MICROBIAL ECOLOGY OF THE MARS 500 HABITAT

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES DER
NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER NATURWISSENSCHAFTLICHEN FAKULTÄT III
- BIOLOGIE UND VORKLINISCHE MEDIZIN -
DER UNIVERSITÄT REGENSBURG

vorgelegt von
Petra Schwendner
aus Regensburg
im Januar 2014

Promotionsgesuch eingereicht am: 22.01.2014

Die Arbeit wurde angeleitet von: **Prof. Dr. Reinhard Wirth** (Universität Regensburg) in Zusammenarbeit mit **Dr. Petra Rettberg** (Deutsches Zentrum für Luft- und Raumfahrt)

Prüfungsausschuss:	Vorsitzender:	**Prof. Dr. Stephan Schneuwly**
	1. Gutachter:	**Prof. Dr. Reinhard Wirth**
	2. Gutachter:	**Prof. Dr. Bettina Siebers**
	3. Prüfer:	**Prof. Dr. Reinhard Rachel**

Action is the foundational key to all success.

Pablo Picasso

TABLE OF CONTENTS

ABBREVIATIONS

Chemical elements and compounds were named the International Union of Pure and Applied Chemistry (IUPAC).

ARISA	Automated Ribosomal Intergenic Space Analysis
AT	Abundance metric
BiSKit	Biological Sampling Kit
BLAST	Basic Local Alignment Search Tool
bp	base pair(s)
BT	Binary metric
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
cm	centimeter
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DLR	German Aerospace Center
ds	double-stranded
ECSS	European Cooperation for Space Standardization
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
EPS	extracellular polymeric substances
ESA	European Space Agency
EU	experimental unit
eOTU	empirical operational taxonomic unit
FB	field blank
g	gram
G2	second-generation
G3	third-generation
gDNA	genomic deoxyribonucleic acid
h	hour
HC-AN	hierarchical clustering using average-neighbor method
HS	heat-shock
IBMP	Institute of Biomedical Problems
i.e.	that is
IsoContrl	isolation control
ISS	International Space Station
iTOL	interactive Tree Of Life
kb	kilo base pair(s)
keV	kiloelectron volt
kHz	kilohertz
l	liter
LC	lab control
LOCAD-PTS	Lab-On-a-Chip Application Development Portable Test System
m	meter
M	molar
MICHAm	Microbial Ecology of Confined Habitats and humAn health, modified
min	minute(s)
ml	milliliter
mM	millimole
mRNA	messenger ribonucleic acid

NASA	National Aeronautics and Space Administration
NCBI	National Center for Biotechnology Information
Nd	not determined
ng	nanogram
NMDS	non-metric multidimensional scaling
NMPDR	National Microbial Pathogen Database Resource
OOR	out of range
OTU	operational taxonomic unit
PBS	Phosphate-buffered saline
PCoA	Principal co-ordinates analysis
PCR	polymerase chain reaction
pg	picogram
pH	potency of hydrogen
PPi	pyrophosphate
PPO	potentially pathogenic organism
R2A	Reasoner's 2A agar
RAS	Russian Academy of Sciences
RH	relative humidity
RNA	ribonucleic acid
RODAC	recovering organisms detecting and counting dish
rOTU	reference operational taxonomic unit
rRNA	ribosomal ribonucleic acid
rpm	rounds per minute
s	second(s)
SDS	sodium dodecyl sulfate
SFINCSS	Simulation of Flight of International Crew on Space Station
SMS	Simulator of the Martian surface
sp	species (singular)
spp	species (plural)
ss	single-stranded
SSU	small ribosomal subunit
TAE	tris-acetate-EDTA
TE	tris-EDTA
TRBA	Technical Rules for Biological Agents
tris	trishydroxymethylaminomethane
TSA	trypticase soy agar
U	unit
UV	ultaviolet
V	volume
v/v	volume per volume
W	watt
w/v	weight per volume
w/w	weight per weight
VBNC	viable but nonculturable
XS	xanthogenate-sodium dodecyl sulface
µl	microliter

ABSTRACT

Biocontamination in confined manned habitats poses a risk for the health of the crew and for the on-board equipment. The reduced immune response of humans that are exposed to extreme environmental conditions, like during long-term confinement, and the degradation capacities of some microorganisms call for continuous monitoring of biocontamination, and measures to mitigate it. Prevention includes taking the acquired knowledge into account for future habitat design.

The MARS 500 project represented a unique opportunity to gain insight into the microbial distribution and its community structure during a long period of time. MARS 500 is the first full duration simulation of a manned flight to Mars, accomplished from June of 2010 to November of 2011. The six-man crew lived, worked, and performed scientific experiments in a closed spacecraft-like environment.

The scientific experiment MICHAm (MIcrobial ecology of Confined Habitats and humAn health, modified) aimed to survey the microbial flora in the MARS 500 facility from the start to the end of the simulation study (520 days), and to investigate the impact of confinement. Therefore, the microbial load and biodiversity in the air and on surfaces as well as their changes over time were monitored. The determination of cultivable microorganisms showed that the overall microbial load in the air and on different surfaces was moderate compared to other non-confined rooms (air: 0 to 716 CFU per m^3 with an average value of 86 CFU per m^3; surface: 0 to 29,760 CFU per 10 m^2 with an average value of 675 CFU per 10 cm^2). The highest number of microorganisms was found in the air of the habitable module, where the crew members spent most of their time. This corresponds roughly to the results obtained from surfaces at certain locations. During the project, fluctuations in microbial load were detected. Those were analyzed in more detail by taking into account the crew activities in the different modules, the cleaning regimes, the air conditioning processes, *et cetera*. However, none of the single parameters revealed a significant influence, thus it is assumed that the combination of all of them led to the site- and time-specific contamination levels.

Phylogenetic investigations indicated a higher diversity in surface samples than in air samples. Dominance of microorganisms associated with humans, especially *Staphylococcus* species, was observed on surfaces and in the air. Environmental microorganisms, mainly spore-forming bacilli, were found to a lesser extent. Besides cultivation-based analyses, the microbial inventory was also studied on the molecular level via DNA isolation, 16S rRNA gene specific amplification, and subsequent PhyloChip analysis, to quantitatively measure the microbiome of two modules of the MARS 500 facility. The findings were surprising since the whole microbiome structure exhibited no significant influence for factor time, with the exception of a few taxa. However, significantly different microbiome structures were identified for both investigated modules, whereas the intragroup dissimilarity among samples was greater in the utility module than in the habitable module.

Furthermore, by applying both cultivation and molecular methods, a deeper understanding of the microbiota and microbiome was obtained that also unveiled potentially pathogenic organisms (e.g. *Staphylococcus* sp., *Propionibacterium* sp., *Enterococcus* sp., and many

more) and microbes with degradative capacities (e.g., *Pseudomonas* sp., *Micrococcus* sp., *Bacillus* sp., *Acinetobacte*r sp., *Flavobacterium* sp., *Escherichia* sp., and *Actinomyces* sp.). However, the majority of microorganisms were not harmful, but belonged to the group of microbes that are even highly valuable to humans. During no point of the confinement study there was any alert and health concern due to potential danger caused by microorganisms.
The scientific information obtained in this thesis is essential to evaluate biosafety risks, predict and mitigate the possible occurrence of biocorrosion, and improve the sanitary and hygienic quality of life for the crew inside closed habitats.

ZUSAMMENFASSUNG

In abgeschlossenen Habitaten, in denen sich Menschen für längere Zeit aufhalten, stellt die biologische Kontamination ein Risiko dar, sowohl im Hinblick auf die Gesundheit des Einzelnen durch mögliche Infektionen als auch im Hinblick auf Materialschäden, die zu einer Fehlfunktion von wichtigen Instrumenten führen können. Geschwächte Immunabwehr bei Menschen, die über einen langen Zeitraum extremen Umweltbedingungen ausgesetzt sind, und durch Mikroorganismen verursachte Biokorrosion erfordern eine kontinuierliche Überwachung der mikrobiellen Kontamination, und die Entwicklung von Gegenmaßnahmen um einer Ausbreitung entgegenzuwirken. Für zukünftige Missionen kann eine Prävention nur dann ermöglicht werden, wenn durch detaillierte mikrobiologische Untersuchungen, Kenntnisse erworben werden, die charakteristisch für den Lebensraum Raumfahrzeug mit den besonderen vorherrschenden Bedingungen sind.

Das Projekt MARS 500 stellte eine einzigartige Gelegenheit dar, um Einblicke in die mikrobielle Verteilung in einem Habitat und die Veränderung über den Zeitraum hinweg zu gewinnen. Es ist die erste Echtzeitsimulationsstudie eines bemannten Fluges zum Mars, die von Juni 2010 bis November 2011 durchgeführt wurde. Die männliche, sechsköpfige Besatzung lebte in diesem geschlossenen raumschiff-ähnlichen Lebensraum und führte wissenschaftliche Experimente durch. In dem Experiment MICHAm (Mikrobiologie und Gesundheit im geschlossenen System) wurde sowohl die mikrobielle Population an Oberflächen und in der Luft des Habitats und deren Entwicklung im Verlauf der 520 Tage währenden Isolationsstudie verfolgt, als auch der Einfluss des Menschen auf die mikrobielle Diversität erfasst. Dabei wurden neben der Gesamtkeimzahl auch die Biodiversität in der Luft und auf Oberflächen, sowie deren zeitlichen Veränderung überwacht. Nach Bestimmung der kultivierbaren Mikroorganismen zeigte sich eine mäßige Keimbelastung (Luft: 0 bis 716 CFU pro m³ und einem Mittelwert von 86 CFU pro m³; Oberflächen: 0 bis 29.760 CFU pro 10 m² und einem Mittelwert von 675 CFU pro 10 cm²), verglichen mit anderen nicht-geschlossenen Räumen. Die höchste mikrobielle Belastung fand sich in der Luft des Wohnbereichs, die niedrigste im weniger frequentierten Lager und dem medizinischen Modul. Eine ähnliche Verteilung der vorhandenen Mikroflora lieferte auch die Analyse der entsprechenden Oberflächenwischproben. Während der gesamten Isolationsstudie wurden Schwankungen in der Gesamtkeimzahl beobachtet. Diese wurden genauer analysiert unter Berücksichtigung der Aktivitäten der Probanden in den verschiedenen Modulen, der Reinigung, der vorherrschenden klimatischen Gegebenheiten und so weiter. Keiner der einzelnen Parameter ergab einen signifikanten Einfluss, so dass die Kombination aller Faktoren zu den ortstypischen und zeitspezifischen Kontaminationen geführt haben musste. Die Identifizierung der Isolate mittels Gensequenzanalyse ergab sowohl verschiedene menschen-assoziierte Stämme, vor allem *Staphylococcus*-Arten als auch Umweltkeime. Die mikrobielle Diversität lag jedoch deutlich höher bei den Oberflächenproben als bei den Luftproben. Ein Pasteurisierungsschritt (15 min bei 80°C) eines Aliquots von den Oberflächenwischproben erlaubte die selektive Anreicherung von hitzetoleranten Mikroben - darunter befanden sich meist sporenbildende *Bacillus*-Arten.

Parallel zu den kultivierungsbasierten Analysen wurde die mikrobielle Population auch auf molekularer Ebene untersucht. Nach DNA-Isolierung und 16S rRNA-Gen-spezifischer Amplifikation wurde eine PhyloChip Analyse angewandt, um eine quantitative Aussage über die vorherrschenden Mikrobiome in zwei Modulen treffen zu können. Die Ergebnisse waren überraschend, da - mit Ausnahme einzelner Taxa - kein signifikanter Einfluss des Faktors Zeit auf die mikrobielle Zusammensetzung festgestellt werden konnte. Allerdings wurden in beiden Modulen deutlich unterschiedliche Mikrobiomstrukturen identifiziert, wobei sich die Proben innerhalb des Lager- und Sportbereiches weniger untereinander ähnelten als die aus dem Wohn-Modul entnommenen Poben.
Darüber hinaus wurde durch die Anwendung von Kultivierungs- als auch molekularer Analysen, ein tieferes Verständnis der Mikrobiota und des Mikrobioms erreicht. Zusätzlich lieferten beide Methoden Hinweise auf die Anwesenheit von potenziellen Krankheitserregern (z.B. *Staphylococcus* sp., *Propionibacterium* sp., *Enterococcus* sp., *et cetera*) sowie von Mikroben (wie z.B. *Pseudomonas* sp., *Micrococcus* sp., *Bacillus* sp., *Acinetobacte*r sp., *Flavobacterium* sp., *Escherichia* sp., and *Actinomyces* sp.), die die Fähigkeit besitzen Metalle und andere Baumaterialien zu schädigen. Bei der Mehrheit der identifizierten Mikroorganismen handelt es sich jedoch nicht um schädliche, sondern im Gegenteil um lebensnotwendige Symbionten für den Menschen.
Zu keinem Zeitpunkt der Studie gab es ein erhöhtes Gesundheits- oder Sicherheitsrisiko und somit war die Gesundheit der Probanden nicht gefährdet.
Die aus dem MARS 500-Projekt MICHAm erzielten Erkenntnisse sind essentiell für künftige bemannte Weltraummissionen, um die Gesundheit durch eine hohe sanitäre und hygienische Lebensqualität zu gewährleisten und die Funktionalität lebenswichtiger Geräte sicherzustellen, die durch die Akkumulation von pathogenen Organismen und das mögliche Auftreten von Biokorrosion gefährdet werden könnten.

I. INTRODUCTION

I.1. HUMAN EXPLORATION OF SPACE

Human exploration of our Solar System started in October of 1957 with the launch of the first-ever satellite Sputnik by the Soviet Union. Shortly thereafter, the first animal, a dog named Laika[1], followed in November onboard the Sputnik representing the first living creature in orbit. She survived the launch, but unfortunately died due to overheating of the capsule and the lack of oxygen in space (Morey-Holton *et al*., 2007).
Eagerly awaited, on April 12th, of 1961 the first human was sent to space - Yuri Gagarin. He was carried into space by a spherical Vostok spaceship and successfully completed the mission (Gagarin and Lebedev, 1969). Alan Shepard, a NASA astronaut, was the second person in space (1961) travelling into the suborbital level as part of the Mercury program (Shepard and Slayton, 1994). In the same year, the Apollo lunar exploration program was launched and reached its peak as the "Eagle has landed" on the Moon (Neil Armstrong) with Apollo 11 on July 20th, of 1969. This event marked the arrival of humans on the Moon. Buzz Aldrin and Neil Armstrong spent 22 h on the surface and two and a half hours outside the Eagle module, where they collected 22 kg of rock and dust samples (Armstrong, 1970).
Once landed on the Moon, going to Mars, which is one of the oldest dreams of the human race, neared. The belief that a human Mars expedition would happen someday came into range. However, as far as Mars is concerned, recent history shows that the whole task is difficult to realize and a tremendous effort will be necessary in order to make reaching Mars feasible. There are several key challenges that must be overcome. Using today's technologies only a few launch windows for manned missions exist, which run through a continuous cycle lasting about 15 years. The duration of the mission depends on the proximity of Earth and Mars to each other. A conjunction-class Mars mission and opposition-class Mars mission can be distinguished. The former takes about 1,000 days and includes a low-energy transfer from Earth to Mars followed by a long stay on Mars, whereas the latter can be carried out in approximately 520 days (Heppener, 2008). Moreover, the opposition-class scenario offers a short stay on the Martian surface, but requires a high-energy transfer when departing from Mars, which in turn demands more propellant than long-term missions (Manned Exploration Requirements and Considerations, 1971). Once launched, there is almost no possibility for mission abort and fast return. Furthermore, the crew has to arrange and manage with delays of up to 45 minutes in bi-directional communication. Very long-lasting zero gravity level during interplanetary transfers trigger adverse effects on the human physiology and immune system, e.g., degradation of muscle mass, bone loss, and changes in the cardiovascular system. Enhanced radiation exposure due to solar and galactic cosmic radiation potentially causing early and late radiation effects is another major concern. Reduced pressure on Mars and the impact of very high gravity levels on landing and ascent techniques have to be taken

[1] http://www.spacetoday.org/Astronauts/Animals/Dogs.html

into account. A major step forward in reducing risks is the inclusion of advanced propulsion techniques, improved protection against cosmic radiation, continuous microbial monitoring of the manned habitat and its life support systems, and the installation of gym equipment to overcome the effects caused by weightlessness (Horneck *et al*., 2010).
The longer the mission duration, the greater are the risks astronauts are exposed to and thus, the *sine qua non* is to perform test-runs on Earth and a plethora of scientific experiments. Consequently, the major technical, scientific, financial, and policy-related challenges that need to be overcome to send humans to Mars by 2030 have to be addressed. This will include Mars mission architecture and challenges, science goals, planetary protection[2], international cooperation and ground-based experiments.

I.2. Isolation Experiments in Moscow

I.2.1. History[3]

In the late 60s, the idea was launched to conduct ground-based experiments at the Institute for Bio-Medical Problems (IBMP) in Moscow, Russia to simulate long-duration spaceflight. The major goal was to mentally and physically prepare the cosmonauts and astronauts for demands of prospective space travels. The first isolation experiment, called “A year in Earth Spaceship” was realized in November of 1967. During this period, three volunteers were isolated to test the operationality of life support systems. By then, little was known about the feasibility of such a project and the influences on the human itself when being isolated in a hermetical facility.
However, simulation of a spaceflight demands a mock-up spacecraft that mimics conditions prevalent during a real spaceflight as closely as possible. Therefore, the medical-technical facility was built in the period between 1964 and 1970. Initially, the facility consisted of the experimental units EU-100, EU-150, and EU-50 that fulfilled all requirements, from the possibility of complete isolation to an operative biomedical support system for collection and processing medical information.
Upon completion, several campaigns were performed from 1971 to 2000 to assess whether and how humans adapt to conditions they would face during spaceflight, and to verify the methodologies used for those experiments. Initial investigations on the reaction of the human body during 60 to 120 days of confinement were performed in the first four years. Later on, the main focus was on psychological issues in order to unveil group dynamics of the isolated individuals. More and more, physiological aspects were also under investigation.
The first long-duration project of 240 days was called SFINCSS-99 (Simulation of Flight of International Crew on Space Station). Between February and September of 1999, 80 different national and international experiments were conducted during a simulated flight to

[2] Planetary protection is the practice of protecting a planet from unintended terrestrial contamination and also protecting Earth’s biosphere from possible extraterrestrial life forms.

[3] History of Isolation Programs: http://mars500.imbp.ru/en/history.html

the International Space Station (ISS) with three main and three visiting crews that were of mixed nationalities and gender.
Immediately upon completion of the SFINCSS-99 project, the focus of interest changed from space capsules and stations to manned flights to other planets. Since missions to Mars differ tremendously from flights around Earth, investigation of new medical, psychological, physiological, microbiological, and sanitary-hygienic aspects are inevitable.

I.2.2. MARS 500 Program[4]

To answer the wide circle of questions that arose when announcing Mars as a future goal for human exploration, the MARS 500 program was developed. Therefore, the medical-technical facility had to be extended. In 2007, an additional fourth module (EU-250) was built, followed by a fifth module in 2008, which simulates the Martian surface.

I.2.2.1. 14-days Isolation

The simulation of a long journey to Mars started with an acceptance trial in two of the modules (EU-150 and EU-100) for only 14 days in November of 2007. This pilot run was performed to test the working capacity and reliability of technical, medical and communication systems, and operational procedures. Furthermore, demonstration that all required specifications were met and suitable conditions for crew's life were created during isolation as closely as possible to real space conditions, should be done.

I.2.2.2. 105-days Isolation

The second stage of the flight to the red planet was a 105-day confinement study of a crew consisting of six males, and was performed from March 31st to July 14th, of 2009. The purpose of this dry-run was to obtain and analyze scientific and technical information for optimal organization of training sessions and effective implementation of preliminary experiments, while simulating all stages of a manned flight to Mars. More than 70 Russian and foreign experiments were conducted regarding clinical-physiological, psychological, biochemical, immunological, biological, and operational technological issues. The obtained data were used as baseline information for preparation and conduction of a 520-day experiment. During the confinement, microbiological and sanitary-hygienic studies were performed by Russian investigators.
The main purpose of microbiological surveys was to test technologies (electronic nose) that allow rapid diagnosis of the microbial contamination and its influence on the artificial habitat. Therefore, microbiological markers of various substrates were applied. Furthermore, the composition of microorganisms was monitored by sampling and further cultivation according to the Russian standard.

[4] ESA activities: http://www.esa.int/Our_Activities/Human_Spaceflight/Mars500/ESA_and_isolation_studies

I.2.2.3. 520-days Isolation

The successful completion of preliminary test runs paved the way for the real-time simulation of a manned mission to Mars, which finally started on June 3rd, of 2010. When the hatch was closed, the six participants, namely the two ESA (European Space Agency) crew members Diego Urbina (Italy, Columbia) and Romain Charles (France), three Russians (Alexey Sitev, Sukhrob Kamolov, Alexandr Smoleevskiy) and one Chinese (Wang Yue) were sealed in the isolation chamber (Fig. I.2.2.3.1).

Fig. I.2.2.3.1 MARS 500 crew seconds before ingressing the hermetically sealed modules for a 520 day stay in June of 2010. © IBMP/Oleg Voloshin

During the following 520 days, these candidates, which are also called marsonauts, lived and acted like astronauts and were responsible for their health. They had to monitor and control all systems and to carry out cleaning and maintenance tasks. Furthermore, they actively performed scientific experiments, but were parallel subjects to assess psychological and physiological aspects. Even communication with the control center was more and more delayed similarly to a real flight to Mars, with a maximum uni-directional retardation of 20 minutes. The crew was split into two groups of three people each when preparation for landing on the Martian surface started (February 1st to 27th, of 2011), and one group entered the Martian simulation module (EU-50). On November 5th, the crew “landed safely on Earth” and the marsonauts left the isolation facility after successfully completing the study.

These ground-based activities ensure safety and success of a future manned mission. Missions that endure longer than six months and target planets beyond low Earth orbit implicate new challenges for the astronauts’ mentality and physiological state, as well as the need for full autonomy, provision, and reprocessing of resources. Furthermore, a crew’s daily life and operation capabilities might be affected adversely by hazardous space environment. During the test in the mock-up facility, scientists and the test subjects unveiled possible difficulties, peculiarities, and drawbacks caused by long-term isolation and confinement. Thus, countermeasures can be developed against any adverse effects that might be envisaged during a long duration manned mission. More than 100 experiments,

carried out by collaborators world-wide, were performed during the confinement to gather data, knowledge and experience for improving mission design. Beyond that, astronauts and the involved scientists were prepared as effectively as possible, but without the influence of weightlessness.
One of these experiments is called MIcrobial ecology of Confined Habitats and humAn health (MICHAm, modified) and was proposed by Dr. Petra Rettberg (DLR, Germany) in cooperation with Prof. Dr. Canganella (University of Tuscia, Italy) and Dr. Viacheslav Ilyin (IBMP, Russia). Besides supplementing marsonauts with microbial probiotic food products (*Enterococcus faecalis*) to investigate the effects on the mouth and intestinal microflora, antimicrobial surfaces were installed and their effectivity was tested (University of Tuscia). Furthermore, the microbial population that was present in the habitat was monitored and investigated at IBMP and DLR.

I.3. Microbial Monitoring – Why, How and Where?

Microorganisms are ubiquitous, highly diverse due to adaptation to almost all environments, and play a crucial role in all systems. They colonize all substrates and also humans, even though every (e.g., extreme, confined) habitat has its own specificities.
Microbial monitoring is inevitable in pharmaceutical industries as well as in spacecraft-associated clean rooms with respect to planetary protection considerations. Furthermore, it is performed in production and laboratory facilities for both sterile and non-sterile products. In the food industry, monitoring is also essential for evaluation of microbiological hygiene (Salo *et al*., 2000). In order to fulfill alert and action levels, the frequency and type of monitoring is dependent on the sampled product and/or location. In general, the established monitoring program should be meaningful, manageable, and defendable (Moldenhauer, 2005). To keep the sample size manageable, a decision has to be made whether a broad sampling of a diverse set of different sites or a deep sampling of a small number of sites has to be performed. Broad sampling allows general statements about principles that control community structure and function, whereas deep sampling is necessary to obtain insights into the distribution of species and genera (Turnbaugh *et al*., 2007). A huge set of standard methods are currently available in which different tools are applied. However, each of the applied protocols has both advantages and disadvantages. Since there is no general advice, pros and cons have to be weighed for the selection of the best-suited system, depending on the project.
In order to obtain qualitative and quantitative insights that are representative of a habitat's microbial community, an ideal sampling device has to be used. Ultimately, the sampling tool must have the ability to both absorb and release as much of the microbial matter as possible. Thus, of critical importance is the type of material that makes up the sampling device and the physical structure of the sampling device material (Madhusudhan, 2009; Venkateswaran *et al*., 2012b).

I.3.1. Air Sampling

Over the years, a plethora of different air samplers were developed, indicating diverse shortcomings involved in efficient collection. This specifically applies to matter containing biological material, where a particular concern is the survivability. The sampling process itself, in which, depending on sampling mechanism, severe physical forces are applied, should not decrease the ability to thrive by drying out. Furthermore, there is a distinction regarding the sampling procedure, which can be done by passive or active techniques. Therefore, a broad variety of different collection principals were suggested, ranging from impingement, impaction, filtration, sedimentation, centrifugation and electrostatic or thermal precipitation (Cox, 1987).

- **Passive**

Fall-out (sedimentation) plates are encountered as passive air monitoring. These sedimentation plates, which are petri dishes filled with nutrient agar, are laid out for a certain period of time in order to get a first snapshot of the airborne microbial contamination. This method is very easy and requires no additional sampling tool. However, it is also biased due to the certain sedimentation speed of rods, cocci and spores on Earth (Schleibinger *et al*., 2004).

- **Active**

As distinguished from passive sampling, active air monitoring is a quantitative method. This can only be reached by sampling a known volume of air, followed by enumeration of microorganisms that were deposited on an agar surface or filter (during the filtration process). Widely used sampling techniques include impaction and filtration methods. Cascade impact samplers, for example, subdivide airborne particles into different sizes, which are drawn through holes and are subsequently impacted onto agar plates. The slit sampler applies another type of impactor methodology. A turntable is mounted on the sampling tool, which rotates once per sampling interval to distribute the sampled microbes over the agar plate. Air sampling procedures are usually of short-time type and general endure 2 to 20 min, where flow rates can be adjusted and range from a few to thousands of liters per minute (Cox, 1987). A lot of techniques sample directly onto nutrient dishes to eliminate further treatment steps, which may result in loss of biomass. Another possibility is the use of water-soluble gelatine filters, instead of the insoluble polycarbonate or cellulose nitrate filters (AirPort device). However, one has to keep in mind that each active sampler gives different results (Pasquarella *et al*., 2000).

I.3.2. Surface Sampling

Generally, it is known that bacteria are able to survive on surfaces and in dust for some time (Schleibinger *et al*., 2004). Surface sampling is conducted to determine the amount of viable microorganisms present on a certain surface. In order to gain insights into the real environmental microbial community, successful collection of biological matter is critical. The tools used for sampling should be appropriate for testing all distinct types of surfaces that are present in the monitored facility. Depending on the project and purpose, more than one method may be applied since different sampling tools show a large variability in recovery

rates (Moldenhauer, 2005). Typical methods include contact plates, swabs, wipes, and Biological Sampling Kits (BiSKits).

- **Contact plates**

Contact samples can be divided into two different subgroups, i.e., cultivable/viable contact culture samples and non-viable samples. In order to determine the viable fraction of the microbial contaminants, petri dishes are filled with nutrient agar over the rim, which is also known as “recovering organisms detecting and counting dish” (RODAC). The agar plate is pressed onto the area of interest so that the resident bacteria stick to the agar and form visible colonies during incubation.

For the nonviable technique, a clear adhesive tape is pressed onto the surface, removed and transferred to a microscope slide. Staining and subsequent microscopically analysis follows in order to determine microbial contamination (Schleibinger *et al*., 2004).

- **Swabs**

Small areas (up to 25 cm²) with irregular surface structures such as skin (Grice *et al*., 2008), equipment, or spacecraft can most effectively be sampled by the use of sterile swabs. Cotton swabs are used for spacecraft sampling and consist of approximately 95 % cellulose. The remaining 5 % are proteins, ash, sugar, organic acids, and other chemicals. Additionally, the outer surface of cotton fibers is covered by a protective barrier of wax. The higher the wax content on cotton fibers, the higher is the surface area of the cotton (Venkateswaran *et al*., 2012b).

Nylon-flocked, macrofoam-based, and polyester swabs are also available and used for collection of microbial samples of various surfaces (Sanderson *et al*., 2002; Rose *et al*., 2004; Hodges *et al*., 2006; Hodges *et al*., 2010; Probst *et al*., 2010a).

The perpendicularly sprayed fibers of the nylon-flocked swab consist of long molecular chains of amides and result in a brush-like texture (Venkateswaran, 2012b). Generally, nylon-flocked swabs are used for nasopharyngeal sample collection, but are also commonly utilized in sampling of spacecraft and their surrounding clean rooms (Rettberg *et al*., 2006; Stieglmeier *et al*., 2009; Stieglmeier *et al*., 2012; Moissl-Eichinger *et al*., 2013; Schwendner *et al*., 2013).

