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Part B: Axeny in long term microbial cultures.
State of the art and definition of the critical points

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Abbreviations

MELISSA	Microbial Ecological LIfe Support System Alternative
BHR	Broad Host Range (plasmids)
FISH	Fluorescent In Situ Hybridization
ISS	International Space Station
MALDI-TOF	Matrix Assisted Laser Desorption Ionization -Time of Flight
PAR	Photosynthetic Active Radiation

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1. Introduction

For a lunar base or mission to Mars, it is essential to rely on a safe life support system including food and water supply, gas management and waste management. Such life support systems are based on several regenerative techniques and processes that can consist of biological, physico-chemical or hybrid systems.

For this purpose the MELISSA concept was conceived by ESA to be the European model for ecological life support system applications. The compartmentalised structure of MELISSA makes possible the use of an engineering approach and the use of deterministic control laws. However, bio-processes are usually subject to genetic evolution and consequently the use of classical control theories is contingent on the assumption of behavioural stability during long-term operation. In terms of efficiency and safety, the risks undergone in case of a genetic evolution in the compartments are critical for the far accomplishment of this human-life-sustaining system. This must take into account also the high doses of radiations that are experienced in the space, outside the Earth's atmosphere. Therefore, in recognition of the well-advanced results of the project on-ground, it seems that time is now appropriate to investigate microbial robustness and more specifically microorganism performance and evolution from generation to generation. Moreover, the potential genetic transfer from one compartment to another has to be investigated, as well as the control of axenic conditions during long-term operation.

Thus, the main objectives of this activity are to establish and validate a method and its associated hardware, and to detect genetic instability and microbial contaminants in the MELISSA compartments.

2. Why bother?

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).

2.1. Sources of contamination

2.1.1. 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). In this context it is absolutely necessary to characterise the organisms of the mineralization compartment (C1), so that the nature and extent of contamination by these organisms to other compartments can be adequately established. In addition, it is equally important to regard the C1 consortium as a likely source of human 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. Ideally, all organisms in C1 should be identified, and all other compartments, especially C3 and C4, should be checked for the absence of every single one of these organisms.

2.1.2. Surface and airborne contaminants

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 *et al.*, 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 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.

2.1.3. 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 biome 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.

The tables 1 to 4 give several lists of human viruses, pathogenic bacteria, pathogenic fungi and protozoans found in wastes that may enter water on Earth (Rose & Grimes, 2001). Though the conditions in spacecrafts and space habitats will be designed to minimize the carry-over from Earth of these problematic contaminants, it seems interesting to consider their diversity.

Table 1 -. Human viruses found in waste materials that enter water. (Rose & Grimes, 2001)

VIRUSES	NUCLEIC ACID	DISEASE(S)	WASTE (S)
Adenoviruses	DNA		
Human adenovirus		acute respiratory, pharyngitis, acute hemorrhagic cystitis	wastewater
Enteric adenovirus		gastroenteritis	wastewater
Caliciviruses	DNA		
Calcivirus		gastroenteritis	wastewater
Norwalk virus		gastroenteritis	wastewater
Coronaviruses	RNA		
Enteric coronavirus		intestinal disorders	
Orthomyxoviruses	RNA		human, swine,
Influenza virus		influenza	and fowl wastes
Picornaviruses	RNA		
Coxsackievirus A		meningitis, herpangia, common cold	wastewater
Coxsackievirus B		mycarditis, pleurodynia, rash, meningitis, paralysis	wastewater
ECHO virus		paralysis, diarrhea, meningitis	wastewater
Hepatitis A virus		infectious hepatitis	wastewater
Poliovirus		Poliomyelitis	
Reoviruses	RNA	respiratory, gastroenteritis	
Reovirus		infantile diarrhea	wastewater
Rotavirus			wastewater

Table 2.- Pathogenic bacteria found in waste materials that enter water. (Rose & Grimes, 2001)

