microbes of interest (including a variety of indicator or pathogenic microbes and even viable but non-culturable bacteria).

6.2.4. Biochemical and immunological assays

The highly specific binding of antibody to antigen plus the simplicity and versatility of this reaction, has facilitated the design of a variety of antibody assays and formats, which comprise the largest group of rapid methods being used in food and water testing (Bernasconi *et al*. 2004).
Depending on the taxonomic level of the targeted antigens, immunological methods permit antigen detection at family, genus, species and/or serotype levels.

The possibility to conjugate antibodies to fluorescent molecules is used in immunofluoresence, which allows the visualisation of bacteria at the single-cell level under non-destructive conditions and independently of the cell growth rate.
The Enzyme-Linked ImmunoSorbent Assay (ELISA) is the most prevalent antibody assay format used for pathogen detection in food. It is usually designed as a 'sandwich' assay in which an antibody bound to a solid matrix is used to capture the antigen from enrichmen cultures. A second antibody conjugated to a enzyme is used for detection.

6.2.5. Nucleic acids based technology

Although there are many DNA-based assay formats, only probes based assays, like Polymerase Chain Reaction (PCR), are suitable developed for waterborne pathogens detection. Nucleic acid probes are single strands of DNA or RNA, which bind to their complementary sequence when present within a mixture of different nucleic acid molecules. Limitation of the technique is the initial low concentration of pathogens in water samples. Gene probes rely on specific stringency requirements and are also limited by low sensibility (10^3-10^4 CFU).
Fluorescent *in situ* hybridization (FISH) is based on the hybridization of fixed cells with fluorescently labeled oligonucleotide probes. They hybridize specifically to their complementary target sequence within the intact cell. Different fluorescent labels can be used

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

enabling multicolor reactions. A typical probe is between 15 and 30 base pair in length and is generated on an automated synthetizer.
Probe assays generally target ribosomal RNA, (5S rRNA, 16S rRNA and 23sRNA) taking advantage of the fact that the higher copy number of bacterial rRNA (ribosomes vary between 10^3 and 10^5 per bacteria) provides a naturally amplified target. Still, only weak fluorescent hybridization signals can often be expected when no artificial amplification is used. Genes for instance cannot be detected by *in situ* hybridisation unless some *in situ* PCR step is used, as some 10 000 labeled molecules are typically required for 'visualisation'.

PCR is a method for in vitro amplification of specific regions of DNA through repeated cycles of duplication driven by the enzyme DNA-polymerase. The amplified nucleic acid could then be further analyzed by e.g. DNA sequencing.
Unlike the culture method, detection by standard PCR does not provide information related to the viability of the bacteria. To overcome this limitation, an indirect approach has been developed: analysis of the samples before and after culture on non-selective media. This will reduce the amount of water sample but increases the assay time significantly. However, this method will only monitor viable and cultivable bacteria.

Another technique that offers potential for assessing viability by means of detecting mRNA is reverse transcriptase PCR (RT-PCR). It should be taken into account that in contrast to DNA, mRNA is very labile with a half-life of only a few minutes. RT-PCR is a two-stage process, in which a target mRNA sequence is first transcribed into a complementary DNA (cDNA) sequence, either using random hexanucleotide primer or sequence-specific primers. The cDNA sequence may then be used to generate a second-strand cDNA, or serve directly as a template for a PCR, resulting in an exponential amplification and subsequent detection of the original complementary target.

Real-time PCR combines amplification of target DNA with detection of amplicons in the same reaction vessel. This technique eliminates the need for further determination by e.g. DGGE. One approach for real-time monitoring of amplicon production is to use fluorescent DNA intercalant dyes, such as SYBR-Green I, which bind non-specifically to double-stranded DNA generated during amplification. Multiplex PCR enables the simultaneous detection of several target sequences by incorporation of multiple sets of primers.
Table 12 provides some commercially available real-time amplification platforms.

PCR is very flexible and allows highly specific detection of particular (sub)species, certain groups of microorganisms or can be used to study aspects of biodiversity in water samples. Since the nucleic acid region that is flanked by the primers does not have to be known completely , uncultured pathogenic microorganisms might be discovered in broad-range PCR approaches.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 12. Real-time amplification platforms (Vernet, 2004)

Manufacturer	Platform
Applied	ABI PRISM 7700
Biosciences	ABI PRISM 7900
Roch diagnostics	LightCycler
	COBAS TaqMan48
bioMerieux	EasyQ
Stratagene	Mx3000/Mx4000
Bio-Rad	iCycler iQ
Cepheid	Smart Cycler II
Cepheid/Biomérieux	GeneXpert

PCR techniques also have limitations. Although PCR is very sensitive, samples have, in most cases, to be concentrated. For PCR assay on a water sample containing low concentrations of pathogens typically a sample of 1 to 3 liters is filtered using a 0,2 µm Millipore membrane. More and more, centrifugation is used for concentration. When DNA is concentrated, several PCR assays can be executed as only 0,5 to 1 µl is used per PCR detection.
The PCR method may generate false positive results, especially when carried out without a pre-cultivation step of the original water sample. False positive results can for instance be generated by free DNA fragments that survive in the environment for long periods.

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) have been introduced in the mid-nineties in environmental microbiology and are now routinely used for genetic fingerprinting.
The two techniques essentially consist of the amplification of the genes encoding the 16S rRNA from the matrix containing different bacterial populations, followed by the separation of the DNA fragments. Separation is based on the decreased electrophoretic mobility of PCR amplified, partially melted, double stranded DNA molecules in polyacrylamide gels, containing a linear gradient of DNA denaturants (DGGE) or a linear temperature gradient (TGGE). Molecules with different sequences may have different sequences may have different melting behavior and will stop migrating at different positions along the gel.