Macrofoam-tipped swabs are produced by turning aqueous foam into a macrofoam sponge. The liquid is mostly composed of water, surfactants, alcohols, and polymers (Archuleta, 1995). Macrofoam sponges are very effective in sample-uptake and can therefore be used for sampling even larger surfaces.

The head of polyester (polyethylene terephthalate) swabs consists of long, ester-linked polymer chains that are double-knitted. The features that make this swab ideal for keeping clean rooms debris-free are an encumbrance for the purpose of sample release (Venkateswaran *et al*., 2012b).

- **Wipes**

A diverse set of different wipe types has been designed for critical-process cleaning of large rough, abrasive, or irregular surfaces of up to 1 m² (initially 0.036 cm²), and also meet diverse clean room class requirements. These wipes are deployed in electronic, pharmaceutical, biotechnological, chemical, and environmental industries. ESA standard for monitoring of spacecraft hardware and their surrounding clean rooms intended to use 100

% polyester wipes (ECSS-Q-ST-70-55 Working Group, 2008). Cotton disinfection wipes, which contain either a mixture of hydrogen peroxide and a quaternary ammonium compound ('Fungistat' kit - supplied by Russian space agency) or a sole quaternary ammonium compound (supplied by National Aeronautics and Space Administration [NASA]), are the tool of choice suggested by the Russian Federal Space Agency (Roscosmos) and NASA (NASA, 2010).

- **Biological Sampling Kit (BiSKit)**

Macrofoam BiSKits are also used to collect biological material from surfaces of up to 1 m². Due to the special design of the unit, where the sample is directly delivered in a liquid with sufficient volume for further testing, the following improvements can be reached. Bias due to cross-contamination when multiple samples are taken is minimized. Furthermore, handling and transportation after sampling is amended since the liquid is safely captured in a collection vial, which in turn leads to an increase in quality and performance of the unit (Buttner *et al.*, 2004). In order to steadily improve the performance of microbial “bioburden” estimation with special regard to spacecraft-associated clean rooms and planetary protection considerations, standard operating procedures for use of BiSKits have also been developed by ESA and NASA (ECSS-Q-ST-70-55 Working Group, 2008; NASA, 2010).

I.4. State-of-the-Art Analysis Techniques for Environmental Community Structures

The estimation and characterization of microbial diversity is still a challenge for microbiologists (Hughes *et al.*, 2001; Cases and de Lorenzo, 2002; Curtis and Sloan, 2004; Loisel *et al.*, 2006; Mohapatra and La Duc, 2013).

Microbial screening of the environment involves the use of different sampling tools as described above. Following sampling, various approaches based on different analysis methodologies are available, and will continuously be improved and extended to fulfill the overall goal which is to obtain the most complete view on a microbial community of a certain monitored environment.

I.4.1. Cultivation Approaches

Studying microorganisms in a habitat was traditionally done by cultivation. To maximize the identified diversity, a manifold set of microbial media that reflect all different kinds of requirements were established. Cultivation media are generally distinguished between rich nutrient, minimal, selective, and differential medium. Despite the huge knowledge about growth requirements of microorganisms, only 1 % can be cultivated (Amann *et al.*, 1995). The remaining 99 % consist of bacteria and archaea that cannot be cultivated, either due to the lack of optimal enrichment conditions or because they can only grow in co-culture with other microbes dependent from their metabolites (Jahn *et al.*, 2008). Another possibility is that the cells are present in a viable but nonculturable (VBNC) physiological state (Oliver, 2005).

The monitoring strategies developed by the space agencies with regard to planetary protection requirements are based on the determination of “vegetatives”, “bioburden” (spore-formers and heat-tolerant microorganisms), and extremophiles. Cell counts of heterotrophs are performed either on Trypticase Soy Agar (TSA) or Reasoner’s 2A agar (R2A) by pour plate or streak plate techniques.

I.4.2. Molecular Techniques

Since 99 % of all microorganisms cannot be targeted by cultivation under laboratory conditions, molecular techniques gained more and more importance when describing environmental microbial communities.
Recently, the amount of available tools and techniques increased rapidly in order to provide possibilities to further investigate the ecology and potential metabolic capabilities of the unculturable fraction. When using molecular techniques, the microbial community can be studied in its natural habitat and changes in response to environmental factors can be detected, whereas, cultivation approaches utilize optimum conditions, which do not always reflect the original environment. Not uncommonly, strains loose metabolic capabilities and structural features when continuously cultured in the laboratories (Wick *et al*., 2001; Bellack, 2011; pers. comment: Prof. Dr. Reinhard Wirth).
A prerequisite for molecular phylogenetic characterization of microbial communities is, however, that stable biomarkers are identified that are conserved and ubiquitous in all prokaryotes. The most notable biomarker is the 16S rRNA gene, which is a component of the small 30S subunit of the prokaryotic ribosome. With a size of about 1.5 kb, the 16S rRNA gene consists of conserved regions and nine “(hyper-) variable regions” termed V1-V9 (Van de Peer *et al*., 1996). The latter are often flanked by stretches that are more conserved, and therefore can easily be targeted by domain-specific primers (McCabe *et al*., 1999; Baker *et al*., 2003), whereas the conserved regions are used for universal PCR primer design. Ultimately, a single “(hyper-)variable region” is not sufficient for general distinction on the species level (Chakravorty *et al*., 2007) with the exception of a limited number of different species or genera (Choi *et al*., 1996; Becker *et al*., 2004; Stohr *et al*., 2005). To access the 16S rRNA gene for further analysis with different tools, the entire genomic nucleic acids have to be extracted from a pure culture or from environmental samples. The 16S rRNA gene can then be amplified by species- or domain-directed primers and subjected to further down-stream analysis, e.g., the generation of a clone library.

I.4.2.1. Cloning

The transfer of a DNA (deoxyribonucleic acid) fragment from one organism into a host organism via self-replicating genetic elements (cloning vectors), such as bacterial plasmids, is called cloning. Once the transformed cells are screened for positive colonies with DNA inserts, subsequent polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis, and/or sequencing allows the identification of the quality, effectivity, and specificity of the inserted amplicon and its phylogenetic classification.

Furthermore, all sequences have to be checked for occurrence of chimeric[5] inserts as negative side effects (Schmidt *et al*., 1991).
The amount of clones that should be analyzed per sample location is dependent on the microbial community structure of the investigated samples. The higher the diversity, the more clones have to be screened to reach a high coverage rate in order to obtain reliable results.
In literature there are hundreds of examples of studies that describe the bacterial and archaeal microbial community of a broad range of habitats via clone libraries. These range from space-related studies (Stieglmeier *et al*., 2009; Stieglmeier *et al*., 2012; Venkateswaran, 2012a; Venkateswaran, 2012b; Schwendner *et al*., 2013) to soil (Sekiguchi *et al*., 2002; Jones *et al*., 2009), human skin (Grice *et al*., 2008), and marine (Moeseneder *et al*., 2001) and airborne (Maron *et al*., 2005) environments, just to mention a few.

I.4.2.2. Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) is a reproducible and high-fidelity, high-throughput methodology that assesses subtle genetic differences between strains by analyzing the polymorphism of a certain gene. Since it measures the size polymorphism of terminal restriction fragments from a PCR-amplified marker and has a high resolution of approximately one base, this method is suited for comparative analyses (Marsh, 1999; Courtney *et al*., 2012). Once the DNA of a sample is isolated, the gene of interest is amplified by PCR using fluorescently labeled primers. This step results in amplicons having a fluorescent label at one end that are digested with a restriction enzyme after purification. Fragments of different sizes are generated and separated by gel or capillary electrophoresis. A laser reader detects the labeled fragments, and the digital output is a profile based on fragment length that is compared to an internal size standard.
This method was used in the past for determination of complex soil communities (Marsh, 1999; Dunbar *et al*., 2000; Smalla *et al*., 2007), fecal deer pellets (Clement *et al*., 1998), and for identification of airborne bacterial community structures in an urban area (Lee *et al*., 2010). Furthermore, T-RFLP can also be applied to analyze microbial diversity of extreme habitats, such as hypersaline environments (Øvreas *et al*., 2003).

I.4.2.3. Automated Ribosomal Intergenic Space Analysis (ARISA)

ARISA, another culture-independent, rapid and low-cost fingerprinting technique which involves a capillary electrophoresis system, was developed by Fisher and Triplett in 1999. Here, the 16S-23S intergenic space region from the bacterial rRNA (ribosomal ribonucleic acid) gene is amplified. Fluorescently tagged oligonucleotide primers are added to the PCR assay resulting in amplicon fragments between 200 and 1,200 base pairs (bp) which are applied to denaturing polyacrylamide gels and resolved by use of an automated sequencer with laser detection (Ranjard *et al*., 2001).

[5] Chimera: a DNA molecule unintentionally formed by laboratory manipulation with sequences derived from two or more different organisms

ARISA was proven to be a useful method to investigate the microbial community of different environments like soil (Ranjard *et al.*, 2000; Ranjard *et al.*, 2001), oysters (Gonzales *et al.*, 2003), and aquatic environments (Fisher and Triplett, 1999; Newton *et al.*, 2006; Kent *et al.*, 2007).

However, while community fingerprinting methods such as T-RFLP and ARISA are useful for comparative analyses, they are not suitable to assess the richness or diversity metrics of complex communities (Danovaro *et al.*, 2006). Hence, ARISA represents a valid starting point for more in-depth exploration of the community composition when complemented by the detailed taxonomic description offered by 454 sequencing, Illumina, or microarray methodologies (Gobet *et al.*, 2013).

I.4.2.4. 454 Sequencing

454 methodology is a next-generation sequencing approach introduced in 2005 that uses one or two hyper-variable regions of the 16S rRNA gene for microbial community analysis.

In the first step (Fig. I.4.2.4.1), the genomic DNA (gDNA) is sheared and oligonucleotide adaptors are added resulting in the attachment of each fragment to a bead. Thereafter, the beads are PCR-amplified with droplets of an oil-water emulsion so that numerous copies of the initial fragment are generated and bound to the bead. The beads are then filtered to remove all beads without DNA attachment, whereas the others are captured in wells along with enzyme beads and primer to initiate pyrosequencing. Once the polymerase and primer attach to the DNA fragment, inorganic pyrophosphate (PPi) is released leading to the enzymatic generation of photons via luciferin-luciferase assay. Light is emitted when a base is incorporated in replication. The higher the intensity, the higher is the number of bases that were incorporated iteratively. The average read length is about 400 bp, which can be decoded by plotting the emission rate sequentially (Medini *et al.*, 2008).

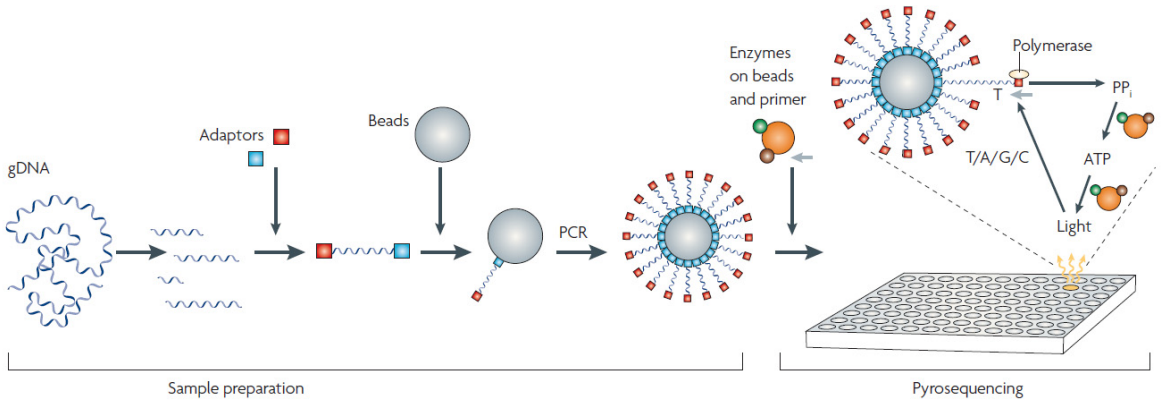

Fig. I.4.2.4.1 454 sequencing method a highly parallel, two-step approach. © Medini *et al.*, 2008

Again, there is a broad range of applications. It has been used to survey microbial community of spacecraft-related areas (Venkateswaran *et al.*, 2012a), a biogas production plant (Schlüter *et al.*, 2008), deep sea (Sogin *et al.*, 2006), and the human intestine (Claesson *et al.*, 2009).

I.4.2.5. Illumina

In 2006, Illumina sequencing debuted, and has been used to investigate the human population history (Li and Durbin, 2011) by screening only one hyper-variable region of the 16S rRNA gene, namely V4.

The first step is the library preparation. This is initiated by breaking the double-stranded (ds) DNA into smaller pieces, and followed by repair of the end and generation of an adenosine overhang. Adaptors are ligated to the fragment (Fig. I.4.2.5.1). The second step is the cluster generation where the selected ligated DNA fragments are attached to a slide (flow cell), which is then put onto a lawn of primer. The DNA bends over, finds a complementary primer on the flow cell, and bridge amplification is performed. This replication process is repeated several times to generate dense clusters. Once the strands are split apart, one type of strand is discarded to increase efficiency. The third step includes sequencing, which proceeds after sequencing primers are annealed and polymerase and fluorescent nucleotides are added. The first base is extended, read, and deblocked, and the cycle starts again. Fluorescence is activated by a laser and base calls are generated.

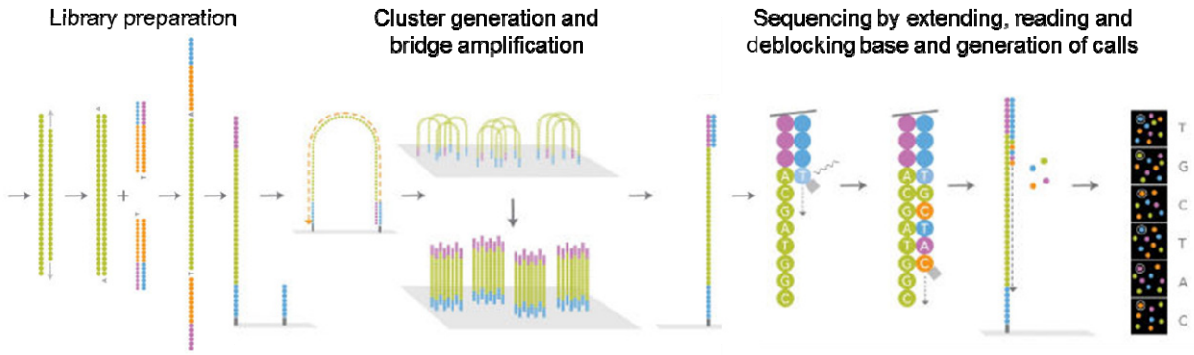

Fig. I.4.2.5.1 Workflow of next-generation sequencing method Illumina. © 2008, Illumina Inc. (edited)

Illumina offers a variety of application possibilities that enables characterization of microbial genomes in a complex community. Therefore, it was utilized to analyze soil (Capraso *et al.*, 2012), permafrost (Mackelprang *et al.*, 2011) oral microbiota (Lazarevic *et al.*, 2010), and the human gut (Qin *et al.*, 2010).

I.4.2.6. PhyloChip

In general, microarray technologies - a generic term for modern molecular systems that allow parallel analysis of thousands of individual gene signatures in a small amount of biological sample material - have been available since 1995. Different forms of microarrays are known.

PhyloChip, a DNA microarray for profiling microbial populations, was invented in 2004 (DeSantis *et al.*, 2007) and continues to be improved upon in terms of phylogenetic resolution. The current third-generation (G3) PhyloChip contains 1.1 million probes for 16S rRNA genes. These multiple DNA oligonucleotide probes target taxa specific regions of the 16S rRNA genes and are able to determine the presence and relative abundance of more

than 50,000 microbial operational taxonomic units (OTUs[6], both, bacterial and archaeal) present in a microbial DNA preparation of an environmental sample (Hazen *et al*., 2010). On the species level, an OTU has been defined as containing 16S rDNA sequences that are ≥ 97 % identical (Gevers *et al*., 2005).
The actual size of an array is 1.28 x 1.28 cm², which is divided into 8 x 8 micron areas (probe spots) on the glass surface, thus providing over 1.5 million locations (Fig. I.4.2.6.1). Each single spot contains several million copies of one the 994,980 specific probes, consisting of 25 nucleotide bases. This 25-base sequence interacts with a unique region of a 16S rRNA gene to categorize microorganisms. 16S rRNA amplicons of an environmental sample are labeled with fluorescent molecules and hybridized on the array. Exposure to laser irradiation causes glowing of those DNA fragments that matched to a complementary probe, indicating a positive interaction. The intensity is equivalent to the taxa's abundance.

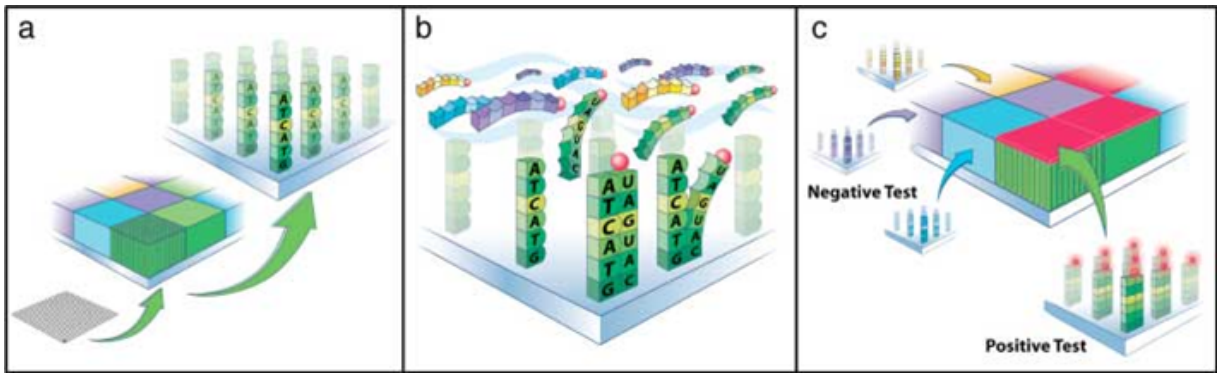

Fig. I.4.2.6.1 Workflow of PhyloChip; a: multiple tests conducted on a single glass surface; b: isolated and labeled gDNA adheres to complementary probes („hybridization"); c: laser scanning identifies positive matches. © Affymetrix Inc., Santa Clara, CA

The PhyloChip G3 was applied to determine indigenous oil-degrading bacteria of a deep-sea oil plume (Hazen *et al*., 2010) or the bacterial diversity of terrestrial crystalline volcanic rocks derived from Iceland (Kelly *et al*., 2011). Furthermore, PhyloChips are also used when investigating low-biomass samples originating from spacecraft-associated clean rooms (Cooper *et al*., 2011).

I.4.2.7. Omics

The neologism "omics" informally refers to all biological data sets originating from one organism or one habitat. This includes information on DNA, RNA, protein, or even on the metabolic level (Ishii and Tomita, 2009). Moreover, phylogenetic classification is not only based on 16S rRNA genes but also on whole genome-fragments, which in turn leads to more stable taxonomy. Due to the large amount of generated data, statistical tools and computational effort are inevitable (Zhang *et al*., 2010).
In brief, omics can be said to be a data-driven, holistic, and top-down approach, as opposed to traditional approaches (Ishii and Tomita, 2009).

[6] Taxonomically classified set of probes

- **Metaomics**

Metaomic approaches expose genes, transcripts, and eventually proteins and metabolites from thousands of microbes to analyze biochemical function and systems-level microbial interactions (Segata *et al*., 2013). This tool provides new ways to study complex ecosystems, since it allows simultaneous examination of thousands of genes, proteins, and metabolites (Blankenburg *et al*., 2009).

- **Metagenomics**

Metagenomics involve the characterization of all microbial genome sequences of a community, as well as their corresponding messenger RNA (mRNA), and proteins. Furthermore, potential metabolic properties can be predicted (The Committee on Metagenomics, 2007) by extraction of gDNA and its further sequencing. Metagenomic data sets of different microbial ecosystems can be compared and provide the basis for other omics (Tringe *et al*., 2005; Podar *et al*., 2007). Since 2002, a variety of sequence-based metagenomic projects were performed starting with the marine viral community. Following studies investigated human feces viral community, drinking water, the Sargasso Sea (Venter *et al*., 2004), human (distal) gut microbiome (Tringe *et al*., 2005; Gill *et al*., 2006), and global ocean sampling (Hugenholtz and Tyson, 2008), just to mention a few. However, the rapidly advancing technology is still limited due to the lack of available and suitable reference genomes, which provide the basis for profound and confident statements. Sequencing of reference genomes is inevitable to improve the quality, to determine the role of horizontal gene transfer, and to obtain information on the microbiome's phylogenetic history (Xu *et al*., 2007).

- **Metatranscriptomics**

Measurements of the whole set of all RNA molecules (mRNA, rRNA, transfer RNA, and other non-coding RNAs) of an environmental sample are embraced by the term metatranscriptomics. This includes high-throughput sequencing of complementary DNA (cDNA) and quantitative measurement of dynamic gene expression on mRNA levels (Leininger *et al*., 2006).

- **Metaproteomics**

Metaproteomic studies involve all proteins and peptides being expressed by a cell, tissue or organism in a specific physiological constitution (Zhang *et al*., 2010).

- **Metabolomics**

Metabolomics unveil chemical processes and display differences in the concentration level of metabolites that are involved therein. Since metabolites are functional entities, their concentration levels vary as a consequence of alterations in an organism's genetics or physiology. Therefore, the small-molecule diversity in a cell can be defined (Zhang *et al*., 2010), but does not provide reliable results regarding phylogeny.

I.5. MICROORGANISMS IN SPACECRAFT ENVIRONMENTS

Every manned mission will be unpreventably accompanied by microorganisms. In order to determine the influence of microorganisms towards human health and biocorrosion processes, monitoring programs were launched during Viking missions and are still ongoing (Ilyin, 2000; Pierson, 2001; Castro *et al*., 2004; La Duc *et al*., 2004; Novikova, 2004; Ilyin, 2005). The joint NASA-Mir Program of the late 1990s provided an extensive database from long-duration missions, which was supplemented by measurements being performed during short-duration flights of over 100 space shuttle missions (Horneck *et al*., 2010). Another focus was laid on the microbial inventory of the ISS, where regular housekeeping activities, visual inspection, and microbial monitoring are on the agenda. In the frame of microbial monitoring of manned space stations, the American "Health Stabilization Program" and the Russian "Plan for Sanitation, Hygiene and Epidemic Prevention in Spacecraft", both designed by space agencies, declare the parameters for screening frequency, locations, and sample type during pre-flight, in-flight and post-flight periods. Monitoring and mitigation measures of humans and their immune status are inevitable in order to investigate if the quality standards were met and if additional prevention is necessary. Confinement and the prevalent conditions during spaceflight might alter microbial growth and lead to undesirable accumulation and potential formation of biofilms onboard the space capsule (Novikova, 2004; Ott *et al*., 2004). A shortened lag phase along with enhanced exponential growth was demonstrated for bacteria enriched in liquid medium under microgravity conditions (Mermel, 2013; Kacena *et al*., 1999; Klaus *et al*., 1997). Once spacecraft components are damaged due to biocorrosion, adverse effects on avionics and spacecraft systems might be the result. The prevalent microflora consortium in a closed environment originates from microbes carried in by the crew and by sticking to medical and technical equipment, and all kinds of supply material. Observations suggest that the microbial behavior is affected in numerous ways. As shown by Roman *et al*. (2006), accumulation took place and the resultant contamination was measured in the air and water as well as on exposed surfaces within the vehicle. Another potential adverse effect for astronauts' health occurs by the activation and aggregation of opportunistic pathogens, which have been described for long-duration spaceflights (Ilyin, 2005). This observation is also supported by *in vitro* studies (Klaus *et al*., 2004). Additionally, conditions being prevalent during spaceflight might have a negative impact on the immune systems (Rykova *et al*., 2008) depending on the individual and the mission.

Reviewing the so-far published data, it is evident that control of microbial level within the vehicle and throughout the flight period is needed to maintain a desired environment.

To round out the diverse roles of microorganisms onboard space vehicles or stations, the aspect of synergistic bioprocesses in life-support systems and bioregenerative systems should also be mentioned. A well-directed and well-aimed deployment of microorganisms can be beneficial onboard, which includes waste degradation, water recovery, and even food and oxygen production, and becomes more and more likely as longer missions demand for self-sufficiency (Roberts *et al*., 2004; Hendrickx *et al*., 2006; Moissl *et al*., 2007; Horneck *et al*., 2010).

I.6. HUMAN BACTERIAL FLORA

The human body consists of approximately 10 to 100 times less human cells compared to the around 10^{14} microbial human-associated cells. These microbial symbionts are collectively defined as the microbiota (Turnbaugh *et al*., 2007). They colonize the surface and deep layers of skin, and are found in saliva, the oral cavity, the conjunctiva, and in the gastrointestinal tract (Fritz *et al*., 2013). There are only few regions in the human body that provide a sterile environment, such as tissue, body fluid, the bladder, tubes, the middle ear, and the paranasal sinus. Another example is the uterus, so that the physiological colonization of the newborn starts immediately after birth with bacteria from the mother and/or from the environment. Generally, only slight variations can be detected in the composition of the physiological bacterial flora from individual to individual when viewed on the genus level, which differs from findings on the species level (Capone *et al.*, 2011; pers. comment: Prof. Dr. Francesco Canganella).

The regulation of bacterial flora is undertaken by the human body itself. This includes the formation and maintenance of differences between skin (32°C) and core body temperature (37°C), moisture gradients, and acidity. Furthermore, the human body provides different nutrient sources through food intake or from the host metabolism itself, as well as habitats with anaerobic or aerobic conditions, further influencing the bacterial community. The conditions in one of the wealth of niches define the bacterial composition, and favor those species that show their respective growth optima and are consequently adapted at the best possible rate to the prevailing environment (Baer, 2012).

On the other hand, certain bacterial interactions also have a determining influence on the composition of the flora. Different regulators in terms of bacterial growth can be distinguished. Substrate competition, e.g., leads to mutual growth restriction. The same antagonistic effects occur when waste products (passive) are released and when bacteriocins are actively formed. Both cases can lead to growth inhibition through the metabolized products. However, synergistic effects also define the diversity of the bacterial community. Microbial succession provides the basis for stepwise further utilization of metabolites. Additionally, the cross-detoxification of the milieu has consequent benefits for all microorganisms involved. It can also be envisaged that the transfer of growth factors and resistance factors (plasmid-encoded) can result in positive growth effects.

In order to accurately analyze the microbial diversity and composition that reside on or in the human body, culture-independent molecular biological methods are now applied. The climax was reached by initiating the Human Microbiome Project with the major goal to comprehensively characterize and identify a core human microbiome, which is defined as the set of genes that are present in a given habitat in all or the vast majority of humans. This habitat can be determined as the entire body or as a specific surface area on or in the human. The variable human microbiome is the set of genes that are present in a given habitat in a smaller subset of humans. These differences in the microbial community might originate in a combination of factors, such as the host's genotype, physiological status, pathobiology, lifestyle, environment and the presence of transient flora that does not persistently colonize the habitat (Turnbaugh *et al*., 2007).

Taken together, a broad range of studies on the human microbiome provided the following insights into the normal bacterial colonization of each individual body region:

- **Skin**

In general, the skin is cool, acidic, and desiccated and therefore represents a rather inhospitable environment for microbial growth (Grice and Segre, 2011). However, skin still serves as an elaborate host for microorganisms due to the microhabitats formed by wrinkles and furrows that cover a broad range of pH levels, temperatures, and moisture content, so that one cm² can contain up to 1 billion germs (Grice *et al*., 2008). Many of those, either commensals or symbionts, play beneficial roles that inhibit thriving of pathogenic species but also further process skin proteins, free fatty acids, and sebum (Roth and James, 1988). Furthermore, hair follicles, sebaceous, and glands are additional features of the skin that are colonized by their own unique microbiota (Marples 1965; Kearney *et al*., 1984). Gao *et al*. (2007) suggested a low level of interpersonal consensus but an extremely dynamic microbial diversity with great fluctuations in a brief span. Furthermore, the diversity is influenced by the birth process and the post-natal environment (Capone *et al*., 2011). Diverse studies unveiled representatives of Proteobacteria (*Pseudomonas*, *Janhinobacterium*, *Serratia*, *Halomona*, S*tenotrophomonas*, *Delftia*, and *Comamonas*), Actinobacteria (*Corynebacterium*, *Kocuria*, *Propionibacterium*, *Microbacterium*, and *Micrococcus*), Firmicutes (*Staphylococcus* and *Clostridium*), and Bacteroidetes (*Sphingobacterium*, *Chryseobacterium*; Grice *et al*., 2009). For further reading about the historical overview and the current knowledge of microbial diversity of skin please refer to the review article by Grice and Segre (2011), since the list is not complete.