SPECIES	DISEASE(S)	WASTE(S)
Acinetobacter calcoaceticus	nosocomial	water, human skin and mouth
Aeromonas hydrophila	septicemia, wound infections, diarrhea	water (fresh and estuarine)
Aeromonas sobria	septicemia, wound infections, diarrhea	water (fresh and estuarine)
Aeromonas caviae	septicemia, wound infections, diarrhea	water (fresh and estuarine)
Bacteroides fragilis	Intra abdominal abscesses	animal feces
Bacteroides melaninogenicus	orofacial	human mouth and feces
Brucella spp.	brucellosis	animal feces, urine, and milk
Campylobacter fetus	septicemia	animal feces
Campylobacter jejuni	enteritis	animal feces
Chromobacterium violaceum	septicemia and diarrhea	soil and water
Citrobacter spp.	nosocomial	water
Clostridium botulinum	botulism	soil, sediment, and fish
Clostridium difficile	pseudomembranous colitis	vagina and gastrointestinal tract
Clostridium perfringens	gangrene, wound abscesses, and food poisoning	animal feces
Clostridium sporogenes	gangrene	soil and animal feces
Clostridium tetani	tetanus	soil and animal feces
Coxiella burnetii	Q fever	milk and animal wastes
Enterobacter spp.	nosocomial	wastewater
Erysipelothrix rhusiopathiae	erysipeloid	animal feces and fish slime
Escherichia coli	gastroenteritis	wastewater
Flavobacterium meningosepticum *Francisella tularensis*	nosocomial, meningitis tularemia	freshwater rodents and freshwater
Fusobacterium necrophorum	liver and soft tissue abscesses	wastewater and animal feces
Klebsiella pneumoniae	pneumonia, bacteremia, and nosocomial	water, feces, soil, and plants
Legionella pneumophila	Legionnaires' disease	freshwater, cooling tower water, and hot water tanks
Leptospira interrogans	leptospirosis	urine
Listeria monocytogenes	listeriosis	soil and feces
Morganella morganii	urinary tract and nosocomial	water, feces, and decaying animals
Mycobacterium tuberculosis *Mycobacterium marinum*	tuberculosis swimming pool granuloma	wastewater water and fish
Plesiomonas shigelloides	gastroenteritis	water, fish, and aquatic animals
Proteus spp.	urinary tract and nosocomial	water, feces, and decaying animals
Pseudomonas aeruginosa	burn, wound, corneal, ear, urinary, lung, skin, and	water, wastewater, plants, sediment, and fish

	gastrointestinal tract	
Pseudomonas pseudomallei	meliodosis	water and soil
Salmonella typhi	typhoid fever	wastewater
Salmonella enteritidis	gastroenteritis and septicemia	wastewater, animal wastes and feed, and compost
Serratia marcescens	nosocomial	water, plants, insects, and feces
Shigella boydii	bacillary dysentery	primate feces and wastewater
Shigella dysenteriae	bacillary dysentery	primate feces and wastewater
Shigella flexneri	bacillary dysentery	primate feces and wastewater
Shigella sonnei	bacillary dysentery	primate feces and wastewater
Staphylococcus aureus	abscesses and food poisoning	mammalian skin and ocean water
Streptococcus faecalis	endocarditis	animal feces
Vibrio alginolyticus	wound infection	ocean water and aquatic animals
Vibrio cholerae	Asiatic cholera	wastewater, shellfish, and saltwater
Yersina enterocolitica	Gastrointestinal, acute mesenteric lymphandenitis	water, milk, mammalian alimentary canal

Table 3. - Pathogenic fungi associated with waste materials that enter water. (Rose & Grimes, 2001)

FUNGUS	DISEASE(S)	WASTE(S)
Aspergillus fumigatus	aspergillosis	decaying vegetation, especially grains
Candida albicans	candidiasis	animal feces
Cryptococcus neoformans	cryptococcosis	pigeon and bird feces, cellar dirt
Geotrichum candidum	geotrichosis	tomatoes, fruits, dairy products

Table 4. - Waste associated protozoa pathogenic for humans (Rose & Grimes, 2001)

SPECIES	DISEASE(S)	WASTE(S)
Mastigophora (flagellates)		
Chilomastix mesnili	diarrhea?	primate feces
Giardia lamblia	giardiasis	human feces
Sarcodina (amebas)		
Entamoeba histolytica	amebic dysentery	human and other animal feces, wastewater
Dientamoeba fragilis	mild diarrhea	human feces
Naegleria fowleri	primary amebic meningoencephalitis	human feces, wastewater
Acanthamoeba spp.	amebic meningoencephalitis	human feces, wastewater, and heated water
Sporozoa		
Cryptosporidium spp.	cryptosporidiosis	animal feces
Sarcocystis spp.	sarcocystosis	animal feces
Toxoplasma gondii	toxoplasmosis	animal feces, especially cats
Ciliata		
Balantidium coli	balantidiasis	animal feces, especially swine