Microarrays and biosensors are the emerging technologies. Biosensors in the medical area have largely been based on antibody technology, with an antigen triggering a transducer or linking to an enzyme amplification system. DNA microarray has attracted tremendous interests among biologists. This technology promises e.g. to monitor the whole genome on a single chip so that researchers can have a better picture of the interactions among thousands of genes simultaneously. An array is an orderly arrangement of samples. It provides a medium for matching known and unknown DNA samples based on base-pairing rules. The sample spots in

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

microarray are typically less than 200 microns in diameter and these arrays usually contains thousands of spots. Microarrays require specialized robotics and imaging equipment. There are two variants of the DNA microarray technology, in terms of the property of arrayed DNA sequence with known identity:

- ✓ probe cDNA (500~5000 bases long) is immobilized to a solid surface such as glass using robot spotting and exposed to a set of targets either separately or in mixture;
- ✓ an array of oligonucleotides or peptide nucleic acid probes is synthetised either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilization. The array is exposed to labeled sample DNA, hybridized, and the identity/abundance of complementary sequences are determined.

The microarray under development by bioMérieux (using Affymetrix Inc. GeneChip technology) for an international water company is expected to reduce test time to four hours. The cost will decrease as well drastically. The high resolution DNA chip technology is expected to target a range of key microorganisms in water.

6.3. Limitations of microbiological methods

Table 13 reviews briefly several classical and molecular techniques indicating their characteristics and limitations.

Table 13. Overview microbiological detection methods (based on Köster *et al.*, 2002)

Method	Characteristics	Limitations
Cultivation of bacteria	- inexpensive - easy to perform - qualitative and quantitative - detection of low concentrations	- time consuming - not all bacteria cultivable - attention should be paid to biosafety issues
Flow cytometry	- faster than cultivation - detection of non-culturable organisms	- expensive - limited reliability for organisms at low concentrations
FISH	- faster than cultivation - detection of non-culturable organisms - can detect individual cells when ribosomal RNA is target	- lack of sensitivity with chromosomal genes or mRNA as target - detection strictly taxonomic - differentiation between living and dead organisms difficult

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 13 (continued)

Immunological detection	- qualitative and quantitative - relatively specific for target organism	- often needs time-consuming pre-cultivation step - lack of sensitivity - selectivity can be a problem due to cross-reacting antibodies
PCR	- in general highly sensitive - specific - can detect non-culturable micro-organisms - fast	- sufficient nucleic acids required - negatively affected by certain environmental conditions - no information on viable and non-viable organisms
RT-PCR	- as PCR - good indicator for living organisms	- RNA is instable molecule
Microarray	- testing of up to several thousand sequences on single chip - fast - sensitive, selective and specific	- at present very cost intensive - highly trained personal needed - absolute quantitative determination

The often low number of target organisms in microbiological analyses increase measurement uncertainty. It is nearly impossible to determine the true number of viable target organisms that are present in a sample. Therefore, absolute recovery cannot be defined and for a new method only a relative recovery can be given by relating it to that obtained with other methods. Microbiological methods are not robust in the sense that chemical methods are. The target and many contaminants in the sample are living entities and therefore unexpected effects and phenomena can occur. Robustness is affected by many different factors, including the physical, chemical and microbiological properties of the sample itself.

6.4. Bacteria

The Standard Methods (1997) provide well-known culture methods for the determination of groups of bacteria or specific species. Section 9215 describes e.g. the heterotrophic plate count, formerly known as the standard plate count. It is a procedure for estimating the number of living heterotrophic bacteria.
Selective agars are prepared by adding certain selective agents that stimulate or reduce growth of specific bacteria species. Beside the procedures for the detection of indicator organisms, media descriptions are given for *Salmonella*, *Shigella*, pathogenic *Escherichia*, …

Fast test kits are available for the simple presence/absence monitoring of indicator organisms. A 100ml water sample is added to a bottle, containing defined substrate. If total coliforms are

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

present, the organisms cleave a chromogenic analog of lactose that turns the media from clear to yellow. Methylumbelliferyl galactoside (MUG) can also be present in the media and is specifically cleaved by *E. coli.* If MUG is cleaved, the media appears fluorescent blue under long-wave UV light. Again, these types of tests are often insufficient as other pathogens can be present in the absence of the indicator organisms.

Furthermore PCR technologies, in real-time or combined with genetic fingerprinting, as described in the previous paragraph are introduced rapidly nowadays for molecular determination of bacteria.

Currently, there are at least 30 assays each for *E. coli* and for *Salmonella* detection. Such a large number of options can be confusing and overwhelming to the user, but more important has limited the effective evaluation of these methods (Bernasconi *et al.,* 2004). Almost all the so-called rapid methods are designed to detect a single target, which makes them ideal for use in quality control programs to quickly screen large numbers of food/water samples for the presence of a particular pathogen or toxin. A positive result however, is only regarded as presumptive and must be confirmed by standard methods.

6.5. Viruses

Viruses are often the most dilute pathogens in water. Volumes in excess of 100 liters for surface water and up to 1000 liters for drinking water are frequently required in order to be reasonably confident in an assay.
The currently accepted sample collection method is filtering water through a positively charged filter on which the viruses are trapped. The nominal pore size is 0,2 µm. The principle involved is that viruses carry a particular electrostatic charge that is predominantly negative at or near neutral pH levels. This charge can be modified to predominantly positive by reducing the pH level to about 3,5.
Gantzer *et al.* (1997) proved that, in order to prevent inhibition of PCR amplification of target RNA, concentration by adsorption onto glass powder or glass wool supports is suitable for detecting viral genomes via PCR afterwards.

The standard method for the detection of viruses in water samples relies on the higher mentioned concentration of the viruses from large water volumes, followed by inoculation of specific cell culture. However, many viruses of concern (Rotavirus, Calicivirus, Norwalk virus) can not be propagated on cell cultures.

The ASTM Standard test method (2001) for low levels of coliphages in water is simple, inexpensive and fast. However, it only tests one specific type of virus. Coliphage organisms serve as an indicator of fecal contamination.
This test method can determine coliphages in water down to 1 coliphage per volume sampled. The test is based on the principle of coliphages infected *E. coli* growth on agar. Only infected bacteria form plaques. For this test, typically a volume of 1 liter is used.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Use of Electron Microscopy (EM) has allowed the identification of viruses on the basis of morphology. The limiting factors are the inadequate preparation techniques, the highly trained personnel required and the high costs. To be detected in suspensions, viruses must be present in large numbers (10^5-10^6 per ml).