- **Oropharynx**

1 ml of saliva is colonized by approximately 10^8 bacteria (mainly α-hemolytic streptococci and *Neisseria*). The oral and pharyngeal mucosa is settled by a dense flora of anaerobic and aerobic microorganisms, whereas the colonization of the teeth greatly depends on the dietary habits of the individual. Up to 10^{12} bacteria per ml can be found in the gingival furrow of which the majority are anaerobes, such as different α-hemolytic streptococci, staphylococci, *Eikenella corrodens*, various *Vibrio* species (*Campylobacter sputorum*, *Selenomonas* sp., *Wolinella* sp.), and O_2-sensitive bacteria such as *Capnocytophaga* sp., *Leptotrichia* sp., and *Treponema* (Baer, 2012).

- **Stomach**

Due to its low pH value, the stomach is sterile except for the transient flora caused by food intake and saliva. *Helicobacter pylori* survives the migration through the acidic stomach environment by production of ammonia (urease) and lives in the pH neutral crypts (Bik *et al*., 2006).

- **Small intestine**

Whereas the upper part of the small intestine is sterile, the lower part of the small intestine consists of several microhabitats, i.e., the bacterial flora differs in the villi, in the deep crypts, and in the lumen. The microbial community of the latter is composed of mainly fast-growing, non-adherent bacteria like *Clostridia* and lactobacilli. On the villi themselves, an increasing amount of gram-negative rods can be detected. The crypts are also characterized by strong motile, non-adherent, and usually not obligate anaerobes (Baer, 2012).

- **Colon**

The vast majority of the human gastrointestinal tract microbiome lives in the colon. Up to 10^{12} microbial cells are present per gram feces. Thus, it is the environment with the highest density, but reveals a relatively low diversity. More than 90 % of all identified phylotypes belong to just two of the 70 known phyla, namely Firmicutes and Bacteroidetes (Eckburg *et al*., 2005). The luminal colonic flora differs from the parietal and covers up to 500 species. About 95 % thereof are obligate anaerobic bacteria that consist of three-quarters of *Bacteroides*, *Bifidobacterium* and *Eubacterium*. The remaining 25 % are composed of *Clostridia*, anaerobic cocci, *Fusobacteria*, and lactobacilli. Based on the total flora, 5 % is made up of *Enterobacteria* and enterococci, which are facultative anaerobic representatives. Since the parietal habitat provides a better oxygen supply, a shift occurs in its bacterial composition towards the benefit of the facultative anaerobic flora. In addition, many of the species have adhesive characteristics. *Bacteroides* spp., *Clostridium* spp., and *Eubacterium* spp. are representatives of the anaerobic proportion (Baer, 2012). Ley *et al*. (2006) discovered the microbial structure to differ greatly between individuals, but to be generally very stable in each host.

Altogether, it is of considerable value to comprehensively characterize the healthy human's microbiota in order to understand its role in disease predisposition and pathogenesis (Turnbaugh *et al*., 2007; Grice *et al*., 2008). On the one hand, the normal microflora is controlled by the innate immune defense system, and on the other hand, it impedes pathogens the access to the host by release of bacteriocins (Heesemann, 2012). However, the normal flora may also have a negative impact. In immune suppressed patients the majority of the infectious agent derives from the patients' own bacterial flora (Baer, 2012).

I.7. Pathogenic Microorganisms

Robert Koch developed a concept of causality to differentiate between pathogens and non-pathogenic microorganisms based on his work on infectious diseases, such as anthrax and tuberculosis. He found a specific association of a certain microbial agent with the disease state of the host, where it originates (Heesemann, 2012). In 1890, Koch presented his postulates for the first time (Koch, 1891), which were summarized by Fredericks and Relman (1996) as follows:

1. The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease.
2. The parasite occurs in no other disease as a fortuitous and nonpathogenic parasite.
3. After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.

Some scientists added a fourth criterion stating the reisolation of the microbe from the host that was inoculated on purpose. These postulates were successfully applicable for identification of *Mycobacterium tuberculosis* as a trigger for tuberculosis (Koch, 1891; Koch,

1942). As it soon turned out, Koch found that his postulates are not appropriate for all infectious agents. *Vibrio cholerae* was isolated from patients with cholera, but was at the same time enriched from healthy subjects. This finding is not congruent with the second postulate. Furthermore, until now, *Mycobacterium leprae* cannot be enriched, even though it is known as the etiologic factor to cause leprosy and therefore, the third postulate is not fulfilled.

Due to the newly invented technologies and methodologies, limitations of these guidelines were revealed and explanations for appearances that caused the problems were found. At that time viruses were not distinguished from microbial lineages. Furthermore, phenomena like host-specificity were uncovered for pathogenic organisms such as *Plasmodium falciparum*. This is why representatives of those pathogenic agents cannot be grown alone. Furthermore, *Neisseria meningitidis* is capable of a carrier state providing an example of a pathogenic microorganism that can cause disease in one host organism but not in another (Fredericks and Relman, 1996). Nowadays, the occurrence of subclinical, which is characterized by a silent infection without developing active disease infection, is better known. This, in turn, is not congruent with the second postulate.

In terms of the observation of the above-mentioned phenomena that include host, environment, microbial adaptation, and the complexities of host-parasite relationships, the original postulates were adapted and are now known as the molecular version of Koch's postulates based on causation theory (Falkow, 1988).

1. The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
2. Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence.
3. Reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity.

By application of molecular techniques in microbiological diagnostics, potentially pathogenic bacteria are tested for the presence of pathogenicity genes, an approach referred to as pathotyping. There is a growing trend towards reliance on genotypes, since they are more specific and easier to quantify and standardize among different organisms compared to the traditionally used phenotypic markers (Fredericks and Relman, 1996). Meanwhile, whole genome sequences of host organisms and most infectious agents have been identified and are universally accessible (Heesemann, 2012). In 2006 for example, the National Microbial Pathogen Database Resource (NMPDR) uploaded a genomics platform based on subsystem annotation for approximately 50 pathogenic strains belonging to Category B pathogens as well as more than 400 supplementary genomes for comparative analysis (McNeil *et al*., 2006). Due to complete sequence information, it was shown that bacterial pathogens have a unique set of pathogenicity genes. However, the genomes of pathogens prove to be highly plastic, i.e., the proportion of the stable core genome is about 70 %, whereas the remaining portion is variable. Furthermore, the pathogenicity of a microorganism can usually not be attributed to only one toxin (one virulence factor), but is caused by a larger repertoire of different virulence factors (including toxins). Ultimately and obviously, not all genes play a role in pathogenicity (Falkow, 1988). Therefore, the

identification of potentially pathogenic bacteria is not sufficient to predict their pathogenic potential (McNeil *et al*., 2006).

I.8. AIM OF THIS WORK

As described above, exceptional environmental conditions are prevalent during prolonged human confinement in a hermetically sealed habitat. This leads to the assumption that the microbial community might undergo changes beyond expectation. The therein-encountered particular features regarding waste disposal, water purification, personal hygiene, preventative housekeeping practices, and lack of fresh air supply can influence the selection and development of the microbial inhabitants. Until now, only little is known about the exchange of human microbial flora with the environmental flora within the isolation facility that mimics the living arrangement within a space capsule.

The major aim of this work was to experimentally investigate air and surface samples that were taken in three habitat modules during the MARS 500 project in the frame of the MICHAm (MIcrobial ecology of Confined Habitats and humAn health, modified version) experiment. Both, the cultivable and the molecular microbial community were taken into account. This should include quantitative approaches by enumeration of colony forming units (CFU) according to the ESA standard procedure, and qualitative methodologies by sequencing and phylogenetic studies. Furthermore, the influence of long-term confinement and other environmental factors on the overall microbiome should be evaluated. Additionally, of great interest was whether changes occur in the diversity of the microbial consortium over time, as no information is currently available about how time factors in when combined with confinement. Last but not least, the fraction of potentially pathogenic organisms should be determined in order to make predictions regarding their effect on astronauts' health.

To reach the above-mentioned goals, initially, procedures had to be defined which allow isolation of sufficient DNA amounts from the MICHAm samples for molecular analysis. Therefore, effective and sensitive protocols for the molecular processing of the samples, herein swabs, needed to be developed.

Moreover, in order to get an impression of the prevalent microbial level onboard the MARS 500 facility, comparative measurements should additionally be performed.

II. MATERIALS AND METHODS

II.1. BACTERIAL STRAINS AND CULTIVATION MEDIA

II.1.1. Bacterial Strains

All bacterial strains that were used for optimization experiments and methodology testing were enriched from samples taken at the MARS 500 complex in Moscow (Russia) during the isolation experiment. *Staphylococcus cohnii* (M_A_07/10_3(3); strain identifier code Fig. II.5.1.1) was chosen as a representative strain since first results from the cultivation approach indicated a vast majority of various *Staphylococcus* species being present in all three complexes over the whole timeframe. Furthermore, *S. cohnii* is not considered as a pathogenic species and can be handled in laboratories without specific restrictions. Additionally, *Bacillus safensis* (M_Sw_oHS_09/10_1_1(2); strain identifier code Fig. II.5.1.1), a spore-forming strain, served as a test organism and originated from samples of the MICHAm campaign.
Both strains were be enriched in liquid R2A (Reasoner's 2A) medium or on R2A plates at 32°C.

II.1.2. Media

The manufacturer's information for chemicals used is listed below:
[a] Difco Laboratories, Sparks, USA
[b] Merck KGaA, Darmstadt, Germany
[c] Fluka, Sigma-Aldrich Chemie GmbH, Steinheim, Germany
[d] AppliChem GmbH, Darmstadt, Germany
[e] Sigma-Aldrich Chemie GmbH, Steinheim, Germany
[f] VWR International GmbH, Darmstadt, Germany

Medium used for the cultivation and further processing of sampled microorganisms was prepared according to specifications by the ECSS-Q-ST-70-55C standard (2008).

- **R2A medium**

Liquid medium:

Bacto tryptone (casein hydrolysate)[a]	0.5 g
Bacto yeast extract[a]	0.5 g
Bacto proteose peptone[a]	0.5 g
D-glucose x 1 H_2O[b]	0.5 g
Starch, soluble[b]	0.3 g
Sodium pyruvate[c]	0.3 g
$MgSO_4$ x 7 H_2O[b]	0.024 g
ddH_2O	ad 1000 ml

Solid medium:
18.12 g from the ready-to-use R2A Agar[c] were suspended in 1 l ddH_2O.

Final pH was 7.2 without any adjustments. The medium was autoclaved at 121°C, 20 min.

II.1.3. Buffer and Solutions

- **Tris acetate EDTA (TAE) buffer (50x) pH 8.0**

TAE buffer was used for agarose gel electrophoresis.

Tris[d]	224.0 g
Na_2-EDTA [e] (0.5 M)	100.0 ml
Glacial acetic acid[b]	57.1 ml
ddH_2O	ad 1000 ml

The solution was adjusted to pH 8.0 with NaOH and autoclaved at 121°C, 20 min.

- **Phosphate-buffered saline (PBS)**

PBS buffer is an isotonic solution maintaining physiological conditions and was used for the purpose of washing bacterial cells and as storage solution.

Na_2HPO_4 x 2 H_2O[b]	7 g
KH_2PO_4[b]	3 g
NaCl[f]	4 g
ddH_2O	ad 1000 ml

The solution was adjusted to pH 7.5 with HCl and autoclaved at 121°C, 20 min.

- **Xanthogenate-sodium dodecyl sulfate (XS)-buffer (2x)**

XS-buffer was used for the extraction of genomic DNA (gDNA) from environmental surface samples, cell suspensions or swabs that were spiked with *S. cohnii* cells or *B. safensis* spores.

Tris/HCl [d], 1 M, pH 7.4	4 ml
Ammonium acetate[b], 7 M	4.56 ml
Na_2-EDTA[e], 250 mM	3.20 ml
SDS[e], 10 % (w/v)	4 ml
Potassium ethyl xanthogenate[e]	0.40 g
PCR grade water[a]	4.99 ml

The solution was composed freshly from single stock solutions and prior to use sterilized by filtration (cellulose acetate filters, 0.2 µm, Whatman GmbH, Dassel, Germany).

II.2. Description of Sampling Locations

Throughout the study, air and surface samples were taken at three different sampling sites and subjected to different further analysis:

- Surface and air samples were taken during a long-term (520 days) human isolation experiment, called MARS 500, at a facility of the Institute for Biomedical Problems (IBMP) in Moscow, Russia. The major goal was to study the ecological dynamics in microbial populations from start to end in terms of long-term confinement. Sampling location selection was based on the research proposal MIcrobial ecology of Confined Habitats and humAn health, modified version from April of 2009 (MICHAm) by Dr. Petra Rettberg. During the whole MARS 500 campaign, Charles Romain (French marsonaut) collected, labeled and placed all samples directly after sampling in the hatch of the MARS 500 complex.
- In order to compare, validate and interpret the results of the surface and air samples taken during the MARS 500 experiment, parallel samples (surface and air) were taken at the German Aerospace Center (Deutsches Zentrum für Luft- und Raumfahrt, DLR, building 24, Cologne, Germany) as well as at two private households in Cologne and suburbs at comparable locations (living room, rest room, bed-room) to the sample sites chosen in the isolation facility.
  Additionally, outdoor air samples were taken to match indoor values with those received from external environments.
- Little information is available about the air circulation system in the simulation complex in Moscow and intensive search for more information did not support reliable conclusions about air flow, filtration, and exchange rate. The air filtration system was thought to influence the microbial load. Additional samples from locations with a known air circulation rate (fermentation facility of the University of Regensburg, Germany) were necessary and a set of samples was taken.

II.2.1. MARS 500 Isolation Facility, Moscow, Russia[7]

The medical-technical facility is located at the Institute of Biomedical Problems within the Russian Academy of Sciences (IBMP RAS) site in Moscow, Russia. This complex consists of the isolation facility itself and includes also the operation room, technical facilities, and offices. The layout of the isolation facility is comprised of four hermetically sealed habitat modules that are interconnected with each other and serve as experimental units (EU; Fig. II.2.1). The total volume of the habitat modules is 550 m^3.

[7] http://mars500.imbp.ru/en/nek.html

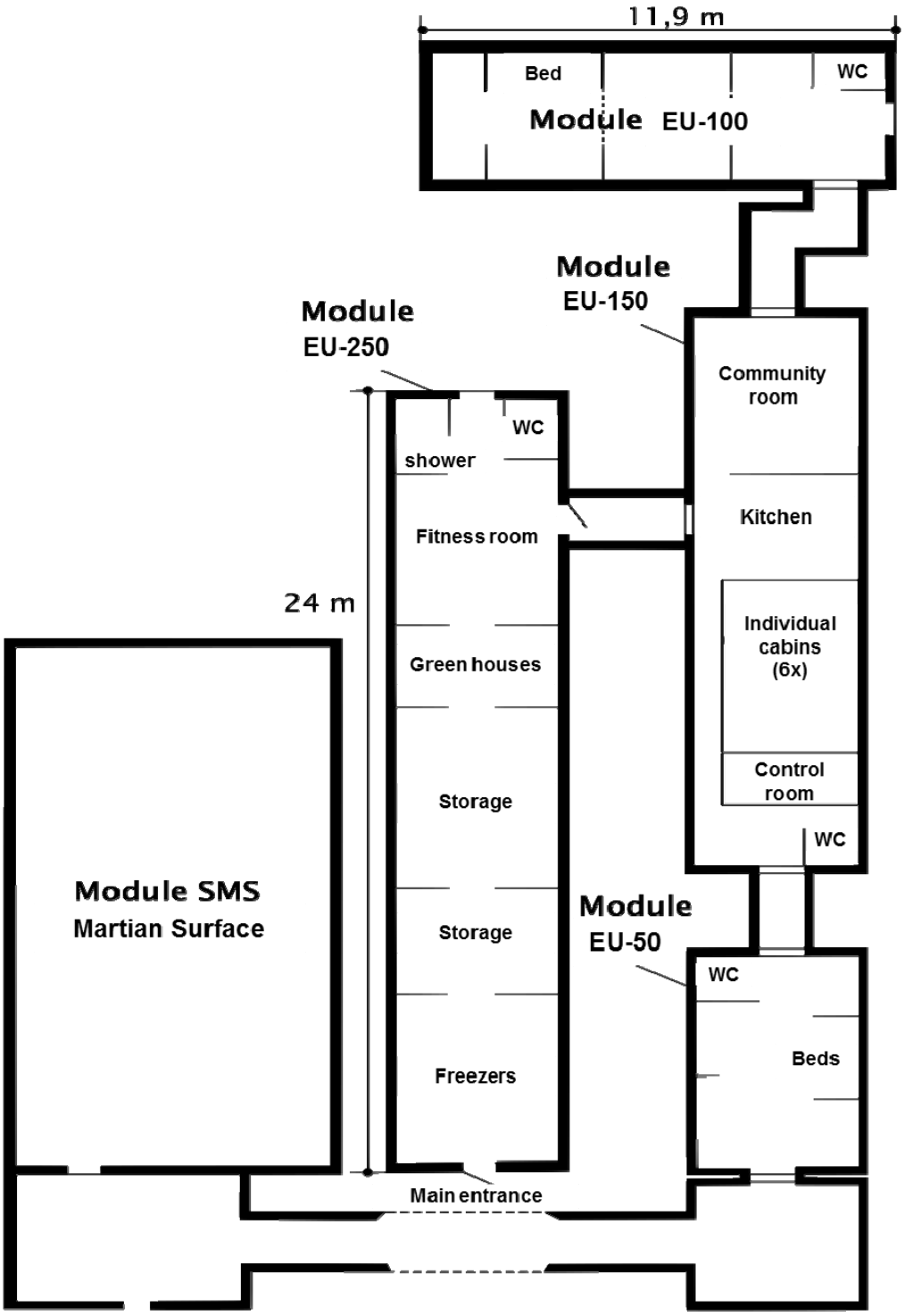

Fig. II.2.1 Schematic drawing of the MARS 500 facility (medical-technical facility - IBMP RAS) with its four EU (experimental unit) modules and the simulated Martian surface module.

- **Technical data of modules**

The technical data of individual modules, where sampling events took place (EU-100, EU-150, and EU-250) are described in more detail as follows:

In all habitable modules a life support system was installed, consisting of a gas analysis system, air-conditioning and ventilation system, sewage system, water supply, as well as a fire alarm and suppression system.

i) Module EU-100 – Medical module

Volume: 100 m³
Size of the floor area: 3.2 x 11.9 m²
Purpose: conduction of medical and psychological experiments
Inventory: This module housed two medical berths, a toilet, and equipment for routine medical examinations and telemedical, laboratory and diagnostic investigations. In case a marsonaut would have been ill, he would have been isolated and treated there. However, this module was frequented more often than planned since the bed located there was bigger than the ones in the individual compartments (pers. comment: marsonaut).
Passage from EU-100 to EU-250, and EU-50 was not accessible directly, since only one transfer tunnel was built that connected EU-100 with EU-150. At one end of the module a hermetical door was installed whereas an emergency door was located at the opposite site.

ii) Module EU-150 – Habitable module

Volume: 150 m³
Size of the floor area: 3.6 x 20.0 m²
Purpose: main living quarters for the crew
Inventory: The module housed six individual compartments for the crewmembers, a kitchen-dining room, a living room, the main control room, and a toilet. The individual compartments (bedrooms 2.8 - 3.2 m^2) had a bed, a desk, a chair, and shelves for personal belongings.
EU-150 possessed three hatches and transfer tunnels that were hermetically sealed, but providing the possibility to reach every experimental unit directly.

iii) Module EU-250 – Utility module

Volume: 250 m³
Size of the floor area: 3.9 x 24.0 m²
Purpose: storage of food, clothes, supplies, as well as location of the fitness area and the greenhouses
Inventory: This module was divided into compartments consisting of a fridge for storage of food, a compartment for storage of non-perishable food, experimental greenhouse, the bathroom, sauna, and gym.
The technical installations included all necessary equipment for running the study (communications and control, ventilation and air supply, water supply, electrical installations, sewerage, air and water quality monitoring and partial recycling, medical equipment, fire and other safety monitoring systems, emergency equipment, etc.). Waste was disposed through lock chambers.
One hermetical door was installed to connect to module EU-150 via a transfer tunnel. Two additional hermetical doors with stairs were located at both ends of the module for loading the complex with supply material in advance.

iv) Module EU-50 – Simulator of the landing Martian ship
Volume: 50 m³
Size of the floor area: 6.3 x 6.17 m²
Purpose: simulation of the Martian landing module for three crew members, only used during the 30 days long 'Mars orbiting' phase
Two transfer tunnels with hatches allowed passing into module EU-150 and into the lock of the chamber of the simulator of the Martian surface.

v) External Module SMS (Simulator of the Martian surface)
Purpose: simulation of the Martian surface
This module included a non-hermetical chamber. The transfer tunnel with stairs (hermetically sealed) connected the SMS module with EU-50.

- **Life support system**

The life support system consisted of four independent systems (one in each module) that guaranteed the autonomy of each module; all four included main and auxiliary systems to ensure a long-term stay for people in this closed environment. The crew stayed in those modules under an artificial atmospheric environment at normal barometric pressure. The main system controlled module climate, gas supply and maintenance of the indoor atmosphere, purification of the atmosphere, water supply and sewerage, video monitoring, supply of electric power, communication, and information. The auxiliary system included following equipment: a computer-aided system for collection and reflection of parameters within the inhabited zone, a thermoregulation system, a dehumidification system for removing condensate from the atmospheric moisture, and a system for climate control.

- **Air control system and water processing unit**

The following section provides more details regarding the indoor air control system as well as about the drinking water and liquid waste water processing unit to understand and interpret the obtained results. Maintenance of the required air quality within the respective four experimental facilities (EU-50, EU-100, EU-150, and EU-250) was realized by independently operating air conditioning systems. Either the external “ground-based” control crew or the marsonauts themselves were allowed to regulate the habitat’s environmental conditions within specified limits. Readings about the current environmental data were taken from screens installed in each module.
Each system was equipped with a ceiling-mounted tunnel air conditioning system made by Wolf GmbH (Mainburg, Germany), which collected the moisture by use of a hermetic cover. The air channels were equipped with gates to prevent airflow; the latter were mounted at the inlet and outlet points of the air conditioner, as well as in the air ducts inside the experimental units in the sub-ceiling of the rooms. The purification of the atmosphere was performed at the pilot plant, and the stagnant air was replaced by use of two carbon filters and an additional filter containing calcium carbonate to absorb carbon dioxide. The replacement of the filter cartridges should be performed on average every 36 h. The allowed relative concentration of carbon dioxide in the atmosphere should be within the

limits of 0.1 to 0.6 %. The air flow rate through the filter systems was carried out by a fan and was regulated by the duty engineer; it should be within the limits of 50 to 300 m³ per h. To remove dust, microorganisms and fungi from the atmosphere, an industrial photocatalytic system, AIRcomfort AC-3020[8] (Namyangju-City, Kyonggi, Korea), with replaceable filters (Fig. II.2.1.2) and the Potok 150-M-01 (Potok Inter, Moscow, Russia) were installed. These units were placed inside the module and were serviced by the crew. The flow rate could be adjusted to high (320 m^3/h) or turbo (440 m^3/h).

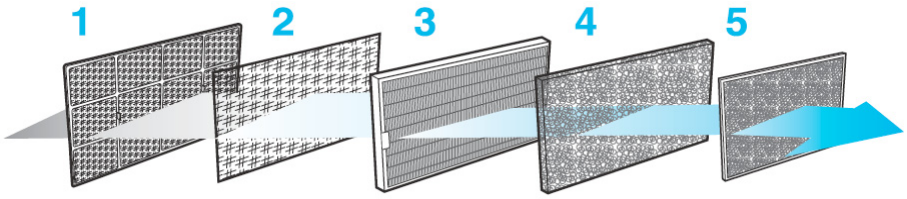

Fig. II.2.1.2 Schematic drawing of the series connection containing five filters with different properties to improve air quality and remove airborne matter. 1: Pro filter; 2:NANO filter; 3:improved TRUE HEPA filter; 4:Deo filter; 5:Foamy photolytic catalyst © 2004-2014 КиМ Онлайн

Components of the industrial photocatalytic system are outlined as follows:

1. Pro filter
   - The pre-cleaning filter holds back the large particles of dust which pass through the front panel to the filter.
   - Cleaning of the front panel is strongly recommended every month or in shorter periods depending on the amount of dust present in a room.
2. NANO filter
   - Blocking system for the passage of microparticles. Air filter with a high purification level for microparticles >0.3 µm keeping back fine dust, dirt, and pollen.
   - Changing of the filters should be performed every three months according to the manufacturer's instructions.
3. Improved TRUE HEPA filter
   - The filter cleaned the air near-completely from fine dust particles, allergens, bacteria, danders, *et cetera*. Removal of particles <0.3 µm (99.97 % efficiency rate) and matter with sizes <0.1 µm including microorganisms (95 % efficiency rate according to results of the AEC Laboratories, USA) was achieved.
   - Exchange of the TRUE HEPA filter, the UV lamp, and the deo filter should be performed in order annually; this task is indicated on the screen of the unit.
4. Deo filter
   - Disinfection filter consisted of a granular coal absorbent (higher efficiency than fabric filters) that removed bad odors from the air.
5. Foamy photolytic catalyst
   - Catalyst performed effective removal of organic compounds, odors, and intercepts viruses. This filter is unique based on a 10-fold coating with a special aluminum metal mixture.

[8] http://de.slideshare.net/guestb07a5c/aircomfort-ac3020-manual-in-russian

Furthermore, each of the modules (EU-50, EU-100, EU-150, and EU-250) was equipped with a system for disposal of household water, potable water supply, and the sewerage system for the removal of food waste and for disposal of excreta of the crew.
The potable water supply system made of stainless steel pipes was designed to provide purified drinking water used for beverages and food. The cold water of the urban water supply flowed through the input unit, which was equipped with the input filter for coarse purification and went on into water tanks that were intended for toilets and showers as well as for the system of post-purification. The tap water was integrated in the cleaning cycle and sanitized by addition of ionized silver, installation of an additional filter set and a UV lamp, to reach the mandatory purification level. Exchange vessels were used for removal of dissolved heavy metals.
The sanitation of the lavatory flush, the sinks of the experimental facilities and the showers was conducted through the sewerage system. Removal of sewage was accomplished by passing the following stations: a storage tank equipped with a membrane - a toilet bowl (basin) - gate valve with electric drive - storage container - fecal pump - valve with electric drive - dumping into the municipal sewage system. The configuration of the above-mentioned structured scheme eliminated the direct connection to the outdoor atmosphere.

II.2.2. Cologne, Germany

- **DLR**

Whenever the DLR is mentioned herein, it refers to the site in Cologne, Germany, in building 24 where offices and laboratories of the Institute of Aerospace Medicine are located. The complex, where the samples were taken, three separate restrooms for men and women, a library used also for lunch, an open kitchen, offices, microbiological laboratories as well as the ultrahigh vacuum chamber and the simulation facility of the Radiation Biology Department are situated on three levels (basement, first floor, and second floor). These rooms (floor and desks) are cleaned on a daily basis. However, it should be noted that there is no air ventilation system and the rooms are frequented irregularly, and the restrooms in the first floor were also used by visitors at the DLR site.

- **Private households**

Two private two/three-room apartments inhabited by two persons (male and female) located in the suburban area of Cologne were sampled. The household sizes are 50 m² and 70 m², respectively. Air samples were taken indoors in the living room, the bed-room and the restroom. Additionally, air sampling was performed at the balcony to obtain outdoor samples.

II.2.3. University of Regensburg, Germany

In more detail, the samples were taken at the Biotechnikum of the Institute of Microbiology and Archaea Center, University of Regensburg, Regensburg, Germany for analysis.

This room contains eleven different bioreactors (fermenters) of various sizes, ranging from 7 to 300 l capacity for mass cultivation of hyperthermophilic and anaerobic microorganisms. The fermenters can be pressurized with pure gas or mixture of gases from a central gas supply being also installed at the Biotechnikum. Furthermore, a work bench and four autoclaves are located therein.
The laboratory is rectangular with a length of 15 m and a width of 8 m, resulting in a floor area of 120 m² and a total volume of 360 m³ with a ceiling height of 3 m. The gas exchange rate is 19.4 times of the room volume per h (equals 7,000 m³ of air). The ventilation system is in operation 24 h a day, 365 days per year. The manufacturer of the ventilation system is the DSD Steel Group GmbH, Saarlouis, Germany; the air filters have been purchased from Camfil KG, Reinfeld, Germany with a filter size of 592 x 592 x 300 mm, Type F 7 (pers. comment: Thomas Hader).
A set of air samples was taken at the bottom of the facility and at 1.5 m height.

II.3. Air Sampling

Air samples were used to gather data about airborne germs present in the interior of a room. The applied assay determined aerobic mesophilic microorganisms (bacteria and fungi) and was based on the ECSS-Q-ST-70-55C (2008) standard.