3. 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 5: Microbial diversity – known and estimated species. From Cowan *et al.* (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

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 6: Estimates of the proportion of 'unculturable' micro organisms in various terrestrial and aquatic biotopes. From Cowan *et al.* (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, 1998, 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 w hat 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. 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** (Olson, 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 and 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 and Çiftçioglu, 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 and Çiftçioglu, 1998). Nonetheless, a recent paper by 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.* (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.

4. Problems that may arise by mechanisms of 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 *Rhodobacter*, *Rhodospirilum*, and *Nitrosomonas*, 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). Phages play an important role in the transfer of pathogenic determinants (Dobrindt and Hacker, 2001). Recent work in the past few years has shown that cholera toxins, shiga toxins, diphteria toxins, and the botulinum toxins all reside on phages, and the recent completion of the genome sequence of two pathogenic *E. coli* O157 strains revealed the presence of > 20 prophages in their chromosome (Ohnishi *et al.*, 2001), encoding a variety of virulence-related proteins such as Shiga toxins (Stx), zinc/copper-type superoxide dismutases (SOD), and Bor proteins and many Lom homologs (implicated in host serum resistance and cell adhesion, respectively). 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 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 axeny disruption. Disruption of axeny may occur either via C I or via direct outside contamination. In this

respect, two categories of genes that are associated to mobile genetic elements require special attention: 1) genes involved in human pathogenesis and, in a lesser extent, 2) genes for resistance to antibiotics (dissemination of the latter genes in confined environments could affect the therapeutic possibilities in the case of pathogenic outbreaks).

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, van Elsas, 2001) and pMOL96/98 (Gstalder and al, in preparation, Gstalder, 2001). 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, 2002) that was found in a *Sinorhizobium* by biparental exogenous isolation (Bale et al, 1988). 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).

Taking into account these considerations about virulence genes , antibiotic resistance genes and the determinants involved in efficient gene dissemination, we have to give special attention to **the plasmid status** of the four Melissa strains and their behaviour towards exogenous plasmids (plasmids coming from outside). Some of the considerations that follow are also of relevance for TN1 (Genetic Stability) 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 P et al, 1993 Ren L, 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). 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.

9 different strains of *R. rubrum* contain a 55 kb plasmid (Kuhl et al, 1983, 1984): the curing of this plasmid irreversibly damaged the capacity to grow photosynthetically and the production of pigment. The plasmid has likely a narrow host range and no information about its transfer capabilities is directly available. The biology and the maintenance of this plasmid are obviously alsoof special relevance for WP1 and TN1.

5. Control of axenicity

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

5.2. Microscopic techniques

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).

5.3. Flow cytometry techniques

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 et al., 2001).

5.3.1. Definition.

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.

5.3.2. Applications of flow cytometry to determine contaminants.

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.

Detection by flow cytometry of anaerobic bacteria in human feces.

The human colon harbours about 10^{11} bacteria per gram contents. It is important to realise that over 99.9% of the colonic microflora consists of a stable ecosystem of possibly as many as 400 different species of anaerobic bacteria in a characteristic individual composition. Potentially pathogenic aerobic bacteria such as Enterobacteriaceae spp (e.g., *Escherichia coli*) make up less than 0.1% of the colonic flora. Faecal anaerobic bacteria are difficult to study.

Culturing and identifying anaerobic bacteria by biochemical properties are very time consuming. Moreover, for immunological studies, culture of bacteria may change their antigenic expression and harbours the danger of a bias towards easily culturable bacteria. Flow cytometry has been recently shown to offer an alternative methodology to the traditional ones. Flow cytometry was used for the analysis of noncultured anaerobic bacteria present in human faecal suspensions. Nonbacterial faecal compounds, bacterial fragments, and large aggregates could be discriminated from bacteria by staining with propidium iodide (PI) and setting a discriminator on PI fluorescence and by exclusion of events with large forward scatter (size).