A first type of rapid methods in virology is represented by the development of immunoassays using antibodies labeled with fluorescein, radioisotopes or enzymes (e.g. ELISA as described above).
A second type of rapid assays is again related to the introduction of nucleic acid methods, allowing for the first time the detection of unculturable, fastidious and slow-growing viruses. Molecular information on the nature of their genomes has only recently become available.
The PCR technology has been adapted to virus detection by using reverse transcription of RNA into DNA (RT-PCR). The formed DNA is further amplified and determined as compared to a classical PCR.
It should be mentioned that RT-PCR does not provide any information about the infectious nature of the virus isolated. Secondly, an important bottleneck remains the need of concentrate large volumes (10 to 400 liters) of water samples to a final volume milliliters.

6.6. Fungi, protozoa, parasites

Sampling for fungi analysis should be stored refrigerated for 24 h at maximum according to Standard Methods (1997). Culture-based methods are based on plating dilutions on specific growth media. In case low numbers are expected, appropriate water volumes need to be filtered over 0.45 or 0.8 µm and the filter transferred to dishes.
The ASTM standard test method (1998) for the enumeration of *Candida albicans* in water states that sample volumes will vary depending on the water sampled: 10 to 40 ml may be appropriate for raw sewage while up to 1 liter or more of relatively clean and clear recreational water should be examined.

For protozoa, the methods for recovery and detection require large sample volumes. In the USEPA method, 10 l of (surface) water is minimally passed through a filter to capture the parasites. The filters are then eluted and the protozoa separated from the matrix using immunomagnetic techniques (Straub and Chandler, 2003).
Currently available methods for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in water are at best tentative and the recovery is low and variable (Bernasconi *et al*., 2004). The procedure consists of the following stages: sample collection and concentration to recover the low number of cysts usually found and separation of cysts from debris through density gradients or immunomagnetic separation. Detection occurs through microscopy, enzyme-linked immunoassays, fluorescent-labelling with monoclonal antibodies, etc. Major drawbacks of conventional detection methods include lack of sensitivity and specificity. Some detection problems have been overcome using flow cytometry. Molecular methods based on PCR have been shown to increase detection sensitivity at least 1000-fold compared to

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

immunofluorescence microscopy. This assay can be used to determine genus, species and genotype of the parasite. The major disadvantage of molecular methods is the inhibitory effect of polysaccharides and humic material in water samples. Deere *et al*. (1998) have pioneered a FISH assay to label protozoa specifically.

Sampling requirements and the need for preconcentration of the samples is similar for the analysis and detection of nematodes.

6.7. Toxins

In food samples, organic solvent are used for extraction of mycotoxins, produced by certain fungi. Determination of the toxins can by done by chromatographic means like High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC). Aflatoxins are relatively easily detectable as they are fluorescent. Alternatively mycotoxin can be detected in a similar way as pathogens, using immunological response techniques like ELISA.
An endotoxin-induced coagulation cascade of the limulus amoebocyte lysate (LAL) has been used for more than 25 years. When micro-organisms invade an animal, the immune system responds by initiating a highly specific enzyme cascade in its blood cells. This cascade is initiated by the presence of lipopolysaccharides (LPS) in Gram-negative bacteria. A LPS-based microbial detection assay exploits this principle (as it occurs in hors-shoe crab (*Limulus polyphemus*) coupled with a chromogenic substrate.
Although LAL is specific to endotoxin, many non-pyrogenic substances interfere with the assay. Furthermore, given that LAL is composed of a series of coagulation enzymes, pH and temperature have a crucial influence on its reactions. Ding and Ho (2001) recommend recombinant Factor C (rFC) as a basis of a novel micro-enzymatic assay for high-throughput screens of endotoxin. As a proenzyme, rFC becomes catalytically activated by trace levels of endotoxin. The resulting activated rFC hydrolyses a synthetic substrate to form a quantifiable product, which measures the level of endotoxin.

Bacterial toxins like botulinum toxin are mostly determined using mice as test animals.

6.8. Non-conventional transmissible agents

Little literature information is available on the direct detection of prions in aqueous samples. Narang *et al.* (2005) have developed a simple, rapid method for the reliable detection of host-encoded PrP in urine from normal subjects by Western blotting. The method can reliably detect PrP in apparently healthy individuals using less than 1ml of urine in which the amount of urinary PrP is estimated to be in the range of low micrograms/liter. A Western blotting is based on the principles of gel electrophoresis. A single protein in a mixture can be selected by using a high-quality antibody
Numerous other analytical methods, mainly enzyme-linked imunosorbent assays (ELISA), have been developed. Virtually all of these are post-mortem assays, requiring the death of the

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

animal or person and removal of brain, spinal column, pituitary gland, and/or related tissues for analysis (Li *et al*. 2004).

Specific probes exist for the PCR amplification of plasmids, especially for those plasmids that encode for antibiotics resistance. In general, the amount of sample needed for PCR is limited.

With respect to free genetic elements, the uptake of free RNA or proteins has not been described in literature. Both compounds are quite unstable. Free DNA may be more stable and cycle through the loop.
Analytical methods for free DNA and RNA are available and are based on appropriate extraction methods, followed by molecular techniques for determination and quantification.

Methods for bacteriophage detection were described in paragraph 6.5.

Mobile genetic elements (MGE) such as pathogenicity islands, can also be transferred among bacterial strains. Currently, no primers are available to detect MGEs or related groups of genes in general (Larissa Hendrickx, oral communication). Sometimes small sequences could be detected though, which give an indication of the presence of MGEs. Or some microarrays exist which contain the genes currently known.

