MELiSSA

TECHNICAL NOTE

Memorandum of Understanding TOS-MCT/2002/3161/In/CL

TECHNICAL NOTE: 80.11

PRELIMINARY REQUIREMENTS FOR GENETIC STABILITY AND AXENICITY STUDY

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reference/*réference*	11206/05/NL/SFe
issue/*édition*	1
revision/*révision*	0
date of issue/*date d'édition*	
status/*état*	Draft
Document type/*type dedocument*	Technical Note
Distribution/*distribution*	C. Paillé, N. Dorval, H. De Wever, H. Witters, W. Verstraete, D. Demey, M. Mergeay

CONFIDENTIAL DOCUMENT

APPROVAL

Title *titre*	Preliminary requirements for the genetic stability and axenicity study	issue 1 *issue*	revision 0 *revision*

author *auteur*	L. Hendrickx, B. Pycke, P. Janssen, M. Mergeay	date *date*

approved by *approuvé by*		date *date*

CHANGE LOG

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

CHANGE RECORD

Issue: 1 Revision: 0

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

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ABBREVIATIONS

AHL	Acyl-Homoserine Lactone
BHR	Broad Host Range
BPA	Bisphenol A
CFU	Colony Forming Units
DAPI	4', 6-diamidino-2-phenylindole
DDT	Dichlorodiphenyltrichloroethane
DLDP	Direct Labeling and Detection Procedure
DNA	DesoxyriboNucleic Acid
dsDNA	Double stranded DNA
E1	Estrone
E2	(17β-)Estradiol
E3	Estriol
EDC	Endocrine Disrupting Chemicals
EE2	(17α-)Ethynylestradiol
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GC-MS	Gas Chromatography - MS
GTA	Gene Transfer Agent
HPLC	High-Performance Liquid Chromatography
ICM-MS	Intact Cell MALDI-TOF-MS
LPP	*Lyngbya-Phormidium-Plectonema* group
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-time of flight
MeEE2	Methyl-Ethynylestradiol
MELGEN	MELiSSA Genetics
MELiSSA	Micro Ecological Life Support System
mRNA	messenger RNA
MS	Mass Spectrometry
NCTC	National Collection of Type Cultures
PCR	Polymerase Chain Reaction
PNA	Peptide Nucleic Acid
PPCP	Pharmaceuticals and Persobal Care Products
PQS	3,4-dihydroxy-2-heptylquinoline
rDNA	ribosomal DNA
RNA	RiboNucleic Acid
rRNA	ribosomal RNA
TN	Technical note
UV	UltraViolet
VBNC	Viable but Non Culturable
WHO	World Health Organisation
2D	2 Dimensional
3OH PAME	3OH Palmitic Acid Methyl Ester

1 INTRODUCTION

In the last two decades, studies have indicated the potential widespread occurrence of hormones, pharmaceuticals, personal care products, and other organic sewage contaminants and their metabolites in low-level concentrations in the aquatic environment (Ternes *et al.*, 1999; Ternes *et al.*, 1999; Boyd *et al.*, 2003; Ternes *et al.*, 2005), and been bundled in the term, micropollutants. Since these compounds showed in some instances a biologically disrupting activity, even in low-level concentrations, their presence in the environment is considered alarming and should, thus, be eliminated from the ecosystem. In the MELiSSA, micropollutants originate from various sources, and can be differentiated in natural and synthetic compounds. The natural compounds originate from both animals and plants; animals excrete a lot of compounds and their metabolites through urine and faeces, whereas phytochemicals simply enter the MELiSSA-loop when plant material is thrown in the liquefying reactor. The synthetic compounds are man-made compounds (*e.g.* synthetic estrogen EE2) and mostly enter the MELiSSA through excretion. Both types of micropollutants can disturb the proper functioning of the MELiSSA loop either directly by influencing bacterial metabolism or indirectly via accumulation, thereby endangering the MELiSSA consumer, the crew.

In this technical note the preliminary requirements will be described to asses genetic and metabolic stability and axenicity in the Bellissima loop. A literature overview is given to present the state of knowledge on the impact of shortage and/or surplus of minerals as well as the presence of micropollutants on the MELiSSA organisms.

2 GENETIC AND METABOLIC STABILITY

2.1 The necessity of minerals, trace elements and vitamins

Chemical elements are used by living organisms and an extensive list of these elements is essential. The traditional criteria for essentiality are that absence or deficiency of the element from the medium or diet produces either functional or structural abnormalities and that the abnormalities are related to, or a consequence of, specific biochemical changes that can eventually be reversed by the presence of the essential element(Bennett, 1993; WHO, 1996). Carbon (C), hydrogen (H) and oxygen (O) are in all organisms referred to as the ‘building block’. Other elements, called ‘primary elements’ or macronutrients are eg. nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), but may differ between groups of organisms. Macronutrients are those elements that are needed in relatively large quantities, compared to micronutrients or secondary elements. The latter also also essential elements, but they are needed in relatively small amounts. Micronutrients are therefore often referred to as trace elements. Except for inorganic elements, vitamins too belong to the group of trace elements or micronutrients.

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According to(Schlegel, 1988), the elementary composition of microorganisms is divided into ten macroelements which are present in all cells and a number of microelements or trace elements. The macroelements are C, H, O, N, S, P, K, Ca, Mg and Fe. Trace elements are Mn, Mo, Zn, Cu, Co, Ni, V, B, Cl, Na, Se, Si and others which are not required by all organisms. Dunn (Dunn, 1985) distinguishes different groups of trace elements, based on their occurrence in organisms (Table 1). Burgess *et al.* (Burgess *et al.*, 1999) mention as most important trace elements Mn, Zn, Co, Mo, Ni, Cu, V, B, Fe and I.

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

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

The role of different trace elements in microorganisms, including the MELiSSA organisms, is summarized in Table 2. They are

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

Vitamins are organic compounds and have much higher toxicity thresholds than inorganic micronutrients. The most important ones are K, B_1, B_2, B_6, B_{12}, biotin, niacin and panthotenic acid.

Essential trace elements do have to some extent homeostatic mechanisms involving regulation of absorption and excretion, which enable organisms to adapt to varying nutrient intakes to ensure safe and optimum systemic supply for the performance of essential functions and growth.

Micronutrient supply must be sufficient to activate cellular enzymes and to provide general nutrients for growth and metabolism. Deficiencies will reduce growth rates and can eventually prevent growth. Excess micronutrients may exert toxic effects by interference with physiological pathways. Addition of micronutrients does not guarantee bioavailability. Essential micronutrients, among them especially metals, can be precipitated or adsorbed before bacteria can assimilate them. Even when ions are present in soluble form, deficiencies may occur as a result of antagonistic interactions. Unfortunately, interactions are impossible to predict because they are influenced by a number of factors, such as type and concentration of the ion, physico-chemical conditions of the medium (pH, conductivity, ..), operating conditions (temperature, retention time,..), etc.

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

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

Micronutrients are often required at doses below 1 mg/l. In addition, they occur as impurities in salts of macronutrients and reach media via contamination of glass vessels and dust particles. Their requirements are therefore difficult to demonstrate. Moreover, they are influenced by organic and hydraulic loading rates, cell growth rate, the nature of the waste and the cell residence time (Burgess *et al.*, 1999). The mineral requirements of plants are discussed in TN80.12.

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2.2 *Micropollutants introduced by the human crew*

2.2.1 HORMONES

A hormone is a signal molecule produced by a multi-cellular organism to signal (environmental) changes and translate these changes in a differential gene expression (transcription). Hormones have been identified for animals and plants, animal hormones can be subdivided into peptide hormones (proteinacious) and steroid hormones. Plant hormones are referred to as phytohormones. Some molecules and/or their metabolites are referred to as endocrine-disrupting chemicals (EDC) because they can show a degree of hormonal activity, by mimicing a natural hormone and subsequently cause cells to over-respond to the stimulus, or respond at inappropriate times. Other EDCs block the hormonal receptor on a cell; and still others directly stimulate or inhibit the endocrine system and cause overproduction or underproduction of hormones. In most cases the endocrine-disrupting activity is not the prime objective of the EDCs (Oberdörster *et al.*, 2001; Xie *et al.*, 2003; Van de Wiele *et al.*, 2005) and have a lower estrogenic activity than the naturally occurring steroid hormones (Krishnan *et al.*, 1993). Presently, the steroid hormones and EDCs are the most important issue concerning the contamination of the MELiSSA with micropollutants.

Several studies have indicated the presence of low-level concentrations of steroid hormones in the environment (Ternes *et al.*, 1999; Ternes *et al.*, 1999; Johnson *et al.*, 2000; Ying *et al.*, 2002; Johnson *et al.*, 2005). Due to the continuous excretion of steroid hormones by humans and livestock their respective concentrations have reached alarming levels in aquatic environments. Chemicals showing endocrine-disruping activity have a wide variety of applications, some are used as fungicide (Bisphenol A), whereas other as pesticides (DDT), *etc.* (Brotons *et al.*, 1995). It has been shown that the presence of hormones and endocrine-disrupting chemicals in the environment causes a range of unwanted alterations in both animals and plants (Shore *et al.*, 1995; Jobling *et al.*, 1998; Oberdörster *et al.*, 2001; Fox, 2004; Fox *et al.*, 2004). Although steroid hormones have been detected in different locations it can be said that the data with regard to the behaviour and fate of the steroid hormones is insufficient. Subsequently, the knowledge related to the risks coinciding with the exposure to hormonally active compounds are too limited (Ying *et al.*, 2002).

2.2.2 PHARMACEUTICALS AND PERSONAL CARE PRODUCTS

Pharmaceuticals and personal care products (PPCPs) describe a large class of chemical contaminants that originate from human usage and excretion, and veterinary use of a variety of products. The applications of PPCPs range from over-the-counter and prescription medications, and fungicides and disinfectants used for industrial, domestic, agricultural and livestock practices (Daughton & Ternes, 1999). Most of the compounds used in medicine are only partially metabolized by patients and are then discharged into the hospital sewage system or directly into municipal wastewater if used at home. Along with excreta, they flow with municipal wastewater to the sewage treatment plant. They may pass through the sewage system and end up in the environment, mainly in the water compartment. Anti-bacterials used

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for livestock enter the environment when manure is applied to fields. These antibiotics may either end up in soil or sediment or in ground water (Kümmerer, 2004). PPCPs and their metabolites have also been observed in low-level concentrations in aquatic environment (Zuccato *et al.*, 2000; Kuch & Ballschmiter, 2001; Ternes *et al.*, 2004; Ternes *et al.*, 2005). Because PPCPs are used throughout the year and have been conceived for their specific biological activity, they have been subject of some concern with regard to the undesired effect in the environment (Oaks *et al.*, 2004) because they appear to be prevalent at detectable concentrations (Kolpin *et al.*, 2002). Additionally, due to the recalcitrance of some of the PPCPs in sewage and wastewater treatment plants, humans are being exposed to low-level concentrations of PPCPs present in drinking water. It has been shown that the indirect human exposure to pharmaceuticals, via drinking water, is three orders of magnitude lower than the standard therapeutic dose (Webb *et al.*, 2003). However, no data is available with regard to the long-term exposure to low-level concentrations of PPCPs (Daughton & Ternes, 1999), and this type of exposure is of growing concern due to the increasing use of potable water and the increasing importance of water reuse.

Another issue regarding the PPCPs is their potential as EDC. Since many of the PPCPs are suspected or potential endocrine-disrupting chemicals, the issue regarding the environmental contamination with PPCPs is increasingly alarming.

2.3 Micropollutants introduced by the MELiSSA organisms

2.3.1 MICROORGANISMS-PRODUCED BIOACTIVE PRODUCTS

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar bacterial strain(s). They are phenomenologically analogous to yeast and paramecium killing factors, and are structurally, functionally, and ecologically diverse. Bacteriocins were first discovered by Gratia in 1925. He was involved in the process of searching for ways to kill bacteria, which also resulted in the development of antibiotics and the discovery of bacteriophage, all within a span of a few years. He called his first discovery a colicine because it killed *E. coli*.

Bacteriocins are categorized in several ways, including producing strain, common resistance mechanisms, and mechanism of killing. There are several large categories of bacteriocin which are only phenomenologically related. These include the bacteriocins of gram + bacteria, the colicins, the microcins, and the bacteriocins of Archaea. Bacteriocins are of interest in medicine because they are made by non-pathogenic bacteria that normally colonize the human body. Loss of these harmless bacteria following antibiotic use may allow oportunistic pathogenic bacteria to invade the human body.

There are many ways to demonstrate bacteriocin production, depending on the sensitivity and labor intensiveness desired. To demonstrate their production, technicians stab inoculate multiple strains on separate multiple nutrient agar Petri dishes, incubate at 30 °C for 24 h., overlay each plate with one of the strains (in soft agar), incubate again at 30 °C for 24 h. After

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this process, the presence of bacteriocins can be inferred if there are zones of growth inhibition around stabs. This is the simplest and least sensitive. It will often mistake phage for bacteriocins. Some methods prompt production with UV radiation, Mitomycin C, or heat shock. Cross streaking may be substituted for lawns. Similarly, production in broth may be followed by dripping the broth on a nascent bacterial lawn, or even filtering it. Precipitation (ammonia sulfate) and some purification (e.g. column or HPLC) may help exclude lysogenic and lytic phage from the assay.

Bacteriocins can be very stable components, not seldomly they can withstand autoclavation at 121°C (Ogunbanwo *et al.*, 2003). On the other hand, there are a number of bacteriocins that are very sensitive, even when preserved in the refrigerator. On the basis of the identified organisms in C1, a literature will need to be made to give an inventory on the possible bacteriocins together with the respective stability, which can be produced within C1.