Detection by flow cytometry of anaerobic bacteria in food samples.

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Detection by flow cytometry of the marine biomasses of small bacteria at low concentrations in a mixture of species.

Measurement of marine biomasses can be performed with the help of flow cytometry (more sensitive than optical density measurement e.g.). Flow cytometry has been used to analyse very diluted concentrations of cells and can differentiate bacterial subpopulations or discriminate organisms from debris. Flow cytometry can determine marine biomass from the intensity of the forward scatter (reflecting the size) of single cells. It even shows adequate sensitivity for organisms <0.1 μm^3 in size.

5.3.3. Use of fluorescent probes and dyes to label rare contaminants and to the sensitivity ofmolecular probes.

This section presents the different probes used to assess different physiological functions and cellular structures. Figure 1 summarises the different physiological target sites of these probes and table 7 gives the characteristics of the fluorescent dyes exposed in the text.

Fluorescence-based methods have remained very useful for a wide diversity of applications ranging from industrial to environmental microbiology. These tools are used for viability/activity assessment in food, pharmaceutical and cosmetic industries, and in the natural environment, including fresh and marine waters. The increased use of fluorescent probes is also due to improvements in the quantitative and qualitative sensitivity of instruments and in particular flow cytometry.

The argon ion laser is the most widely used light source for flow cytometry. Argon ion lasers provide emission lines at several wavelengths ranging from 351.1 to 514.5 nm: the most widely used is the single line at 488 nm. The excitation wavelength is fixed and the strategy for staining is limited to the range of probes and stains excitable at this wavelength. The use of probes with contrasting wavelengths is usually required for multiparameter measurement (e.g, combination of a nucleic acid dye for the quantification of total bacteria with physiological and taxonomic probes). In this instance, contrasting wavelength means a combination of excitation and emission wavelengths, which allows discrimination of each probe in the presence of the others. Double staining procedures with a single laser excitation

source (often 488 nm) are limited since both dyes may have a common excitation wavelength and different emission wavelengths with a minimal overlap.

Table 7. - Characteristics of some fluorescent dyes

Characteristic	Absorption (nm)	Emission (nm)	Molecular weight
Membrane integrity			
SYTO-9 (membrane permeant stain)	*(blue)	*(green)	*
SYTO-13 (membrane permeant stain)	488	509	400
Propidium iodide	535	617	668
Sytox Green	502	523	600
PO-PRO-3	539	567	605
CSE	*(blue)	*(orange)	*
Membrane potential			
Rhodamine 123	507	529	381
DiOC(6)	484	501	573
DiBAC4	493	516	517
Oxonol VI	599	634	316
Esterase activity			
FDA Fluorescein diacetate	473	514	416
CFDA Carboxyfluorescein diacetate	492	517	460
CFDA-AM	492	517	532
BCECF-AM	482	520	615
Calcein-AM	494	517	995
Chemchrome	488	520	*
Dehydrogenase activity			
CTC (CTC formazan, CTF)	450	580-660	332

* Unspecified by the manufacturer. Source of information: Molecular Probes (Eugene, Oregon,, USA), Polysciences Europe (Germany)