6.9. Characterization and monitoring of microbiota in ISS

All attempts to describe the microbial species present aboard Apollo, US space shuttles and the Russian MIR have relied on traditional culture-dependent methods for the isolation of microbes and on molecular techniques for the species identification. A study by NASA (La Duc *et al*. (2003)) focused on the development of procedures to characterize the microbial quality of the ISS and shuttle drinking water at various stages of water treatment. In addition to traditional culture-based techniques, ATP, endotoxin and DNA targeted microbial enumeration procedures were employed. Samples were transported to earth after several months, so results were possibly not representative, but the study mainly aimed to evaluate the different techniques. The techniques described below are of course also applied on terrestrial samples.
Adenosine triphosphate (ATP) is used by all living organisms and can be exploited as an indicator for the presence of living organisms. In an *ATP-based bioluminiscent assay,* firefly luciferase catalyzes the reaction of luciferin with ATP to form the intermediate luciferyladenylate. Its subsequent reaction with oxygen leads to a cyclic peroxide, the breakdown of which yields CO_2 and the oxyluciferin product in the electronically excited state from which a proton is emitted. The bioluminescence generated is directly proportional to the amount of ATP, and as a result to the number of micro-organisms, in the sample being assayed. Potentially present extracellular ATP can be eliminated enzymatically, so that the remaining ATP is attributable only to intact microbial cells.
In a *LPS-based endotoxin assay*, the limulus amoebocyte lysate assay (LAL) was used. This was already described in the section on toxins.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

6.10. Determination of pathogens in sludge

A large number of micro-organisms can be found in sewage sludge and other biowastes. Problems in monitoring the pathogens occur due to

1. insufficient knowledge on relevance of sampling (the only guideline on sampling from ISO contains for example no information on precision that can be expected)
2. strong background of sludge matrix interfering with analysis.

Lepeuple *et al.* (2004) state that at present the important difficulty is standardization of the entire monitoring procedure from one lab to the other and from one country to the other. Lots of data are available but comparisons are difficult.

Yu *et al.* (1999) suggest the following method for the simultaneous extraction of DNA and RNA from activated sludge:

1. mini-bead beating, which is most efficient in breaking bacterial aggregate flocs and cells,
2. protection of RNA with diethyl pyrocarbonate and
3. precipitation of impurities with ammonium acetate.

Phenol/chloroform extraction and column purification are not necessary. The resulting DNA and RNA are suitable for PCR and reverse transcriptase PCR respectively. The efficiencies of cell lysis and nucleic acid recovery were high enough to permit detection by PCR of 10^2 cells/ml of mixed liquor.

Onuki *et al.* (2000) described the use of molecular methods to analyze microbial community structures. They used the FISH method to analyze activated sludge from waste water treatment plants. An effective characterization on group level appeared feasible. A PCR-DGGE method was used for the investigation of laboratory nitrifying sludge. This method characterized the community at species-level. For determination of the bacteria by PCR-DGGE 1ml of sludge was washed and centrifuged. The supernatant was eliminated and the pellet stored at -20°C. Genomic DNA was extracted and purified for further research. Whether these methods are sensitive enough to detect pathogens against a large background of organisms is unsure.

Garrec *et al.* (2003) describe as well sampling of sludge for comparison of cultural method and PCR-ELISA methods for the enumeration of *Listeria monocytogens* in naturally contaminated sewage sludge. Here again 1 ml of sludge was used for the molecular technique, as well as for the plating method.

The ASTM standard practice (2002) for recovery of viruses from wastewater sludges (2002) suggests to sample 100 ml of the liquid sludge. The procedure relies upon adsorption of viruses from the liquid phase to the sludge solids, which are concentrated by centrifugation. The supernatant is discarded. Viruses are desorbed from the solids by physicochemical means and further concentrated by organic flocculation. Decontamination is accomplished by filtration.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

A comparative study on analysis of bacteriophages proposed to take between 1 and 10 ml or 10 g of wet weight for solid samples to quantify viruses. Samples needed to be stored at 4°C. After extraction, elution, homogenization and solid separation, the samples are often decontaminated before actual detection. Methods used were cell culturing, reverse transcriptase PCR, negative staining or sequencing. Procedures were as described in ISO norms for selected bacteriophages.
(www.ecn.nl/docs/society/horizontal/hor_desk_29_Bacteriophages_Critical_Review.pdf)

A sample of maximal 2 g of sludge or biomass will probably be sufficient for a molecular analysis of bacteria. At a sludge concentration of e.g. 1 g/l within the reactor this would mean a sample volume of 2 l. For viruses, higher volumes are needed of minimal 10 g dry weight.to minimize heterogeneity of the samples.

6.11. Requirements for design

Preference is given to continuous operation of the different MELiSSA compartments rather than batch culture because the former allows to evaluate system performance under steady state conditions. However, transition conditions may be of importance as well. Membrane filtration units for instance will show a quite different elimination rate for certain organisms during start-up than during stable operation when a cake layer has been formed and retention of elements smaller than the membrane pore diameter are retained as well. Since the membrane unit is associated with compartment I where most pathogens are expected to occur, its performance both during start-up and during stable operation should be evaluated.

First, the currently used culture mediums should be used for evaluation of the presence of pathogens and the study of pathogen transfer. It can be anticipated however that a less optimal medium composition – as is expected during loop closure – will affect the microbial community stability, diversity and performance. As mentioned before, a decrease in community diversity may improve the chances for pathogens to establish themselves.

Sterile cultivation and operation has to be aimed for. Provisions to sterilize equipment or to remove biofilms in interfaces where pathogens may proliferate should be taken. Especially for the filtrate side of CI in-place sterilisation will be necessary. The feeding of the compartments should be filtered by means of a sterility filter with pore sizes less than 0,05 µm. Those filters should be easily accessible and replaceable. Prevention of reverse flow is required. Sampling ports before and after filtration step will be useful to determine the efficiency of this sterilisation device.

Zones within the reactors, interfaces, sampling ports,… where retention of biomass may occur should be minimized.

The appendix of the ASTM Standard practice for aseptic sampling of biological materials (1999) gives some additional sterilization guidelines:

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

- ✓ know the organisms to adjust the sterilization method effectively;
- ✓ saturated steam gives best result for bacterial spores removal. Heated steam requires cooling to reach saturation quality for maximum effectiveness. Wet steam has a lower heat content and adds water to the system that must be removed;
- ✓ remove air from the sample system to avoid steam dilution and air pockets that prevent full sterilization.

The choice of acids and bases for pH correction in the individual compartments is of little importance for future biosafe investigations. However the choice of chemical used may imply formation of certain toxic sub-products and thus requires special attention. The pH as such at which the compartments are operated may affect as well the degree to which pathogens survive, toxins are produced, or the time during which they occur in the loop.

To be able to evaluate the removal mechanisms of different units for particular pathogens or hazardous compounds, at least inlet and outlet water samples are required. Sludge or biomass samples may provide information on the degree of sorption and on the expected danger related to food intake.