Quorum sensing bacteria produce chemical signal molecules, called autoinducers. Their external concentration increases as a funstion of increasing cell-population. These quorum sensing molecules, bacteria signal to synchronize certain behaviors. The first signal described quorum sensing system, is the system of the bioluminescent marine bacterium *Vibrio fischeri*. The squid uses these bioluminescent organisms, which produce light when grown in high density in the light organ, to mask its shadow and avoid predation. The autoinducing compound is the acyl-homoserine lactone (AHL) 3OC6-homoserine lactone and is produced by the action of the LuxI protein (Waters & Bassler, 2005).

Other signalling molecules are likewise produced by bacteria. *Staphylococcus aureus* produces autoinducing peptides (Tenover & Gaynes, 2000). The molecule 3,4-dihydroxy-2-heptylquinoline, termed PQS, is a signal that is integral to the *P. aeruginosa* quorum –sensing cascade (Pesci *et al.*, 1999). Furthermore the compound 3OH palmitic acid methyl ester (3OH PAME) can signal through the two-component sensor histidine kinase-response regulator pair, PhcS-PhcR, to cause the plant pathogen *Ralstonia solanacearum* to switch from a motile to an infective state(Flavier *et al.*, 1997). Finaly, the presence of cyclic dipeptides have been shown to anatgonize AHL binding to cognate receptors at high concentrations(Holden *et al.*, 1999).

2.3.2 PHYTOHORMONES

Although detection of phytohormones in the environment has not yet been reported, they can become hazardous for the proper functioning of the MELiSSA-loop. If these phytohormones (that occur naturally in all plant material) are not degraded by the first three compartments, the phytohormones will reach the fourth compartment where they will be able to disturb the normal growth of the higher plants. It has been shown that the addition of phytohormones to the growth medium of higher plants increases plant growth (Clouse, 1996). In addition, phytochemicals are able to disturb the normal symbiosis in nodulating plants (Fox, 2004; Fox *et al.*, 2004). These changes can endanger the functionality of the MELiSSA and subsequently endanger the manned space mission.

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The most intensely studied phytohormones are the isoflavones present in soy. Many studies have shown that these nonsteroidal estrogens posses potent biological activities that have beneficiary effects on the consumer. The threshold intake of dietary estrogens, necessary to reach a biological effect in humans appears to be 30-50 mg/d, which is easily consumed by the inclusion of modest amounts of soy protein in the average Western diet (Burgess *et al.*, 1999). Research on the production and effect of bioactive phytochemicals in other MELiSSA plants is limited and therefore no information can be given at this point.

Plants can also produce chemical substances that resemble the character of bacterial toxins. These are called phytotoxins. The best know human effecting phytotoxins are curare (toxic alkaloid found in certain tropical South American trees that is a powerful relaxant for striated muscles; used by South American indians as an arrow poison); hemlock (from evergreen trees); strychnine (an alkaloid plant toxin extracted chiefly from nux vomica; formerly used as a stimulant); brucine (a bitter alkaloid poison resembling strychnine and also extracted from nux vomica); and nicotine (an alkaloid poison that occurs in tobacco; used in medicine and as an insecticide). In the MELiSSA loop, however it will be more important to check for toxins produced by fungal organisms, residing on the plants.

2.4 The problem of micropollutants in MELiSSA

2.4.1 COMPARTMENT I

The first compartment of the MELiSSA-loop (C_I) is the compartment where the raw biomass is degradated through liquefaction of hydrolysis. Several parameters are kept fixed to insure the proper functioning of the reactor: (i) pH is kept acidic to exclude methanogenese, (ii) the operating temperature is kept at 55°C to exclude the stable settling of pathogenic bacteria (to eliminate prions several days at 55°C is good to pasteurize the mixture), (iii) the reactor is run under anaerobic conditions to allow anaerobic fermentation yielding ammonium and volatile fatty acids.

Micropollutants will enter Compartment I via: human waste (urine and faeces) and plant waste (inedible plant material).

Literature regarding the elimination of steroid hormones under anaerobic conditions is limited because endocrine-disrupting chemicals are associated with municipal wastewater, in most cases this water is treated in aerobic wastewater treatment plants. A small number of available studies suggest that during anaerobic liquefaction steroid hormones will remain unaltered (Ying *et al.*, 2003; Ivashechkin *et al.*, 2004). No degradation of the synthetic estrogen 17α-ethynylestradiol (EE2) was observed, while the natural estradiol (E2) was very slowly degraded in a 70-day period (Ying *et al.*, 2003). Steroid hormones are secreted in a conjugated (bioinactive) form, Johnson and Williams (2004) hypothesised that mainly the anaerobic bacteria would cut the sulphate-molecule from the sulphate-conjugated hormones (Johnson & Williams, 2004). The degradation of the sulphate-molecule would endow the release of a bioactive steroid hormone in the environment. The potential of the C_I-microorganisms to

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degrade or activate the steroid hormones is actually unknown, physical degradation of thermolabile compounds does remain possible in the thermophillic compartment.

As for the steroid hormones, the potential for biological/chemical/physical transformations of PPCPs is largely unknown for microorganisms living under anaerobic thermophillic conditions. However, it can be stated that biodegradation will be highly dependent on the molecular structure of the respective compounds. Some antibiotics can be enzymatically deactivated by specific bacteria, detected in the human gut. Hence, these pathways might also be observed in the first compartment of the MELiSSA. Adsorption of compounds can also be a significant factor in the removal of micropollutants, more specifically the non-polar compounds.

2.4.2 COMPARTMENT II

The second compartment of the MELiSSA-loop (C_{II}) is the compartment where the volatile fatty acids are mineralized to carbon dioxide, which can subsequently be fed to the photosynthesizing compartments as a carbon source. *Rhodospirillum rubrum* is a bacterium with a large spectrum of metabolic pathways, but uses the photoheterotrophic metabolism in the MELiSSA.

Micropollutants will enter Compartment II via: Effluent originating from Compartment I
Rhodospirillum rubrum possesses a wide spectrum of metabolic pathways. The bacterium is able to use photoheterotrophic, photoautotrophic, chemoheterotrophic, chemoautotrophic growth, fermentation, and anaerobic respiration. However, to date, there is no reason to assume the elimination of steroid hormones by *Rhodospirillum rubrum* through biodegradation.

No biological transformations of PPCPs are expected in Compartment II if they did not occur in Compartment I. Non-biological processes, such as adsorption and photochemical transformations, do remain possible for both steroid hormones and PPCPs.

2.4.3 COMPARTMENT III

The third compartment of the MELiSSA-loop (C_{III}) is the compartment where the ammonium is oxidized to nitrate via the nitrification reaction. The nitrifying reactor is the first aerobic reactor of the loop and thus might endow new mechanisms to degrade micropollutants.

Micropollutants will enter Compartment III via: Effluent originating from Compartment II, and maybe human urine.

Compartment III is the most promising compartment with respect to hormone degradation. In humans, E2 is readily oxidised to E1, which can subsequently be transformed to E3, the main excretion product in urine. MeEE2 is immeadiately transformed to EE2, once it is taken up (Ternes *et al.*, 1999). Steroid hormones are mainly excreted as inactive conjugates of sufphate- and glucuronic acids. These conjugates lack biological activity but can act as precursors, which can be bioactivated by bacteria that are present in the environment (Ternes *et al.*, 1999; Baronti

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et al., 2000). Different studies show that effluent of wastewater treatment plants still contains many micropollutants. In aerobic batch cultures with activated sludge, the fate of steroid hormones was studied; E2 appeared to be transformed to E1, which was subsequently eliminated with no degradation products remaining (Ternes *et al.*, 1999). On average 88% of the E2 and 74% of the E1 is eliminated when treated with activated sludge (Johnson *et al.*, 2000). In batch cultures of activated sludge, EE2 appeared to be persistent while MeEE2 was observed to be readily eliminated and only a small fraction was demethylated to EE2 (Ternes *et al.*, 1999; Weber *et al.*, 2002). Activated sludge eliminates on average 85% of the E2, E3, and EE2, while the degradation of E1 appears to be less efficient and with high variance (Johnson & Sumpter, 2001). Extremely interesting is the study of the hormone degrading ability of *Nitrosomonas europaea* in comparison of activated sludge, both are able to degrade estrogens but the pure culture of *Nitrosomonas europaea* was observed to be less efficient than the activated sludge (Shi *et al.*, 2004).

2.4.4 COMPARTMENT IV(a)

In current literature nothing has been described on the effect or a possible interaction with hormones on cyanobacteria.

2.5 The influence of micropollutants on microorganisms

Several studies report on a measurable effect of the presence of PPCPs and hormones in microbial ecosystems (Moursi, 1966). Microorganisms have been shown to posses binding capacity with hormones (Sugarman & Mummaw, 1990). Even though the capacity to bind hormones strengthens the hypothesis that estrogens might alter virulence of prokaryotes, there exist no unequivocal indications that actual hormone-responsive receptors occur in Bacteria. High affinity, saturable estrogen-binding sites were found in fresh isolates and stock cultures of several species of pathogenic bacteria. More than 95% of these sites were destroyed by incubation with trypsin (Sugarman & Mummaw, 1990).

Moursi showed, by means of in vitro studies, that the responsiveness of the micro-organisms differs according to the hormonal substances used. On the one hand, two synthetic hormones (hexoestrol and stilboestrol) possessed antibacterial action on Micrococcus pyogenes var. aureus when applied in relatively high concentrations (0.05 to 10 mg/ml). On the other hand, the natural estrogens neither promoted nor reduced bacterial growth of Micrococcus pyogenes var. aureus significantly. Androgens (testosterone, testosterone propionate, and methyltestosterone) and progestational compounds (progesterone and ethisterone) promoted the growth of M. pyogenes, and Corynebacterium pyogenes significantly (Moursi, 1966). Kisidayová and Váradyová reported on the increased

Other studies also suggest the existence of a hormonal effect on the microbial cell (Buetow & Levedahl, 1964), where these compounds might be of importance on the evolution of staphylococcal infection or on the growth and metabolism of staphylococci (Yotis & Fitzgerald, 1968). It has been shown that progesterone (µg/ml-range) interferes with the

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cytochrome system, resulting in a decrease of the available energy to the cell, and thus could explain the bacteriostatic action of androgens on staphylococci (Yielding *et al.*, 1960).

Fox et al. reported on the endocrine disruption in organisms lacking an estrogen receptor. Leguminous plants such as soybean and alfalfa produce phytoestrogens to deter herbivores, to ward against fungal and bacterial pathogens, and as signalling agents to recruit soil bacteria to the plant's root system for nitrogen-fixing symbiosis (e.g. with Synorhizobium meliloti). However, they reported that EDCs can disrupt this symbiotic process, by disrupting the symbiotic gene activation (Fox *et al.*, 2004).

The peptide hormone, insuline, was shown to significantly increase methane production and dry matter degradability in whole protozoan cultures (consisting of protozoa plus bacteria) (Kisidayová & Váradyová, 2005).

Microbial decomposition and transformation of steroids has already been reported (Schatz *et al.*, 1949; Naguchi & Fukushimo, 1965; Carlstrom, 1967; Shi *et al.*, 2004; Shi *et al.*, 2004; Li *et al.*, 2005), it is unknown however, whether these steroids have a purpose as energy or carbon source or whether the transformations merely occur due to co-metabolism.

The use of antibiotics have been coined as another concern related to the dessimination of micropollutants in the environment. Not only has it contributed to the spread of bacterial resistence to antibiotics, but also it has been noted to have a negative effect on important ecosystem bacteria (Costanzo *et al.*, 2004). The rates of denitrification were observed to decrease in response to some antibiotics, but not to others. Keeping in mind that between 30% and 90% of an administered dose of most antibiotics to humans and animals are excreted in the urine as an active substance (Rang *et al.*, 1999), the key concern was the risk associated with the transfer of resistant genes from one bacterium to another (Costanzo *et al.*, 2004) and which on its turn could introduce shifts in microbial ecosystems.

2.6 The influence of micropollutants on plants

Only very few is known about the effects of hormones and PPCP's on plants. Interesting however are the studies of bisphenol A phytoremediation. Bisphenol A mono- and di-beta-D-glucopyranosides are major plant metabolities of bisphenol A (BPA) degradation. These compounds have estrogenic activity and can enter the food chain (Morohoshi *et al.*, 2004). Synthesis involved the glucosidation of unprotected BPA with glucose penta-acetate with phosphorus oxychloride as catalyst. The estrogenic activity of BPA and its mono- and di-beta-D-glucopyranosides were measured with an enzyme-linked immunosorbent assay (ELISA)-based estrogen receptor competitive binding assay and with a yeast two-hybrid assay adapted to a chemiluminescent reporter gene (for beta-galactosidase). Both methods showed that the estrogenicity of BPA was eliminated by formation of the di-glucoside, but whereas the ELISA-based method indicated that reduced activity remained in the monoglucoside, the yeast two-hybrid method showed the monoglucoside to be inactive (Morohoshi *et al.*, 2004).

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2.7 Analysing the effects of the presence of micropollutants in the MELiSSA compartments

2.7.1 METHODS TO DETECT STRESS RESPONSE OF THE MELISSA ORGANISMS IN THE PRESENCE OF MICROPOLLUTANTS

2.7.1.1 Measuring metabolic stability and stress response using flow cytometry

Certain morphological changes have been reported in the literature following temperature, oxidative or pH stress. These include compression of gas vacuoles, cell elongation or shortening, separation of the membrane from the cell wall, pore formation, alterations of the cytoskeleton, nucleus, and cell organelles, coagulation of plasma proteins and release of cell constituents to the exterior. Temperature, H_2O_2 or pH treatment is also known to induce alterations in the composition of the cell membrane, with resultant phase transitions from liquid into gel.