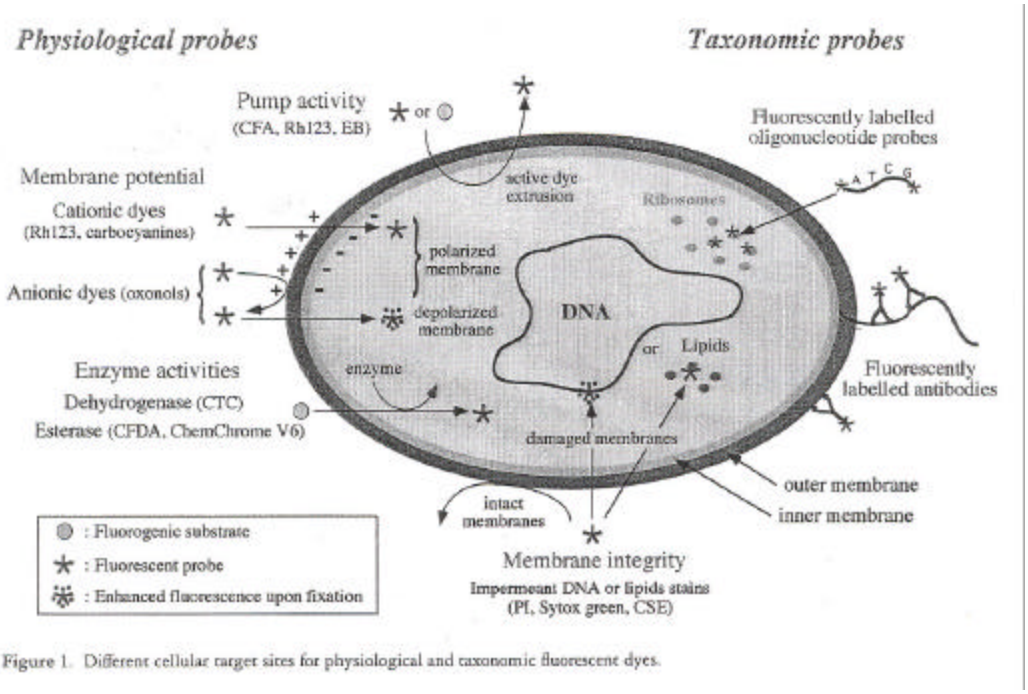

Figure 1. Different cellular target sites for physiological and taxonomic fluorescent dyes.

Membrane integrity:

The loss of membrane integrity represents significant damage for cells due to multiple functions linked to the plasma membrane (permeability barrier, transport, respiratory activity, etc...). The maintenance of membrane integrity is commonly measured in eukaryotic cells as an indicator of cell damage or cell death. Membrane integrity analysis is based on the capacity of the cells to exclude fluorescent dye compounds, which when used at low concentrations do not normally cross intact membranes. Most of the membrane integrity assays use nucleic acid stains, due to the high concentrations of nucleic acids within the cells and the large fluorescence enhancement exhibited by nucleic acid stains upon binding, leading to a clear separation between intact and dead cells.

A wide variety diversity of impermeant nucleic acid stains can be used among which propidium iodide (PI) which is the most commonly used. In order to simultaneously detect dead and intact cells, Molecular Probes has developed the Live/Dead BacLight kit containing two nucleic acid stains (SYTO-9 and PI) which differ in their spectral characteristics and their ability to penetrate intact bacterial membranes. SYTO-9 penetrates inside cells with both intact and damaged membranes, staining the cells green, whereas PI only penetrates cells with damaged membranes, staining the cells red. When the dyes are used in combination, cells with intact membrane show a green fluorescence while cells with damaged membranes show a red fluorescence (SYTO-9 emission contributes to the excitation of PI by energy transfer).

Membrane potential:

The electrochemical potential occurring through the plasma membrane of metabolising bacteria is generated by respiration or by ATP hydrolysis. It results from the selective permeability of biological membranes to a variety of cations and anions leading to a difference of electric potential across the membrane. Inside, the cell is negatively charged

compared with outside the cell, and membrane potential plays a central role in different cell-life processes (ATP synthesis, active transport, mobility, regulation of intracellular pH, etc.) Voltage-sensitive dyes have been developed to measure membrane potential in bacteria. Depending on the charge of the dye, they are accumulated in polarised (cationic dyes) or depolarised (anionic dyes) cells. In appropriate conditions, the amount of dye taken up can be directly related to the level of energy metabolism in the cell.

Rhodamine 123 (Rh-123) is a lipophilic, cationic dye commonly used to detect membrane potential. However, careful calibration of the staining procedure is required to avoid false Rh-123 positive signals.

Membrane potential can also be determined by the anionic lipophilic oxonols. Accumulation inside bacterial cells is favoured by a reduction in the magnitude of the membrane potential, allowing dye molecules to concentrate within the cell, and bind to lipid-rich components. Bis-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4(3)$) has been reported to be useful to detect depolarised cells of numerous Gram-positive and Gram-negative bacterial species.

Enzyme activity:

Dehydrogenase activity.