Sampling conditions should be axenic, avoiding any contact with foreign nucleic acids and micro-organisms. Grab samples should be sufficient to evaluate removal efficiencies provided that hydraulic retention times are taken into account.

Sample volumes have been indicated before (see also Table 14). Depending on the analytical technique used and mainly on the number of organisms of a specific class which is present in the sample, volumes may vary. As can be seen from the above descriptions of several available techniques it can generally be stated that the required sample volume should be 100ml for bacterial determination via culture methods or amplification. As viruses are often present in small amounts, a concentration step is required on a large volume (at least 10liters) in order to prepare the sample for further detection with e.g. RT-PCR technology. For coliphage detection by means of a classical *E. coli* infection method, 1liter is sufficient.
Sampling frequency is proposed to be weekly or biweekly. Intensive sampling campaigns may be needed e.g. during start up of a membrane filtration unit.

Samples should preferably be collected through sampling ports. For influent and effluent collection this will however be difficult and the respective tanks may need to be used. In any case, the tank or sampling material has to be selected in such a way that it does not affect cell numbers or microcompound concentration (through sorption or leakage of inhibitory compounds). The material needs to be sterilizable and provisions need to be taken to collect the samples in an axenic way. Ideally, samples should be protected from light and cooled to below 10°C. Samples should not be stored longer than 24 h, so the buffering capacity has to be chosen to accommodate this.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

The ASTM Standard practice for aseptic sampling of biological materials (1999) provides following guidelines for the design of sampling systems:

- ✓ sufficient agitation of the bulk material is required to ensure uniformity.
- ✓ change of process conditions (temperature, pressure, mixing efficiency,…) from the bulk phase towards the sampling device needs to be considered. The change should not affect sample quality.
- ✓ the quantity of sample required should be considered.
- ✓ sample valves should minimize holdup of sample within the valve. Diaphragm and ball valves generally have less holdup than gate or globe valves. Diaphragm valves are normally sterilized easier than other standard design valves.
- ✓ sample valves should have minimum lengths of piping connection on suction and discharge ports to avoid trapping of sample material.
- ✓ velocity considerations are important to avoid settling of solids. Inert, sterile gas could be used for blowback of the sample tube to minimize stale sample accumulation.
- ✓ rough surfaces where micro-organisms can grow uncontrolled should be avoided. Welds should be ground smoothly and top quality welding procedures used.

The sampling devices/ports on liquid, solid and gas phases should prevent the release of microorganisms. The European CEN standards give guidance on sampling procedures related to the risk level of the system (EN 13092). This standard differentiates between devices for use where release of microorganisms should be minimized and where it should be prevented . In general it states that i. special sampling probes are required, ii. a special combination of valves and couplings must be used on pipes and tubes, iii. an external loop of a vessel via a barrier for organisms should be included or iv. a septum that can be pierced by a syringe must be foreseen. An non-exhaustive list of examples, including figures, is given in this standard.

The design of the sampling device should ensure to limit egress of microorganisms to the environment in all modes of operation, despite, of course, the egress into the sampling receptacle.
No specific sample treatment is needed which may have an impact on loop design. Samples may be filtered or centrifuged off line, i.e. after collection.

With regard to measurements and instrumentation on the MELiSSA compartments, only those which are needed to monitor regular reactor operation should be provided. However, a few extra ports should be provided in case additional measurements need to performed or in case new sensors developing in the coming year which may have an added value for biosafe studies. Minimally, temperature, pH, oxygen (when appropriate) and flow rates need to be monitored.

As mentioned before, material selection should be such that it does not release inhibitory compounds, that no sorption occurs and that it is sterilizable.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Attention should go to those organisms and elements that are likely to pass the first compartment and its ultrafiltration unit. Protozoa and the aerobic fungi will certainly be retained in the anaerobic waste compartment. Bacteria will be retained largely. However, the ultrafiltration unit does not guarantee 100% retention. As the waste compartment deals with a high input of bacteria monitoring on the effluent should be foreseen. Many types of viruses will pass easily through CI and its filtration unit.

Table 14. Overview of sampling volumes related to study item and used analytical method

Study item	Analytical method	Volume
Bacteria	Classical culture methods	100 ml
	Molecular technique	100 ml (normal)
	(PCR, DGGE)	1 to 3 l (low concentration of pathogens)
Viruses	Difficult to measure	1 to 10 l depending on concentration
	Concentration step, followed by: - cell culture -molecular technique or - immunoassay	
Fungi	Culture based methods	100 ml (normal) several l (low concentration of pathogens)
Protozoa	Concentration step, followed by: - microscopy - immunoassay Cysts are difficult to measure	1 to 10 l depending on concentration
Toxins	Immunoassays Cell cultures	up to several l depending on concentration level

In case of a normal follow up and during steady state, a weekly sampling of 100 ml of the effluent from the different compartments for classical culture analysis of bacteria should be sufficient. During transitional phases and depending on the kinetics of the system, more frequent monitoring may be needed.

A monitoring of diluted pathogens (bacteria or viruses) by PCR, requires larger volumes. This would be almost impossible in a closed loop system at laboratory scale. During the test phase on individual compartments an intensive measuring campaign would imply a sampling of 1 to 3 l of effluent weekly or twice a week. Again, this depends on the kinetics, hydraulics and status of the system.

The above information is summarized in Table 15 for the analysis of aqueous samples and in Table 16 for biomass determinations.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 15. Requirements for aqueous sample analysis in the BELISSIMA loop for biosafe investigations

Parameter	Comp. I	Comp. II	Comp. III	Comp. IV
operation mode: batch < > continuous	continuous	continuous	continuous	continuous
culture medium: as is, optimal nutrient concentrations, limitations, ..	as is	as is	as is	as is
Sterile cultivation: Y/N	N	Y	Y	Y
Choice acid/base: A/B	as usual	as usual	as usual	as usual
Samples: influent (I), effluent (E), biomass (B), supernatant, (S) diff reactor locations (L), gas (G)	I, E, S	I, E, S	I, E, S	I, E, S
Sampling conditions: axenic (A), continous (C) grab samples (G)	A, G	A, G	A, G	A, G
Maximum total sample volume	3 l	3 l	3 l	3 l
Sampling frequency: weekly (W), monthly (M), intensive sampling campaign (I)	W or I	W	W	W
Sample treatment: centrifugation, sterilization, ...	generally off line (see text)			
Sample preservation: keep light (L), keep dark (D), temperature (T)	D, cooled	D, cooled	D, cooled	D, cooled
Required measurements/instrumentation: as for normal reactor operations (Y/N), on-line measure-ments (which)	as usual	as usual	as usual	as usual
Required control: pH, T	normal	normal	normal	normal
Material selection	inert	inert	inert	inert