In phase 1 of MELGEN, we were interested in the application of flow cytometry to physiological studies of some of the Melissa bacterial strains following stresses that can happen in space. The results presented in MELGEN, phase 1, indicated that the *R. metallidurans*, *R. rubrum* and *Arthrospira* sp. strains showed different staining behaviours with a series of various fluorochromes meaning that physiological characterisation of the strains reveals a difference in the resistance of the strains to oxidative, temperature or pH stress. *R. rubrum* seemed to show higher sensitivity to temperature and oxidative stresses than *R. metallidurans*. Concerning *Arthrospira* sp., small and big filaments did not react the same way and temperature or oxidative treatment yielded varying results. In all three strains, a correlation was observed between membrane integrity and potential. Following oxidative stress, the membrane permeability and potential increased in function of the concentration of H_2O_2.

In conclusion, flow cytometry has been shown to be a fast means of obtaining information about physiological status and metabolism of the MELiSSA strains that provides individual cell information. It makes it an ideal tool not only to understand the influence of various stresses (for example, X and UV-irradiations, pressure, starvation or physical stress) on homogeneous (or heterogeneous) populations but also to be used in routine controls of the strains present in the MELiSSA bioreactors. Finally, flow cytometry with specific fluorescent probes, for example specific mRNA probes, could be used to follow changes in gene expression patterns that are consequent to stresses.

2.7.1.2 Measuring metabolic stability and stress response using 2D-Proteomics

A proteomic approach based on two-dimensional gel separation and mass spectrometry protein identification was used to detect and identify protein modifications in different stress conditions (temperature, pH variation and oxidative stress).

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First, 2D maps of *C. metallidurans* were build to validate the method of 2D proteomic mapping as a method to detect stress. In this study, we constructed a database of total cellular proteins from *Ralstonia metallidurans* CH34. This database will serve in the future as a reference to which proteins differentially expressed, consecutively to exposure to a variety of stress and environmental conditions (i.e. space conditions), will be compared. Protein identification was carried out via N-terminal amino acid sequencing, MALDI-TOF MS and tandem MS. So far 1400 different proteins were characterized out of 1600 protein spots. 99.9 % of proteins tested with the MALDI-TOF MS have been identified with success. Although the proteome map is still not complete, one could appraise the importance of proteomics for genome analyses (especially by the MALDI-TOF approach) through (1) the identification of previously undetected open reading frames, (2) the identification of proteins not encoded by the already sequenced genome fragments, (3) the characterization of protein-encoding genes spanning two different contigs, enabling their merging, and (4) the precise delineation of the amino-terminus of several proteins. Finally, this map proved a useful tool, firstly, in the identification of proteins differentially expressed in the presence of low amounts of different heavy metals and secondly, in the identification of the mechanism to use acetone as carbon source in *R. metallidurans*. These last results are the first demonstration of the ability of *R. metallidurans* to use the acetone as carbon source.

This approach and technology, particularly appropriate to begin a proteomic study from uncompleted bacterial genome will be used to detect and identify protein modifications in different stress conditions (temperature variation, oxidative stress, space conditions…) especially with the MELiSSA strains as *Arthrospira* sp. PCC8005 and *Rhodospirillum rubrum* ATCC25903.

With the DNA sequences of more genomes being completed, the sequence of *Ralstonia metallidurans* CH34 and *Rhodospirillum rubrum* genomes, a major challenge in modern biology remains the understanding of the expression, function, and regulation of the entire set of proteins encoded by a micro organism: proteomics. This information will be valuable for understanding how complex biological processes occur at the molecular level, how they differ in various microorganisms, and how they are altered in different growing conditions as space conditions particularly in the case of the MELiSSA project. The spectrum of proteins expressed in a microorganism provides that cell with a unique identity, elucidating how the protein complement changes in a cell type during development in response to environmental stimuli as oxidative stress, temperature or pH variation. Recent years have witnessed a revolution in the development of new approaches for identifying large numbers of proteins expressed in cells and also for globally detecting the differences in levels of proteins in different cell states. In recent years, protein separation methods as the two-dimensional gel electrophoresis coupled with various mass spectrometry (MS) technologies have evolved as the dominant tools in the field of protein identification and protein complex deconvolution. This approach is probably the best approach to characterise the proteome especially if the genome is partial or unknown. Among the different steps of this approach, the protein extraction is crucial for the outcome of this approach.

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In this context, an extraction protocol compatible with a proteomic approach using the two-dimensional gels electrophoresis separation was initiated for the two MELiSSA strains: *Rhodospirullum rubrum* and *Arthrospira* sp.. This approach should allow as it already has successfully been used for *C. metallidurans* to study the up- or down regulated proteins in bacteria growing in different environmental conditions as oxidative stress, temperature variation, space conditio by a differential comparison. The preliminary results showed already more known proteins up-regulated during temperature variations (GroEs, GroEL, Dnak and HtpG) and oxidative stress (GroES, Thioredoxine, RecA and Alkyl hydroperoxyde reductase subunit C). For *Rhodospirillum rubrum* ATCC25903 a number of stress-induced proteins were already identified. Under conditions of thermic stress, the chaperone proteins GroEL, GroES, DnaK and HtpG were induced. Under oxidative stress the induced proteins were identified as thioredoxin, alkylhydroperoxyde reductase, HtpG and RecA. Thioredoxin and alkylhydroperoxyde reductase are proteins known to be induced under oxidative stress. HtpG on the other hand is a typical heat shock protein, and RecA is involved with gene repair. Unexpectedly there was no induction of superoxide dismutase observed. To obtain an induction of superoxide dismutase it was probably necessary to increase the concentration of H_2O_2 or the time of incubation. The induction of HtpG during oxidative or thermic stress has already been observed in the literature and indicates that certain proteins can be induced as a general response against stress.

In conclusion, the proteomic approach based to 2-DE coupled to mass spectrometry has been shown to be a excellent means of obtaining information about physiological status and metabolism of the MELiSSA strains that is almost impossible to obtain in any other way, especially with an non or partially characterise genome. It makes it an ideal tool to understand in the molecular level the influence of various stresses (for example, X and UV-irradiations, pressure, starvation, pH stress) on the strains present in the MELiSSA bioreactors. Finally, proteomic approach could be used to understand the behaviour of the MELiSSA bioreactors in different growth conditions.

2.7.1.3 Measuring metabolic stability and stress response using MALDI-TOF-MS

In the context of the MELiSSA loop, temperature, pH and oxidative stresses are a perplexing risk for *Rhodospirillum rubrum* ATCC25903 and *Arthrospira* sp. PCC8005 and understanding the physiological mechanisms induced by those stresses. In phase 1 of MELGEN we reported the success of mass spectrometry as a powerful technique to monitor with a high degree of statistical resolution, cell modifications during temperature, oxidative ad pH stress of *R. rubrum* and *A. platensis*. Moreover, these results confirmed identical observations obtained by another approach based to the flow-cytometry analysis.

A variety of characteristics of microorganisms (both phenotypic and genotypic) are used to differentiate between strains and often to determine the relationship between strains of bacteria present on the bacterial cell wall are surface components, which give rise to a unique pattern of biomarkers. These surface components are important since they mediate the contact between

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the cell and the environment. This unique population of molecules can be rapidly desorbed from the cell surface; ionised and analysed by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS) resulting in a mass fingerprint. This approach should be rapid and yet be based on a sufficiently large group of proteins so that a unique mass spectral fingerprint for the organism or strain can be obtained. Some of the difficulties involved in the MALDI-TOF mass spectral analysis of bacteria have been the complexity of the spectra, the large mass ranges used, and the subtle differences that may be observed in spectra from related stains. Moreover, reproducibility is a difficult problem in experiments that involve MALDI of cells, and large variations have been seen in spectra obtained under different conditions. There are many experimental parameters that can have a strong effect on the observed mass spectra (minor variations in the sample/matrix preparation, in the experimental conditions used to bacterial extraction or analysis). In MELGEN, phase 1 we reported on the success of the intact mass spectrometry to study statistically and with a high reproducibility *Rhodospirillum rubrum* ATCC25903 and *Arthrospira* sp. PCC8005.

In phase 1, the following stresses were detected: temperature, oxidative and pH stresses. Another initially perplexing issue was the time dependence of spectra noted upon analysis of bacteria from cultures. Bacteria respond rapidly to environmental changes, and the production of stress proteins or other similar changes in cellular processes results to a modification in the spectra for reasons associated with the biology of bacteria. Certain morphological changes have been reported in the literature following temperature, pH or oxidative stress. These include compression of gas vacuoles, cell elongation or shortening, separation of the membrane from the cell wall, pore formation, alterations of the cytoskeleton, nucleus, and cell organelles, coagulation of plasma proteins and release of cell constituents to the exterior. Temperature and pH treatment is also known to induce alterations in the composition of the cell membrane, with resultant phase transitions from liquid into gel.

Our results demonstrated that MALDI-TOF of intact cells could be used to monitor biological changes, such as those that occur during oxidative, temperauture and pH stress. This whole-cell approach to the monitoring of biological changes would, of course, only allow a fraction of the environmental-response related proteins to be detected, compared to similar studies with isolated or fractionated protein samples, but the rapidity of the analysis often offsets this limitation. Moreover, our results indicate that the *R. rubrum* and *Arthrospira* sp. strains showed different behaviours to oxidative, pH and temperature stresses. *R. rubrum* seemed to show higher sensitivity to temperature, pH and oxidative stresses than *Arthrospira* sp. Interestingly, these results were in good concordance with those obtained by flow cytometry. Moreover, in both strains, a correlation was observed between membrane integrity and the mass spectrum modifications observed during stress variations.

Nevertheless, the MALDI-TOF-MS approach is not able, without a prefractionation, to monitor specific physiological modifications from heterogeneous populations of a bacterial strain such as *Arthrospira* sp., which is constituted of small and big filaments.

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3 AXENICITY STUDY

It is important to control the axenicity of the MELiSSA compartments because contaminants constitute a major concern in the proper functioning and maintenance of a closed artificial ecosystem. Contamination by biomolecules, normally not produced in a given compartment, may result into instabilities or even the collapse of the system, rendering the biomasses of *Arthrospira* and *Rhodospirillum* unsuitable for human consumption. In addition, contamination of C4 by pathogenic organisms almost certainly will pose serious risks to human health. One should also keep in mind that the liquid medium is recycled, causing an enrichment in organics and metabolites. This may favour the development of contaminants or may give rise to unstable reactor conditions.

Biological contaminants can be of bacterial, fungal, protistic, or viral origin. Metazoan contamination (e.g. molluscs, nematodes, rotifera, etc.) of the MELiSSA cycle is rather unlikely. However, the risks mediated by emerging pathogens and other infectious agents (e.g. prions) should be assessed (Rose & Grimes, 2001).

3.1 Sources of microorganism contamination

3.1.1 MELISSA BACTERIA

MELiSSA bacteria may escape from their intended compartment, causing severe problems in other parts of the cycle (e.g. by affecting the growth of indigenous strains).

It is equally important to regard the C1 consortium as a likely source of human or plant pathogens. Especially in prolonged occupation of the space craft or station, where human waste is recycled into food and food recycled (by the humans) to waste, one should be aware of the fact that some candidate pathogens may become actually highly virulent after repeated passage through a human host (Walther & Ewald, 2004; Andre & Hochberg, 2005).

3.1.2 SURFACE AND AIRBORNE BACTERIA

Surface and airborne contaminants, like those that can be found in space craft and space stations (Guarnieri *et al.*, 1997; Klintworth *et al.*, 1999; Pierson, 2001), may have been introduced by previous visits of humans or the use of test animals. They may have escaped detection and removal due to inadequate diagnostica and insufficient filtration (or other means of sanitation). In this respect, studies at the Institute for BioMedical Problems (IBMP, Moscow) have shown the presence of Gram-positive bacteria (i.e. bacilli and cocci) and fungi (i.e. aspergillic conidia) in air and surface samples of the MIR station. Samples were taken in 12 locations of the station during a 10-period ('87-'96) and analysed on microbial content using the VITEK60 system (BioMerieux, France). In total, 40 bacterial and 25 fungal different genera could be recognized, with 108 and 126 species detected, respectively. Some of these species have clear pathogenic potential (e.g. *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Aspergillus fumigatus,* etc.). The most abundant bacterial genus found in both surface and air

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samples was *Staphylococcus*, followed by *Corynebacterium* and *Bacillus*, while the fungus *Penicillinium* could be found in nearly 80% of all samples. Air contamination levels for both bacteria and fungi fluctuated in time between 100 and 10,000 colony forming units per cubic meter (cfu/m^3). Surface samples reached contamination levels that were usually between 10^2 and 10^6 cfu/m^2 but surface probed contaminations of 10^7 cfu/m^2 or higher were occasionally reported. In addendum the survey of the environmental biocontamination on board the International Space Station by Novikova *et al.* (2006) on the current state of knowledge on airborne and/or surface recovered samples in MIR and the ISS, has been added.

3.1.3 HUMAN BEINGS

It is well known that the human body harbours hundreds of different microbial species, but very little is known about their occurrence, abundance, and spread, in particular under space conditions. The presence of a bioreactor in which some of these organisms may actually flourish (due to the abundance of nutrients), is of considerable concern, taking into account the possible propagation of dangerous pathogens in a long-term self-contained environment. Recently, a call has been made to consider a 'second human genome project' (Relman & Falkow, 2001), i.e. to undertake a large-scale genomic sequence survey of the major microbial niches within the human body (i.e. mouth, intestinal tract, skin, etc.). In the words of Relman & Falkow: "The human gut microbiome is as much an unexplored frontier as the collection of life found at deep-sea thermal vents, if not more so.". MELiSSA, and manned space exploration on a whole, would certainly benefit by such an inventory.