II.3.1. Sampling Procedure

Samples were taken using the active air sampler Sartorius AirPort MD8 (Sartorius AG, Goettingen, Germany, Fig. II.3.1).

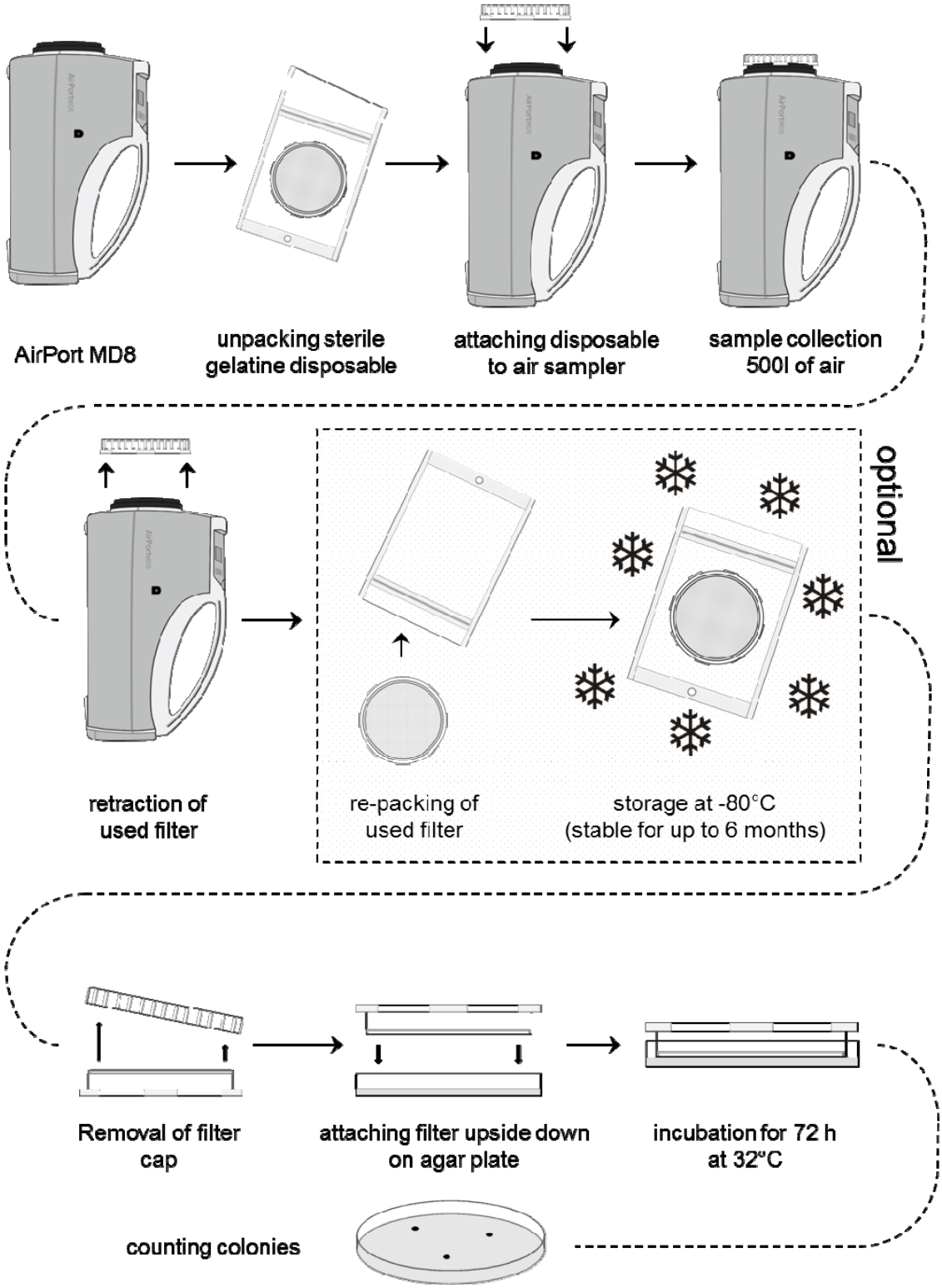

Fig. II.3.1 Workflow of air sampling according to the ESA ECSS-Q-ST-70-55C standard (2008) for assessment of vegetative mesophilic aerobic microbial load; illustrated with the help of A. Parpart.

A gelatine air filter (17528-80-ACD, Satorius AG, Goettingen, Germany) was removed aseptically from its packaging and mounted on the sampler. The air sampling device was oriented vertically at all times during sampling. At each sampling site, the collection of air took 16.67 min and was performed with a flow rate of 30 l per minute, resulting in a total filtration of 500 l air. Afterwards, the filters were placed back in the sterile envelope.

II.3.2. Controls

Additional samples were taken at the sampling location and in the laboratory under a laminar flow (Hera safe KS12, Thermo Scientific, Langenselbold, Germany). Therefore, air samples were taken without mounting the gelatine filter on the air sampler but simply by waving the filter through the air for a few seconds that represented field blanks (FB) and lab controls (LC), respectively. This procedure was performed at least once per sampling event. The FB and LC served as proof of lack of contamination during assaying and analysis. Whenever a control was positive, it indicated contamination that could have been caused for example by the sampler through unsterile handling or by production errors.

II.3.3. Transportation and Storage

Depending on the origin of the samples, transportation and storage conditions (length) differed.

- Samples from MARS 500: Upon completion of the air sampling all samples from one sampling event were put into the hatch. After that, they were removed by the responsible person of the IBMP and stored at -80°C (pers. comment: Svetlana Poddubko). After being stored at -80°C in the laboratories of the IBMP for periods of at least 4 days up to 6 months the samples were sent via World Courier. Shipping from Moscow to DLR, Cologne was performed in three batches on dry ice to avoid repeated freeze-thaw cycles which lead to reduction of microbial viability. Upon arrival, samples were kept at -80°C until processing.
- Parallel samples taken at the DLR were directly stored at -80°C for either at least 4 days or half a year in order to evaluate the influence of time on survivability of collected bacterial cells.
- Samples taken at the DLR and surrounding areas to compare indoor and outdoor microbial contaminations were transported to the laboratory, stored at 4 to 8°C and were processed within 24 h.
- Samples for analysis of the microbial contamination in a steadily filtered room were taken at the University of Regensburg, were kept on dry ice for transportation (approximately 48 h) to DLR and were processed directly.

II.3.4. Sample Processing

After gentle and slow thawing of the samples (including negative controls) the filter holder was removed under sterile conditions. The gelatine filter was aseptically pressed slightly onto the surface of R2A plates. The plates were inverted just after the gelatine started to dissolve and stuck to the surface. Incubation was performed for 72 h at 32°C (+/- 1°C). The plates were examined by eye every 24 h, the colonies were counted and the data was recorded (Fig. II.3.1).

II.4. SURFACE SAMPLING

II.4.1. Sampling Tools and Procedure

The sampling was performed according the ECSS-Q-ST-70-55C standard (2008) applied in spacecraft-associated clean rooms. Being widely adopted and increasingly used, the obtained results could be compared and interpreted more easily.

The swab (552C regular swab; ethylene oxide sterilized, Copan, Brescia, Italy) was prepacked in single containers, removed from the protective cover and dipped in an Eppendorf tube (VWR International GmbH, Darmstadt, Germany) containing 1 ml sterile PCR grade H_2O. To eliminate redundant water, the swab tip was squeezed against the wall of the tube. An area of 5 x 5 cm² was sampled in three directions (horizontal, vertical and diagonal). During this procedure the swab was turned several times.

The following procedure differed depending on whether samples were subjected to subsequent cultivation studies or to molecular assays:

- For downstream cultivation analysis, the swab was broken at the predetermined breaking point and was transferred to a sterile 15 ml Falcon tube (VWR International GmbH, Darmstadt, Germany) containing 2.5 ml PBS (Fig. II.4.1.1).
- For downstream molecular analysis, the swab was put back in its original, still sterile container without addition of water and was stored under dry conditions.

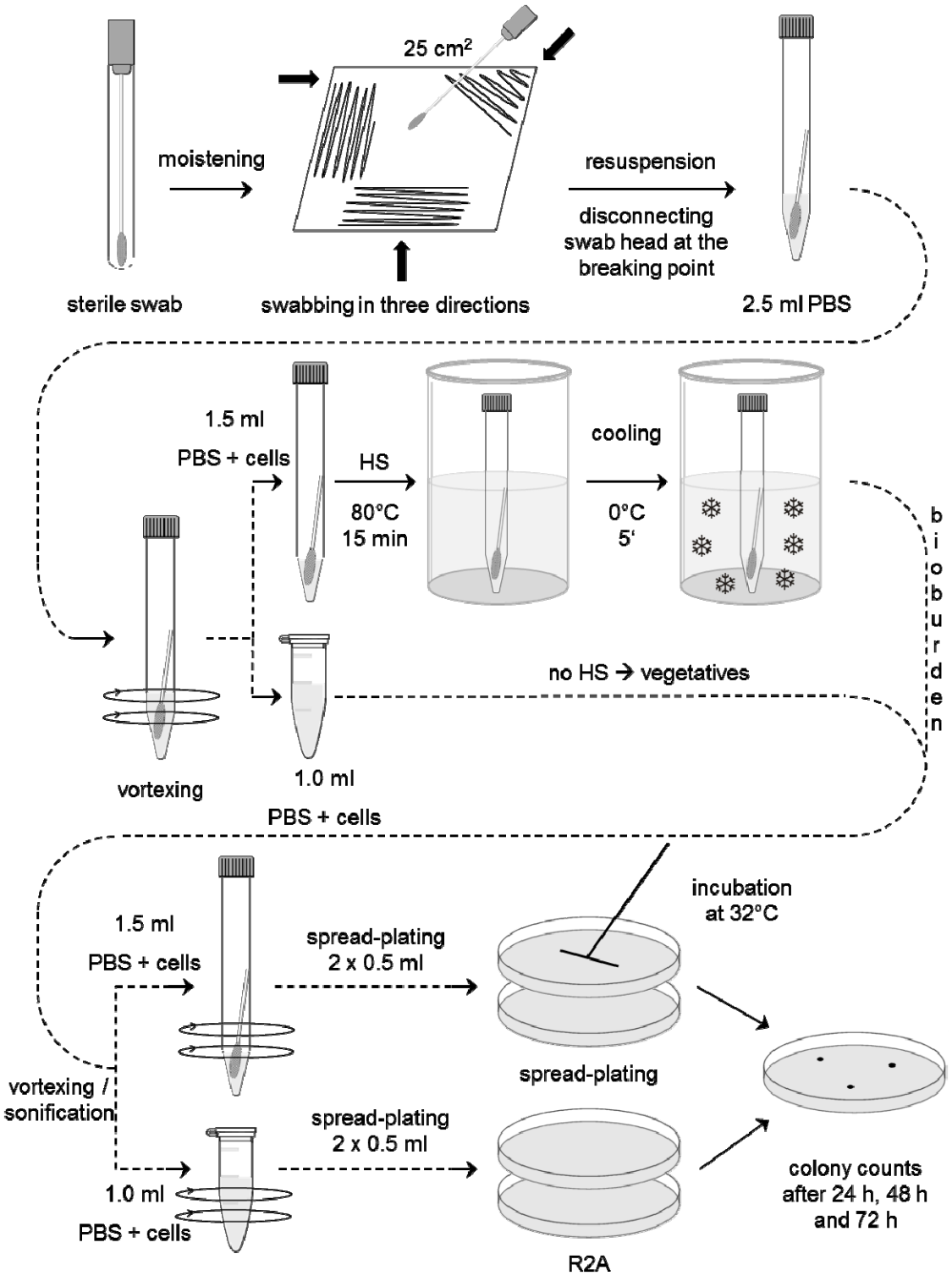

Fig. II.4.1.1 Workflow of surface sampling according to the ESA ECSS-Q-ST-70-55C standard (2008) for assessment of vegetative mesophilic aerobic microbial load and "bioburden" measurements; HS=heat-shock; illustrated with the help of A. Parpart.

II.4.2. Controls

Similar controls were taken for surface samples as described in section II.3.2. with the difference that the swab was removed from the sterile cover, was waved through the air and was put back in its initial package.

II.4.3. Transportation and Storage

Transportation and storage conditions (length) varied depending on sample origin and further processing.
Surface samples from MARS 500 were shipped together with the air samples (section II.3.3.). Parallel samples taken at the DLR were directly stored at -80°C for at least 24 h.

II.4.4. Sample Processing - Cultivation Studies

After a gentle thawing process, each tube containing 2.5 ml water and a swab was vortexed at maximum power for 5 to 6 s and the liquid was divided into two aliquots (1 ml and 1.5 ml; Fig. II.4.1.1). 1 ml was subjected to the determination of the overall microbial inventory, whereas the remaining 1 ml was used to investigate the “bioburden”.

II.4.4.1. Determination of Overall Microbial Inventory “Vegetatives”

Aliquots of 0.5 ml each were aseptically pipetted onto the surface of two R2A petri plates (Fig. II.4.1.1). Using a sterile disposable spreader (VWR International GmbH, Darmstadt, Germany), the solution was spread over the surface as evenly as possible. Upon absorbance of the moisture into the agar, the plates were incubated inverted at 32±1°C. The plates were examined after 24, 48, and 72 h and visible colonies were counted (reveal to CFU). The final count was done at 72 h and colonies were selected for further processing (section II.5.).

II.4.4.2. Determination of “Bioburden”

The vortexed, sonicated swab in the Falcon tube containing the remaining 1.5 ml water was placed in a water bath at 80±2°C for 15 minutes (heat-shock), as determined by a pilot tube containing a thermometer according to the standard protocol (Fig. II.4.1.1). The Falcon tube was cooled rapidly (stored in an ice box) to bring the contents to 30-35°C. Following heat-shock treatment, the Falcon tubes were vortexed again at maximum power for 2 seconds and further proceeded as described in section II.4.4.1. Thus, 0.5 ml were still left in the tube.

II.4.5. Sample Processing - Molecular Studies

The main goal of applying molecular approaches was to further develop our understanding of both, diversity and abundance of microbial communities and their changes over time in a confined manned habitat.
For the sake of completeness, the sample processing of dry swab is briefly mentioned here, but the precise further handling procedure is described in section II.8.1.3 (MICHAm samples) and in section II.8.1.1f (DLR samples). Following gentle thawing, the gDNA was extracted from the swabs and sent to Second Genome, Inc. (South San Francisco, CA, USA) to perform PhyloChip analysis (section II.8.6) in case of MICHAm samples, whereas DLR samples were further subjected to PCR assay (section II.8.3.).

II.5. PROCESSING OF ISOLATES

II.5.1. Purification of Microorganisms

Bacterial specimens were isolated and purified from mixed environmental cultures using the streak plate method. Therefore, single, morphological different colonies (size, shape, texture, color, raised, concave, etc.) were picked from each plate with a sterile inoculation loop (VWR International GmbH, Darmstadt, Germany), streaked onto a new agar plate and incubated at 32°C until growth occurred. This step was done at least two times and provided the basis for further processing like storage and phylogenetic analysis.
The following Figure II.5.1.1 represents the code that was invented to catalog every single cultivable isolate in order to provide a uniquely defined and recognizable assignment for each identified strain. The code includes sample location, type, treatment, date, sampling site as well as the corresponding aliquot and the strain-ID itself.

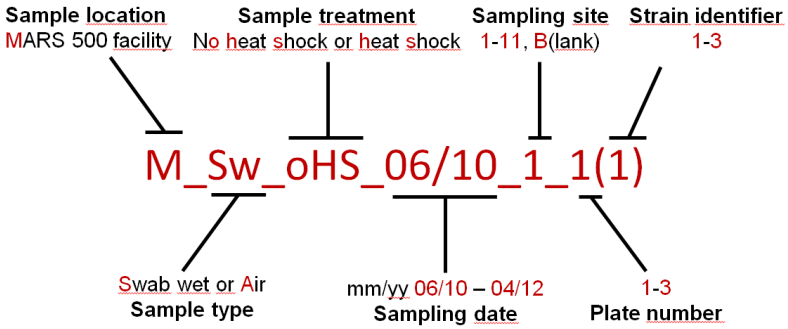

Fig. II.5.1.1 Strain identifier code scheme

II.5.2. Long-term Conservation of Pure Cultures

Cryostocks of each single isolate were created in order to archive the purified organisms from the MARS 500 facility sampling site. This long-term storage allowed further identification and testing of physiological capacities at a future date. Therefore, a single colony of the purified isolate was picked from the R2A plate and transferred into a 2 ml Eppendorf screw cap tube (VWR International GmbH, Darmstadt, Germany), which contained 1 ml of sterile liquid R2 medium completed with 10 % (v/v) glycerin. The cryostocks were vortexed and stored at -80°C.

II.5.3. Phylogenetic Analysis

- **Sequencing**

LGC Genomics (Berlin, Germany) performed traditional Sanger sequencing on the fully automated and high throughput ABI 3760 XL platform. Therefore, specific 96-well plates containing solid medium were prepared freshly for shipment of the bacterial strains. Long-dated storage was not possible due to quick desiccation of the medium.

200 µl of still liquid R2A medium (section II.1.2.) was pipetted in each well of the sterile 96-well plate (VWR International GmbH, Darmstadt, Germany) and stored in the refrigerator until the medium was solidified. Each well was then spiked with a purified isolate, the 96-well plate was closed with an appropriate lid and parafilm (Parafilm „M"® Laboratory Film, American National Can™, Chicago, Illinois, USA) was wrapped around to avoid contamination and rapid drying up. Before shipping, the plates were incubated at 32°C overnight.
Upon arrival, each isolate was subjected to subsequent DNA extraction and PCR. The 16S rRNA gene was amplified with the primer set 27F and 1492uR (sequences; section II.8.3.) followed by a sequencing step starting from the 27th base pair position. The unaligned results were downloaded from the company's website.

- **Sequence data analysis**

The sequence data were returned to DLR and analysis was firstly performed with Chromas 2.4 (Technelysium, South Brisbane, Australia), followed by alignment with the publicly available database BLAST[9] (Basic local alignment search tool) in order to get a rough idea about the classification and diversity. With this tool, the 16S rRNA sequences were correlated and aligned with the public sequence database GenBank of NCBI[10] (National Center for Biotechnology Information).
All sequences were submitted to the chimera check program Decipher (Wright *et al*., 2012), which allowed the detection of possible chimeric artifacts consisting of sequence fragments. Discovered chimeras were not integrated in microbial community structure analysis.
Re-classification was done by Second Genome, Inc. (South San Francisco, CA, USA) in order to provide comparable results between data acquired by PhyloChip analysis and cultivation approach.
In sum, sequences were manually trimmed and the minimum sequence length was set to 700 bp. The 16S rRNA genes of the isolates were classified using the Bayesian classification method (Wang *et al*., 2007) against an updated, widely accepted Greengenes taxonomy (McDonald *et al*., 2012).
After classification, the sequences were grouped and due to the high amount of obtained sequences, only one sequence per identified species served as representative strain. In order to receive an accession number, the sequence was submitted to Genbank using Sequin 12.30[11].

[9] http://blast.ncbi.nlm.nih.gov/Blast.cgi

[10] www.ncbi.nlm.nih.gov/genbank/

[11] www.ncbi.nlm.nih.gov/Sequin/

II.6. Estimation of Cell Numbers

The cell density is often determined spectrophotometrically, however, that form of estimation does not allow an assessment of cell integrity, nor can cell types be distinguished. Furthermore, this method is not as exact as a Thoma counting chamber (VWR International, Darmstadt, Germany) used to determine the number of cells per volume. One µl cell suspension was filled into the counting chamber and the cover glass was fitted tight to avoid volume variations. Using the phase contrast microscope (magnification: 400x), cells or spores of seven mini squares were counted. By help of this number, the amount of cells per ml can be calculated according the formula below. Since there is no differentiation between dead and alive cells, the cell number corresponds to the total cell count.

$$\frac{\text{Cell number}}{\text{ml}} = \frac{\text{Number of counted cells/spores}}{\text{Number of mini squares}} \times 2 \times 10^7$$

II.7. Preparation of Spiked Swabs

To investigate the extraction efficiency of swab samples, swabs were spiked with a freshly prepared overnight culture of a single *S. cohnii* colony (section II.1.1.). The total cell number was determined as described above followed by the preparation of dilution series containing 10^7 to 1 cell(s) per 50 µl. The swab was removed aseptically from its cover and 50 µl were applied by carefully dropping single droplets onto the head of the swab. The liquid was then dispensed all over with the pipet tip. Previous experiments showed that 50 µl is the most suitable amount without loosing any excess liquid not being absorbed by the swab. Once the liquid was absorbed, the swab was carefully put back in its original package and stored at -80°C. For each dilution step, five swabs were prepared as described above.

II.8. Molecular Techniques

All centrifugation steps were carried out using the Z216Mk centrifuge with rotor V11 from HERMLE, Labortechnik GmbH, Wehingen, Germany.

II.8.1. DNA Extraction

Isolation of gDNA was mainly achieved by chemical cell lysis. To improve the success, physical disruption via ultrasonic treatment was performed prior to the application of detergents, such as SDS, potassium ethyl xanthogenate (XS) and/or enzymatic treatment with lysozyme.

II.8.1.1. Evaluation of Ultimate DNA Extraction Method (general)

For comparative purposes and evaluation of the best suitable method, one test series was performed with a purchased, widely used DNA extraction kit, whereas a phenol extraction process using the XS-buffer method was applied to a parallel sample series.
10-fold diluted *S. cohnii* cell suspensions with a starting concentration of 10^8 cells/ml were subjected to both extraction methods and the gDNA content was compared.
Based on the result of this experiment, all following DNA extractions were performed with the method that achieved the highest efficiency.

- **PeqGOLD Bacterial DNA Kit**

A rapid DNA extraction was performed from pure cultures (as liquid suspensions or from spiked swabs) using the PeqGOLD Bacterial DNA Kit (PEQLAB Biotechnologie GmBH, Erlangen, Germany). The kit uses the reversible binding properties of PerfectBind silica filters in spin columns where DNA binds selectively to the silica matrix. DNA is washed and finally eluted. The extraction process was done according to manufacturer's instruction for gram-positive bacteria and lysis was achieved by adding lysozyme (Merck KGaA, Darmstadt, Germany). Furthermore, two elution steps were performed to reach a recovery rate of 90 %, instead of 70 % after only one elution step. Prior to extraction, TE buffer (10 mM Tris/HCl pH 8, 1 mM Na_2-EDTA) was prepared. The protocol was changed regarding incubation of the cells at 30°C from 10 min to 30. Afterwards, an additional ultrasonic treatment (120±5 s with a power of 240 W and a frequency of 35 kHz) was applied to enhance cell disruption. As a last step the DNA was eluted from the column using different volumes of water which will be indicated separately in each result section.

- **XS-Buffer (Tillett and Neilan, 2000)**

For each preparation, a swab was directly put into 1 ml of pre-warmed 2x concentrated XS-buffer, followed by a sonication step in an ultrasonic bath (120±5 s with a power of 240 W and a frequency of 35 kHz) and by vortexing. The next step included incubation in a water bath at 65°C for 2 h. During this time the samples were mixed every 30 min by hand and lastly vortexed for 10 s. The tubes were kept on ice for 10 min and centrifuged (5000 rpm, 5 min, 4°C) to separate lysed cell components. The supernatant was transferred into a PhaseLock Gel tube (2 ml, Eppendorf AG, Hamburg, Germany) and the same volume of phenol:chloroform:isoamylalcohol (25:24:1) was added. The tubes were mixed thoroughly and centrifuged (5000 rpm, 5 min, 15°C). The aqueous, DNA containing layer was recovered in a new tube. For DNA precipitation the same volume of ice cold 100 % isopropanol and 1/10 volume of 4 M ammonium acetate were added and mixed gently. To finalize the precipitation step, the samples were kept at -20°C overnight. The next day a centrifugation step (13000 rpm, 30 min, 4°C) followed and the supernatant was discarded. The pellet was washed twice with ice cold ethanol (70 %) without vortexing, was dried and dissolved in 20 µl (if not otherwise stated) PCR grade water.

II.8.1.2. Evaluation of the Best-Suited Method (for Low-Biomass Swab Samples)

Results of the CFU determination revealed an unexpected low-biomass per swab leading to the decision to pool swabs for subsequent DNA extraction with XS-buffer method.
In order to optimize the recovery of DNA, two different swab pool protocols were tested:

- extract the DNA of each single swab and pool the gDNAs of appropriate samples after dissolving in H_2O.
- pool the swabs (up to five) already in the extraction buffer (vortexing, ultrasonic treatment and removal of the swabs is done consecutively) and carry on with the protocol (water bath), thus having only one tube per certain number of swabs.

Each of these approaches was performed using either spiked swabs with a known concentration of *S. cohnii* cells to minimize the bias of cell number variation and increase comparability or with swab surface samples (sampling description; section II.4.1.) that were taken at locations next to each other in order to simulate the real sampling conditions at MARS 500 (influence of dust, particles and hairs).

II.8.1.3. DNA Extraction from MARS 500 Surface Samples

Molecular analysis of surface samples taken at different locations from the isolation facility was performed to get an overall (alive and dead specimens) insight into the microbial community structure that is present during different points in time of the experiment. Therefore, a microarray based-technique, PhyloChip, was the method of choice. To maximize the gDNA yield and receive reliable results, dry swabs taken per module per sample event were grouped together and pooled.
After gently thawing the dry swabs (section II.4.1.), the first swabs from each pool (containing only samples from one module taken at a certain date) were removed consecutively from their original packaging and directly transferred into each one Falcon tube containing 1 ml of pre-warmed XS-buffer (section II.1.3.). The tubes were vortexed, sonicated, and removed. To eliminate redundant XS-buffer and minimize the loss of biological content, the swab tips were squeezed against the wall of the tube and were rotated at the same time. After removal of the first swab the second swab was transferred into the same tube and the procedure was repeated. This was done four times for surface samples from module EU-250 (utility) and five times for surface samples from module EU-150 (habitable, section II.2.1.). Subsequently, the DNA extraction using XS-buffer method was carried on as described in section II.8.1.1. The final step included the dissolving of the gDNA in 100 µl PCR grade H_2O and storage at 4°C.

II.8.2. DNA Concentration Determination

The dissolved DNA was measured via two different methods:

- **NanoDrop ND-2000c spectrophotometer**

1.5 µl of the extracted DNA was used for measuring the DNA concentration with the NanoDrop ND-2000c (Thermo Fisher Scientific, Waltham, MA, USA), a spectral photometer to estimate the DNA concentration in ng/µl.

- **Qubit® 1.0 Fluorometer**

This system is based on a fluorometric technology using fluorescent dyes emitting signals only when bound to DNA or other targets (different kits are available for RNA, single-stranded [ss] or double-stranded [ds] DNA, and proteins with specific detection ranges). Furthermore, the minimal detection limit is 10 pg DNA per µl, providing a high sensitivity.
In the current work, i.e., analysis of low-biomass samples, Qubit® dsDNA HS Assay with a detection range of 0.2 to 100 ng and a sample starting concentration of 10 pg/µl to 100 ng/µl was used. All required reagents are included in the assay.

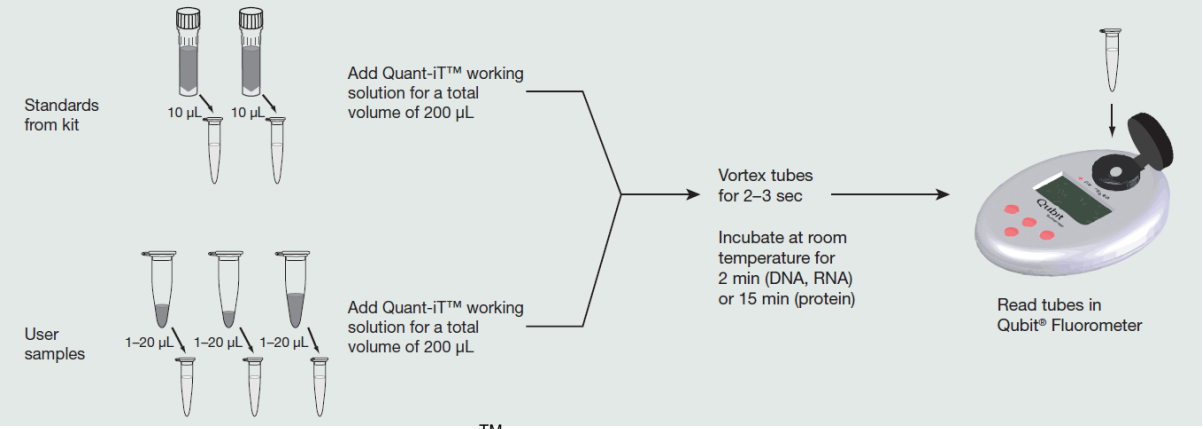

Fig. II.8.2.2.1 Workflow for the Quant-iT™ assay using the Qubit® 1.0 Fluorometer (Invitrogen, Life technologies, Carlsbad, CA, USA). © 2010 Life Technologies Corporation

All Quant-iT™ assay reagents were mixed in 500 µl thin wall PCR tubes (Axygen, Inc. Union City, CA, USA) as described in the manufacturer's instructions (Fig. II.8.2.2.1). For each measurement a standard (supplied) was run in addition to calibrate the instrument. The measured DNA concentration was given in µg/ml.