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Table 16. Requirements for biomass analysis in the BELISSIMA loop for biosafe investigations

Parameter	Comp. I	Comp. II	Comp. III	Comp. IV
operation mode: batch < > continuous	continuous	continuous	continuous	continuous
culture medium: as is, optimal nutrient concentrations, limitations, ..	as is	as is	as is	as is
Sterile cultivation: Y/N	N	Y	Y	Y
Choice acid/base: A/B	as usual	as usual	as usual	as usual
Samples: influent (I), effluent (E), biomass (B), supernatant, (S) diff reactor locations(L), gas (G)	B	B	B	B
Sampling conditions: axenic (A), continous (C) grab samples (G)	A, G	A, G	A, G	A, G
Maximum total sample volume	2-10 g	2-10 g	2-10 g	2-10 g
Sampling frequency: weekly (W), monthly (M), intensive sampling campaign (I)	W	W	W	W
Sample treatment: centrifugation, sterilization, ...	generally off line (see text)			
Sample preservation: keep light (L), keep dark (D), temperature (T)	D, cooled	L, cooled	D, cooled	L, cooled
Required measurements/instrumentation: as for normal reactor operations (Y/N), on-line measure-ments (which)	as usual	as usual	as usual	as usual
Required control: pH, T	normal	normal	normal	normal
Material selection	inert	inert	inert	inert

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

For biomass analysis, it is important to store the biomass samples of compartment II and IVa in light. For all other conditions, storage in the dark is preferred.

Evaluating the impact of biosafe studies regarding toxins and other non-conventional transmissible agents on the loop is hampered by the fact that no specific analytical methods are described in literature. However, taking into account the expected low concentrations, it is assumed that even higher sample volumes may be needed for detection of some of these agents than the ones mentioned in Table 15. Precautions with respect to sterile sampling, choice of material, sample preservation and general monitoring of the loop will most probably be similar to those for microbial studies. Operation in continuous mode with the usual culture media is advisable.

7. Risk management

In the light of the risks described in the previous chapters, a number of non-exhaustive list of countermeasures is described.

As a starting point all precautions described in the WHO guidelines for Biosafe handling should be addressed during operation of the MELiSSA loop. Proper hygiene measures have to be implemented. Good laboratory practices shall be applied.

Additional measures are the following:

Vaccination of the crew against certain bacterial or viral pathogens e.g. *Clostridium* could be an option.

The filtration systems currently available in the MELiSSA loop have cut-off values which will not retain nanobacteria, viruses, prions or genetic elements. Hence, the risk exists that these elements circulate in the loop. A way to address this problem may be to include additional technology in the loop which has been validated for elimination of these substances. Regular sterilization of highly challenged (sub)compartments may be required. The use of antimicrobial agents is not desirable in the MELiSSA loop, due its closed loop nature and its dependence on various microbial populations in the individual compartments.
Alternatively, cooking of the food provides a barrier for uptake of these elements by the crew.

According to Clarke and Waters (2005) phytopathogens can either be managed through

- Sterilization: this turns out to be difficult and expensive. Both chamber, equipment, plants and seeds need to be treated, while human contact needs to be minimal. Furthermore, sterilization may render plants more susceptible to remaining pathogens. Sterilization may be achieved through heat, UV, ozone, hydrogen peroxide or filtration depending on the application.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

- sanitation and inoculation: this implies starting with clean equipment and then inoculating it with beneficial microbes, which could improve plant growth and compete with pathogens. It is the preferred option for most hydroponic operators on earth because it is low in cost and efforts.
- plant selection and genetic analysis and engineering: certain plants may show a lower risk of being populated by pathogens or genetic engineering may increase or introduce plant resistance against pathogens.

In general, the hardware materials should be selected in such a way that the risks for (bio)corrosion and the associated risk of release of hazardous chemicals and/or malfunctioning of the system is minimized or eliminated. Ideally, it should reduce the potential for biofilm formation.

A sufficient level of automation of the system should reduce the possibility of errors made by operators.

The gas outlets of the loop must be sterile to prevent the release of micro-organisms into the surrounding environment.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

8. References

American Public Health Association, American Water Works Association, Water Environment Federation (1998). "Standard methods for the examination of water and wastewater, 20th ed." Washington, DC.

ASTM D4249-83 (1998) "Standard test method for enumeration of *Candida albicans* in water"

ASTM D4994 (2002) "Standard practice for recovery of viruses from wastewater sludges"

ASTM D6734 (2001) "Standard test method for low levels of coliphages in water."

ASTM E1287-89 (1999) "Standard practice for aseptic sampling of biological materials"

Bauer H., Fuerhacker M., Zibuschka F., Schmid H. and Puxbaum H. (2002). "Bacteria and fungi in aerosols generated by two types of wastewater treatment plants." Water research Vol.36, Number 16: 3965-3970.

Beech I..B. (2004) "Corrosion of technical materials in the presence of biofilms – current understanding and state-of-the-art methods of study." International Biodeterioration & Biodegradation 53: 177-183

Beech I.B. and Sunner J. (2004) "Biocorrosion: towards understanding interactions between biofilms and metals." Current opinion in Biotechnology 15: 181-186.

Belpomme, D. (2005) "Consultancy study: Introduction to biosafety issues of regenerative life support systems" Presentation at MELiSSA meeting, November 2005, Paris.

Bernasconi C., Ghiani M., Daverio E. and Camozzi M. (2004) "Molecular-based methodologies for the assessment of microbiological status of drinking and surface" EUR 21085 EN: European Commission – Joint Research Centre .

Breitbart M., Hewson I., Felts B., Mhaffy J.M., Nulton J. Salamon P. and Rohwer F. (2003). "Metagenomic analyses of an uncultured viral community from human feces" Journal of bacteriology Vol.185, No.20: 6220-6223.