3.2 Sources of other contaminants

Other contaminants like bacteriophages and free DNA, which can create disturbance of the genetic or metabolic stability of the MELiSSA compartments, are important to be considered and will play a role in horizontal gene transfer between bacteria (3.3.1.).

A bacteriophage is a virus that infects bacteria, which could eventually induce lysis, or dissolution of the cell. Bacteriophages, or phages, have a head composed of protein, an inner core of nucleic acid - either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) - and a hollow protein tail. A particular phage is very specific and can usually infect only one or a few related species of bacteria. A virus infects a bacterial cell by first attaching to the bacterial cell wall by its tail.

Some DNA phages, called temperate phages, only lyse a small fraction of bacterial cells; in the remaining majority of the bacteria, the phage DNA becomes integrated into the bacterial chromosome and replicates along with it. In this state, known as lysogeny, the information contained in the viral nucleic acid is not expressed. A lysogenic bacterial culture can be treated with radiation or mutagens, inducing the cells to begin producing viruses and lysogeny. Lysogenic phages resemble bacterial genetic particles known as episomes. Incorporated phage genes are sometimes the source of the virulence of disease-causing bacteria (Columbia encyclopedia).

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The risk on the presence of human virusses is described in TN80.13.

3.3 Problems that may arise from contamination of the axenic compartments in MELiSSA

More research would be necessary to determine the precise effects of contaminants on the biomass quality of *Arthrospira* and *Rhodospirillum*. If the cultures collapse, the effect is the disappearance of the food source. However, it is conceivable that contaminants might have more subtle effects on biomass quality. This remains to be analysed.

3.3.1 THE PROBLEM OF HORIZONTAL GENE TRANSFER

Transfer of genetic material from one microbial cell to another may involve one of the following three mechanisms: transformation, conjugation and transduction. Transformation is a process by which certain bacterial cells import soluble DNA from their surroundings. Bacteria known to be capable of natural transformation (i.e. that are naturally competent) include *Helicobacter pylori* and *Streptococcus pneumoniae*. Conjugation (which, unlike the other two forms of transfer, requires cell-to-cell contact) is often mediated by plasmids (self-replicating, double stranded extrachromosomal DNA elements). In transduction, a small chromosomal fragment of the host is incorporated into a maturing phage particle, which upon release may infect a new host, injecting the genetic material from the former host into the new host.

From the three forms of gene transfer, transformation is by far the unlikeliest to occur in any environment (but the test tube). In transformation, DNA molecules need to encounter the recipient microbial cell, and the recipient cell needs to be competent. Natural competency of the four principle MELiSSA strains (thus excluding the unknowns of C1) is not well documented. In addition, the presence of extracellular endonculeases (i.e., from lysed cells) would dramatically lower the concentration of free ‘naked’ DNA in the medium or culture. Although very little is known about the existence of restriction-modification systems in *Rhodospirilum*, *Nitrosomonas*, and cyanobacteria generally produce large amounts of potent endonucleases.

Transduced DNA is better protected by the phage coat proteins, and phage particles are more abundant, both in open as well as in closed environments, so the chance for an encounter between the phage and its cellular host-to-be is much larger (there is a multiplication factor involved because one phage infected cell may release tens to hundreds of new phage particles). Double-stranded DNA (dsDNA)-containing bacteriophages are very likely the most numerically abundant group of similar organisms in the biosphere, and nearly 4,500 different dsDNA phages - capable of infecting a large diversity of bacterial hosts – are known. The vast majority of these phages have common ancestry and they undergo profuse exchange of functional genetic elements drawn from a large shared pool (Hendrix *et al.*, 1998). Clearly, they play an important role in microbial evolution. Studies carried out by various laboratories throughout the world have demonstrated that both chromosomal and plasmid DNA can be

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successfully transduced in natural environments ranging from sewer plants to rivers and lakes. Two important environmental factors which affect virus-host interactions are the metabolic state of the host and the exposure of the host to DNA-damaging stresses such as solar UV light (reviewed by (Miller, 2001)).

The monitoring of gene dissemination via plasmid-mediated conjugative transfer is of special importance in confined environments, especially in the case of axenicity disruption. Disruption of axenicity may occur either via CI or via direct outside contamination. The disseminating vehicles also deserve close attention and especially the conjugative plasmids with a broad host range (BHR plasmids) need to be scrutinised. While most plasmids have a narrow host range, generally restricted to the genus or the species to which the host where they were found belongs, plasmids are able to easily cross taxonomic boundaries. Not only are they able to self transfer and to disseminate the genes they carry, but they also often mobilise other plasmids that are unable to self transfer or even may trigger release of chromosomal bound genes or transposable elements. This feature could be very critical if the mobilised information is related to virulence determinants. BHR plasmids are also able to capture genes from other bacteria to the advantage of their hosts (for a review, (Szpirer *et al.*, 1999)).

Conjugative BHR plasmids belong mainly to IncP and IncW families: they generally carry genes for resistance to antibiotics and/or to mercury (IncP and IncW) or genes involved in the degradation of recalcitrant organics (IncP). In addition, novel BHR plasmids have been directly selected from environmental samples (Top *et al.*, 1994) by so-called triparental exogenous isolation. The selective action of this method does not rely on resistance or catabolic markers as it is the case with the isolation of natural plasmids but directly acts on the plasmid capacity to mobilise genes. BHR plasmids found by triparental exogenous isolation are often cryptic as it is the case with pIPO2 (van Elsas *et al.*, 1998) and pMOL96/98 (Gstalder *et al.*, in preparation). pIPO2 and pMOL96 share some characteristics with IncP plasmids, but clearly belong to another Inc group or plasmid class. This group also includes a BHR mercury resistance plasmid (Schneiker *et al.*, 2001) that was found in a *Sinorhizobium* by biparental exogenous isolation (Bale *et al.*, 1987). These three plasmids (pMOL96/98, pIPO2 and pSB102) were isolated from various nonclinical environments (soils polluted with oil, wheat rhizosphere and legume nodules respectively) and illustrate the presence, in various soil environments, of unnoticed BHR plasmids that may play a role in gene dissemination or capture. In this respect, pMOL96/98 and pIPO2 were shown to have a retrotransfer phenotype that even looks more efficient that the retrotransfer phenotype described in IncP plasmids (Szpirer *et al.*, 1999). Cyanobacteria may contain conjugative plasmids (Billi *et al.*, 2001). They are accessible to BHR plasmids that were used to introduce vectors or transposons, but restriction is clearly an important barrier limiting the access of foreign DNA (Wolk *et al.*, 1984; Kreps *et al.*, 1990; Sode *et al.*, 1992) (Marraccini *et al.*, 1993; Ren *et al.*, 1998). Concerning nitrifying bacteria, there is up to now only one report that describes the presence of plasmids in one strain of *Nitrosomonas* (Yamagata *et al.*, 1999). There is no report of any plasmid-mediated conjugative transfer from or to these bacteria. *Rhodospirillum rubrum* is very accessible to BHR plasmids (Olsen & Shipley, 1973) and to conjugative plasmids that were currently used to introduce transposons in this strain (Bao *et al.*, 1991; Jiang *et al.*, 1998).

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Among the MELiSSA strains, *R. rubrum* is certainly the strain that looks the most permeable to plasmid–mediated gene dissemination. This feature is also enhanced by the crucial position of *R. rubrum* in the C2 compartment just downstream of the C1 compartment.

Furthermore two particularly interesting phenomena can be highlighted as well: (1) The presence of R-bodies was reported in *Rhodospirillum centenum*. R-bodies were known mainly as diagnostic features by which (bacterial) endosymbionts of paramecia were identified as kappa particles ("killing particles"). (Pond *et al.*, 1989; Heruth *et al.*, 1994). R-bodies have no plasmid or phage features and are probably not of eukaryotic origin. (2) The eukaryotic transposable elements of the "mariner" family are now known to transpose to bacteria: this was reported for *E. coli* and mycobacteria (Rubin *et al.*, 1999). It is not yet known if the mariner elements may directly transpose from insects or other eukaryotes to bacteria. Therefore it is too early to conclude if the observation would deserve some attention in the context of MELiSSA.

3.3.2 THE PROBLEM OF BACTERIOCIDAL SUBSTANCES

Some possible contaminants are producers of toxins that can be detected by proteomics (see above). A screening of 146 strains from the Pasteur Culture Collection for genes of peptide synthetases (hepatotoxins), failed to reveal the presence of these genes in *Arthrospira* strains PCC8005 and PCC7375, whereas they were found in 75,3% of the tested (Christiansen *et al.*, 2001). Thus, it is likely that the MELiSSA strain does not contain the genetic information to produce this particular hepatotoxin. However, caution should be taken. While only a few cyanobacterial genera produce hepatotoxic microcystins, the production of hepatotoxic nodularins appears to be limited to a single genus. Generally, production of neurotoxins has been considered phylogenetically unpredictable (Cox *et al.*, 2005). Recently a study was published, which reported that the neurotoxin β-*N*-methylamino-L- alanine may be produced by all known groups of cyanobacteria (Cox *et al.*, 2005). The production of this neurotoxin should therefore be tested in the MELiSSA cyanobacterial strain *Arthrospira* sp..

Even if no known phages were described in the literature, it is possible that the MELISSA strains harbour them (especially the inhabitants of C1). Cyanophages were found in marine *Synechococcus* (Fuller *et al.*, 1998; Lu *et al.*, 2001), filamentous heterocystous *Anabaen*a and *Nostoc* strains (Bancroft & Smith, 1988), and LPP strains (*Lyngbya-Phormidium-Plectonema*). Mass lytic processes were also observed in microbial communities colonised by filamentous cyanobacteria (van Hannen *et al.*, 1999). Lysogeny has been observed in a marine *Synechococcus* (McDaniel *et al.*, 2002). This may be of relevance for the Melissa cyanobacteria as the lysogenic mode of life (where the bacteriophage is integrated in the chromosome of the host) may revert to the lytic mode of life under induction of UV light or other radiations. Phage induction should be tested in *Arthrospira* sp. under a variety of conditions. On the other hand sunlight could also inactivate fecal bacteria and bacteriophages (Sinton *et al.*, 1999; Sinton *et al.*, 2002).

There is no report about the presence of phages in *Nitrosomonas*; phage-like bodies were reported in a series of German papers published in 1974/1976 for *Nitrobacter* (Bock *et al.*,

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1974; Peters, 1974; Westphal & Bock, 1974). No phage was reported for *R. rubrum*, although a rhizobiophage may integrate in the chromosome of *R. rubrum* in a tRNA gene. In related bacteria: two observations on *Rhodobacter capsulatus* are worth mentioning: (1) This bacterium carries a gene transfer agent (GTA) which is a defective prophage able to "constitutively" transduce bacterial DNA fragments (Lang & Beatty, 2000; Lang & Beatty, 2001; Lang & Beatty, 2002). It would be of interest to check the presence of such a GTA in *R.rubrum* or at least of the corresponding genomic sequences. (2) It also produces cis-vaccenic acid, which is an antiviral substance (Suwanto & Kaplan, 1991). In the same perspective of bacterial self-defence against competitors or parasites, a review about the production of bacteriocins in *Athiorhodaceae* (a former name for non-sulfur purple photosynthetic bacteria, thus including *Rhodobacter*, *Rhodospirillum* and the like) has to be mentioned (Guest, 1974).

3.3.3 THE PROBLEM OF PLANT PATHOGENS

An in depth description of viral, fungal and microbial plant pathogens is described in TN80.13.

3.4 Controlling axenicity

3.4.1 THE PROBLEM OF UNCULTURED AND UNCULTURABLE MICROBES

In 1990 about 10 divisions of the domain *Bacteria* were known. Now nearly 50 have been described and this remarkable expansion in our knowledge of bacterial biodiversity has occurred entirely due to the recent explosive growth of molecular approaches (Amann *et al.*, 1995; Hugenholtz & Pace, 1996; Pace, 1997) (Table 1). At least 15 of these divisions are currently known only from sequences - most commonly, from their 16S rRNA - and have no cultured representatives (Hugenholtz *et al.*, 1998; Dojka *et al.*, 2000; Suzuki *et al.*, 2001).

Table 3 Microbial diversity – known and estimated species. From (Cowan, 2000).

Group	Estimated total	Known species[b]	Proportion known of total (%)
Viruses	130,000[a]	5,000	[4] [c]
Archaea	? [d]	<500	?
Bacteria	40,000 [a]	4,800	[12]
Fungi	1,500,000	69,000	5
Algae	60,000	40,000	67

[a]These values are substantially underestimated, possibly by 1 to 2 orders of magnitude
[b]These values date from the mid-1990s and will have increases by 10-50%
[c]Blocked parentheses indicate that these values are probably gross underestimates
[d]16S rRNA sequence analysis of different biotopes suggests that archaeal species represent a much higher proportion of in situ diversity than is indicated by microbial culture studies

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Plate counts of bacteria from natural habitats, such as soil, freshwater and the sea are much lower than direct total counts and it is accepted that less than 1% of these bacteria are actually culturable with the current enumeration methods (if similar effort was put into culturing these bacteria as has been expended on culturing bacteria of medical importance over the last century, then most could probably be cultured) (Table 2).

Table 4. Estimates of the proportion of 'unculturable' micro organisms in various terrestrial and aquatic biotopes. From (Cowan, 2000).