II.8.3. Polymerase Chain Reaction

This technique was used for 16S rRNA gene amplifications (Saiki *et al*., 1985; Saiki *et al*., 1988).
The following two PCR setups were tested based on recommendations of Second Genome, Inc. (South San Francisco, CA, USA) with two major goals:

- To check the quality of the extracted gDNA of swab samples
- To analyze the amount of gDNA needed to obtain 500 ng of amplified DNA for following hybridization on PhyloChip (section II.8.6.). Initial bacterial DNA template was set 30 to 50 ng per 50 µl PCR reaction or 10 to 30 ng per 25 µl reaction.

The results of the above-mentioned experimental setups were necessary to finalize the decision about the method of choice for molecular analysis of the MARS 500 swab samples.

All reactions were carried out using the degenerate forward PCR primer 27F (5'-AGRGTTTGATCMTGGCTCAG-3') and the non-degenerate reverse PCR primer 1492uR (5'-GGTTACCTTGTTACGACTT-3').
For every PCR reaction a negative control was carried along, i.e., Master Mix without DNA but with 1 µl H_2O (LiChrosolv®, Merck Millipore, Darmstadt, Germany).

II.8.3.1. TaKaRa ExTaq® PCR Protocol

TABLE II.8.3.1.1 COMPOSITION OF THE PCR REACTION USING TAKARA EXTAQ® PCR PROTOCOL

Component	Starting concentration	µl per reaction
10x Buffer	10x	5
Forward primer	3 µM	3.5
Reverse primer	3 µM	3.5
BSA (unacetylated)	20 mg/ml	2.5
TaKaRa dNTP Mix	10 mM total	4
TaKaRa ExTaq	5 U/µl	0.25
Template	10 - 30ng	1 - 2
Water		ad 50
Total reaction volume		50

II.8.3.2. Molzym™ 16S Basic Master Mix

TABLE II.8.3.2.1 COMPOSITION OF THE PCR REACTION USING MOLZYM™ 16S BASIC MASTER MIX

Constituents	Volume (µl)
Forward primer 3 µM	3.5
Reverse primer 3 µM	3.5
Molyzm 16S Basic Master Mix	10
Molzym 16S Taq Polymerase	0.8
Molzym DNA-free, nuclease-free water	5.2
DNA at 10 - 30ng/µl	2
Total reaction volume	25

II.8.3.3. Amplification Conditions

Targets for both setups were amplified using the PCR thermocycling program displayed in Table II.8.3.3.1.

TABLE II.8.3.3.1 AMPLIFICATION CONDITIONS

PCR step	Temperature (°C)	Time	Cycle number
Initial denaturation	95	3 min	1
Denaturation	95	30 s	
Annealing	50	30 s	35
Elongation	72	120 s	
Final elongation	72	10 min	1
End	10	Hold forever	1

II.8.4. Agarose Gel Electrophoresis

Genomic DNA and (purified) PCR products were visualized by agarose gel electrophoresis. Depending on the size of DNA, 0.8 - 1 g agarose (Serva Electrophoresis GmbH, Heidelberg, Germany) was added to 100 ml TAE buffer (1x) (section II.1.3.). The solution was heated and swirled gently several times in between until agarose was completely dissolved. The staining occurred either with ethidium bromide (1 µg/ml) (Serva Electrophoresis GmbH, Heidelberg, Germany) or with 1,000x SYBRSafe (1 µg/ml) (Molecular probes, Invitrogen, Carlsbad, CA, USA) before pouring the gel into the horizontal gel slide (dimensions of 5 x 6 cm), which was equipped with a comb. The sample was mixed with 6x loading dye (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA), loaded into the agarose gel slots and subjected to 70 V and 60 mA through 1x TAE buffer for 60 to 120 min. A standard DNA size marker (1 kb DNA ladder, Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) was electrophoresed in a lane next to the DNA sample. After electrophoresis, nucleic acid bands were visualized using ImageQuant LAS 400 digital imaging system (GE Healthcare, Freiburg, Germany). Sample DNA sizes and concentrations were estimated by comparison to band position and intensity of the standard DNA markers.

II.8.5. Purification of PCR Products

MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany) was used as specified by the manufacturer's instructions to purify and concentrate PCR amplicons. All centrifugation steps were carried out at 17,900 g at room temperature. To increase recoveries, the eluate was applied a second time to the MinElute column. Then a final centrifugation step was performed followed by DNA concentration determination (section II.8.2.) and quality check via agarose gel electrophoresis (section II.8.4.).

II.8.6. PhyloChip

PhyloChip is a microarray-based method that relies on the entire 16S rRNA gene sequence information and has been developed by the Lawrence Berkeley National Lab (CA, USA). In the third-generation assays (G3) a total of 1,100,000 DNA probes are hybridized on the array as targets for the identification and measurement of the relative abundance of

individual microbial taxa in any sample. Due to its high sensitivity, parallelism and reproducibility, this tool is highly efficient for analysis of microbial dynamics over time or place, representing a major goal of the thesis regarding the microbial inventory analysis of samples derived from the MARS 500 program.

Second Genome, Inc. (South San Francisco, CA, USA), is worldwide the only company that applies the microarray technique developed by Prof. Dr. Gary Anderson. The service of this company included experiment design support, assay processing including PCR reaction, labeling and hybridization (Fig. II.8.6.1), and statistical analysis.

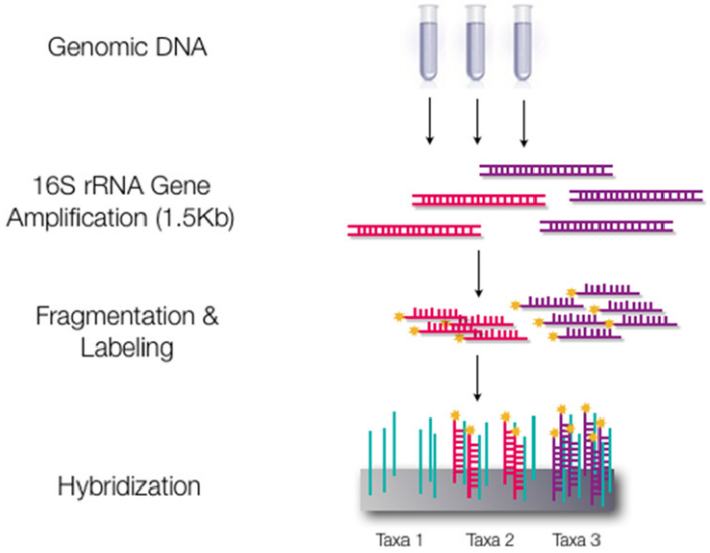

Fig. II.8.6.1 Workflow from extracted genomic DNA to hybridization on the microarray including target amplification, fragmentation and labeling. © Second Genome, Inc.

- **Sample preparation**

Second Genome also provides DNA extraction service, but due to the uniqueness and irretrievability of the MARS 500 samples as well as to the fact that the swabs contain only low-biomass, DNA isolation was done by myself and required several pre- and optimization experiments (as described above).

The general operating procedure of Second Genome is as follows: Upon arrival all samples are quantified. If the samples have greater than 50 ng of DNA, they are carried forward for PCR. After amplification, a maximum of 500 ng of DNA is hybridized. If a sample has less than 500 ng, but greater than 100 ng, then the entire amount of amplified product is hybridized (pers. comment: Christel Chehoud).

To reach higher DNA yields swabs have to be pooled from the same sampling dates in the same module but from different sampling sites within one module. Extraction was performed using the XS-buffer method as described in section II.8.1.3., resulting in high quality DNA. DNA quantity was checked by measuring the concentration as described in Fig. II.8.2.1, DNA integrity was controlled via PCR (section II.8.3.1.) and amplicons were visualized on an agarose gel (section II.8.4.). Furthermore, the PCR products were purified and the PCR yield was measured (section II.8.2.), since 100 to 500 ng were requested for hybridization.

Samples were sent in low-bind screw cap Eppendorf tubes (Eppendorf AG, Hamburg, Germany) on dry ice with World Courier and reached the laboratories of Second Genome within 48 h.

- **Sample treatment and analysis methods at Second Genome**

Once the samples arrived at the service laboratories of Second Genome they were processed as described briefly below:

- gDNA concentration was determined using PicoGreen® method. Bacterial 16S rRNA genes were amplified in duplicate using Molzym™ 16S Basic Master Mix and adding 2 µl DNA template (section II.8.3.2.; input gDNA mass for PCR was 50 ng).
- Amplicons were concentrated via solid-phase reversible immobilization method and purified using PowerClean® DNA Clean-Up Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) for removing PCR contaminants.
- PCR amplification products were quantified by electrophoresis using the Agilent Bioanalyzer® (Agilent Technologies, Inc., Santa Clara, CA, USA) and following criteria were considered for PCR amplicon quality:
  - presence of the specific target (1.5 kb 16S rDNA)
  - relative abundance of the specific target to the total product (>50%)
  - consistent trace pattern
  - PCR yield (>500 ng)
- PhyloChip™ Control Mix was added to each sample.
- Labeled bacterial sequences were fragmented, biotin labeled, and hybridized to the PhyloChip™ Array version G3.
- Arrays were washed, stained and scanned using a GeneArray® scanner (Affymetrix, Santa Clara, CA, USA).
- Affymetrix software (GeneChip® Microarray Analysis Suite) captured the scan of each array and measured hybridization values, and fluorescence intensities.

- **Data analysis of the PhyloChip**

The first step included rank-normalization of fluorescence intensities across probes for each array individually (applied when different amounts of amplicons were hybridized) in order to ascertain comparability of the samples. This step was followed by data pre-processing (detailed information see data CD [folder PhyloChip: Second Genome Microbial Profiling Report]; DeSantis *et al*., 2005; Hazen *et al*., 2010), i.e., filtering for taxa that are present in at least one sample (filter 1) or for taxa that show significant abundance differences (filter 5). Significance testing for filter 5 was done with parametric Welch test. The outcome could be validated (false discovery rates) by calculating q-values using on Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

In the study described here, the operational taxonomic unit (OTU) determination was based on the novel empirical OTU (eOTU) selection process, i.e., directly taxonomically annotated with a Bayesian method from the combination of the 9-mers contained in all probes of the set (Probst *et al*., 2014). Therefore, probe sets were defined on the basis of relatedness of

the probes and their correlation in fluorescence intensity throughout the experiment. Former studies are based on reference OTUs (rOTU) applying a pre-defined taxonomy map.
Once eOTUs have been determined, they were included in further analysis, data were summarized in two distinct ways, i.e., either abundance metrics (AT) or binary metrics (BT) are generated. For detailed information regarding the calculation of AT, please refer to Hazen *et al*. (2010 supplements).
Based on this classification, sample-to-sample distances (inter-sample) were calculated in order to determine similarity or dissimilarity between sample pairs. Therefore, Bray-Curtis Distance[12] statistical tool was utilized for abundance values, whereas it is called Sorensen dissimilarity when incidence values provided the basis for calculation. Multivariate data analysis steps are incorporated into Second Genome's PhyCA-Stats™ analysis software package.
The graphical processing of the dissimilarity scores was done by generating hierarchical clustering maps using the average neighbor (HC-AN[13]) method and two-dimensional ordinations (non-metric multidimensional scaling [NMDS[14]]). Unless stated otherwise whole microbiome significance testing was performed using Adonis[15] test.

In addition to the full data analysis pipeline, custom data analysis was requested including:

- the correlation of OTU trajectories with metadata
  Metadata included information on sampling date (grouped also in early, mid, and late), environmental data (CO_2, O_2, relative humidity (RH), and temperature) and CFU data, obtained from cultivation. Selection of eOTUs which had a significant correlation with different metadata factors was done by Spearman rank correlation, a non-parametric measure of statistical dependence between two variables.
- reclassification of 16S rRNA gene sequences from isolates
- comparison of PhyloChip derived taxa with those obtained by cultivation
- identification of potentially pathogenic organisms (PPO)
  Therefore, a catalogue of pathogens (data CD [folder: TRBA 466]) was provided to Second Genome based on the Technical Rule for Biological Agents (TRBA) 466 document "Classification of prokaryotes (bacteria and archaea) into risk groups" (TRBA 466, 2010), considering risk groups two and higher. All eOTUs that were detected by PhyloChip assay were individually compared to each pathogenic

[12] Statistical tool to quantify the compositional dissimilarity between two different communities which considers differences in the abundance of a species or OTU across two communities (Second Genome).

[13] Uses the Average-Neighbor methods to graphically summarize the inter-sample relationships in a dendrogram. Branch length indicates the biological similarity between samples (Second Genome).

[14] Methods of two-dimensional ordination plotting in order to visualize complex relationships between samples in the form of relative distances (Second Genome).

[15] Determination of significant differences in the whole microbiome among discrete categorical or continuous variables. Samples are randomly reassigned to various samples categories, and the fraction of permutations with larger cross-category differences relative to within-category differences is reported as the p-value (Second Genome).

species noted in the list and were further statistically analyzed applying methods described above.

II.9. Statistical Analyses

The data shown in this study represent mean values with standard deviations. Statistical significant differences between treatments were determined using Student's t-test or stated otherwise. Differences with p-values <0.05 were considered statistically significant.
Linear regression and Pearson correlation was performed using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA) in order to determine the correlation of two factors with each other.

II.10. Questionnaires

After the end of the confinement and the analysis of the majority of samples, several questions were raised. With the goal to get more information and answers in order to be able to interpret the obtained results from the cultivation approach, two questionnaires were compiled successively and sent to the marsonauts themselves (data CD [folder: Questionnaires]. Since this was not requested in the original proposal, it was their voluntary decision to answer the questions. Furthermore, it has to be noted that some marsonauts had only a basic knowledge of English.

II.11. Scanning Electron Microscopy (SEM)

Scanning electron microscopy was performed using the high-resolution JEOL JSM-6510 series microscope by M. Sc. Marcel Fiebrandt at the Institute for Electrical Engineering and Plasma Technology of the Ruhr-University Bochum in Germany.
The aim was to depict the structural texture of nylon-flocked swabs before sampling, i.e., sterile and without application of mechanical force. Of peculiar interest was the configuration change of the nylon fibers that are attached to the head of the swab after surface sampling. Therefore, sampling was performed as described in Fig. II.4.1.1, applying a steady pressure on the head of the swab during rotation. Furthermore, another goal was to visualize the distribution of microbial cells after sampling a surface or when spiked with *S. cohnii* cells (for sample preparation; section II.7.).
The samples were sent under room temperature conditions to Mr. Fiebrandt where the further preparation of the objects was conducted. Samples were fixed with an adhesive carbon to stainless steel discs and then coated with gold. The gold layer thickness could be estimated at approximately 10 nm. This was necessary as swabs should not get statically charged by the electron beam. The acceleration voltage is indicated on the pictures, but was usually set to 10 keV.

III. RESULTS

Microbial monitoring of manned spacecraft and spacecraft-related environments was performed since the 80's on the Mir space station, indicating a high diversity of bacteria and fungi being present (Ilyin, 2000). The occurrence and accumulation of microorganisms can lead to technical and instrumental errors onboard a spacecraft (Novikova *et al*., 2006). Pathogens can even be the elicitor of infections. This threat is quite relevant and causes a high demand to obtain an in-depth understanding of the interaction of microorganisms with materials resulting in biocorrosion, as well as with humans in order to predict potential hazards for astronauts. Thus, obtaining information about resident microbial diversity is inevitable in order to evaluate potential jeopardy. MARS 500, the long-term ground simulation experiment of a manned flight to Mars, provided a unique opportunity to acquire data from a confined manned habitat, since until now little is known about the influence of long-term confinement on the microbial inhabitants and their community structure that might undergo changes with time.

The following chapter presents results from comparative microbiological measurements of air and surface samples. Sampling was performed at the MARS 500 facility in Moscow, Russia, the University of Regensburg, DLR and households in Cologne, Germany. Further, several optimization experiments were conducted regarding DNA extraction processes for low-biomass samples followed by PCR amplification. The main part describes in detail data derived from the proposed MARS 500 experiment MIcrobial ecology of Confined Habitats and humAn health (MICHAm, modified).

In sum, 18 sampling events took place (17 during the confinement and one additional reference sampling, which was performed six months post-confinement; Fig. III.1) at eleven different locations for surface sampling and nine different locations for air sampling in the MARS 500 complex. On the one hand, it was critical to monitor the inhabiting microorganisms on the **cultivation level** since specific traits (e.g., harmful to humans, resistances) of bacterial strains can only be assessed by isolation and further characterization. Additionally, these isolates will function as future test objects in order to evaluate strategies that have been designed to minimize contamination. On the other hand a **molecular** state-of-the-art technique (PhyloChip G3) was applied to monitor the overall microbial small ribosomal subunit (SSU) 16S rRNA gene signatures present in the different modules.

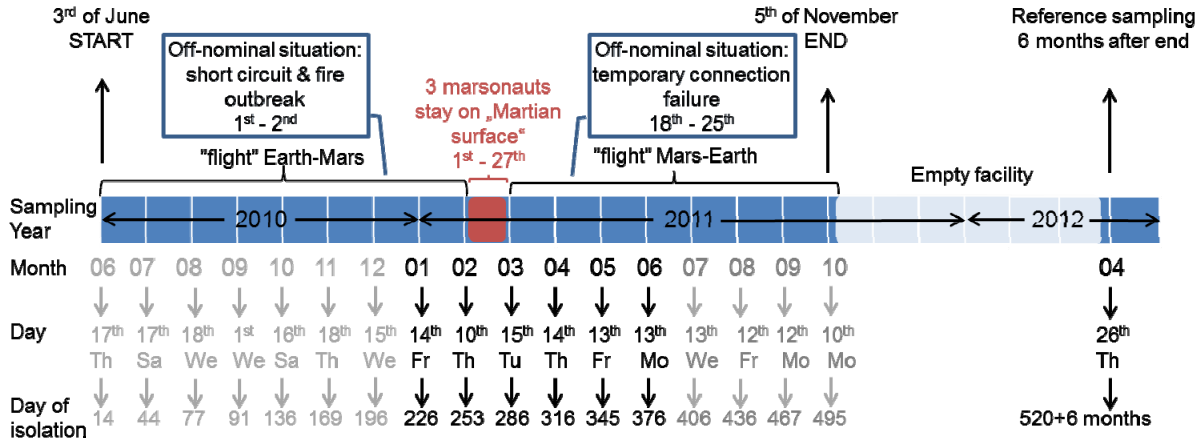

Fig. III.1 Timeline of the MARS 500 experiment from the beginning (June 3rd, of 2010) until the end (November 5th, of 2011) plus six months. The schematic drawing also indicates important steps during the confinement (above timeline) including the two off-nominal situations and sampling dates (below timeline) from 18 sampling events. Red area/font denotes the stay of three marsonauts in the simulated Martian surface complex, whereas light blue area represents the timeframe where the facility was untenanted. Samples were returned to DLR in four batches illustrated by different font colors of sampling month and day.

The experimental strategy employed to address the microbial load is outlined in Fig. III.2.

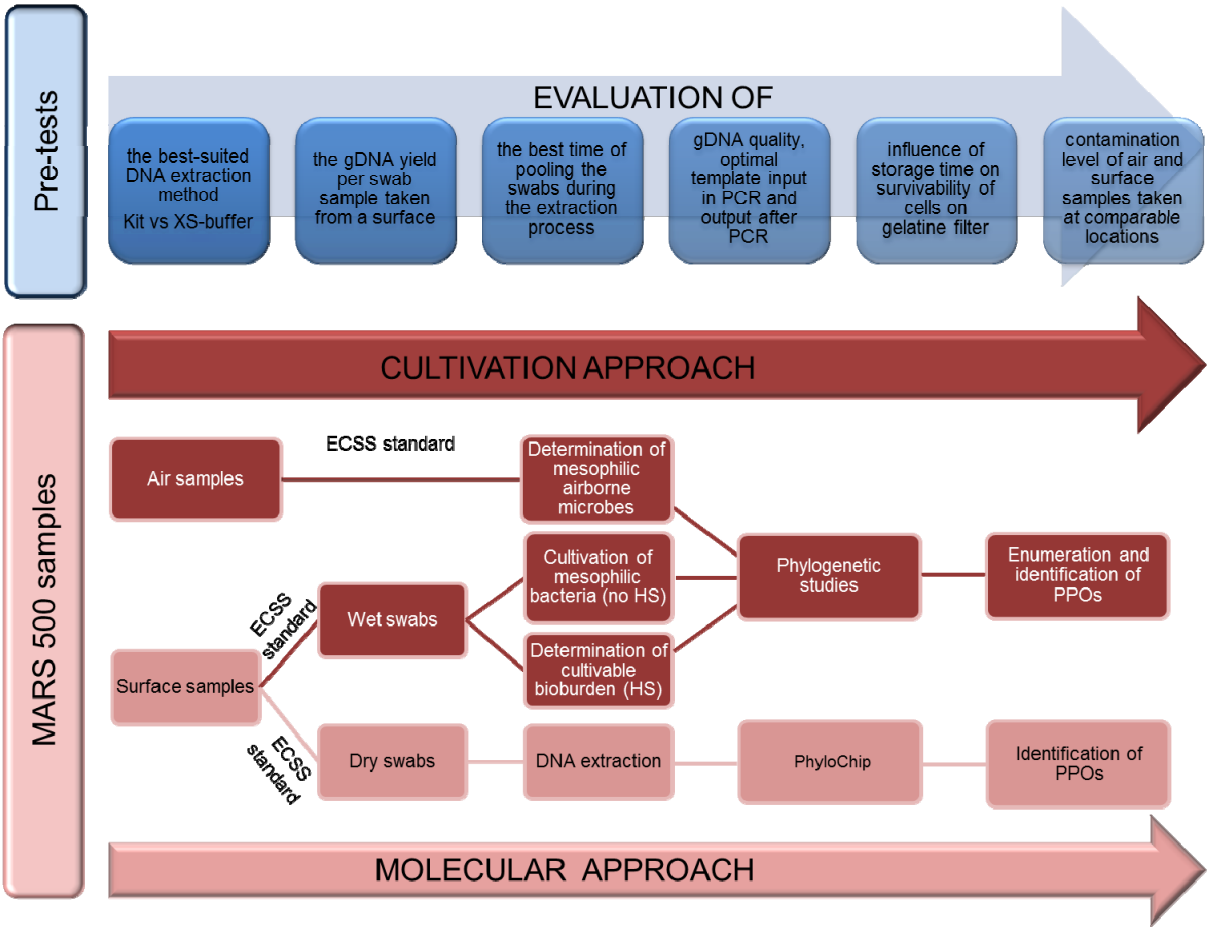

Fig. III.2 Schematic outline of the experimental strategy designed to identify the microbial ecology of the MARS 500 habitat. PPO=potentially pathogenic organism; HS=heat-shock

III.1. CULTIVABLE DIVERSITY

During the campaign, two cultivation approaches were performed to address the airborne microbial specimens and the microbial load that is residing on surfaces. Therefore, ESA's ECSS-Q-ST-70-01C (2008) standard was applied to detect either mesophilic aerobic microbial cells ("vegetatives") or to determine the spore-forming or heat-shock (HS) resistant microbes ("bioburden").

III.1.1. MICHAm: Airborne Microorganisms

The enumeration and evaluation of the diversity of microbes within indoor air is an important first step to prevent risk for human health.
Consequently, air contamination of three different modules was monitored once a month during the confinement with an additional reference sampling. Nine areas, chosen from three different modules (EU-100, EU-150, and EU-250) within the isolation facility were surveyed during this study to compile an overview of the bacterial contamination present in this MARS 500 facility (Table III.1.1.1).
For clarity, a color code was generated to easily distinguish between and recognize the different modules. Blue is assigned to the utility module (EU-250), green to the habitable module (EU-150) and red to the medical module (EU-100).

TABLE III.1.1.1 LIST OF AIR SAMPLES AND SAMPLING AREAS AT THE MARS 500 FACITLIY (EU-250=UTILITY MODULE, EU-150=HABITABLE MODULE, AND EU-100=MEDICAL MODULE)

Module	Sample #	Description
250	1	On the floor in front of the toilet
	6	On the floor of the gym next to the treadmill
	7	On the floor in front of the greenhouse
	8	On the floor between storage racks
	9	On the floor between the fridges
150	3	On the carpeted part of the floor in the community room
	4	On top of the table in the dining area
	5	On the floor of an individual compartment
100	2	On the floor at the working place between desk and bunk

The exact position of the different sample sites is depicted in Fig. III.1.1.1 for the utility module, in Fig. III.1.1.2 for the habitable module, and in Fig. III.1.1.3 for the medical module.