Cadenas S. and Cadenas A. A. (2002) "Fighting the stranger - antioxidant production against endotoxin toxicity." Toxicology 180: 45-63

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Clark, O., Waters, G. (2005) "Possible pathogens in HPC – MELiSSA candidate crops." Document prepared in the framework of BELISSIMA, University of Guelph

Cools D. (2001) "Manure-derived antibiotic-resistant bacteria: survival in soil and contamination of crop roots" Ph.D. Thesis Catholic University of Leuven, Applied Biological Sciences

Cools D., Merckx R., Vlassak K. and Verhaegen J. (2001) "Survival of *E. coli* and *Enterococcus spp.* derived from pig slurry in soils of different texture." Applied Soil Ecology 17: 53-62.

Croci L., De Medici D., Scalfaro C., Fiore A. and Toti L. (2002) "The survival of hepatatis A virus in fresh produce." International Journal of Food Microbiology 73: 29-34.

Deere D., Vesey G., Ashbolt N., Davies K.A., Williams K.L. and Veal D. (1998) "Evaluation of fluorochromes for flow cytometric detection of *Cryptosporidium parvum* oocysts labeled by fluorescent in situ hybridization" Letters in Applied Microbiology 27 (6): 352-356.

Demey D., Van Meenen P. and Van Eyck L. (1995). "Breakdown of human faeces by co-cultures and the characterization of the microbial strains present in human faeces by means of a litarure study.'MELiSSA Technical Note 22.4.

Ding J.L and Ho B. (2001) "A new era in pyrogen testing." Trends in biotechnology Vol.19 No.8: 277-281

Doblhoff-Dier, O. and C. H. Collins (2001). "Biosafety: future priorities for research in health care." Journal of Biotechnology 85(2): 227-239.

Dobrindt U. and Hacker J. (2001) "Whole genome plasticity in pathogenic bacteria." Current Opinion in Microbiology 4: 550-557.

Elorrieta M.A., Suárez-Estrella F., López M.J., Vargas-Garcia M.C. and Moreno J. (2003). "Survival of phytopathogenic bacteria during waste composting." Agriculture, Ecosystems & Environment 96: 141-146.

EN 13312-3 (2001) "Performance criteria for piping and instrumentation - Part 3: Sampling and inoculation devices"

Gantzer C., Senouci S., Maul A. Levi Y. and Schwartzbrod L. (1997) "Enterovirus genomes in wastewater: concentration on glass wool and glass powder and detection by RT-PCR" Joiurnal of Virological Methods 65: 265-271.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Garrec N., Dilasser F., Pourcher A.M., Perelle S. and Fach P. (2003) "Comparison of a cultural method with ListerScreen plus Rapid'L.mono or PCR-ELISA mehods for the enumeration of *L. monocytogenes* in naturally contaminated sewage sludge". Journal of Microbiological Methods 55: 763-773.

Garland J.L., Cook K.L., Johnson M., Sumner R. and Fields N. (1997). "Density and composition of micro-organisms during long-term (418 day) growth of potato using biologically reclaimed nutrients from inedible plant biomass." Adv. Space Res. Vol.20, No.10, pp. 1931-1937.

Giraffa G. and Neviani E. (2001) "DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems" International Journal of Food Microbiology 67: 19-34

Gleeson C. and Gray N. (1997) "The coliform index and waterborne disease. Problems of microbial drinking water assessment", E&FN Spon, Chapman & Hall, London, UK

Goularte L., Martins C.G., Morales-Aizpurua I.A., Destro M.T., Franco B.D.G.M, Vizeu D.M., Hutzler B.W. and Landgraf M. (2004) "Combination of minimal processing an irradiation to improve the microbiological safety of lettuce." Radiation Physics and Chemistry 71: 155-159.

Gu J-D. (2003) "Microbial deterioration and degradation of synthetic polymeric materials: rcet research advances." International Biodeterioration & Biodegradation 52: 69-91

Gu J-D., Roman M., Esselman T. and Mitchel R. (1998) "The role of microbial biofilms in deterioration of spaces tation candidate materials" International Biodeterioration & Biodegradation 41: 25-33

Hart T. and Shears P. (2004). "Medical microbiology" Elsevier Ltd.

Heiser I., Oβwald W. and Elstner E.F. (1998) "The formation of reactive oxygen species by fungal and bacterial phytotoxins." Plant Physiol. Biochem. 36 (10): 703-713.

Heller F. and Duchmann R. (2003). "Intestinal flora and mucosal immune responses." Int. J. Med. Microbiol. 293, 77-86.

Hendrickx L., de Wever H., Pycke B., Janssen P. and Mergeay M. (2006). "Preliminary requirements for genetic stability and axenity study". MELiSSA Technical Note 80.11.

Huyard A., Ferran B. and Audic J-M. (2000). "The two phase anaerobic digestion process: sludge stabilization and pathogens reduction." Water Science and Technology Vol. 42, No. 9: 41-47.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Köster W., Egli T., Ashbolt N., Botzenhart K., Burlion N., Endo T., Grimont P., Guillot E., Mabilat C., Newport L., Niemi M., Payment P., Prescott A., Renaud P. and Rust A. (2003) "Analytical methods for microbiological water quality testing." Chapter 8 from WHO publication "Assessing microbial safety of drinking water: improving approaches and methods".

La Duc M.T., Venkateswaren K., Sumner R. and Pierson D. (2003) "Characterisation and monitoring of microbes in the International Space Station drinking water" – ICES 2003-01-2404

Lay C., Sutren M., Rochet V., Saunier K., Doré J. and Rigottier-Gois L. (2005). "Design and validation of 16 S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human faecal microbiota." Environmental Microbiology 7(7): 933-946.

Lenoir Wijnkoop I. and Hopkins M. (2003) "The intestinal Microflora - Understanding the Symbiosis." Danone Vitapole.

Lepeuple A.S., Gaval G., Jovic M. and de Roubin M.R. (2004) "Literature review on levels of pathogens and their abatement in sludges, soils and treated biowaste" EU Horizontal project.

Li G., Zhou X., Wang Y., El-Shafey A., Chiu N.H.L. and Krull I.S. (2004) "Capillary isoelectric focusing and affinity capillary electrophoresis approaches for the determination of binding constants for antibodies to the prion protein." Journal of chromatography A, 1053: 253-262.