Cell-specific assays to detect respiratory activity of bacteria have been developed based on the use of different tetrazolium salts. Tetrazolium dyes are reduced from a colourless complex to a brightly coloured, intracellular, formazan precipitate by components of the electron transport system and/or a variety of dehydrogenase enzymes present in active bacterial cells. Since electron transport is directly related to cellular energy metabolism in respiring cells, the ability of cells to reduce tetrazolium compounds can be considered an indicator of bacterial activity. A variant approach is the use of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). CTC is reduced by bacteria to a water-insoluble, red fluorescent formazan product. It allows the quantification of the metabolic activity of bacteria under both aerobic and different anaerobic conditions. CTC is commonly used in microbial ecology, for both aquatic and terrestrial systems. Applications include drinking water, biofilms, lake and sea-water and sediments.

Esterase activity.

Detection of esterase activity is measured using lipophilic, uncharged and non-fluorescent fluorogenic substrates. Once within active cells, the substrate is cleaved by non-specific esterases releasing a polar fluorescent product (fluorescein or fluorescein derivatives) retained inside cells having an intact membrane. Esterases are present in all living organisms, and these enzymes can be used to provide information on the metabolic state of bacterial cells. Although enzyme synthesis requires energy, the enzyme-substrate reaction does not, and this assay alone should be considered energy independent. However, dead or dying cells with damaged membranes rapidly leak the dye, even if they retain some residual esterase activity. Consequently, fluorogenic substrates for esterases often serve as activity and cell integrity probes that measure both enzymatic activity, which is required to activate their fluorescence, and cell-membrane integrity, which is required for intracellular retention of their fluorescent products.

Fluorescein diacetate (FDA) is known to give weak fluorescence signals, since fluorescein is poorly retained inside the cells. In contrast, hydrophobic FDA derivatives are cleaved into hydrophilic products that are retained more efficiently inside cells with an intact membrane. Among these, acetoxymethyl ester (calcein-AM) was shown ineffective to label different species, with the exception of Staphyllococcus aureus. A comparison made between different fluorogenic esters shows that carboxyfluorescein diacetate (CFDA) is superior to FDA (fluorophore retention problems) and carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) (solubility problems). However, the best results were obtained with ChemChrome B (from Chemunex) (a commercial preparation of unknown formulation) is superior to FDA, CFDA, and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy methyl ester (BCECF-AM), as it stains the widest diversity of Gram-negative and Gram-positive species.

Nucleic acids:

The detection of damaged DNA, such as breaks in the DNA strands, is often used to characterise apoptosis in eukaryotic cells. The cellular rRNA content of bacterial cells can be quantified by fluorescence in situ rRNA hybridization (FISH) coupled with flow cytometry of oligonucleotides carrying a fluorochrome. Because rRNA content in many bacterial species varies depending on their growth rate and decrease rapidly in inactive cells, FISH coupled to flow cytometry, have been proposed to estimate the physiological state of the cells. Moreover, in the case of complex communities, this assay could be developed to detect activity of specific bacteria using appropriate oligonucleotide probes. However, in dynamic environments and when cells are submitted to stress treatment (e.g., cold stress, acetic acid, or ethanol), the rRNA content is a poor indicator of activity due to the high stability of rRNA. The recent development of FISH techniques using mRNA or pre-rRNAs (precursors in rRNA synthesis) as target molecules and those which determine the expression of specific functional genes may provide more reliable methods to assess the activity of individual cells within complex bacterial communities.

Another application of FISH is the hybridisation of DNA probes, like the ones described in the chapter 4 on molecular methods. The same probes that are used for membrane hybridisation or as PCR primers can also be used in conjunction with Flow cytometry to indicate the presence of a well-defined taxon.

5.4. Molecular methods

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). Innovative molecular tools are listed in Table 8.