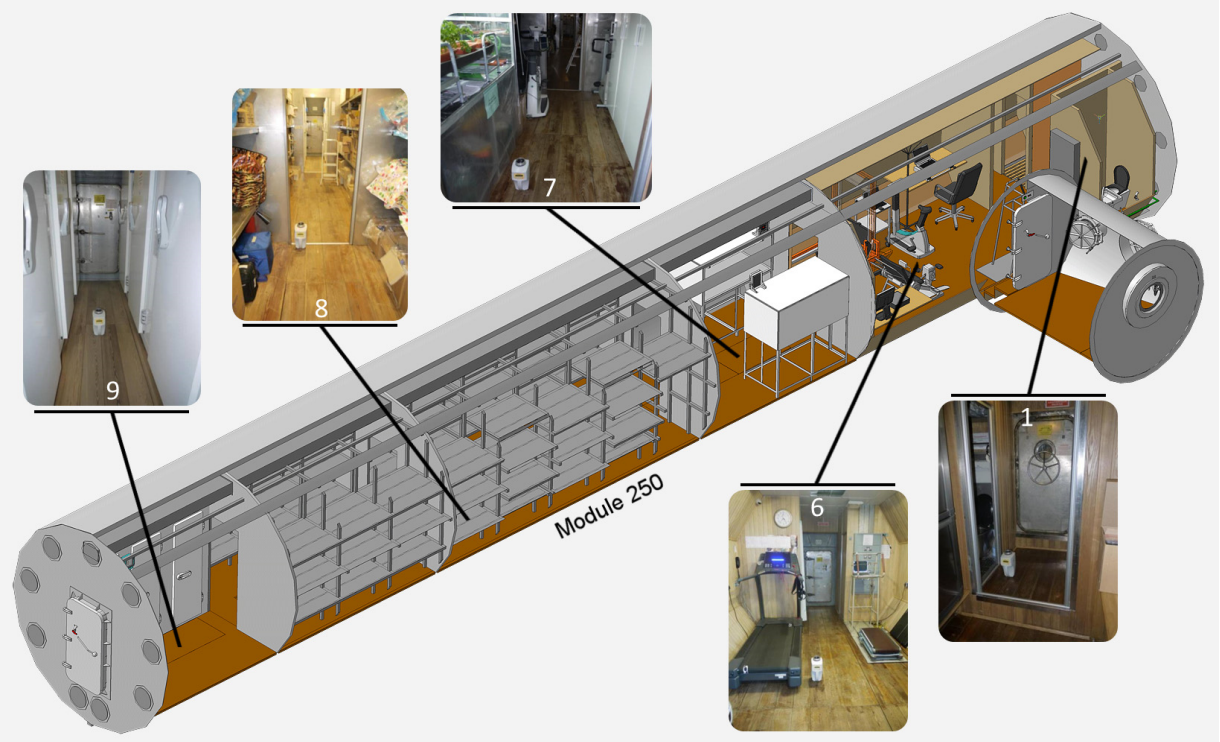

Fig. III.1.1.1 Schematic drawing of the utility module. Photographs of each sampling site are allocated to the specific location. © SSC RF IBMP RAS, Oleg Voloshin (edited)

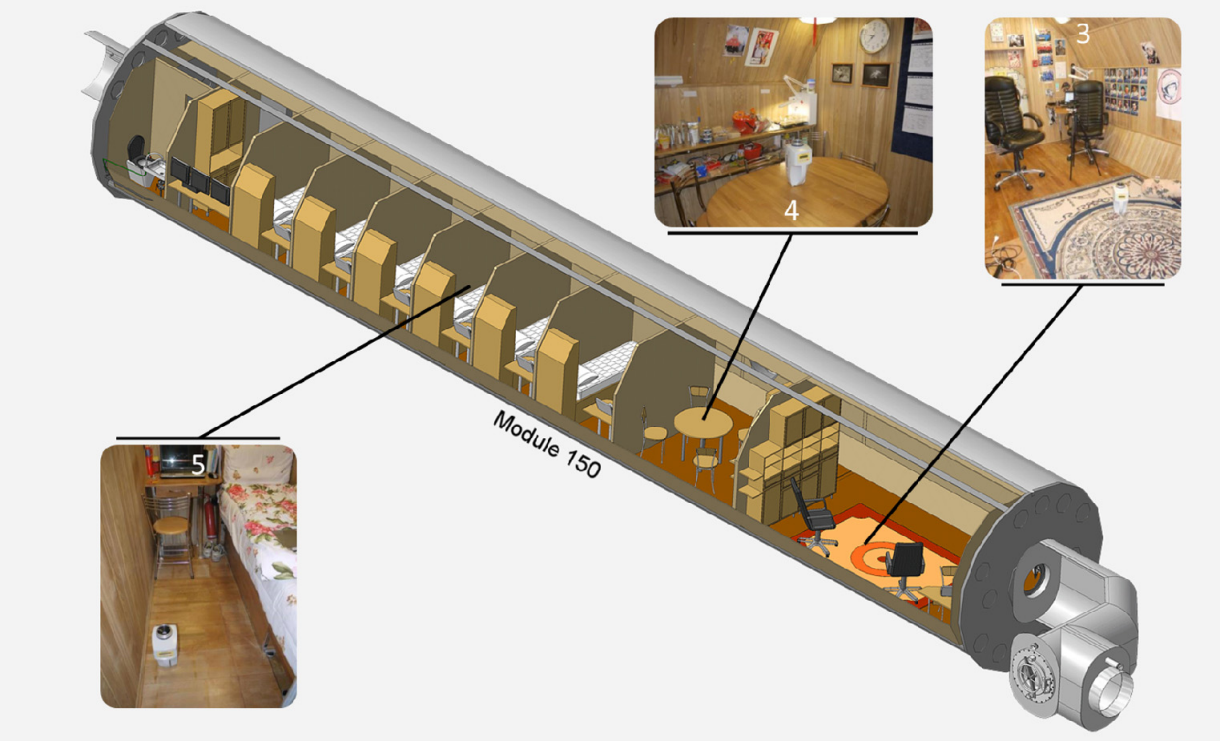

Fig. III.1.1.2 Schematic drawing of the habitable module. Photographs of each sampling site are allocated to the specific location. © SSC RF IBMP RAS, Oleg Voloshin (edited)

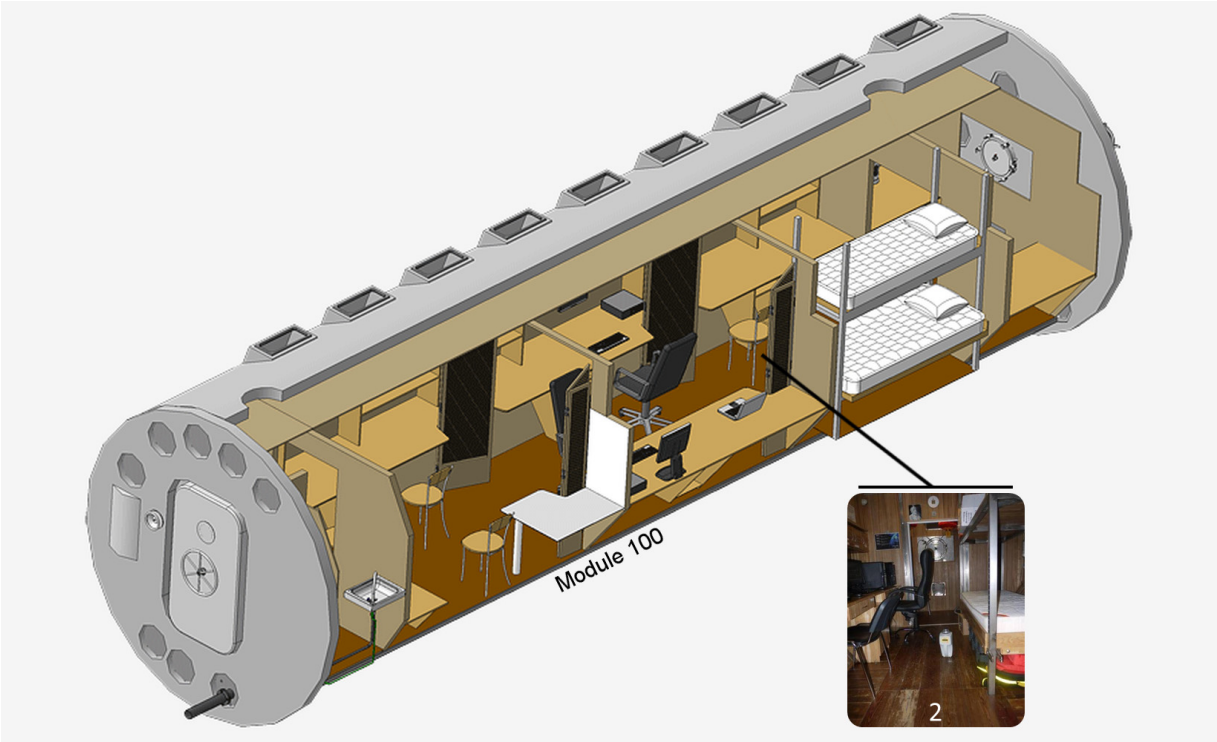

Fig. III.1.1.3 Schematic drawing of the medical module. Photographs of each sampling site are allocated to the specific location. © SSC RF IBMP RAS, Oleg Voloshin (edited)

In general, 500 l (correspond to 0.5 m³) air was sampled with the AirPort MD8 (Sartorius AG, Goettingen, Germany), an active air sampling tool. After collection, done by Charles Romain (marsonaut), storage at -20°C for up to six months and gentle thawing, bacteria were cultivated to identify the fraction of the airborne community that is able to grow under ECSS-Q-ST-70-01C standard procedure conditions (2008), i.e., aerobic cultivation on R2A plates for 72 h at 32°C (section II.3.4.).

- **Results on sample level**

Employing the afore-mentioned protocol, mainly mesophilic, heterotrophic, aerobic bacterial strains were detected. The obtained number of colony forming units (CFU) from each sampling site and per sampling date can be found in Table III.1.1.2. All field blanks were negative, demonstrating a sterile handling of the gelatine filters during sampling procedure.
All absolute cell counts were taken after 72 h incubation at 32°C, preventing bias by counting after different incubation times. Comparisons between the samples were feasible since no overgrowth issues occurred during the incubation time of 72 h. In addition, the proportion of swarming microorganisms was very low and did not have a negative influence on the counts. Even though R2A is not a typical medium for the enrichment of fungi, growth of few fungal colonies was observed, especially in the samples taken six months after the end of the confinement experiment (fungal counts not included in Table III.1.1.2). During this time, the complex was only seldom frequented by humans.

TABLE III.1.1.2 TOTAL MICROBIAL AIRBORNE COLONY FORMING UNIT (CFU) COUNTS ON R2A AFTER 72 H INCUBATION AT 32°C ACCORDING TO 500 L SAMPLED AIR AN DIFFERENT LOCATIONS IN MODULES EU-250 (UTILITY), EU-150 (HABITABLE), AND EU-100 (MEDICAL).

Module	Utility module - Locations 1, 6, 7, 8, 9 CFU/0.5m³						Habitable module - Locations 3, 4, 5 CFU/0.5m³				Medical module - Location 2 CFU/0.5m³
Sampling date	**1**	**6**	**7**	**8**	**9**	**Mean value**	**3**	**4**	**5**	**Mean value**	**2**
June 2010	14	7	2	0	4	5	62	80	9	50	22
July 2010	3	12	33	33	10	18	113	16	269	133	0
Aug 2010	5	87	22	11	2	25	114	63	174	117	7
Sep 2010	10	3	52	65	4	27	61	101	358	173	1
Oct 2010	3	122	17	5	28	35	159	345	208	237	1
Changing of NANO-filters and cleaning of primary filters on 11.11.2010 (day 162 of isolation)											
Nov 2010	7	4	11	26	0	10	175	27	77	93	14
Dec 2010	5	136	10	1	1	31	60	209	32	100	11
Jan 2011	4	18	9	7	9	9	52	73	54	60	0
Changing of NANO-filters and cleaning of primary filters on 02.02.2011 (day 243 of isolation)											
Feb 2011	6	14	3	1	2	5	71	30	45	49	0
March 2011	2	269	23	11	8	63	59	25	84	56	0
Apr 2011	3	68	34	4	1	22	47	40	57	48	2
May 2011	11	2	12	1	2	6	54	7	38	33	2
June 2011	11	25	17	6	4	13	48	12	208	89	9
July 2011	5	20	34	6	1	13	70	75	145	97	1
Aug 2011	2	32	33	4	8	16	60	29	313	134	4
Sep 2011	17	53	39	8	4	24	216	143	210	190	10
Oct 2011	16	24	20	9	9	16	13	30	40	28	2
Apr 2012	13	18	11	7	13	12	9	32	13	18	18
Mean value	8	51	21	11	6	-	80	74	130	-	6
Standard deviation	5.0	67.7	13.7	15.9	6.6	-	54.9	84.9	109.3	-	6.8

In sum, air samples revealed cell numbers from 0 to 716 per m³ with an average value of 86 CFU per m³ (n=162 samples; blanks are not included). In total, 6,966 colonies were counted. Samples from the habitable module showed estimated bacterial cell numbers from 14 to 716 per m³, whereas CFU counts from the utility module ranged from 0 to 538 per m³ and counts for medical module were even lower (0 to 44 per m³).

In all, the number of cultivable bacteria underlied fluctuations over the whole timeframe during the confinement with regard to sampling site and module, respectively. An example is displayed below (Fig. III.1.1.4) but more details on the fluctuations of each sample location can also be found on the enclosed data CD (folder: Air Samples).

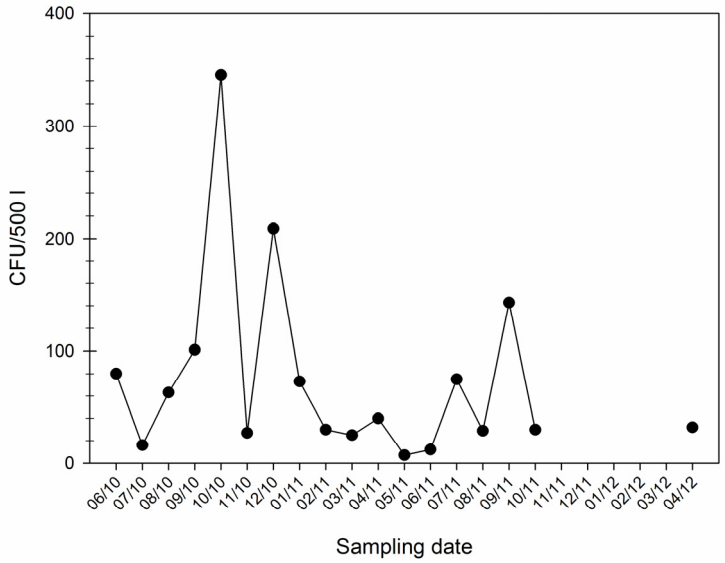

Fig. III.1.1.4 Microbial airborne inventory of one air sample over time: colony forming unit (CFU) counts per 500 l air on R2A after 72 h incubation at 32°C of one sampling site (location 4; dining area) from the corresponding module EU-150 (habitable) of the MARS 500 facility.

However, when comparing the mean values of each location (CFU values from 18 sampling events) over the whole confinement period plus the post-confinement sampling, it is obvious that contamination in the habitable module (EU-150) is significantly higher (p-value <0.05) than in the utility (EU-250) and medical module (EU-100; Fig. III.1.1.5). This effect was observed for all three locations of module EU-150. All samples from the utility module revealed comparatively low CFU counts, other than sample six where the air sampler was placed on the floor of the gym close to the treadmill.

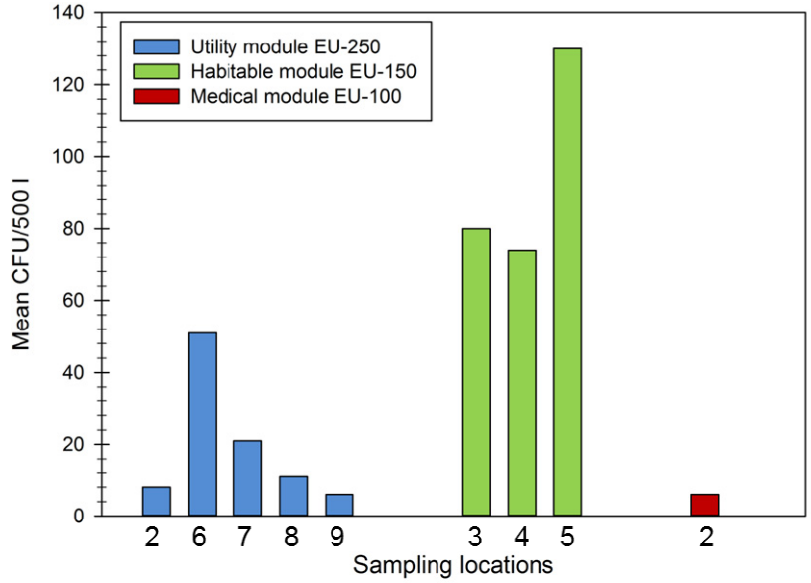

Fig. III.1.1.5 Mean microbial airborne inventory per sample location: colony forming unit (CFU) counts per 500 l air on R2A after 72 h incubation at 32°C grouped by sampling sites from the corresponding module (utility, habitable, or medical) of the MARS 500 facility. Data are expressed as mean (n=18) values calculated by including data from every sampling date beginning in June of 2010 and ending April of 2012 (520 days + 6 months). For reasons of clarity and due to the high variations, standard deviations are not displayed (**Table III.1.1.2**).

➢ **Results on module level**

The following Figure III.1.1.6 refers to mean values comprised of five sample locations (1, 6, 7, 8, 9) from the utility module, three sample locations (3, 4, 5) from the habitable module and only one sample (location 2) from the medical module. The highest number of cultivable bacteria was obtained from samples taken in the habitable module, showing a 6-fold and a 53-fold mean increase compared to the utility module and medical module, respectively.

The same trend was observed for all analyzed samples apart from one sampling event in March of 2011, where the mean CFU value was higher in EU-250 compared to EU-150. This effect resulted from an outlier sample in March of 2011 from location 6. However, the mean values of the whole timeframe of each module were subject to unequally wide margins of deviation. Fig. III.1.1.6 depicts the highest fluctuations in the habitable module with peaks in October of 2010 and September of 2011 whereas the medical module showed the least variation. The utility module displays similar variations compared to the habitable module with the exception of revealing generally lower CFU counts and thus expressed in minor manifestations. Furthermore, there is no concrete trend visible that shows an increase or decrease with time.

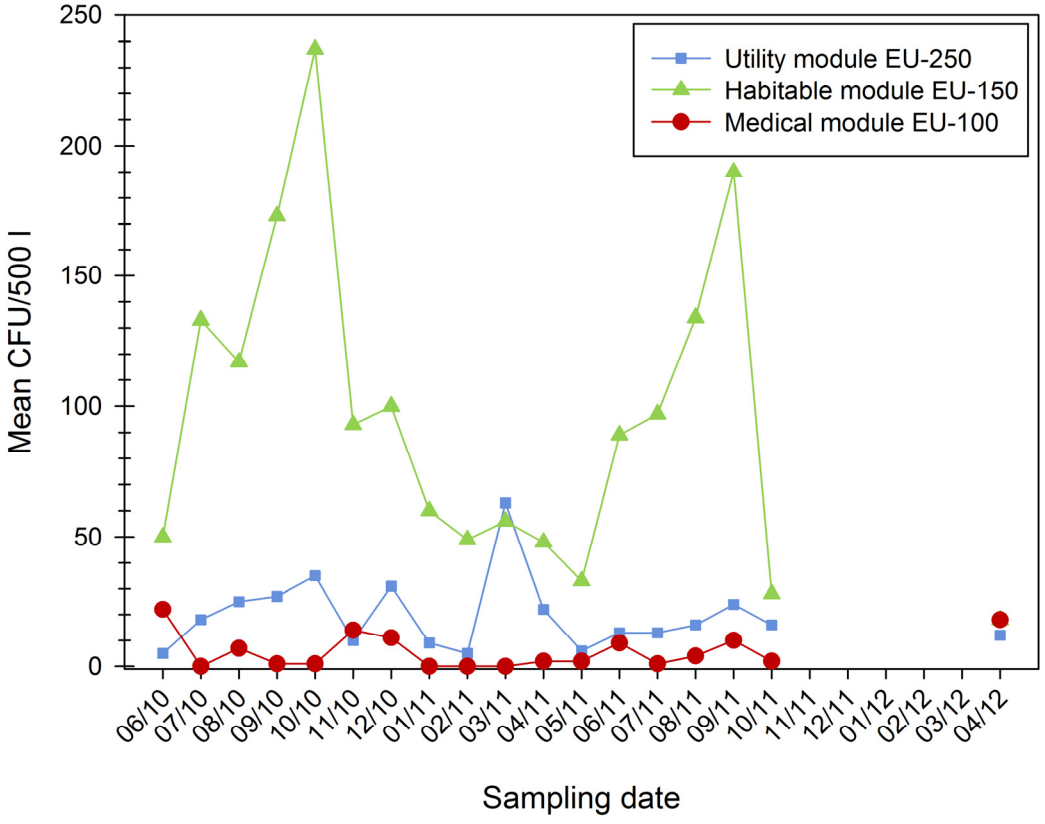

Fig. III.1.1.6 Mean microbial airborne inventory per module over time: colony forming unit (CFU) counts per 500 l air on R2A after 72 h incubation at 32°C of each module (utility, habitable, or medical) of the MARS 500 facility over time. Data are expressed as mean (n=5 for utility module; n=3 for habitable module; n=1 for medical module) values calculated by including data from every sampling site of the corresponding module. For reasons of clarity and due to the high variations, standard deviations are not displayed. An example is shown in **Fig. III.1.1.7**.

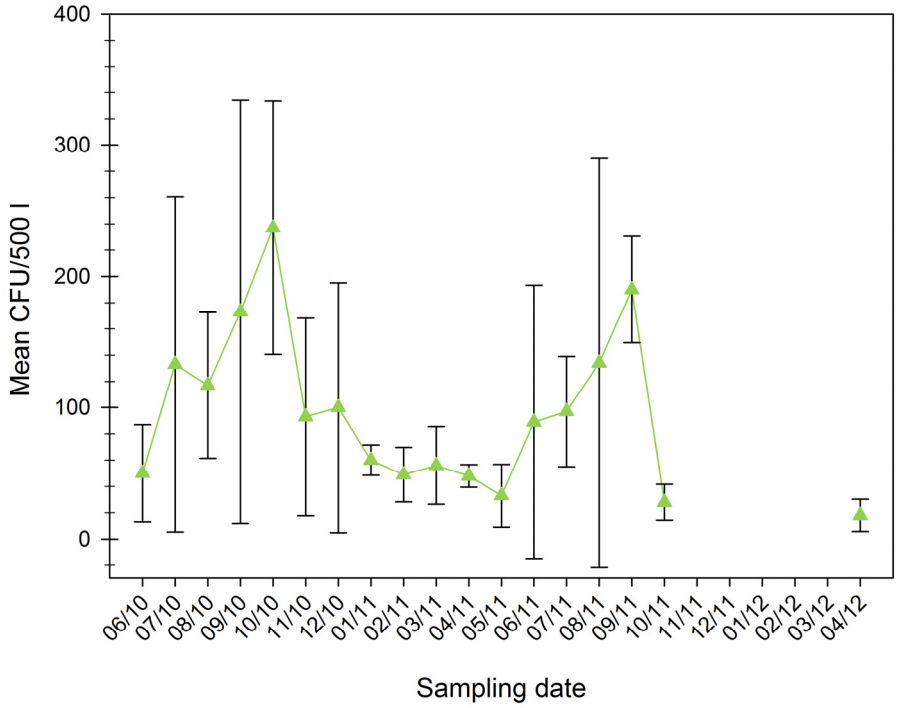

Fig. III.1.1.7 Mean microbial airborne inventory of one module over time: colony forming unit (CFU) counts per 500 l air on R2A after 72 h incubation at 32°C of the habitable module of the MARS 500 facility over time. Data are expressed as mean (n=3) values with standard deviation calculated by including data from every sampling site of the corresponding module.

Due to the observed fluctuations the question arose, whether changing the filter installed in the air ventilation system during the confinement had an influence on the detectable amount of microorganisms. Therefore, inquiries were performed to gather information about changing of the filters. A technician at the IBMP changed the filters only twice as indicated in Table III.1.1.2. Assumptions that the microbial load being present in the air should be reduced after filter change were not verified.
Furthermore, this observed fluctuation over time can be a result of the varying storage time of the filters. To answer this question an additional experiment was setup and results are shown in section III.1.2.

Summary:
Despite fluctuations over time and within sampling locations per module, the majority of airborne contaminants were detected at the following three sampling points in the habitable module: community room, dining area, and individual compartment. These locations represent areas with a high nutrient content (food debris) and are characterized by a high dispersion of dust caused through continual coming and going. A far lower microbial burden was obtained from the medical module and the utility module. No trend was observed regarding the microbial contamination with proceeding confinement.
The question remains open, whether the fluctuations result because of a dynamic microbial community structure, bias due to the different storage duration, or atmospheric conditions.

III.1.2. Effect of Storage Duration on Viability

Operationality of sampling shipment (on dry ice) after each sampling event during the MARS 500 program was not feasible for financial reasons and for the additional need of man-power. Therefore, samples were shipped in three batches after 6 months, 13 months and 19 months resulting in different storage times until processing.

To address the question, whether the storage duration of the air filter samples at -80°C has an influence on the survival of microorganisms, air samples were taken in duplicate at several locations at DLR that were comparable with sample sites at the MARS 500 complex. The optimal case would have been a parallel sampling procedure. However, that was not achievable since only one air sampler was available. To reach the most comparable results, the samples were taken in quick succession (in the following text passages referred to as parallel samples) and were stored at -80°C directly after sampling. One sample of each of the two parallel samples was processed after 4 days (possible minimum storage time of MICHAm samples), whereas the other one was stored for 6 months (maximum storage time of MICHAm samples until analysis).

Nine different locations in building 24 at the DLR were chosen as representative sampling sites (office, restroom, shower, laboratory, Fig. III.1.2.1).

As expected, fluctuations were detected regarding the origin of the samples. Both an increase and decrease in counted CFU values were noticed. Only three samples (all taken from restrooms) revealed a significant decrease after six months of storage. The remaining sampling locations displayed no distinct trend.

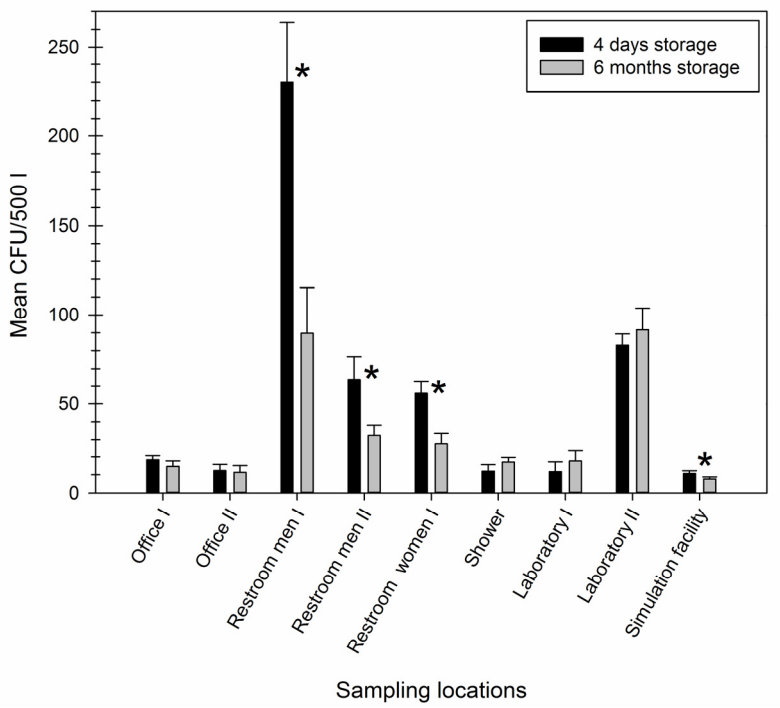

Fig. III.1.2.1 Effect of storage time to air samples at -80°C on survival rate of microbial cells on gelatine filters: colony forming unit (CFU) counts per 500 l air on R2A after 72 h incubation at 32°C. I and II: sampling locations at different levels in building 24 at DLR. Data are expressed as mean (n=3) values after 72 h incubation with standard deviation. Asterisk denotes significant difference between values obtained after 4 days and 6 months storage (p-value <0.05).

Summary:
Storage duration does not necessarily have a positive or negative effect on the viability of microorganisms. Due to this observation, the results of all MICHAm samples can be compared with each other, although the storage time of samples (sent per batch) varied.

III.1.3. MICHAm: Evaluation of Contamination Levels by Comparison

Evaluation of the measured airborne contamination level during the confinement is only achievable when comparing the results with those obtained from selected sampling sites in Germany. Furthermore, the difference between outdoor and indoor values had to be investigated. Therefore, parallel samples (n=4) were taken at two different private households between August and October. The three locations (bedroom, living room, and bathroom) were selected based on comparability with samples from the MARS 500 facility (individual compartment, community room, dining area and area in front of the toilet, Table III.1.1.1; Table III.1.1.2). Obtained results are plotted in Fig. III.1.3.1.

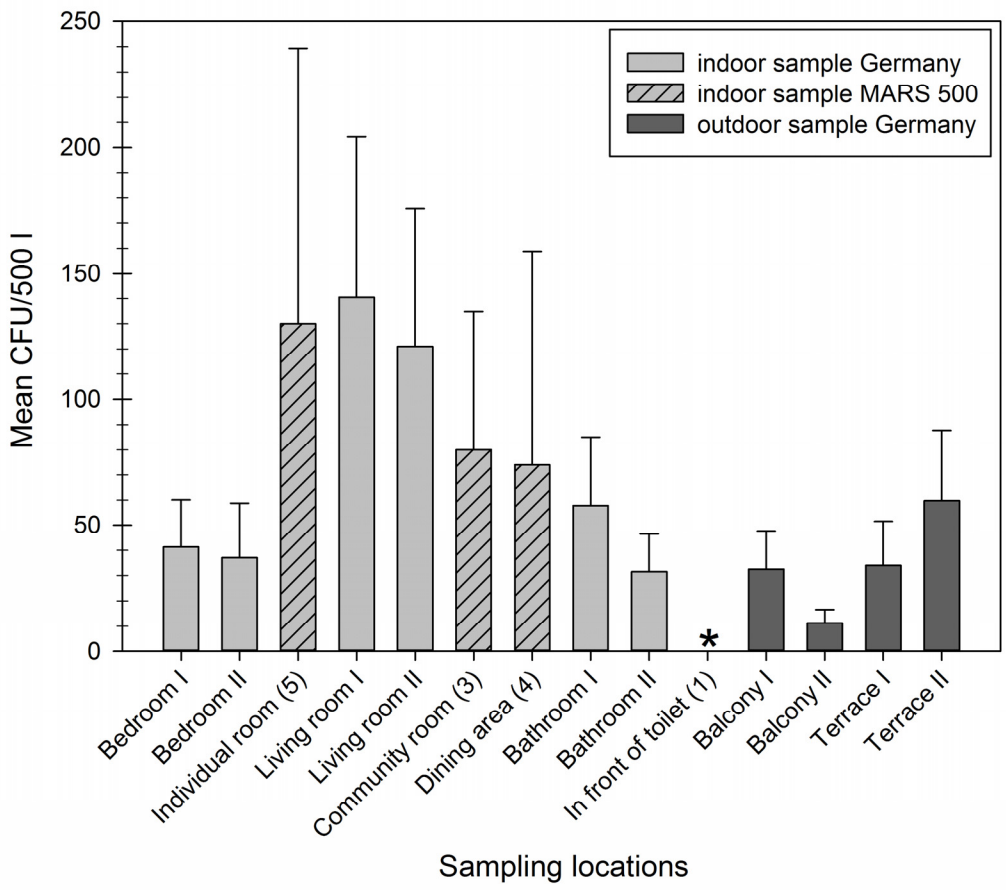

Fig. III.1.3.1 Estimation and comparison of indoor and outdoor airborne contamination levels of air samples taken at several sampling sites at two private households and respective samples from MARS 500: colony forming unit (CFU) counts per 500 l air on R2A after 72 h incubation at 32°C. I and II: sampled household; #: corresponding sample site in the isolation facility. Data are expressed as mean (n=4 for indoor and outdoor samples of private households, n=18 for indoor samples taken during MARS 500 campaign) values with standard deviation. Asterisk denotes revealed CFU count of 8 per 0.5 m³.

As expected and already detected in previous experiments, different levels of contamination were observed regarding the sampling location. The high standard deviation per sampling site pointed to high intra-sample variations.
The mean value of all indoor samples (n=24) taken in private households is more than twice as high as the mean value of the outdoor samples (n=16).

Furthermore, there is no distinct difference between indoor samples from private households and samples from the MARS 500 complex. Thus, assumptions that the level of contaminants might be increased due to the confinement and the lack of exchange with the exterior cannot be confirmed.