Biotope	Proportion of culturability (%)
Seawater	0.001 - 01
Freshwater	0.25
Mesotrophic lake	0.1 - 1.0
Unpolluted estuarine waters	0.1 - 3.0
Activated sludge	1 - 15
Sediments	0.25
Soil	0.3

Molecular approaches have also been used to detect and identify uncultured bacteria in men (Tanner *et al.*, 1999; Rolph *et al.*, 2001). These developments are particular important in the early detection of emerging pathogens (Relman, 1997; Relman, 1998; Relman, 1999) and for systematic surveys of human endogenous bacterial flora (Kroes *et al.*, 1999).

Recently, many new species of *Archaea* (the 'third domain of life') have been characterised by sequence analysis only (Barnes *et al.*, 1996; DeLong, 1997). *Archaea* are evolutionary unique prokaryotes, as genetically distant from *Bacteria* as they are from *Eucarya* ("eukaryotes"). Cultivated and well-characterised archaeal groups include the extreme thermophiles, extreme halophiles, and the methanogens. Lately, less 'extremophilic' archaea have also been found in aerobic marine habitats, and some of these archaea are widely distributed and abundant components of marine plankton (DeLong, 1998). Uncultivated psychrophilic marine *Archaea* even have been found to thrive in Antarctic waters at 1.5°C. Although it is unlikely that *Archaea*, by the standards of what we know today, are present in spacecrafts, let alone in MELiSSA, we can not entirely exclude the possibility of contamination by a hitherto unknown archaeal species, in particular in respect to their natural resilience to environmental factors. We should also keep in mind that space craft (and the people involved in their operation and maintenance) may have been in contact with archaeal species in the context of scientific experiments, past and future.

The term **"**viable but nonculturable**"** (VBNC) has been coined to describe a state from which bacterial cells can not be recovered, but in which they maintain certain features of viable cells, such as cellular integrity and activity. It appears to be a common observation that bacteria enter such a "VBNC" state under environmental or laboratory conditions (reviewed by (Colwell & Grimes, 2000). This "non-recoverable" state has often been interpreted to be a consequence of dormancy. Is it however equally possible that "VBNC" cells dwell in a genetically determined "refractory" state other than dormancy in which cell division may be blocked? Or "VBNC" cells are perhaps merely injured or debilitated cells by the exposure to stressful conditions.

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Non-recoverable cells that have lost their viability may still play significant roles in ecology and epidemiology. Some toxins, for example, may be maintained or even produced in cells long after the ability for proliferation has been lost. Furthermore it is plausible that maintenance of cellular stability could allow for persistence of genetic material in the environment even if the organism itself has definitely lost its capability for propagation. This "surviving DNA" might serve as a pool of genetic traits that can be passed on to other organisms by transformation (see further).

One of the controversies that have plagued modern microbiology in recent years has been the reports on so-called nanobacteria (Olsen, 2000). The term 'nanobacteria' was first introduced by geologists to describe coccoid-shaped particles, with diameters of approximately 0.1 μm, in scanning electron micrographs of rock and minerals (Folk, 1993). Although similar structures were found on the surface of a freshly fractured Martian meteorite, leading to the belief that nanobacteria may be relics of primitive live (Sears & Kral, 1998), no hard evidence was put forward that such particles indeed represent free-living cells. Adding to the feud was the possible biological evidence for the existence of a group of small micro organisms, collectively referred to by biologists as 'nanobacteria'. These bacteria were reportedly isolated from human serum and kidney stones (Kajander & Ciftçioglu, 1998) and were thought to be responsible for biomineralisation and extraskeletal calcification. According to Kajander and co-workers, nanobacteria are present in up to 80% of commercial lots of FBS (Fetal Bovine Serum), most kidney stones and dental pulp stones, tooth surfaces, and saliva. Based on results of 16S rDNA sequencing, bovine and human isolates of nanobacteria have been tentatively assigned to the α-2 subgroup of proteobacteria (which also includes the intracellular pathogens *Brucella* and *Bartonella*) (Kajander & Ciftçioglu, 1998). Nonetheless, a recent paper by Cisar *et al.* (Cisar *et al.*, 2000)showed that the putative nanobacterial 16S rDNA sequences were indistinguishable from those of an environmental micro organism, *Phyllobacterium mysinacearum*, previously detected as a contaminant in PCR. In addition, molecular examination of decalcified biofilms failed to detect nucleic acid or protein that would be expected from a living entity and there was no shred of bacteriological evidence for culturability. Cisar *et al.* (Cisar *et al.*, 2000) however leave open the backdoor by their concluding remark: "…continued studies to identify and characterize the primary nucleators of these important clinical conditions are needed, regardless whether the molecules in question are of host and/or microbial origin". The important lesson to take home here is that the potential existence of unusual microorganisms (such as nanobacteria) should not be formally excluded based on negative evidence; rather, their (suggested) existence must be established by a defining set of unambiguous criteria. In the context of the MELiSSA project, in particular in respect to the unknown composition of C1, the lack of information on possible environmental and intracompartemental contamination, and the possible problems surrounding axenicity, a similar cautious approach is recommended.

3.4.2 METHODS TO CHECK AXENICITY

Cultivation: Inoculation on solid media or in broth is the most classical method to detect the presence of pathogens in the clinical applications. Since Pasteur and Koch, many culture media

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and a great number of tests were developed and specifically enable the microbiologists to identify the micro organisms that grow (Hobson *et al.*, 1996).

Microscopic techniques (light, confocal, electron…) are useful when the organisms have different morphologies. For example, the presence of bacterial contaminants in *Arthrospira* cultures could be detected after staining with DAPI. Using fluorescence microscopy, the *Arthrospira* appear red due to their autofluorescence, whereas the bacteria appear dark blue (stained DNA) and the cell debris that have a similar size to the bacteria appear light blue. The confocal microscopy has been used to study the interactions of bacteria with food, and show their spatial localisation (Takeuchi & Frank, 2001).

Flow cytometry: This method has been applied in conjunction with viability markers for rapid counting of yeast, mould and bacterial cells in food products (Laplace-Builhe *et al.*, 1993), and in milk (Gunasekera *et al.*, 2000). A recent development is using a laser-induced fluorescence coupled with flow cytometry to detect single contaminants in real time (Johnson & Sumpter, 2001).

Flow cytometry is a method for quantitating components or structural features of cells primarily by optical means. Although it makes measurements on one cell at a time, it can process thousands of cells in a few seconds, giving a more representative idea of nature, and allowing more meaningful extrapolation. Since different cell types can be distinguished by quantitating structural features, flow cytometry can be used to count cells of different types in a mixture. Flow cytometry has great potential as a rapid, automated tool for ecological studies of micro-organisms. It is used for detection and characterization of aquatic bacteria, analysis of bacterial populations present in soils and sediments, detection of bacterial food contamination, analyses of intestinal flora and drinkable water. Flow cytometry can detect and separate specific populations of bacteria by using the cell sorting.

However, within the frame of MELGEN, it has been shown that it was impossible to distinguish a contaminant from a *R. rubrum* culture (TN70.11). A fluorescent probe was developped targetting against *R. rubrum*. By visualising *R. rubrum* by immunolabelled rRNA targetted oligonucleotide probes. It was expected to enable the separation between target and contaminating species on the basis of discriminating between fluorescent and non-fluorescent cells. However, the spirillum shaped bacterium intercallated with itself as well as with the contaminant. The cultures where hence difficult to resuspend, resulting in poor separation of the target and contaminating organism. On the other hand, flow cytometry presented to be an excellent tool to detect contaminating single cell organisms in the background of the filamentuous cyanobacterium *Arthrospira* sp..

Another way of detecting the presence of contaminants is to monitor changes in the metabolic parameters of the culture. Detection of respiration, metabolism, enzymes … of the contaminants in the supernatant (for example, metabolisation of a specific substrate) might be used to find particular bacteria, using an indicator that changes of colour or fluoresces when cleaved from the specific substrate. This is used for coliforms (Hobson *et al.*, 1996).

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Detection of contaminants by proteomics: The proteomics techniques (Reverse phase capillary high performance liquid chromatography – electrospray ionisation mass spectrometry, gas chromatography-tandem mass spectrometry, MALDI-TOFS) are offering new developments to detect microbial contamination. Mass spectrometry in combination with novel bio-informatics provides a powerful new strategy for the rapid speciation and typing of microorganisms. This revolutionary Bacterial "Mass-Fingerprinting" approach offers greater sensitivity, selectivity and speed of analysis compared to classical identification methods in clinical microbiology, food science, biotechnology, water quality and pharmaceutical analysis. The method applies proven biopolymer Mass Spectrometry techniques to the analysis of intact bacteria the intact cell MALDI-TOF-MS (ICM-MS). The method allows the unique population of macromolecules expressed on the surface of bacteria to be rapidly sampled and characterised by molecular weight. The resulting mass spectrum provides a unique physico-chemical fingerprint for the species tested.

Mass-Fingerprints of unknowns can be reliably matched against databases of quality controlled reference mass spectra, leveraging this simple analytical method into a powerful new tool for real-time detection and sub-typing of bacteria. Mass-Fingerprinting is very rapid - the entire process from sample preparation to result takes only a few minutes for each test micro organism. Sample preparation is quick and easy - intact cells from primary culture are smeared across a stainless steel target plate and allowed to co-crystallize with a UV-absorbing matrix. After drying, the target is placed into the MALDI-TOF mass spectrometer. The microorganisms in the matrix are illuminated with a pulse from a nitrogen laser (337 nm). The matrix absorbs energy from the laser and macromolecules from the surface of the micro organisms are desorbed and ionised. The resulting ionised macromolecules are mass analysed and the results reported as a mass spectrum - a plot of mass (X axis) versus abundance (Y axis). The Mass-Fingerprint of the test microorganism is then submitted to the MicrobeLynx™ search algorithm, which challenges an appropriately selected database from a range of quality controlled bacterial reference mass spectra. A bacterial fingerprinting database (>1500 different bacterial species) is now accessible from the Manchester Metropolitan University in collaboration with the National Collection of type Cultures (NCTC), Central Public Health. This technique could be used to identify the bacterial population in MELiSSA compartment 1 and to characterize a possible contamination present in the other MELiSSA compartments (Bright *et al.*, 2002). The application of this technique to viruses, bacteria, fungus and spores was reviewed by Fenselau *et al.* and Lay (Fenselau & Demirev, 2001; Lay, 2001). Proteomic approach has been be used to identify the possible contamination from supernatant of bacterial cultures. Mass spectrometry can be used to isolate, quantify and identify proteins (extracellular proteins: toxins, enzymes…) that may be characteristic of some contaminants. This was applied to the exoproteins of a clinical isolate of *Staphylococcus aureus* where 3 exoproteins and 3 toxins were found (Kawano *et al.*, 2000). To concentrate the bacteria from dilute samples before analysis by MALDI-TOF-MS, lectin-derivatized surface was used (Bundy & Fenselau, 1999). To study bacteria in dust, 3 markers were analysed by GC-MS by (Szponar & Larsson, 2001). Ergosterol was a marker of fungal biomass (Saxena *et al.*, 2001), muramic

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acid indicated peptidoglycan (and thus bacteria), and 3-hydroxy fatty acids was a marker of endotoxin. This method allowed detecting trace levels of contaminating organisms.

Molecular methods are versatile tools and can be used (combined or not with visualisation by microscopy or flow cytometry) to detect the nucleic acids of the contaminants. Molecular techniques are already used in clinical medicine and can be adapted for environmental testing. Gene probes are being used that are highly specific and capable of detecting genetic sequences of DNA and RNA common to or conserved in pathogens such as *Salmonella* and *Legionella*, two disease-causing microbes. Researchers have now developed PCR, gene probes, and DNA "fingerprinting" techniques to detect intestinal bacteria and viruses in seawater and seafood" (Rose & Grimes, 2001). In general, PCR amplification must be thoroughly validated, as false-positive and false-negative results could occur (Vaneechoutte & Van Eldere, 1997). It is used to detect rapidly specific pathogens in samples, including viruses, slowly growing bacteria, fastidious or not-yet-cultivable bacteria, fungi and protozoa (Pillai, 1997). It is more difficult to use when the identity of the contaminants is unknown.

Most molecular tools used in clinical settings are targeting well known pathogens and enable a fast detection. Examples are the detection by amplification of 16S rDNA of *Bacillus cereus* group bacteria (Hansen *et al.*, 2001), *Staphylococcus aureus*, *Pseudomonas aeruginosa* and Enterobacteriaceae (Merker *et al.*, 2000), *Campylobacter* species (Magistrado *et al.*, 2000), *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli, Aspergillus niger* (Jimenez et al., 2000).

When PCR of species-specific genes is possible, a modification using Direct Labeling and Detection Procedure (DLDP) was shown by Gorelov *et al.* (Gorelov *et al.*, 1996) to detect less than 20 CFU of bacteria in human fluids. Other methods and kits have been published in the last years, targeting various bacteria in different kind of samples (food, clinical samples, etc). A DNA probe kit, using colorimetric DNA/rRNA sandwich hybridisation in microtiter wells was shown to detect 10^5 CFU/ml in pure culture of *Salmonella* spp (Namimatsu *et al.*, 2000). A chemiluminescent in situ hybridisation (CISH) using Peptide Nucleic Acids (PNA) probes could provide a fast detection of individual *Pseudomonas aeruginosa* or other bacterial microcolonies on membrane filters (Stender *et al.*, 2000; Perry-O'Keefe *et al.*, 2001). PNA molecules are pseudopeptides where the sugar phosphate backbone of DNA was replaced by a polyamide backbone. They behave like DNA for hybridisations but are more specific and stable. With the advent of Rapid-Cycle Real-Time PCR, PCR assays for routine use in clinical diagnostic testing are currently assayed and perform better in speed and sensitivity for most micro organisms tested (streptococci, *Bordetella pertussis*, diverse viruses) (Cockerill & Smith, 2002).