Table 8. A sampling of new molecular tools (Rose and Grimes, 2001)

TOOL	CHARACTERISTICS AND ADVANTAGES	LIMITATIONS
Gene Probes	◦◦Relatively rapid compared to conventional culture methods ◦◦Can be used for quantitative assay, especially for micro organisms [1] ◦◦Can differentiate agents carrying the known virulent genes and, thereby, differentiate potentially virulent strains from nonvirulent strains	◦◦Only applicable to culturable microbes ◦◦Cannot determine the infectivity of the microbe
PCR	◦◦Applicable for detection of specific infective agents and their virulent genes; can target specific genetic elements; can be rapid and specific ◦◦Can be used for quantitative assay for a limited number of pathogens [2] ◦◦Infective agent does not have to be culturable for direct identification ◦◦Can be used for identification of functionality of the virulent genetic element (RT-PCR) ◦◦Can be applied to detect viruses that do not have a defined laboratory animal model ◦◦Can easily be used with other viability methods (e.g., culture techniques)	◦◦Only applicable if sufficient quantity of nucleic acids can be recovered from the targeted harmful micro organisms ◦◦Inconsistencies in performance of this methodology can increase uncertainty of the technique or make it unreliable (in most applications) ◦◦Must validate PCR methodologies (QA/QC) and "troubleshoot" to ensure reliability and optimal conditions prior to implementation ◦◦Currently unable to discern viable from non-viable micro organisms
RAPD, AFLP, AP-PCR, DNA Fingerprint Analyses	◦◦Genetic fingerprints can be generated by PCR amplifications followed by, if necessary, restriction endonuclease treatment ◦◦A disease-causing infectious agent can be traced for its source; this is helpful for discerning the occurrence, distribution, and prevalence of a specific disease-causing agent ◦◦Pulse field gel electrophoresis can also be useful [3]	◦◦Currently unable to discern viable from non-viable micro organisms
BioSensors	◦◦Immunoaffinity step to capture and concentrate bacteria on beads, membranes, or fiber optics probe tips, followed by detection of bound bacteria by laser excitation of bound fluorescent antibodies, acoustogravimetric wave transduction, or surface plasmon resonance ◦◦Rapid, but must have culturable micro organisms	◦◦Currently unable to discern viable from non-viable microbes
Immunomagnetic Capture Approach	◦◦Relatively specific for the targeted harmful microbe	◦◦Sensitivity, consistence, and robustness for application across different environ-mental conditions ◦◦Currently unable to discern viable from non-viable microbes

Gene Chip Technology	◦Visionary approach currently being tested and modified by a group of biotechnology companies for use in microbial water quality ◦4-hour detection ◦Sensitive to the desired level for certain harmful micro organisms ◦Specific ◦Being developed to be ten-fold less expensive for determining expressed genes in the environment	◦Technique not yet available, so limitations cannot be determined

Solid-State Biochip	◦Visionary approach currently being developed for the rapid detection (minutes) of a number of toxins and actual microbial cells ◦Approach does not require isolation and characterization of the genetic elements ◦No capturing of antibody ◦No lengthy incubation times ◦No labeling ◦No washing	◦Technique is not yet available, so limitations cannot be determined

1 For example, *Vibrio vulnificus* or *V. parahaemolyticus* from oyster homogenates enrich in suitable growth media, grow on agar plates, hybridize on filter, using specific gene probes for pathogenic and nonpathogenic strains, non-hazardous colorimetric signal identification, quantify the harmful microbes from the positive signals.
2 For example, *E. coli* by TaqMan assay (PE).
3 Note: These methods can produce inconsistent results unless they are first carefully optimized and validated.
4 Harmful microorganisms can be captured from a complex environmental sample using magnetic beads coated with specific antibodies, followed by detection using gene probes and/or PCR methodologies.

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., 2001), *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. (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. A fluorogenic 5'-nuclease assay to detect the enterotoxin *yst* gene of virulent *Yersinia enterocolitica* in food samples and was efficient for 10^3 CFU/ml (Vishnubhatla et al., 2001). Similar approaches may be used for detection of fungi using rDNA spacer sequences (Turenne et al., 1999). 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)

5.5. Antibody based methods

Antibodies may be raised against known contaminants and different forms of testing (immunoassays, ELISA …) can be used to show their presence. For example, the TECRA *Staphylococcus aureus* Visual Immunoassay is described by Hughes et al. (1999). In addition, antibodies coupled to immunomagnetic beads can serve to concentrate the organisms before any other test; like PCR (Hsih & Tsen, 2001).

5.6. Detection of contaminants' activity

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).