Summary:
The airborne microbiome content in the MARS 500 complex was not increased compared to private two-person households with the possibility to ventilate, taking into account that the facility was inhabited by six people in a small area which continuously breathed recycled air. Furthermore, it could be seen that the percentage of microbial contaminants that are imported from the outdoor environment is less than half of the indoor contamination value, and therefore, humans can be stated as the major contamination source.

III.1.4. Comparative Measurements at Sites with Known Air Circulation Rate

Since little information is available about the air circulation system and the effective air flow rate of the MARS 500 complex, air samples were taken from the Biotechnikum at the University of Regensburg. The gas exchange rate there is set to 19.4 times of the room volume (360 m³) per hour (equals 7000 m³ of air), compared to 320 to 440 m³ that can maximally be reached in the isolation facility. Sampling was performed at four different locations (two at the bottom and two in 1.5 m height, Fig. III.1.4.1) within this room.

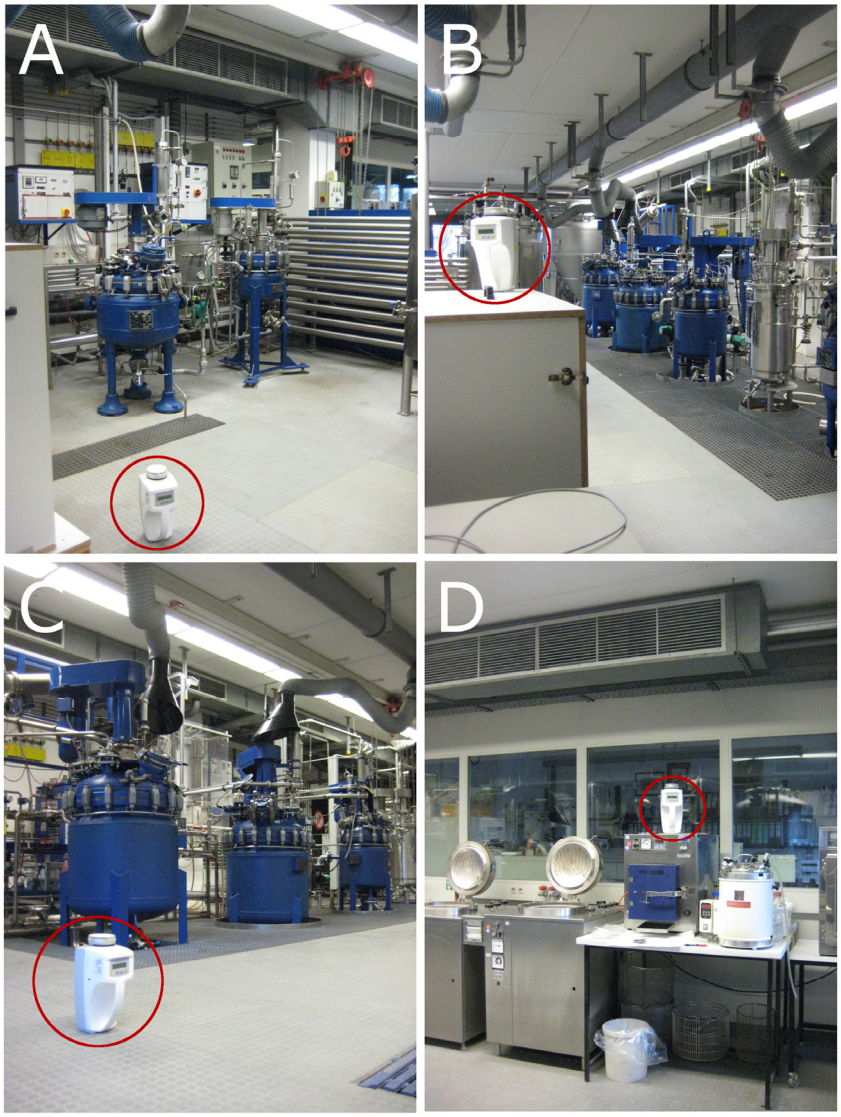

Fig. III.1.4.1 Pictures taken of the Biotechnikum at the University of Regensburg during air sampling; AirPort MD8 was situated A: on the floor close to the main entrance; B: in 1.5 m height on top of a tool cabinet close to A; C: on the floor in opposite direction of main entrance; D: in 1.5 m height close to C.

Following sampling, the samples were kept on dry ice and were transported to DLR where processing started within 4 days. The results after 72 h incubation are listed below in Table III.1.4.1.

TABLE III.1.4.1 LIST OF AIR SAMPLES AND SAMPLING AREA AT THE BIOTECHNIKUM (UNIVERSITY OF REGENSBURG) GIVING THE CORRESPONDING AIRBORNE MICROBIAL COLONY FORMING UNIT (CFU) COUNTS ON R2A AFTER 72 H INCUBATION AT 32°C ACCORDING TO 500 L SAMPLED AIR AT DIFFERENT LOCATIONS.

Sample #	Description of sampling locations (Biotechnikum, University of Regensburg)	Mean CFU values per 500 l air n=4	Standard deviation
A	On the floor close to the main entrance	13.3	8.3
B	In 1.50 m height on top of tool cabinet close to A	14.0	6.8
C	On the floor in opposite direction of main entrance	12.3	6.6
D	In 1.50 m height close to C	11.3	5.7

In general, a low mean airborne contamination of 13 CFU per 500 l air was detected, when considering an observed mean CFU value of 43 derived from all samples taken during the isolation experiment MARS 500 (section III.1.1.). Comparison with samples taken at DLR and private households revealed the same tendency (Fig. III.1.2.1; Fig. III.1.3.1).
Regarding the individual values from the Biotechnikum (Table III.1.4.1), one additional difference was observed when comparing the four sampled locations with each other. The mean CFU values did not differ as much from each other in relation to samples taken at Cologne and during the confinement.

Summary:
Controlling of air flow rate led to more stable results especially regarding different sampling sites in one room. Furthermore, measurement of samples taken at the bottom or in a height of 1.50 m did not reveal significant differences.
The more powerful air ventilation system installed at the Biotechnikum seemed more effective compared to the one mounted in the complexes of the MARS 500 facility in Russia. Higher air exchange rates should lead to lower airborne contamination.

III.1.5. MICHAm: Taxonomic Assignment of the Airborne Isolates

Growth could easily be detected due to obvious formation of colonies on agar plates. Whenever a sample was growth-positive, colonies were further processed to receive pure cultures (section II.5.1.). From each sample, a maximum of three colonies was selected, since the amount of detected colonies was far too high to process all of them due to time limitations and costs. The coverage of diversity was increased via consideration of colonies with different shapes or colors. The assortment of morphologically different colonies resulted in a non-random selection scheme. Incidentally, some of the picked colonies showed no growth after streak outs. Altogether, 302 purified airborne isolates were sent to LGC Genomics (Berlin, Germany) for sequencing. The ultimate classification was done by Second Genome, Inc. (South San Francisco, CA, USA) in order to ensure further alignment comparisons with sequence data gained from PhyloChip analysis (section II.5.3.; section III.3.6.12).
After quality checking, 275 sequences remained that were subjected to further alignment. Detailed information about classification is given in Table III.1.5.1. The representative sequences were submitted to Genbank. The received accession numbers can be found on the provided data CD (folder: Genbank Submission Data).

TABLE III.1.5.1 CLASSIFICATION OF THE AIRBORNE MICROBIAL ISOLATES FROM THE MICHAM CAMPAIGN DISPLAYING ALL TAXONOMIC TAXA FROM PHYLA TO FAMILY, TOTAL NUMBER OF COLONIES AND ISOLATES PROCESSED. GREY AREAS ON FAMILY LEVEL REFER TO GRAM-POSITIVES. THE GIVEN NUMBERS REPRESENT THE ISOLATES OBTAINED FROM ALL SAMPLES OF ONE MODULE.

		Utility module	Habitable module	Medical module
Total number of colonies		1748	5114	104
Number of sequences processed (after quality check)		137 (13 %)	110 (2 %)	26 (25 %)
Phyla	Actinobacteria	4	3	-
	Firmicutes	128	97	26
	Proteobacteria	5	10	-
Class	Actinobacteria	4	3	-
	Bacilli	128	97	26
	α-Proteobacteria	4	6	-
	γ-Proteobacteria	1	4	-
Order	Actinomycetales	4	3	1
	Bacillales	127	97	25
	Sphingomonadales	2	2	-
	Enterobacteriales	1	-	-
	Lactobacillales	1	-	-
	Rhodospirillales	2	-	-
	Pseudomonadales	-	3	-
	Rhizobiales	-	4	-
	Xanthomonadales	-	1	-
Family	*Staphylococcaceae*	122	97	26
	Sphingomonadaceae	2	2	-
	Nocardiaceae	-	2	-
	Acetobacteraceae	2	-	-
	Bacillaceae	5	-	-
	Brevibacteriaceae	2	-	-
	Enterobacteriaceae	1	-	-
	Enterococcaceae	1	-	-
	Micrococcaceae	2	-	-
	Corynebacteriaceae	-	1	-
	Moraxellaceae	-	2	-
	Methylobacteriaceae	-	4	-
	Pseudomonadaceae	-	1	-
	Xanthomonadaceae	-	1	-

In total, three different phyla from 274 airborne isolates were detected that encompasses four classes, nine orders, 14 families and only 15 genera (Table III.1.5.1; for genera Fig. III. 1.5.2). However, approximately 20 % of all DNA sequences could not be specified on the species level and were denoted as “unclassified” microbes. Approximately 90 to 95 % of the isolates from the habitable and utility module were representatives of the gram-positive phyla while gram-negative phyla accounted for 10 to 5 % (Fig. III.1.5.1).

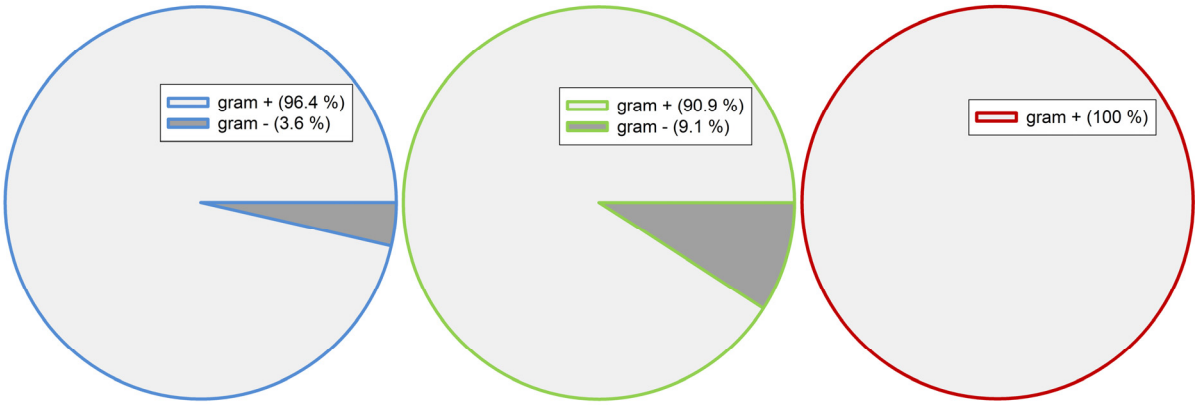

Fig. III.1.5.1 Percentage distribution of the airborne isolates concerning their gram affiliation originating either from the utility module (EU-250=blue), the habitable module (EU-150=green), and the medical module (EU-100=red). Light grey=gram-positives, dark grey=gram-negatives

The majority of the cultivated bacteria (91 % of the isolates) belonged to the Firmicutes phylum. The organisms of this phylum were affiliated with three bacterial families: *Staphylococcaceae* (53 %), *Bacillaceae* (2 %), and *Enterococcaceae* (0.4 %). The second identified phylum Proteobacteria (only α- and γ-Proteobacteria), constituted 6 % of the cultivable species.
The collected specimens belonged to the families *Methylobacteriaceae* (1.6 %), *Sphingomonadaceae* (1.6 %), *Acetobacteraceae* (0.8 %), *Moraxellaceae* (0.8 %), *Enterobacteriaceae* (0.4 %), *Pseudomonadaceae* (0.4 %), and *Xanthomonadaceae* (0.4 %). The third discovered phylum, the Actinobacteria, represented the final 3 % of the cultivables and is comprised of *Nocardiaceae* (1.2 %), *Brevibacteriaceae* (0.8 %), *Micrococcaceae* (0.8 %), and *Corynebacteriaceae* (0.4 %).

On the genus level, as depicted in Fig. III.1.5.2, only staphylococci were detected in all three modules. Staphylococci accounted for the majority (95.5 %) of all processed sequences in the habitable module, whereas the modules that contained a lot of equipment and consisted of areas for storage revealed a lower content (66 % and 62 % for utility and medical module, respectively). Additionally, it can be stated that there is almost no interference regarding the microbial inhabitants residing in each module. Each module revealed its own characteristic airborne community structure (Fig. III.1.5.2), consisting of different genera with only two exceptions besides *Staphylococcus*: *Sphingomonas* was detected in modules EU-150 (habitable) and EU-250 (utility); and *Rhodococcus* was detected in the habitable and the medical module.

Microorganisms that are able to multiply in a strictly aerobic environment or are facultatively anaerobes could be enriched during the campaign. Gene sequences that were assigned to taxonomic groups which can thrive aerobically and under anaerobic conditions were *Staphylococcus*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Kocuria*, *Roseomonas*, and *Bacillus* (the only genera obtained with the ability to form spores). *Enhydrobacter*, *Methylobacterium*, *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas*, *Brevibacterium*, and *Micrococcus* can only survive under strict aerobic conditions. Strictly anaerobic organisms were not isolated due to the experimental setup that selected only for facultatively and strictly aerobic microorganisms.

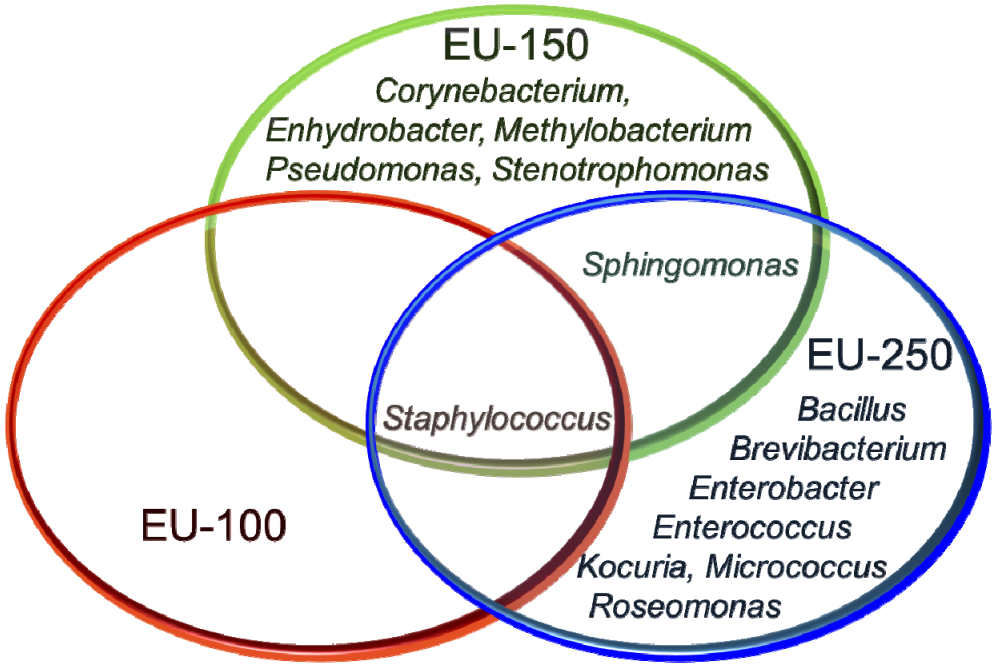

Fig. III.1.5.2 Cultivable airborne bacterial genera distribution analysis. The figure highlights the distribution pattern of bacterial genera within the MARS 500 isolation facility. The genera were grouped into three modules; habitable (EU-150=green), utility (EU-250=blue), and medical (EU-100=red).

Out of 273 sequences, 21 distinct bacterial strains were identified on the species level, while 71 sequences remained unclassified (Fig. III.1.5.3). In all, the medical module revealed the lowest microbial airborne diversity, whereas the microbial communities of the habitable and the utility modules were more manifold. Only members of the human-associated genus *Staphylococcus (S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, Fig. III.1.5.3) were present in all three modules.

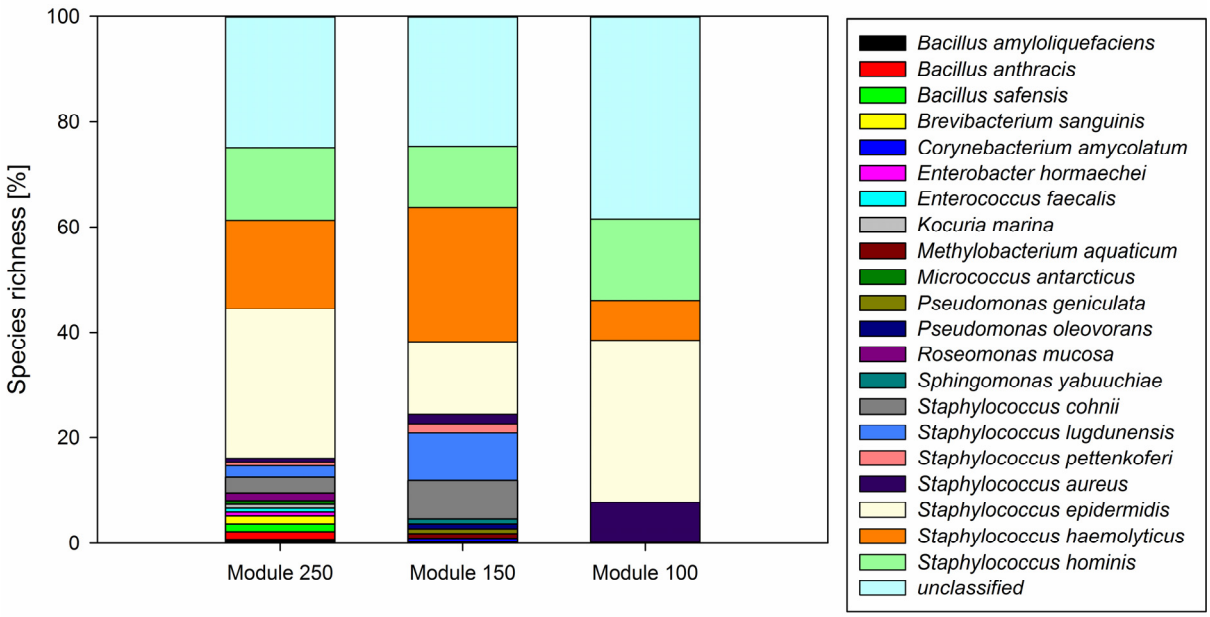

Fig. III.1.5.3 Cultivable airborne microbial diversity from air samples of the MARS 500 facility as detected via 16S rRNA gene sequence analysis. The distribution by percentage of the detected microbial diversity at the three modules (EU-250=utility, EU-150=habitable, EU-100=medical) is shown on species level.

Nevertheless, it has to be noted that all gene signatures except staphylococci were obtained only once or twice per module, and are therefore not prevalent at all sampled sites.

Summary:
The three modules differed in their microbial community structure besides the appearance of staphylococci. Similarities were detected regarding the high percentage of gram-positives. However, approximately 30 % of the obtained 16S rRNA gene signatures remained unclassified at least on the species level.

III.1.6. MICHAm: Cultivable Contamination of Surfaces

While investigating the airborne population being present in the MARS 500 facility, the natural colonization of surfaces and changes over time was also monitored during the confinement. Samples from areas of 25 cm² were taken at eleven selected surfaces monthly (Table III.1.6.1; Fig. III.1.6.1ff) by Charles Romain (marsonaut) in order to assess the biocontamination under confined conditions. At each sampling site, two parallel samples were taken (section II.4.). The dry swab was stored for further molecular analysis (section II.8.1.3; section III.3.6.1.) and the second swab, stored in buffer, was subjected to two different cultivation approaches. The samples were returned in four batches (after 6 months, 13 months, 18 months and 6 months post-confinement) to DLR.

TABLE III.1.6.1 LIST OF SURFACE SAMPLES AND CORRESPONDING SAMPLING AREAS AT THE MARS 500 FACILITY INDICATING PROPERTIES OF THE SWABBED AREA (EU-250=UTILITY MODULE, EU-150=HABITABLE MODULE, AND EU-100=MEDICAL MODULE)

Module	Sample #	Description	Surface character
250	2	Wall above the vanity basin	Stainless steel
	3	Wall under the faucet close to the corner in the per capita shower cabin	Stainless steel
	9	Left hand side of the inside of the greenhouse	Stainless steel
	11	Rack surface from the storage area of clothes	Stainless steel
150	1	External surface of the toilet bowl	Stainless steel
	4	Wall in the corner in the community cabin	Wood
	5	Desktop surface close to the keyboard on the left hand side in the main panel	Wood
	6	Surface of the dining table in the kitchen	Wood
	10	Table surface in individual compartment	Wood
100	7	Table surface close to the insulator zone	Wood
	8	Table surface around the water plum	Wood

The exact positions of the different sample sites are depicted in Fig. III.1.6.1 for module EU-250, in Fig. III.1.6.2. for module EU-150, and in Fig. III.1.6.3 for module EU-100.

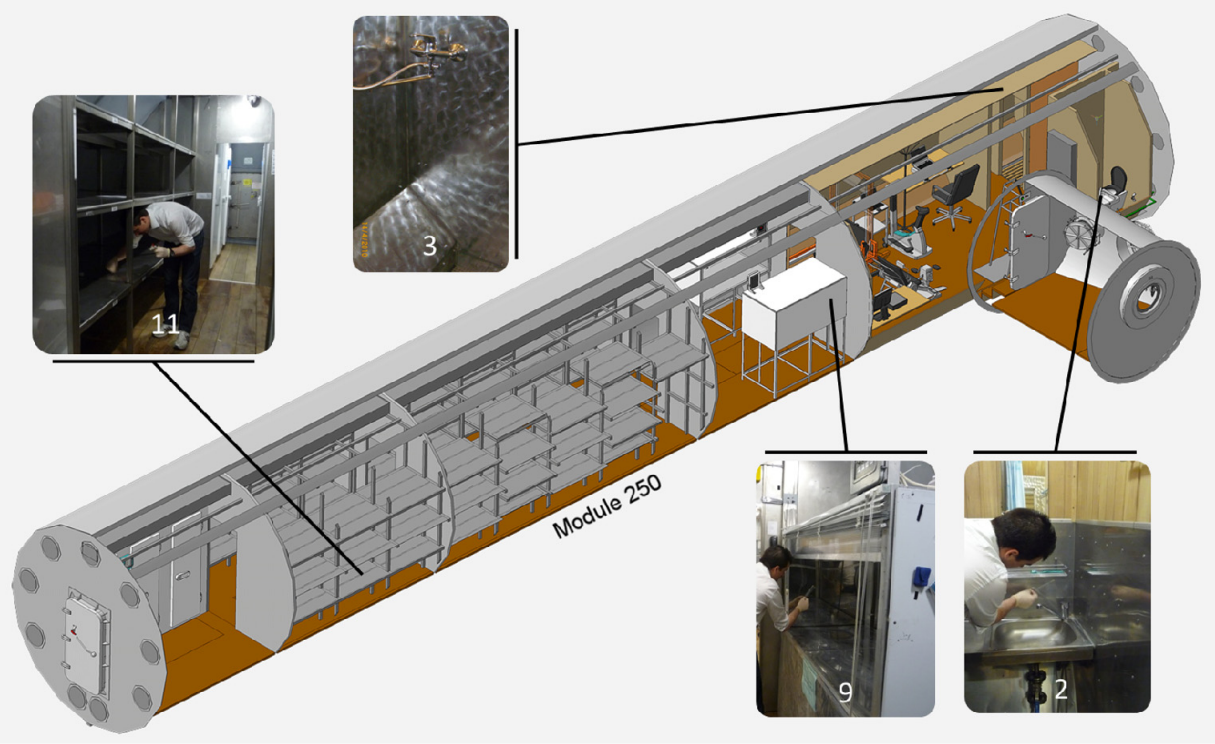

Fig. III.1.6.1 Schematic drawing of the utility module (EU-250). Photographs of each sampling site are allocated to the specific location. © SSC RF IBMP RAS, Oleg Voloshin (edited)

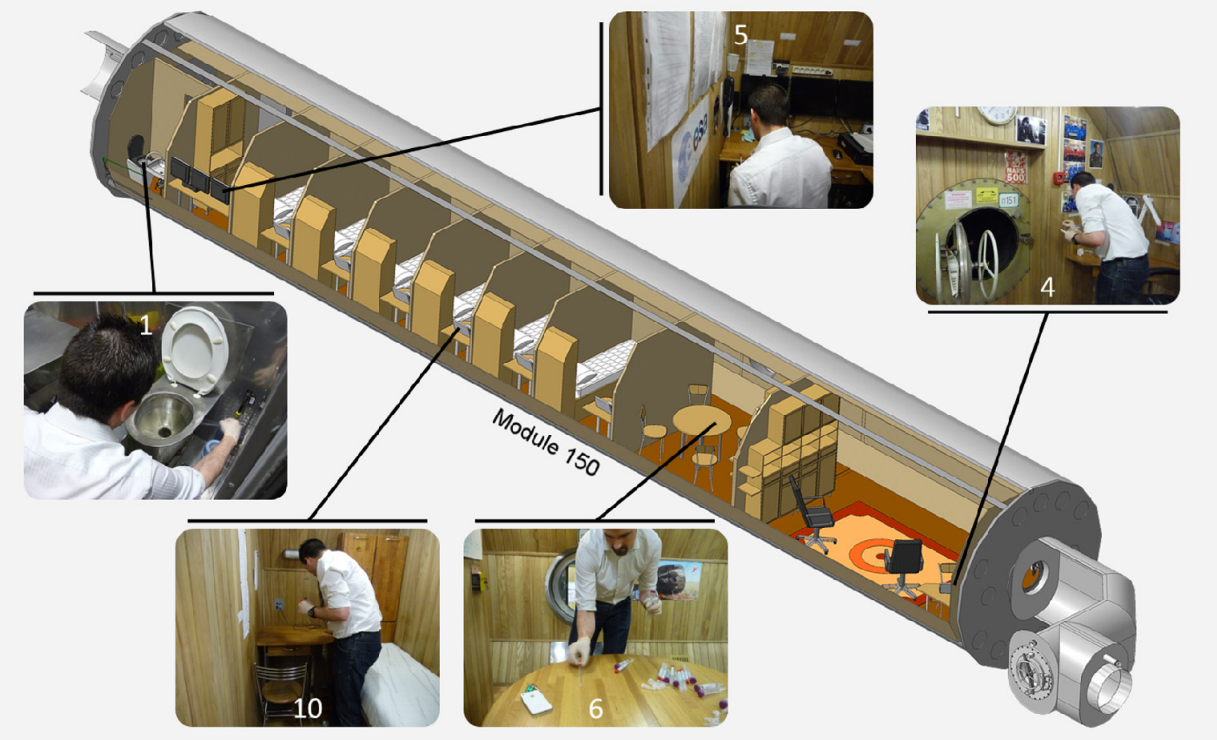

Fig. III.1.6.2 Schematic drawing of the habitable module (EU-150). Photographs of each sampling site are allocated to the specific location. © SSC RF IBMP RAS, Oleg Voloshin (edited)

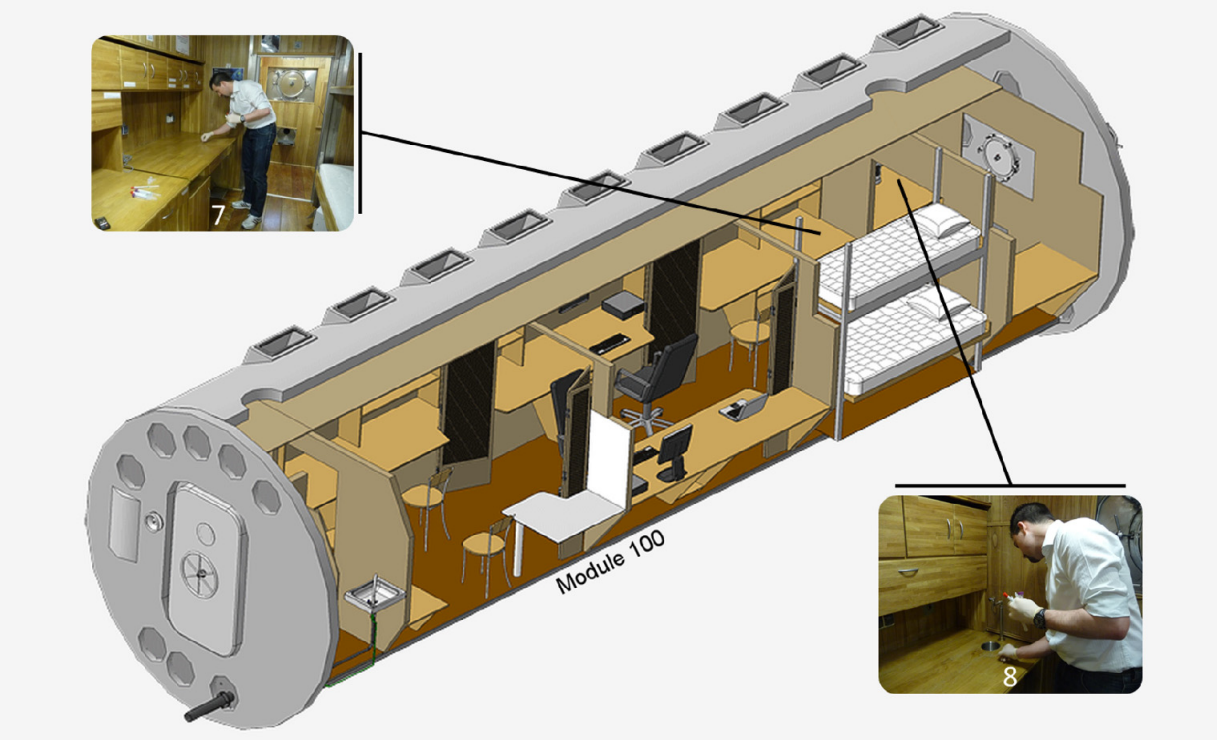

Fig. III.1.6.3 Schematic drawing of the medical module (EU-100). Photographs of each sampling site are allocated to the specific location. © SSC RF IBMP RAS, Oleg Voloshin (edited)

After gentle thawing and vortexing of the Falcon tubes (containing the swabs and 2.5 ml PBS), one aliquot (1.0 ml) of each surface sample was subjected to cultivation to determine mesophilic bacteria (“vegetatives”) on two R2A plates (500 µl each), whereas the other half

(1.5 ml) was heat-shocked prior to spreading on two R2A plates (500 µl each) to obtain spore-forming and/or heat-resistant strains (“bioburden”).
The observation of the plates from the first batch after 24 h of incubation at 32°C already showed indications of swarming microorganisms that might lead to overgrowth within 72 h. In order to minimize bias due to overgrowth and to facilitate colony counting of samples with high “bioburden”, 50 µl per sample and treatment were additionally spread on a separate R2A plate in parallel. This supplementary step was included for the analysis of the second and third batch. Counts of these plates were treated as back up plates and colony counts were performed in the case of swarming microbes or when too numerous amounts of colonies were observed. Furthermore, it has to be noted that during the analysis of the second batch (included samples from December of 2010 until June of 2011), contamination occurred (Fig. III.1.6.4). The contaminant could easily be distinguished from strains that originated from the MARS 500 isolation facility due to the typical colony morphology: The color of the colonies was orange red and a brighter, more yellowish outer ring could occur.