Although methods are increasingly sensitive and fast, there is one major drawback. Rarely studies have been performed on the detection of contaminating species in a background of a large volume of target organisms. The proteomic approach has been tested within the framework of MELGEN. MALDI-TOF mass spectrometry could not detect the presence of

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contaminants to satisfying levels. At best only up to 1 contaminant per 156 targed organisms, could be detected.

Most satisfying where the PCR based detection methods, revealing 1 *R. metallidurans* cell in a background of 100,000 *R. rubrum* cells. However, this technique seems only applicable when the contaminating organism is known. If this technique could be used to detect an unknown contaminant, it would be very powerfull. Therefore a system was necessary to develop an enrichment of the contaminant at any level of the quantification procedure. Preliminary results reveal that discriminatory PCR resulted in the most effective and practical analysis. For example can the use of PNA probes, targeted specifically against the primary organism, thereby allowing a positive discrimination on the amplification of contaminating DNA (TN70.12).

4 SAMPLING, PREPARATION AND REQUIREMENTS FOR THE STUDY ON THE GENETIC/METABOLIC STABILITY AND AXENICITY IN BELLISSIMA

4.1 General precautions

It is an absolute prerequisite that growth of experimental and reference cultures and sampling from the reactors are carried out in a controlled and sterile way, as even minute quantities of contamination. For taking samples from the MELiSSA reactors, different strategies need to be deployed, depending on the compartment, but great care must be taken that at no time there is contact with personnel, and as little as possible contact with the open environment (e.g., by treating reactor outlets and recipient openings with a blue flame during the sample taking).

4.2 Flow cytometry

Ideally, samples should be first analysed fresh. Alternatively, if fresh analysis is not possible, a simple method for the preservation of the samples, that interfere minimally with the cellular properties of cells can be used. The combined analysis of the forward and the side scatters can allow the identification of different groups that differ in terms of size and granularity, respectively. Several aspects are critical to succesful analysis of bacterial samples :

- careful sample preservation (if necessary)
- good discrimination of populations from noise
- accurate identification of populations
- careful determination of flow sample rate

Best results should be obtained on fresh samples run immediately after collection. Fresh samples can be stored at 4°C for up to 12 hours with minimal effect. Fixation (in paraformaldehyde solution) will always result in cell loss (about 10%) and in change of scatters signals.

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Preparation of the fixative paraformaldehyde solution requires special care. It is very difficult to completely dissolve the paraformaldehyde powder, it must be vigourously mixed in distilled water for 2 hours or more at 70°C. The solution obtained can be clarified, after cooling at room temperature, by addition of small amounts of sodium hydroxide 1N. The pH is then adjusted , but the solution must be always filtered on Whatman paper filters and then through 0.2 µm-filter before aliquoting and storage. However, after thawing, paraformaldehyde aliquots must be kept at + 4°C and should not be used beyond a week.

4.2.1 SAMPLE PROCESSING

In sterile conditions 5ml each is harvested from each compartment for the stability study and for the axenicity study. Samples should be send to SCK without cooling (or otherwise indicated).

4.3 Genomic/transcriptomic analysis

For transcriptomic analysis enough sampling material needs to be taken to perform the microarray analysis. Furthermore RNA is unstable and requirements are needed to preserve the RNA of the samples. Ideally the sample should immediately be extracted. In case immediate extraction is impossible, it is possible to add RNA later solution and freeze the sample immediately at -80°C.
For DNA analysis, prodecures are more simple. Direct preservation of the sample at -80°C without treatment will provide for a way to store the sample upon analysis. For *Arthrospira* sampling, -80°C storage is not ideal. Immediate DNA extraction is recommended. Storage at -80°C is only possible for 1 month maximally.

4.3.1 SAMPLE PROCESSING

R. rubrum transcriptomic analysis will require 2 times 80 ml. The axenicity study in all compartments will only requires 2 times 5ml.

4.4 Proteomic analysis

In case flow cytometric and genomic experiments will not provide for enough sensitivity of the to be tested samples, it will be wise to adapt the study on the stress response to a proteomic approach.

4.4.1 GENERAL PRECAUTIONS

The proteome of a bacterial species could be modified by proteases present in the culture or in the supernatant. Whenever possible, a protease cocktail inhibitor will be used (e.g. Complete™ Mini, EDTA-free, Roche) and work at 4 °C. Isolated proteins (ex : filtrated supernatant) can be temporarily stored on ice or refrigerated (2-8 °C) or frozen (-80—20 °C) for long-term storage. Storing protein can cause protein precipitation, especially if the protein solution is repeatedly

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going through freeze-thaw cycles. Thus, it is recommended to freeze multiple aliquots of each preparation.

4.4.2 SAMPLE PROCESSING

For each data point 50 ml is needed to analyse the stress response of *R. rubrum*, the C3 co-culture and *Arthrospira* sp.. For the axenicity study no sampling is required.
Ideally, all experiments are to be performed in sterilized polypropylene test tubes. Liquids must be transferred using sterilized graduated plastic pipettes.
For long term preservation, the samples will be pelletted with specific filtration units (0.2um) (ex: Nalgene disposable filter ware: 150mL filter unit with sterile receiver) just after the sampling. The filtration has to be done in the laminar flow. Before the filtration, a protease cocktail inhibitor (1 tablet for 25 mL of supernatant) is added to the supernatant (in the laminar flow). After filtration, samples are kept at –20 °C (3 months) or -80°C (1 year).

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4.5 Summary on the general requirements for the study on the genetic/metabolic stability and axenicity in Bellissima

Parameter	Comp. I	Comp. II	Comp. III	Comp. IV
operation mode: batch < > 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	A/B	A/B	A/B	A/B
Samples: influent (I), Reactor (R), effluent (E), biomass (B), supernatant, (S) diff reactor locations,(L) gas (G)	I, R, E, B, S	R, E, B, S	R, E, B, S, L	R, E, B, S
Sampling conditions: axenic (A), continous (C) grab samples (G)	A, G	A, G	A, G	A, G
Maximum total sample volume	200 ml	200 ml	200 ml	200 ml
Sampling frequency: weekly (W), monthly (M), intensive sampling campaign (I)	I	I	I	I
Sample treatment: centrifugation, sterilization	See table			
Sample preservation: keep light (L), keep dark (D), temperature (T)	T	L	D	L
Required measurements/instrumentation: as for normal reactor operations (Y/N), on-line measure-ments (which)	Y	Y	Y	Y Oxygen
Required control: pH, T	Y	Y	Y	Y
Material selection	-	-	-	-

Table 5. Parameters and requirements for the study on the genetic/metabolic stability in the Bellissima loop.

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Parameter	Comp. I	Comp. II	Comp. III	Comp. IV
operation mode: batch < > 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	A/B	A/B	A/B	A/B
Samples: influent (I), Reactor (R), effluent (E), biomass (B), supernatant, (S) diff reactor locations,(L) gas (G)	-	R, E, B, S	R, E, B, S, L	R, E, B, S
Sampling conditions: axenic (A), continous (C) grab samples (G)	-	A, G	A, G	A, G
Maximum total sample volume	-	10 ml	50 ml	10 ml
Sampling frequency: weekly (W), monthly (M), intensive sampling campaign (I)	-	2M	I	2M
Sample treatment: centrifugation,	See table			
Sample preservation: keep light (L), keep dark (D), temperature (T)	-	L	D	L
Required measurements/instrumentation: as for normal reactor operations (Y/N), on-line measure-ments (which)	-	Y	Y	Y
Required control: pH, T	-	Y	Y	Y
Material selection	-	-	-	-

Table 6. Parameters and requirements for the study on the axenicity in the Bellissima loop.

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Method	when	Volume (ml)	purpose	storage
Flow cytometry	Upon appointment	5	Stress response	No
	Upon appointment	5	Axenicity	No
Genomic	To be defined (10 data points)	2 × 80	Stress response	-80°C, RNA later treatment
		2 × 10	Axenicity	-80°C, no prep
Proteomic	To be defined (10 data points)	2 × 50	Stress response	-80°C, protein inhibitor treatment
		No	Axenicity	No

Table 7. Sampling requirements for the study of genetic/metabolic stability and axenicity in the Bellissima loop.

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Survey of environmental biocontamination on board the International Space Station

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Received 12 April 2005; accepted 20 July 2005

Available online 1 December 2005

Abstract

The International Space Station (ISS) is an orbital living and working environment extending from the original Zarya control module built in 1998. The expected life span of the completed station is around 10 years and during this period it will be constantly manned. It is inevitable that the ISS will also be home to an unknown number of microorganisms. This survey reports on microbiological contamination in potable water, air, and on surfaces inside the ISS. The viable counts in potable water did not exceed 1.0×10^2 CFU/ml. *Sphingomonas* sp. and *Methylobacterium* sp. were identified as the dominant genera. Molecular analysis demonstrated the presence of nucleic acids belonging to various pathogens, but no viable pathogens were recovered. More than 500 samples were collected at different locations over a period of 6 years to characterize air and surface contamination in the ISS. Concentrations of airborne bacteria and fungi were lower than 7.1×10^2 and 4.4×10^1 CFU/m^3, respectively. *Staphylococcus* sp. was by far the most dominant airborne bacterial genus, whereas *Aspergillus* sp. and *Penicillium* sp. dominated the fungal population. The bacterial concentrations in surface samples fluctuated from 2.5×10^1 to 4.3×10^4 CFU/100 cm^2. *Staphylococcus* sp. dominated in all of these samples. The number of fungi varied between 2.5×10^1 and 3.0×10^5 CFU/100 cm^2, with *Aspergillus* sp. and *Cladosporium* sp. as the most dominant genera. Furthermore, the investigations identified the presence of several (opportunistic) pathogens and strains involved in the biodegradation of structural materials.
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Keywords: International Space Station; Space flight; Contamination; Bacteria; Fungi; Air and surface sampling; Pathogenicity; Biodeterioration

1. Introduction

Man's instinct to explore space can be exemplified by numerous manned missions (Apollo program, construction, and exploitation of the Mir orbital station, etc.) and unmanned missions (Cassini–Huygens mission, Mars Express mission, etc.). The main goal of manned exploration is to achieve a prolonged stay in space, e.g. in an orbital station or in planetary bases on the moon and/or Mars. It goes without saying that such missions can only be realized if the cosmonaut's health and well-being are secured. The characterization of microbiological contamination on board spacecraft and orbital stations is therefore of paramount importance. Russian reports on the exploitation of the orbital station Mir have indicated that microorganisms are ubiquitously present and that they should be considered as indigenous to any spacecraft environment [19,21,25]. Microbial contamination may originate from different sources and includes the initial contamination of space flight materials during manufacturing and assembly, the delivery of supplies to the orbital station, the supplies themselves, secondary contamination during the lifetime of the orbital station, the crew and any other biological material on board, e.g. animals, plants and microorganisms used in scientific experiments [22,25].

The cosmonaut is probably the most important contamination source, as his body contains a large amount of bacteria. These are found on the skin, on mucous membranes, in the upper respiratory tract, the mouth, the nasal passage, and in the gastrointestinal tract. The two major routes through which

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doi:10.1016/j.resmic.2005.07.010

the human microbiota can be spread in the environment are: (i) the air followed by sedimentation on surfaces; and (ii) direct transfer to a surface. Although most microorganisms do not threaten human health, it has been reported that in a confined environment such as a space cabin, microorganisms may produce adverse effects on the optimal performance of the space crew and the integrity of the spacecraft or habitat. These effects range from infections, allergies, and toxicities to degradation of air and water supplies [24]. Biodegradation of critical materials may result in system failure and this may jeopardize the crew. Studies performed in Mir indicated that some equipment and structural materials were prone to the accumulation and proliferation of biodestructive bacteria and fungi [21,22]. Damage to polymers and metals could be observed and this resulted in cases of malfunctioning and even breakage of certain units, e.g. air conditioners, water recycling systems, etc. [10,13].

The International Space Station (ISS) is the orbital station that is being built by the United States in collaboration with Russia, 11 nations of the European Space Agency, Canada, Japan and Brazil (Fig. 1). The ISS is the largest and most complex international scientific project in history. More than four times as big as the Russian Mir space station, the completed ISS will have a mass of about 500 tons. It will measure 108 m across and 88 m in length, with more than 4000 m^2 of solar panels providing electrical power to six state-of-the-art laboratories. The projected lifetime of the ISS after completion is approximately 10 years. During this period, the station will experience periodic visits from international spacecraft for crew exchanges, resupply of food and consumables, and many payloads with scientific experiments [26].

Space agencies attempt to avoid microbiological problems by developing strategies to limit microbial contamination aboard the ISS (disinfection and sterilization of space flight materials during assembly and transport to the ISS, rigorous cleaning procedures, etc.). Nevertheless, monitoring of biological contamination is imperative and the results of the investigations may trigger specific countermeasures when microbial concentrations pass defined thresholds. Over the past few years, a number of research groups have had access to air, water and surface samples taken aboard the ISS. These samples have been characterized using state-of-the-art microbiological analysis techniques.