With a laser scanning instrument, it is possible to quantify the bacteria that were labelled by their own metabolic activity and captured by membrane filtration. For example, esterase activity converts a substrate inside the cell into a fluorescent molecule (Reynolds & Fricker, 1999). Using a step of culture in enrichment broth for bacteria, yeast and moulds, the Adenosine Triphosphate Bioluminescence assay can detect bacteria in about one day (Jimenez, 2001). A commercially available microbial phosphatase test kit (Fast Contamination Indicator; Charm Sciences, USA) uses the heat resistance of fecal microbial phosphatase to test animal carcasses (Kang & Siragusa, 2002).

The examination of more than 150 Microbial Volatile Organic Compounds (MVOCs) from indoor air samples was tested to detect moulds (*Aspergillus fumigatus, A. versicolor, A. niger, A. ochraceus, Trichoderma harzianum, T. pseudokoningii, Penicillium brevicompactum, P. chrysogenum, P. claviforme, P. expansum, Fusarium solani* and *Mucor* sp. It was found that each species has a specific MVOC profile that depends on environmental conditions (Fiedler et al., 2001).

5.7. 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-crystallise 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. (2001) and Lay (2001).

Proteomic approach has been be used to identify e possible contamination from supernatant 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-TOFS, 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 acid indicated peptidoglycan (and thus bacteria), and 3-hydroxy fatty acids was a marker of endotoxin. This method allowed detecting trace levels.

5.8. Detection of contaminants by bioelectrochemical methods

These methods involve measurement of changes in the electrical characteristics of culture media and the micro organisms themselves (Hobson et al, 1996).

5.9. Knowledge of the ecology of the contaminants

From a general point of view, it is interesting to understand the ecology and physiology of the contaminants and to compare it with the different 'niches' that are offered by the MELISSA loop. This includes 'microniches' of the compartments like biofilms on the surfaces of reactors and tubing, interfaces, filters, etc. Such an approach is advocated by Szewzyk et al. (2000) for drinking water safety analysis.

6. Detection of critical points

6.1. Detection of specific metabolite production in response to process culture condition changes

The production of specific metabolites in response to culture conditions remains to be analysed, for example by proteomics (see above).

6.2. Production of toxins

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 strains (Christiansen et al., 2001). Thus, we know that the Melissa strain does not contain the genetic information to produce this particular hepatotoxin.

6.3. Microbial biomass quality (proteins, lipids, carbohydrates)

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.

6.4. Virus development possibilities

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 *Anabaena* 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 Haanen *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 platensis* under a variety of conditions.

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, 1974, 1976; Peters et al, 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:

a) This bacterium carries a gene transfer agent (GTA) which is a defective prophage able to "constitutively" transduce bacterial DNA fragments (Lang & Beatty, 2000, 2001, 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. (This observation is also relevant for WP1 and will be handled in more detail in TN1)

b) 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).

6.5. Plasmid exchanges

The main point is to avoid the contact between the different compartments, and especially between C1 and C2. Contacts between aerobic compartments and external atmosphere (aerosols) should be monitored as well. A study will be required to define the plasmid status of the four Melissa strains (C2 to C4): presence of plasmids, their number, their stability, and their genetic content. The presence of functional restriction endonucleases in the four Melissa strains should be tested as well: it may be important to check in which extent the four strains can "restrict" foreign DNA.

The presence of conjugative plasmids in C1 bacteria will surely deserve a study even if we know that C1 will never go into contact with the rest of the Melissa loop and if the (moderate) thermophilic character of C1 may counterselect some undesired genetic determinants.

6.6. Transmissions of prions and the like

More studies are required to know better how prions and the like can be transmitted. However, they are not in the scope of this study.

6.7. Genetic elements of eukaryotic origin or first found in eukaryotes:

Two lines of information have to be quoted at this stage:

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. Nevertheless, the observation deserves some attention in the context of Melissa

7. Axenicity indicators

A battery of axenicity indicators could be pointed out:

- 'normal' exoprotein patterns and absence of toxins (MALDI-TOFS)
- absence of PCR products after PCR detection with primers targeting possible contaminants
- absence of contaminant cells and viruses detected by Flow cytometry
- absence of contaminant's DNA detected by a DNA-chip containing probes for a whole array of possible contaminants
- microscopy
- plating

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Annexe 1: Interesting articles about flow cytometry and its applications

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Annexe 2: Interesting papers on mass spectrometry

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Annexe 3: Interesting papers on methods to detect contaminants

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