Mackowiak C.L., Stutte G.W., Garland J.L., Finger B.W. and Ruffe L.M. (1997). "Hydroponic potato production on nutrients derived from anaerobically processed potato plant residues." Adv. Space Res. Vol.20, No.10, pp.2017-2022.

Mackowiak C.L., Wheeler R.M., Stutte G.W., Yorio N.C. and Sager J.C. (1997). "Use of biologically reclaimed minerals for continuous hydroponic potato production in a CELLS (Controlled Ecological Life Support System)." Adv. Space res. Vol.20, No.10, pp 1815-1820.

Moss M.O. (1998). "Recent studies of mycotoxines." Journal of Applies Microbiology, Symposium Supplement 84, pp 62S-76S.

Moss M.O. (2002). "Risk assessment for aflatoxins in foodstuffs." International Biodeterioration and Biodegradation 50, pp. 137-142.

Narang H.K., Dagdanova A., XIE Z., Yang Q. and chen S.G. (2005) "Sensitive detection of prion protein in human urine." experimental Biology and medicine 230: 343-349.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Novikova N.D. (2004) "Review of the knowledge of microbial contamination of the Russian manned spacecraft." Microbial Ecology 47: 127-132.

Novikova N., De Boever P., Poddubko S., Deshevaya E., Polikarpov N., Rakova N., Coninx I. and Mergeay M. (2006). "Survey of environmental biocontamination on board the International space Station." Research in Microbiology 157: 5-12.

Ohnishi M., Kurokawa K., and Hayashi T. (2001) "Diversification of *Escherichia coli* genomes: are bacteriophages the major contributors?" Trends in Microbiology 9: 481-485.

Onuki M., Satoh H., Mino T. and Matsuo T. (2000) "Application of molecular community analysis of activated sludge" Water Science and Technology 42:17-22.

Ott C.M., Bruce R.J. and Pierson D.L. (2004) Microbial characterisation of free floating condensate aboard the Mir station." Microbial Ecology 47: 133-136.

Pechère, J.C. (2005) "Microbiological risks associated to the MELiSSA loop." Intermediate report of the Biosafety consulting study.

Pierson D.L. (2001) "Microbial contamination of spacecraft." Gravitational and Space Biology Bulletin 14(2).

Pierson, D.L. (1992) "Microbiology" Spacecraft and the spacecraft environment, Chapter 8, p. 157-166

Pittet A. (1998) "Natural occurrence of mycotoxins in foods and feeds - an updated review" Révue de médecine Vétérinaire 149, pp. 479-492.

Rigottier-Gois L., Le Bourhis A., Gramet G., Rochet V. and Doré J. (2003). "Fluorescent hybridisation combined with flow cytometry and hybridisation of total RNA to analyse the composition of microbial communities in human faeces using 16S rRNA probes." FEMS Microbiology Ecology 43: 237-245.

Roberts M.S., Garland J.L. and Mills A.L. (2004) "Microbial astronauts: assembling microbial communities for advanced life support systems." Microbial Ecology 47: 137-149.

Roossinck M.J.(2003) "Plant RNA virus evolution." Current opinion in Microbiology 6: 406-409.

Schlegel H.G. (1986) "General microbiology" Cambridge University Press.

Secretariat of the Convention on Biological Diversity (2000). "Cartagena protocol on biosafety to the convention on biological diversity."

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Sengelov G., Agerso Y. Halling-Sorensen B., Baloda S.B., Andersen J.S. and Jensen L.B. (2003) "Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig slurry." Environment International 28: 587-595.

Særensen L.K., Elbæk T.H. (2005) "Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry." Journal of chromatography B, 820: 183-196.

Simone G.W. and Momol M.T. (2001) "Vegetable disease recognition and management." Florida greenhouse vegetable production handbook Vol. 3.

Soto C. and Castilla J. (2004) "The controversial protein-only hypothesis of prion propagation." Nature Medicin On-line.

Straub T.M. and Chandler D.P. (2003) "Towards a unified system for detecting waterborne pathogens" Journal of Microbiological Methods 5: 185-197.

Suau A., Rochet V., Sghir A., Gramet G., Brewaeys S., Sutren M., Rigottier-Gois L. and Doré J. (2001). "*Fusobacterium prausnitzii* and related species represent a dominant group within the human fecal flora." System. Appl. Microbiol. 24: 139-145.

Tirranen L.S. (2001). "Formation of higher plant component microbial community in closed ecological system". Acta Astronautica Vol.49, No.1, 47-52.

Tournas V.H. (2005) "Moulds and yeast in fresh and minimally processed vegetables, and sprouts." International journal of Food Microbiology 99, 71-77.

True H.L. (2006) "The battle of the fold: chaperones take on prions." Trends in genetics Vol.22 No.2: 110-117.

Van Campenhout, L. (2004) "Food microbiology". Course taught at the Hoger Instituut der Kempen.

Vernet G. (2004) "Molecular diagnostics in virology." Journal of clinical virology 31: 239-247

Weinbauer M.G. (2004) "Ecology of prokaryotic viruses". FEMS Microbioloy reviews 28: 127-181.

World Health Organization (2004). "Laboratory biosafety manual - third edition." WHO Library Cataloguing-in-Publication Data

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP

Yang S. and Rothman R.E. (2004) "PCR-based diagnostics for infectious diseases: uses, limitations, and further applications in acute-care settings." Lancet Infectious Diseases 4: 337-348.

Yu Z. and Mohn W.W. (1999) "Killing two birds with one stone: simultaneous extraction of DNA and RNA from activated sludge biomass" Canadian Journal of Microbiology Vol. 45, No. 3: 269-272.

Zhang T., Breitbart M., Lee W.H., Run J-Q., Wei C.L, Soh S.W.L., Hibberd M.L., Liu E.T., Rohwer F. and Ruan Y. (2006). "RNA viral community in human feces: prevalance of plant pathogenic viruses." PloS Biology Vol. 4 Issue 1: 0108-0118.

This document is confidential property of the MELiSSA partners and shall not be used, duplicated, modified or transmitted without their authorization
Memorandum of Understanding 19071/05/NL/CP