Investigations into the microbial load of ISS potable water have been mainly reported by La Duc and coworkers [15,16]. The latter authors analyzed ISS potable water samples at different timings (preflight, during flight and postflight) and at various stages of purification, storage and transport. A combination of culture-dependent and culture-independent analyses was used to characterize microbial contamination. A biocide treatment in the form of iodine was responsible for the fact that the preflight potable water had bacterial concentrations below the detection threshold (i.e. less than 1 CFU/100 ml). A water sample collected from the humidity condensate recovery system yielded 5.1×10 CFU/100 ml and isolates of both *Acinetobacter radioresistens* and *Acidovorax temperans* were obtained [17]. Characterization of the microbial content of the humidity condensate is important because this water is used for generation of potable water in the ISS. La Duc and collaborators performed a molecular microbial diversity analysis to reveal that ISS drinking water contained DNA sequences from

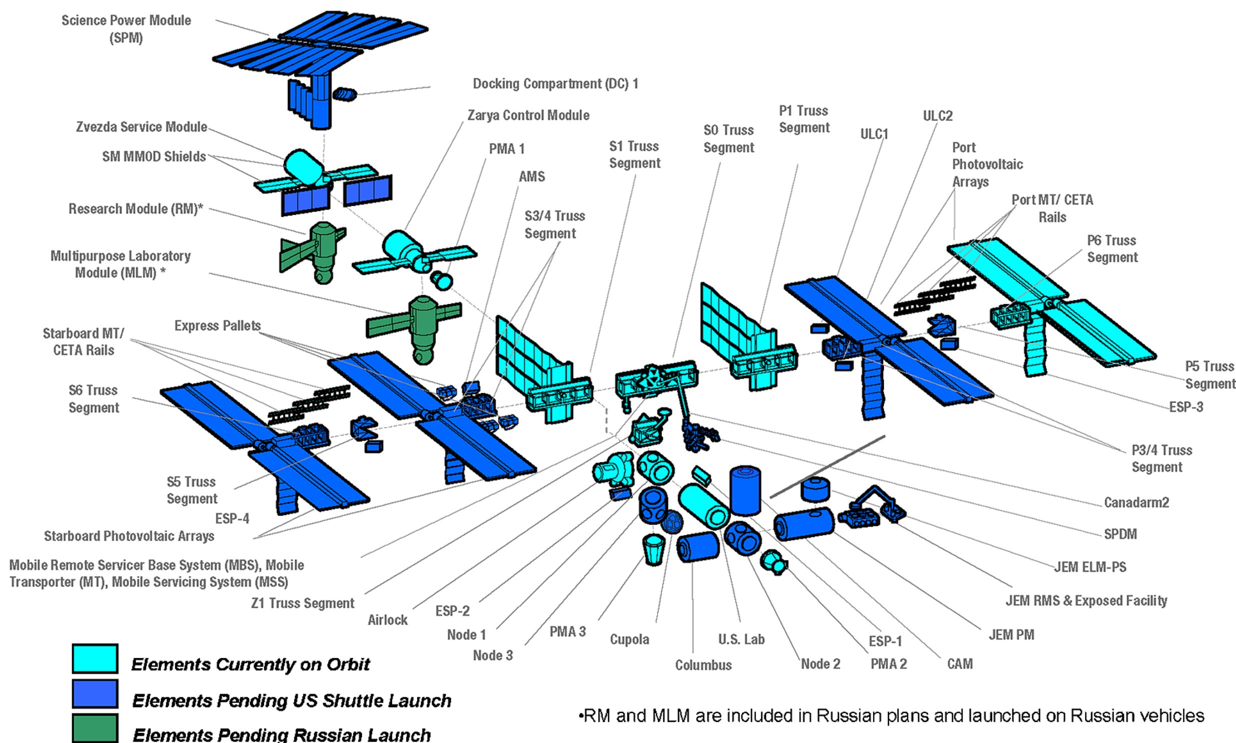

Fig. 1. Overview of the main structural elements of the ISS. The information is taken from http://spaceflight.nasa.gov/station/assembly/index.html (last accession on July 4th, 2005).

many types of bacteria [16]. Bacterial small subunit rDNA fragments were PCR-amplified with the eubacterial primers B27F and B512R. Purified amplicons were cloned and representative DNA inserts were sequenced. A first sample contained DNA sequences from N_2-fixing *Bradyrhizobium* sp., and various biofilm-producing microbes (*Caulobacter* sp., *Hyphomicrobium* sp., etc.). The analysis also confirmed the presence of DNA sequences originating from *Ochrobacter* sp., *Propionibacterium* sp., and *Brevundimonas* species. The latter organisms are catalogued as human pathogenic bacteria. A second sample contained DNA sequences of *Methylobacterium exotorquens* and *Delftia acidovorans*. These organisms are known to colonize and attack a large variety of polymeric and metallic surfaces. Samples originating from the ISS humidity condensate water were rich with sequences of the bacterial pathogen *Afipia broomeae*. La Duc and colleagues were not able to prove the presence of active pathogens in the ISS water samples and hence they could not calculate concentrations of pathogenic bacteria. Their study suggests that pathogenic bacteria may be present in the water systems of the ISS [15,16]. Future research should be directed towards identification of niches in which these microorganisms may accumulate and survive. This will help to assess the health risk for cosmonauts. A second major information source is the research conducted by Castro et al. [4]. Water samples were collected from the hot and cold ports of the humidity condensate recovery system. The potable water supply that is generated by reclaiming humidity condensate consistently provided water with bacterial levels below the US acceptability limit of 1.0×10^2 CFU/ml. Twenty-seven bacterial colony types were isolated within the frame of this study. The identifications were performed by sequencing a 527-bp fragment of the 16S rDNA gene using the eubacterial primers B005F and B531R included in the Microseq 500 16S rDNA Bacterial Sequencing kit from Applied Biosystems (USA). The humidity condensate contained mostly Gram-negative bacteria and the population was predominantly made up of the genera *Sphingomonas* sp. (25%) and *Methylobacterium* sp. (18%).

Publicly available documents about airborne contamination and contamination associated with the ISS interior are sparse. We report in this survey the results of a six-year campaign during which airborne and surface contamination in the ISS was monitored. The investigations have been carried out by the Institute of Biomedical Problems (IBMP, Russia), which is responsible for monitoring biological contamination in the habitable compartments of the ISS for safety and hygienic reasons, and the Belgian Nuclear Research Centre (SCK-CEN, Belgium).

2. Materials and methods

2.1. Surface samples

Surface sampling was done by swabbing a 10×10 cm surface of the interior and equipment using an in-house-made sampling kit (Surface pipette kit). This kit consists of a belt with pockets containing fluoroplastic tubes with swabs impregnated in a phosphate buffer containing vaseline (Fig. 2).

Upon receipt of the samples in the laboratory, aliquots were inoculated on Petri dishes containing various nutrient media (tryptic soy nutrient agar, mannitol salt agar, Mac Conkey agar, Sabouraud chloramphenicol agar, potato dextrose agar, and Czapek–Dox agar). The Petri dishes were incubated at 37 °C for 48 h or 28 °C for five to seven days to recover bacteria and fungi, respectively. Subsequently, the bacterial and fungal concentrations were determined. Morphologically different bacteria were picked for Gram staining and identification by means of the Vitek system (BioMérieux, France). Fungal strains were identified microscopically by their morphological characteristics [11]. Yeasts and yeast-like fungi were also identified using the Vitek system. The Vitek system makes use of tests cards made up of 30 or 45 microwells containing either identification substrates or antibiotics. The growth pattern of axenic isolates in these test cards is recorded and used for species identification using specialized software.

2.2. Air samples

The Russian Ecosphere kit includes a spaceflight-certified SAS air sampler (PBI International, Italy) that collects airborne bacteria by impaction on agar medium, and sets of Petri dishes

Fig. 2. Surface pipette kit for sampling internal surfaces and structural materials of the ISS.

Fig. 3. The Russian Ecosphere kit includes a spaceflight-certified SAS air sampler and sets of Petri dishes containing nutrient-rich media.

containing nutrient-rich media (tryptic soy nutrient agar for bacteria and Czapek–Dox agar for moldy fungi). The equipment is stored permanently onboard the ISS, whereas the sets of Petri dishes are delivered by cargo and transport vehicles (Fig. 3). Air samples of 90 l were taken one to two days prior to the undocking of the cargo vehicle and return to earth. Upon receipt in the IBMP laboratory, the Petri plates were incubated as described above and colonies were counted afterwards. The obtained isolates were identified using the procedures described under the section of surface samples.

Identification procedures using molecular tools started by generating an overnight culture of the axenic bacterial strains. Incubation was done in Luria broth at a temperature of 28 °C. Genomic DNA was extracted using the Wizard SV Genomic DNA Purification system (Promega, USA) according to the protocol supplied by the manufacturer. The genomic DNA was used for performing a PCR reaction. The PCR reaction mixture contained per sample 9.5 µl sterile double-distilled water, 1 µl forward primer, 1 µl backward primer, 2.5 µl bovine serum albumin (stock concentration of 1 mg/ml), 2.5 µl dNTPs (stock solution of 2 mM), 2.5 µl 10× PCR buffer, 1 µl Taq DNA polymerase and a 5 µl sample. The PCR reaction was performed on 25 µl samples with a GeneAmp 2700 (Applied Biosystems, USA) using the following protocol: denaturation (3 min at 94 °C), followed by 34 cycles of denaturation (45 s at 94 °C), annealing (45 s at 56 °C), and extension (45 s at 72 °C). Final extension was done for 7 min at 72 °C. Amplification was performed using the eubacterial primers B008F (AGATTTGATCCTGGCTCAG) and B926R (CCGTCAATTCCTTTRAGTTT). The positions refer to the 16S rDNA of *Escherichia coli*. PCR products were cleaned up with a Wizard SV gel and PCR clean-up system (Promega, USA) using the protocol described by the manufacturer. The sequencing reaction was performed according to the following protocol. Five to twenty ng of PCR product was mixed with 2 µl (±3.2 pmol) F-primer or R-primer (see above) and 8 µl of big dye mix (Applied Biosystems, USA). PCR reaction conditions were 3 min at 96 °C, followed by 25 cycles of 30 s at 96 °C, 30 s at 50 °C, and 4 min at 60 °C. The reaction was stopped by putting the mixture at 4 °C. Purification of the reaction mixture was done using Centri-Sep columns (Princeton Separations, USA) according to the instructions supplied by the manufacturer. DNA sequences were determined by performing capillary electrophoresis with fluorescent detection on an ABI310 automatic sequencer (Applied Biosystems, USA). Data were collected with the Sequencing Analysis software v 3.7 of Applied Biosystems. The DNA sequences were compared by BLAST analysis to all sequences in the Genbank database using the software tool described by Devulder and collaborators [6].

3. Results and discussion

The compositional analysis of microorganisms residing in the ISS environment has been performed over a period of six years. Samples from nine main missions and seven Soyuz taxi-flights to the ISS have been processed from the year 1998 till the present. A total of 419 samples from air and surfaces were screened for the presence of bacteria. Bacteria were recovered in 71% of the cases (i.e. 297 samples). In addition, the fungal contamination aboard the ISS was investigated by analyzing 378 samples from air and surfaces. Fungi were obtained from 92 samples, which is 24% of all the samples. The large species diversity that was obtained from the environmental samples was more pronounced in the surface samples than in the air samples. A total of 36 and 15 bacterial species were isolated from surface and air samples, respectively. A total of 32 and 5 fungal species were isolated from surface and air samples, respectively.

During the monitoring campaign, 243 swab samples were taken from structural elements and internal surfaces at 33 different locations in the Service Module (SM), Functional Cargo Block (FGB), NODE-1, and LAB. The surfaces were sampled one to two days before the end of each mission. Identification of the microbial contaminants was performed as described in Section 2.1. The bacterial concentrations fluctuated within a broad range, i.e. from 2.5×10^1 to 4.3×10^4 CFU/100 cm^2. A temporary increase in bacterial concentrations was registered for some locations, e.g. table surface in SM and behind several panels of FGB. The concentrations of fungi varied between 2.5×10^1 and 3.0×10^5 CFU/100 cm^2. The largest number of viable fungal filaments was discovered on panel 402 of the SM ventilation screen. The data show that the maximum allowable concentrations for surface contamination established in "The International Space Station Medical Operations Requirements Document" (MORD SSP 50260) were exceeded on some occasions. The latter document was enacted at the commencement of the operation of the ISS and serves as a code of standard requirements at the phase of pre-launch treatment and in-flight operation [20]. In this document it is stated that surface contamination levels should not exceed the limits of 1.0×10^4 and 1.0×10^2 CFU/100 cm^2 for bacteria and fungi, respectively. Whenever the contamination threshold was exceeded for a specific surface, the latter was cleaned with the disinfectant fungistat wipes that are routinely used in ISS. These wipes are impregnated with a mixture of a quaternary ammonium salt (N-alcanoylaminopropyldimethylbenzylammonium chloride with urea) and a compound of hydrogen peroxide with urea

Table 1
Bacterial species isolated from the ISS environment and their occurrence (%) in the total number of samples

Number	Bacterial species	Environment	
		Surface	Air
1	*Acinetobacter woffii*	2.8	0.8
2	*Actinobacillus ureae*	0.9	–
3	*Aerococcus* sp.	0.5	–
4	*Bacillus cereus*	0.5	–
5	*Bacillus licheniformis*	2.8	–
6	*Bacillus pumilus*	7.9	0.8
7	*Bacillus* sp.	0.5	–
8	*Bacillus sphaericus*	12.1	–
9	*Bacillus subtilis*	7.9	–
10	*Brevibacterium vesicularis*	2.3	–
11	*Corynebacterium* sp.	8.9	0.8
12	*Corynebacterium xerosis*	0.5	–
13	*Eikenella corrodens*	1.4	–
14	*Flavobacterium indologenes*	1.4	–
15	*Gemella morbilorum*	0.5	–
16	*Micrococcus luteus*	1.4	–
17	*Micrococcus* sp.	6.5	0.8
18	*Pseudomonas putida*	2.3	–
19	*Pseudomonas stutzeri*	2.3	–
20	*Staphylococcus aureus*	3.7	3.2
21	*Staphylococcus auricularis*	23.4	6.3
22	*Staphylococcus capitis*	3.3	1.6
23	*Staphylococcus cohnii*	1.4	0.8
24	*Staphylococcus epidermidis*	22.4	9.5
25	*Staphylococcus haemolyticus*	2.8	3.2
26	*Staphylococcus hominis*	9.3	5.5
27	*Staphylococcus saprophyticus*	3.3	–
28	*Staphylococcus simulans*	6.5	1.6
29	*Staphylococcus* sp.	3.3	7.1
30	*Staphylococcus warneri*	3.7	–
31	*Staphylococcus xylosus*	0.9	–
32	*Streptococcus constellatus*	0.5	–
33	*Streptococcus intermedius*	0.5	–
34	*Streptococcus mitis*	0.5	–
35	*Streptococcus* sp.	2.8	0.8
36	*Xanthomonas maltophilia*	–	0.8

(urea peroxyhydrate). Cleaning with these wipes and mechanical moisture removal always resulted in a drop in the biological surface contamination to below the thresholds established in the MORD document.

The bacterial species that were detected in the ISS and their isolation frequency are reported in Table 1. Bacteria belonging to the *Staphylococcus* sp. genus were isolated from 84% of the surface samples. The two second most commonly identified genera were *Bacillus* sp. (31.7%) and *Corynebacterium* sp. (9.4%). It is apparent from Table 1 that the most prevalent species in surface samples was *Staphylococcus auricularis* (23.4%), followed by *S. epidermidis* (22.4%). *Bacillus sphaericus* and *S. hominis* were encountered in 12.1 and 9.3% of the cases, respectively. A few species that have (opportunistic) pathogenic behavior were isolated, i.e. *B. cereus*, *Eikenella corrodens*, and *S. aureus*. Bacterial species like *Flavobacterium indologenes*, *Pseudomonas putida*, and *Xanthomonas malthophila* that can be involved in biodeterioration of materials were also recovered [3,8,9]. Castro and coworkers [4] also characterized microbial surface contamination. They collected samples from a reusable cargo container, which is used to transport flight hardware and consumables to and from the ISS, as well as from flight hardware present in NODE 1, SM, and LAB. Surfaces of 25 × 25 cm were sampled using calcium alginate swabs with a phosphate buffer as wetting agent. They report that the contamination levels were below the acceptability of 1.0×10^4 CFU/100 cm^2 in more than 75% of the sampling times [24]. Our species identifications are comparable to the results mentioned by Castro and collaborators, who mainly observed *Staphylococcus* sp. and *Corynebacterium* sp. in their swab samples [4]. The latter authors characterized the contaminants using 16S rDNA sequencing and Vitek identification.

Table 2
Fungal species isolated from the ISS environment and their occurrence (%) in the total number of samples

Number	Species	Environment	
		Surface	Air
1	*Aspergillus candidus*	0.5	–
2	*Aspergillus clavatus*	0.5	–
3	*Aspergillus ficuum*	0.5	–
4	*Aspergillus flavus*	–	2.5
5	*Aspergillus janus*	0.5	–
6	*Aspergillus nidulans*	0.9	0.8
7	*Aspergillus niger*	2.7	–
8	*Aspergillus ochraceus*	0.5	0.8
9	*Aspergillus phoenicis*	6.5	–
10	*Aspergillus pulvinus*	0.5	–
11	*Aspergillus sydowi*	3.8	–
12	*Aspergillus ustus*	0.5	–
13	*Aspergillus versicolor*	2.3	–
14	*Candida* sp.	0.5	–
15	*Candida parapsylosis*	0.5	–
16	*Cladosporium sp.*	0.9	–
17	*Cladosporium cladosporioides*	0.5	–
18	*Cladosporium herbarum*	0.5	–
19	*Cladosporium tenuissimum*	0.5	–
20	*Cryptococcus albidus*	0.9	–
21	*Geotrichum* sp.	0.5	–
22	*Lipomyces* sp.	0.5	–
23	*Penicillium aurantiogriseum*	6.0	1.7
24	*Penicillium expansum*	2.3	0.8
25	*Penicillium grabrum*	0.5	–
26	*Penicillium italicum*	0.9	–
27	*Penicillium lividum*	0.5	–
28	*Phoma* sp.	0.5	–
29	*Rhodotorula rubra*	0.5	–
30	*Saccharomyces* sp.	2.8	–
31	*Ulocladium botrytis*	0.5	–

The list of fungi isolated from the ISS surface samples is given in Table 2. *Aspergillus* sp., *Penicillium* sp., and *Saccharomyces* sp. were the most dominant genera and were isolated in 19.7, 10.2, and 2.8% of the samples, respectively. A diverse *Aspergillus* population was recovered (13 species), whereas this was less pronounced in the case of *Penicillium* (5 species) and *Cladosporium* (4 species).

A. phoenicis and *P. aurantiogriseum* dominated the population with occurrence percentages of 6.5 and 6.0%, respectively. Some of the samples contained *A. versicolor* and *Cladosporium* sp., which are known for (i) their capacity to colonize natural and synthetic polymers, and (ii) their involvement in

Table 3
Number of airborne bacterial species recovered from ISS air samples

Species	Frequency	Species	Frequency
Acinetobacter radioresistens	5	*Staphylococcus capitis*	2
Aerococcus viridans	1	*Staphylococcus epidermidis*	14
Bacillus cereus	1	*Staphylococcus haemolyticus*	2
Bacillus subtilis	1	*Staphylococcus hominis*	7
Enterococcus faecalis	12	*Staphylococcus lugdunensis*	1
Kocuria rosea	1	*Staphylococcus xylosus*	5
Psychrobacter urativorans	2	*Xanthomonas* sp.	1
Staphylococcus sp.	7	Unknown bacterium	2
Staphylococcus aureus	2		

Samples were taken within the framework of collaboration between IBMP, SCK-CEN, and ESA. Identification were performed by means of 16S rDNA sequencing considering 98% as the similarity threshold.

the biodeterioration of electronic insulation polymers such as polyimides [8]. Aggressive metabolites such as organic acids produced during outgrowth of species such as *P. aurantiogriseum* and *C. herbarum* may compromise the integrity of metallic surfaces [27]. Hence, these fungi can be considered as potential biocorrosive agents. These events may ultimately lead to biodegradation of space flight materials with short circuits and malfunctioning as a consequence [3,8,9]. Such examples have been described during the exploitation of the Mir orbital station [10,13,21]. Furthermore, pathogenic saprophytes have been recovered. These are known to provoke mycoses and mycotic intoxications in the case of an impaired immunological response. Ten species out of 31 can be classified as relatively pathogenic and all of them can cause allergic reactions. Species that can cause fungal infections such as *Candida* sp. (in particular *C. parapsylosis*) and species that can produce toxins such *Aspergillus* sp. (in particular *A. versicolor* and *A. ochraceus*) were encountered [2,7,14].

Over the period 1998 up to the present, the airborne contamination was characterized by analyzing 278 air samples from 16 different sites onboard the ISS. Sampling and analysis by means of the Vitek system have been performed as described in Section 2.2. The results indicate that the airborne microbial contamination was low at all sampling locations. The highest concentration was encountered in the toilet area of SM where a maximum level of 7.1×10^2 CFU/m^3 was reached. Fungal concentrations ranged between 1.1×10^1 and 4.4×10^1 CFU/m^3. The contamination levels measured in the air were within the limits described in MORD SSP 50260. The thresholds for air contamination have been fixed at 1.0×10^3 and 1.0×10^2 CFU/m^3 for bacteria and fungi, respectively [20]. Airborne contamination could be kept to a minimum by the use of the POTOK 150MK system that efficiently filters particles and microorganisms from the air [1].

Staphylococci were dominant and they were isolated in 38.8% of the samples (Table 1). *S. epidermidis* and *S. auricularis* occurred in the highest concentrations. The human pathogen *S. aureus* was isolated in 3.2% of the cases (Table 1). All other genera occurred in much lower concentrations. Fungi were recovered in only 7.4% of the air samples. Most of the fungi belonged to the genus *Aspergillus* or *Penicillium* and only five different species were identified (Table 2). *A. flavus*, which is known to cause nosocomial infections, was isolated with a frequency of 2.5%.

Air samples have also been analyzed at the Belgian Nuclear Research Center (SCK-CEN) within the framework of a collaboration between IBMP and the European Space Agency (ESA). A total of 33 samples were taken at different locations in the ISS (i.e. SM, NODE 1, and FGB) during three different Soyuz taxiflights (October 2003, April 2004, and October 2004). The sampling was done using the Ecosphere kit with air volumes ranging from 30 to 240 l. After incubation of the Petri dishes for a period of 7 days at 28 °C, 48% of the plates scored positive for the presence of bacteria. The highest bacterial concentration was found in FGB and amounted to 1.1×10^2 CFU/m^3. A total of 66 isolates has been obtained and the bacteria were identified using 16S rDNA sequencing (see Section 2.2.). Positive identifications were made based on a 98% or better alignment with database entries. It is apparent from Table 3 that the majority of the population consists of different *Staphylococcus* sp. (at least 60%) and *Enterococcus faecalis* (18%). Generally, the contamination levels reported by IBMP and SCK-CEN are comparable to observations made by others who captured air samples (84.9 l) with a Burkard microbial air sampler [4,24]. Castro and coworkers [4] characterized the microbial isolates by a combination of 16S rDNA sequencing and Vitek identification.

4. Conclusion and perspectives

The environmental biocontamination of the ISS has been followed up since its early construction days. The main emphasis has been placed on the air quality and the surface contamination of internal structures. The total number of samples analyzed by IBMP and SCK-CEN amounts to 554. The microbial and fungal concentrations were in most instances below the acceptability limits established in the International Space Station Medical Operations Requirements Document (MORD SSP 50260). An occasional rise in microbial surface colonization could be eliminated by cleaning that particular surface with wipes impregnated with disinfectants. The microbiota recovered from the ISS environment is clearly dominated by residents of the human mucous membranes and skin (i.e. *Staphylococcus* sp. and *Corynebacterium* sp.). Members of the rotating crews are the most likely source of the environmental contamination. In

addition to the typical representatives of the human microbiota, spore-forming *Bacillus* sp., pathogenic bacteria (e.g. *S. aureus*), and species involved in biodeterioration have been isolated. The biocontamination experienced in ISS is to a great extent comparable to results obtained from the Mir orbital station. Biocontaminants isolated from the Mir environment were mainly identified using the Vitek system. Novikova [21] reports that airborne contamination remained fairly stable during the occupation of Mir and that the bacterial concentration was below 5.0×10^2 CFU/m^3 in 95% of the air samples. The most predominant airborne bacterial genera were *Staphylococcus* sp., *Corynebacterium* sp., and *Bacillus* species. The concentration of airborne fungi was variable and fluctuated between 2 and 5.0×10^4 CFU/m^3, with *Penicillium* and *Aspergillus* as the dominant genera. Contamination levels of surfaces and equipment aboard Mir were also variable, with bacterial and fungal concentrations between 1.0×10^2 and 1.0×10^7 CFU/100 cm^2. The dominant bacterial and fungal genera were the same as for the airborne contamination.

The presence of several (opportunistic) pathogens and species involved in biodeterioration has been confirmed in the Mir environment [21] as well as on board the ISS (this survey). Although onboard cleaning procedures restrict the level of these harmful organisms to a minimum, there is always a risk of an increase in the concentration of these potentially harmful organisms. Continuous environmental monitoring during the lifetime of the ISS is of paramount importance to ensure (i) the cosmonaut's health and (ii) the integrity of the spatial hardware. IBMP and SCK-CEN will therefore continue their efforts to monitor environmental contamination in the ISS during the coming years. One disadvantage of the current strategy is that samples need to be returned to earth for analysis. This usually generates a time gap of almost one week between sampling and the availability of the first results. Such an information delay may be detrimental when formulating countermeasures in the case of a pathogen outbreak. In this respect, the efforts for developing on-line monitoring systems (e.g. for air and water) and on-site analysis systems (e.g. for surfaces) should be intensified. Only by using such systems will it be possible to warn cosmonauts at an early stage about an increase in biological contamination. This will give them the opportunity to take appropriate countermeasures in time (e.g. replacing air filters, cleaning surfaces with specialized surfactants and disinfectants, etc.). A second big challenge is linked to better characterization of microbial contamination using culture-independent molecular analysis. Up to now, samples are grown on agar media and bacterial and fungal isolates are identified afterwards. This approach may lead to a biased view of environmental biocontamination, as many organisms are non-cultivable [28]. A molecular analysis should target nucleic acids in order to determine the total biodiversity pool and the biologically active microorganisms [5, 12]. Analysis protocols can be adapted fairly easily in the case of water samples and swab samples. Aliquots of the samples can be used immediately for molecular analysis. The situation is somewhat more complicated in the case of air samples. Defined volumes of air are being collected by impaction on Petri plates containing culture media. This collection technique is, however, not suitable for direct recovery of nucleic acids. The applicability of collection techniques such as collection of the airborne contamination on 0.22 μm filters and filtration techniques using alginate filters should be exploited [18,23]. These methodologies enable isolation of contamination and consecutive extraction of nucleic acids without cultivation. However, these methods need to be evaluated thoroughly and must pass a number of safety and compatibility tests before their usage is allowed aboard the ISS.

In conclusion, an intensive monitoring strategy in combination with novel analysis methods and possibly new sampling techniques should allow correct mapping of biocontamination aboard the ISS. This will help to define how microbial communities evolve in confined habitats and whether there are microbial risks (such as pathogen outbreak, biodeterioration, malfunctioning of hardware, etc.) during prolonged exploitation of an orbital station or any envisaged moon or planetary base.

Acknowledgements

Collaboration between IBMP and SCK-CEN was funded by ESA (contract number 17401/03/F/DC).

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