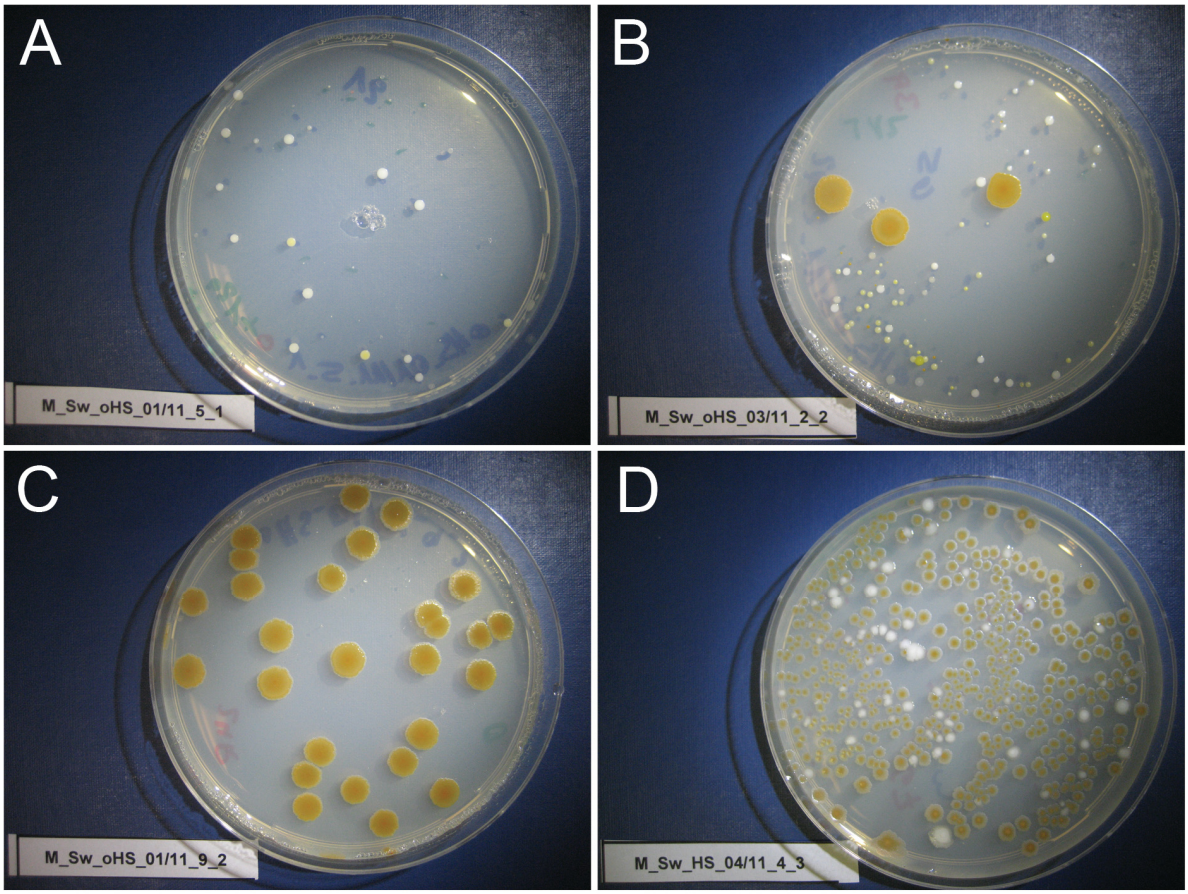

Fig. III.1.6.4 Pictures taken of agar plates from the second batch of surface samples after 72 h incubation at 32°C. Plates indicate different levels of contamination (orange colonies). Samples can be assigned to the sampling location and date by the identifier code (**Fig. II.5.1.1**). A: no contamination; B: less than ten contaminants; C: less than fifty contaminating colonies, D: too many contaminants to count

Due to this characteristic shape, the contaminant was supposed to be *B. atrophaeus*, an obligate aerobic, gram-positive bacterium that has the property to form spores and hence also the ability to survive HS treatment. This presumption was confirmed by purification of

the contaminant and subsequent sequencing. Not all plates were affected (Fig. III.1.6.4A) by the contamination, but this fraction was low compared to the ones that were hit. Both, samples that were not treated with HS as well as the ones that were heated to 80°C for 15 minutes were contaminated. Interestingly, the number of contaminants per plate and per sample revealed great variations. Fig. III.1.6.4B to D displays representative plates for samples with low contamination (<10 CFU), medium-level contamination (10<x<100, still countable), and high-level contamination (uncountable), respectively. However, it was nearly impossible to draw inferences about the origin of the contamination source from the pattern of plates that were affected. Furthermore, contamination was also observed on plates of blank samples. Interestingly, it transpired that one plate showed contamination whereas the parallel plate of the same sample was not impacted. Therefore, it can be excluded that the contamination is derived from the MARS 500 habitat and/or through the sampling procedure that was performed by Charles Romain. This statement was confirmed since this sort of colony was not observed on plates from air samples taken in parallel at the same modules. Furthermore, contamination of the agar plates due to unsterile preparation can also be excluded for the very same reason.

On this account, a set of witness plates, i.e., uncovered agar plates, were put down in the laminar flow, on the laboratory benches and in the incubator. Following incubation, it turned out that on two out of nine plates one to three colonies were detected. However, this observation cannot be the explanation for the origin of the contaminating specimen (due to the low occurrence rate, which was not consistent with the amount of colonies observed on the plates). Therefore, the contamination source remained unclear. Fortunately, all plates (no HS) were analyzable. The distinction, whether the observed colony is a contaminant or not, was harder to make for the heat-shocked samples (swarming occurred and the brighter rings were bigger) but colony counting was performed in all conscience.

Analysis of the third and fourth (reference sampling) batch was performed in the same manner as the two previous ones. Here no contamination occurred.

III.1.6.1. Swab Assay: “Vegetatives”

In total, approximately 1.3 x 10^5 colonies were counted of the aliquot without special treatment (no HS) from 198 surface samples (representing an area of 1,980 cm²). Cell numbers from 0 to 29,760 per 10 cm² (of 25 cm² sampled surface area) were observed generating a mean value for all samples of 675 CFU per analyzed sample.

In all, the number of cultivable “vegetatives” per sampling site underlied fluctuations over the whole timeframe during the confinement. An example for one sampling site is given below (Fig. III.1.6.1.1). The remaining plots can be viewed on the enclosed data CD (folder: Swab Assay Vegetatives).

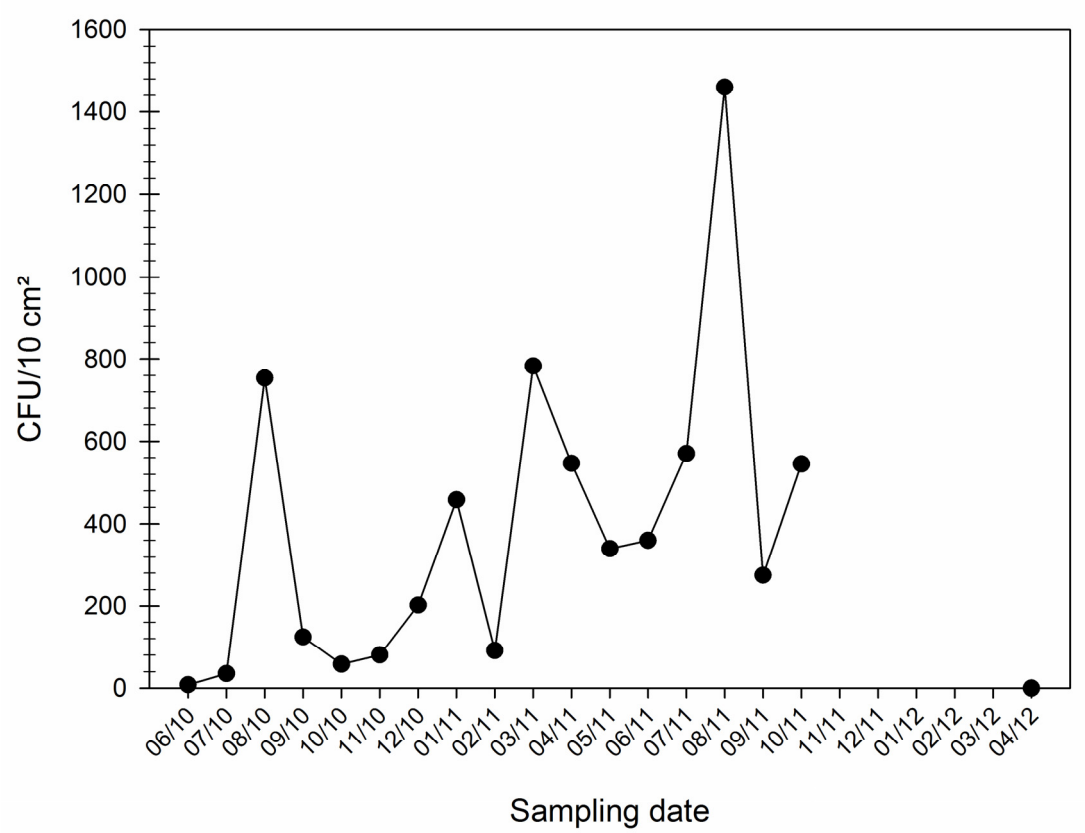

Fig. III.1.6.1.1 Microbial "vegetative" inventory of one surface of over time: colony forming unit (CFU) counts per 10 cm² sampled surface on R2A after 72 h incubation at 32°C of one sampling site (location 6; dining table) from the corresponding module EU-150 (habitable) of the MARS 500 facility.

Comparisons of the mean values of all samples taken over the whole timeframe per location revealed the highest values from samples (1 [toilet], 5 [desktop], 10 [individual compartment]) originating in the habitable module (Fig. III.1.6.1.2). Mean CFU values (n=18) from the habitable module ranged between 36 and 4,472 per 10 cm², whereas the utility module revealed CFU counts of 5, 30, 162, and 380. As already seen from the air samples, the medical module also revealed the lowest amount of microorganisms being present on surfaces (mean CFU values per 10 cm²: 14 and 103). However, the plot clearly depicts differences in abundance of microorganisms of samples taken in the same module. At least one sample per module uncovered extremely low colony counts. For example the accumulation in the habitable module was up to 100 times higher in sample 1 (toilet) compared to sample 4 (table in community room).

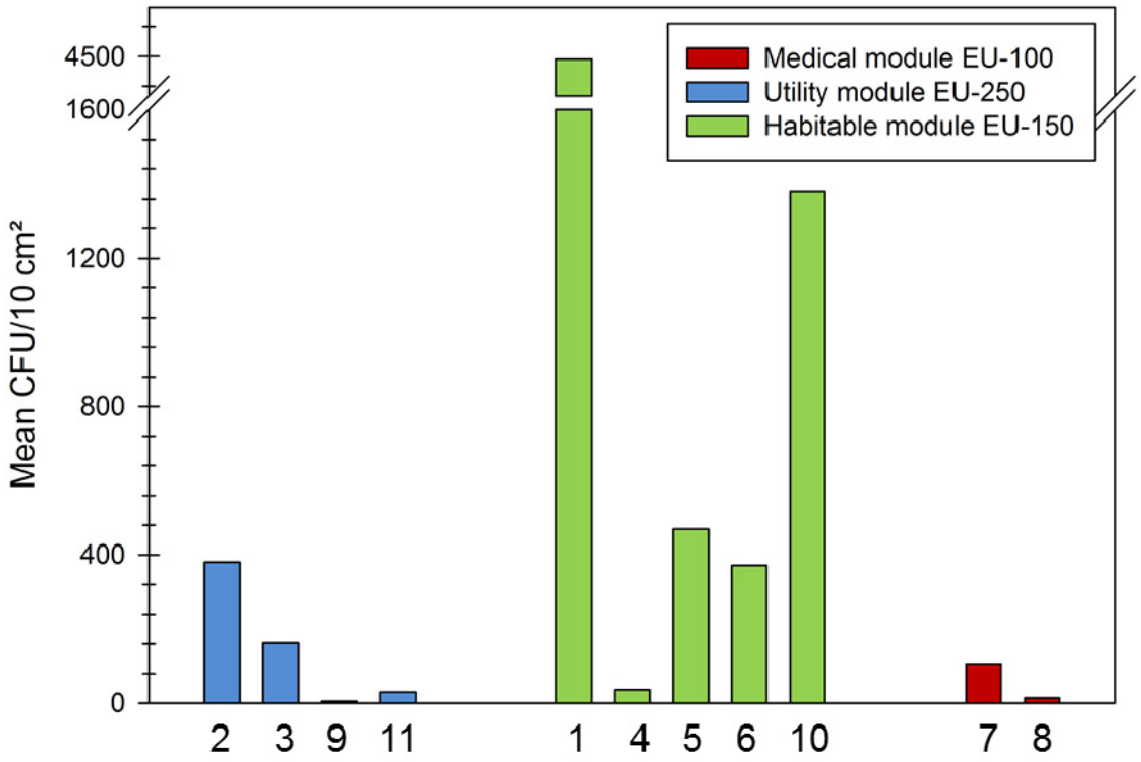

Fig. III.1.6.1.2 Mean microbial "vegetative" inventory of surfaces per sample location: colony forming unit (CFU) counts per 10 cm² surface area on R2A after 72 h incubation at 32°C grouped by sampling sites from the corresponding module (utility, habitable or medical) of the MARS 500 facility. Data are expressed as mean (n=18) values calculated by including data from every sampling date beginning in June of 2010 and ending in April of 2012 (520 days + 6 months). For reasons of clarity and due to the high variations, standard deviations are not displayed (values are listed in **Table III.1.6.2.1**).

In order to compare the mean CFU values obtained per module and to observe the changes of the microbial contamination over time, the following plot (Fig. III.1.6.1.3) was generated. The curve of the habitable module (green) displays the mean values of the five sampling locations 1, 4, 5, 6, and 10, that of the utility module (blue) was calculated from the values obtained from the four sampling locations 2, 3, 9, and 11, and that of the medical module (red) was comprised of CFU counts from samples 7 and 8. In general, samples from the habitable module revealed estimated bacterial cell numbers from 0 to 10,880 per 10 cm², whereas CFU counts from the utility module ranged from 0 to 1,880 and counts for medical module were even lower (0 to 315 per 10 cm²).

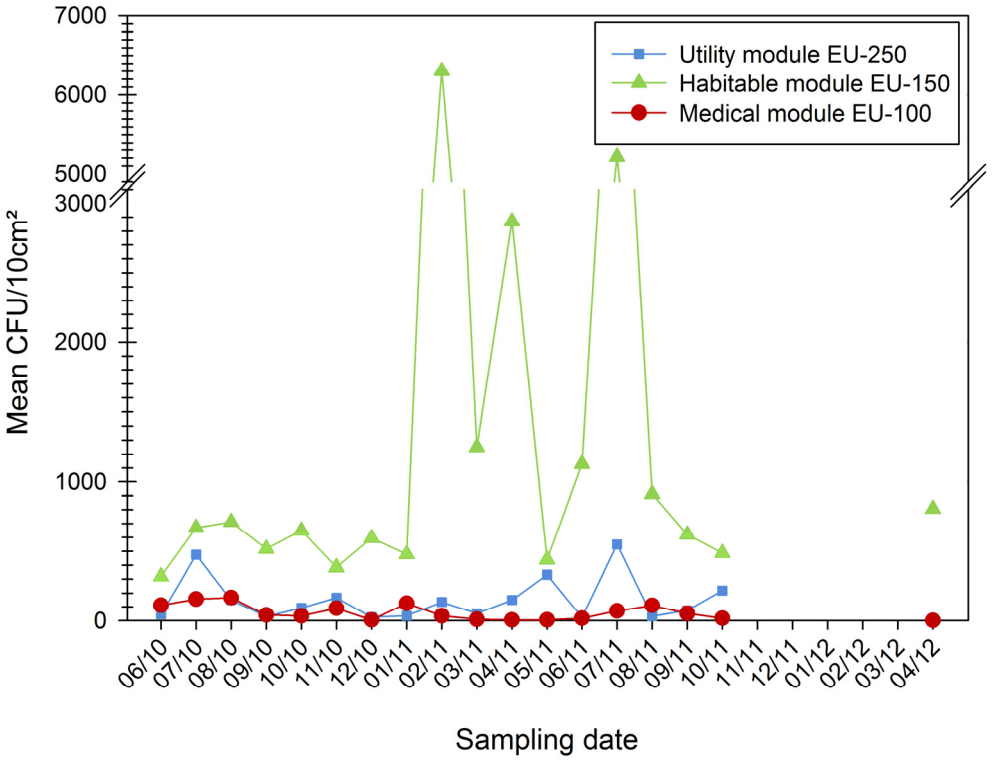

Fig. III.1.6.1.3 Mean microbial "vegetative" inventory of surfaces per modules over time: colony forming unit (CFU) counts per 10 cm² surface area on R2A after 72 h incubation at 32°C of each module (utility, habitable, or medical) of the MARS 500 facility over time. Data are expressed as mean (n=4 for utility module; n=5 for habitable module; n=2 for medical module) values calculated by including data from every sampling site of the corresponding module. For reasons of clarity and due to the high variations, standard deviations are not displayed. An example is shown in **Fig III.1.6.1.4**.

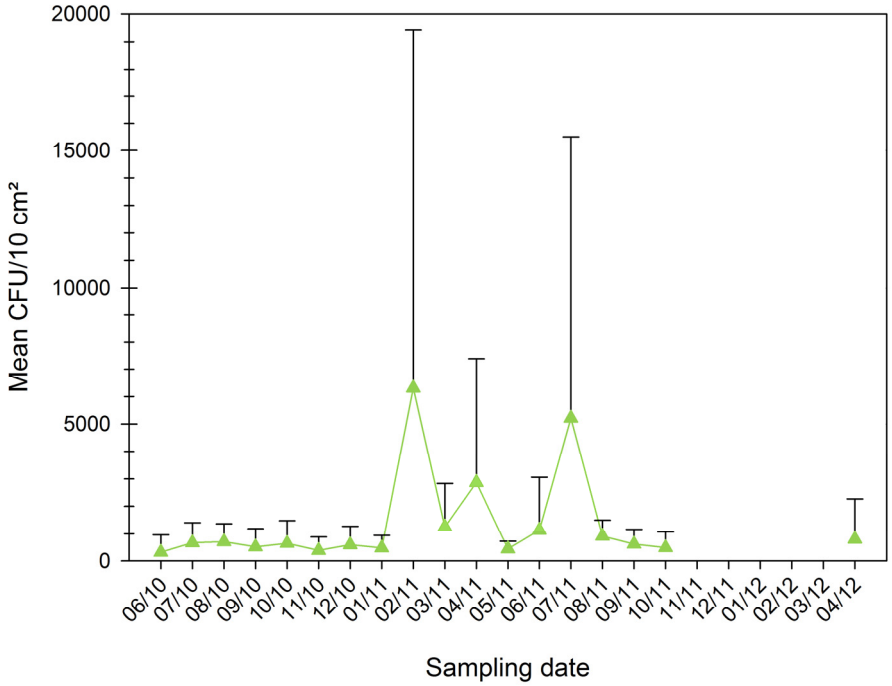

Fig. III.1.6.1.4 Mean microbial "vegetative" inventory of one surface over time: colony forming unit (CFU) counts per 10 cm^2 surface on R2A after 72 h incubation at 32°C of the habitable module over time within the MARS 500 facility. Data are expressed as mean (n=5) values with standard deviation calculated by including data from every sampling site of the corresponding module.

In general, fluctuations over the whole timeframe were observed for all three modules, but three major peaks were only obtained in module EU-150 in February of 2011 (6,305 mean CFU per 10 m²), April of 2011 (2,871 mean CFU per 10 m²), and in July of 2011 (5,215 mean CFU per 10 m²). A slight enhancement of contamination was observed in the utility module in July of 2010 (475 mean CFU per 10 m²), May of 2011 (331 mean CFU per 10 m²), and also in July of 2011 (549 mean CFU per 10 m²). The values obtained from the medical module revealed only slight variations from 1 to 167 mean colony counts per 10 m². The habitable module showed a 23- and 9-fold mean increase compared to the medical and the habitable module and thus indicating the highest microbial contamination per surface area. This trend was detectable for the whole period of time.
In the course of the work, an explanation for the occurrence of the observed peaks in module EU-150 in the latter period of confinement, starting in February of 2011, was found. It turned out that the different surface materials and the procedure for their purification played a crucial role. Four out of five sampled surfaces from module EU-150 were wooden, while all surfaces from the utility module were made of steel. The following answer from the questionnaire was received regarding the question which cleaning materials and chemicals they used. “Initially, during 5 or 6 months we used a solution of Katamin AB and water. After this period we discontinued its use for everything, and used it only for metallic surfaces (i.e., bathrooms). After February (approx.), we ran out of it, and used dish washing liquid for everything.” (pers. comment: marsonauts; data CD [folder: Questionnaire]). Katamin AB is a transparent, slightly yellowish liquid made of approximately 50 % (w/w) alkyl dimethyl benzyl ammonium, 1.7 % (w/w) tertiary amine and amine hydrochloride and 0.5 % (w/w) tertiary amine and a solution of 1 % (v/v) was used (pers. comment: Svetlana Poddubko). The pH is 6 to 7.

Summary:
In general, the contamination levels varied strongly over sample locations, modules, and time. The values did not exceed 29,760 CFU per 10 cm². An overall mean value (n=198) of 675 CFU per 10 cm² was detected. The highest microbial load was monitored in the habitable module, whereas the lowest was observed in the medical module. These results reflected the same trend as in case of the airborne community.

III.1.6.2. Swab Assay: “Bioburden”

Simultaneously to the determination of the mesophilic microorganisms, which was performed to obtain an overall insight, the proportion of “bioburden” thereof was of interest. In the field of planetary protection and space science, “bioburden” is defined as the number of viable spores and heat-tolerant bacteria being present on a distinct pre- and post-sterilized surface area. This definition should not be confused with the general definition referring to the number of bacterial cells inhabiting a surface. The herein mentioned swab assay is usually performed for determining the bioburden of spacecraft relevant for planetary protection as defined by the COSPAR planetary protection guidelines and in the ECSS-Q-ST-70-55C standard (2008). The amount of viable spores was received by

subjecting the samples to heat (80°C) for 15 min prior to plating. HS killed the vast majority of vegetative cells so that only spores and/or heat-resistant vegetative cells remained. To outline the effect of the HS treatment, the results (mean CFU counts per sampling location over the whole timeframe) of the untreated samples were graphed comparatively (Fig. III.1.6.2.1).

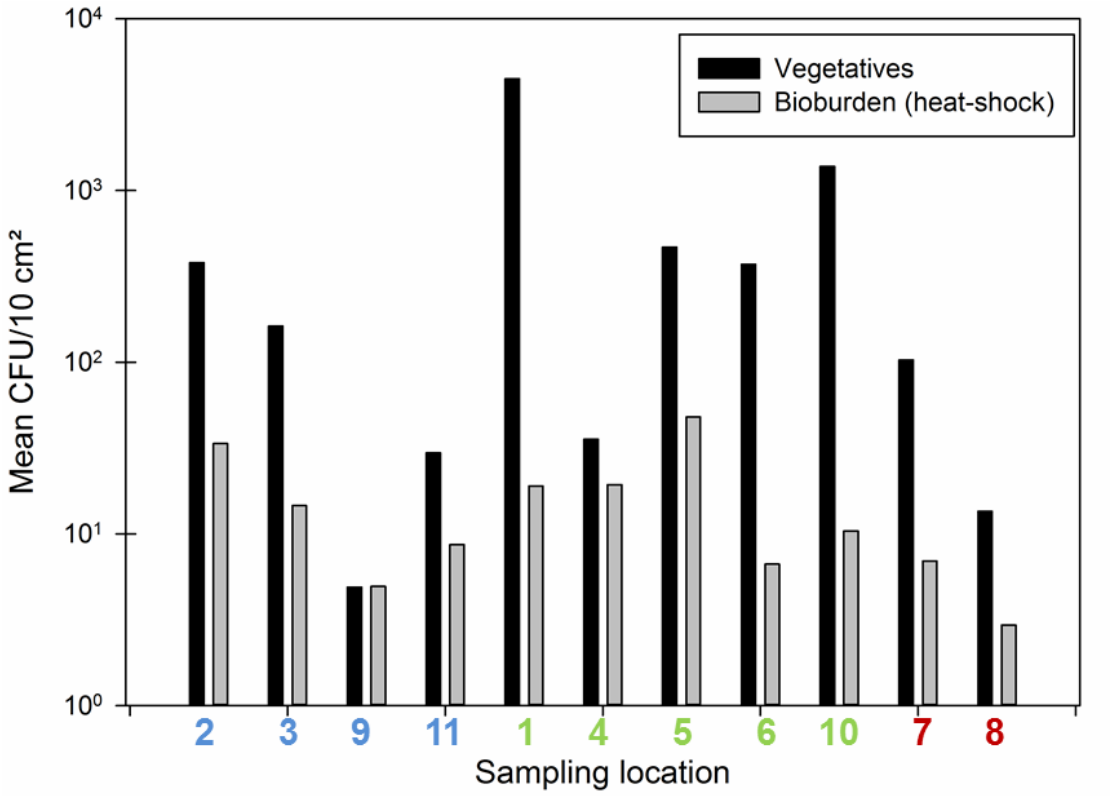

Fig. III.1.6.2.1 Comparison between microbial "vegetative" inventory (black columns) and "bioburden" (grey columns): colony forming unit (CFU) counts per 10 cm² surface area on R2A after 72 h incubation at 32°C grouped by sampling sites from the corresponding module (utility=blue; habitable=green; medical=red) of the MARS 500 facility. Data are expressed as mean (n=18) values calculated by including data from every sampling date beginning in June of 2010 and ending in April of 2012 (520 days + 6 months). For reasons of clarity and due to the high variations, standard deviations are not displayed.

For all sample locations a reduction of culturable microorganisms was observed after HS treatment. An overall mean reduction of 85 % was measured. However, the measured effect of reduction varied per location. Almost no reduction was detected for sample location 9 (greenhouse). Sample location 4 (table in community room, Fig. III.1.6.2.2) revealed a "bioburden" proportion with respect to planetary protection of more than 50 %. Less than 2 % of the overall microbial load survived HS treatment applied to samples 1 (toilet), 6 (dining table), and 10 (table in individual compartment). The remaining samples revealed a reduction between 70 and 90 %.

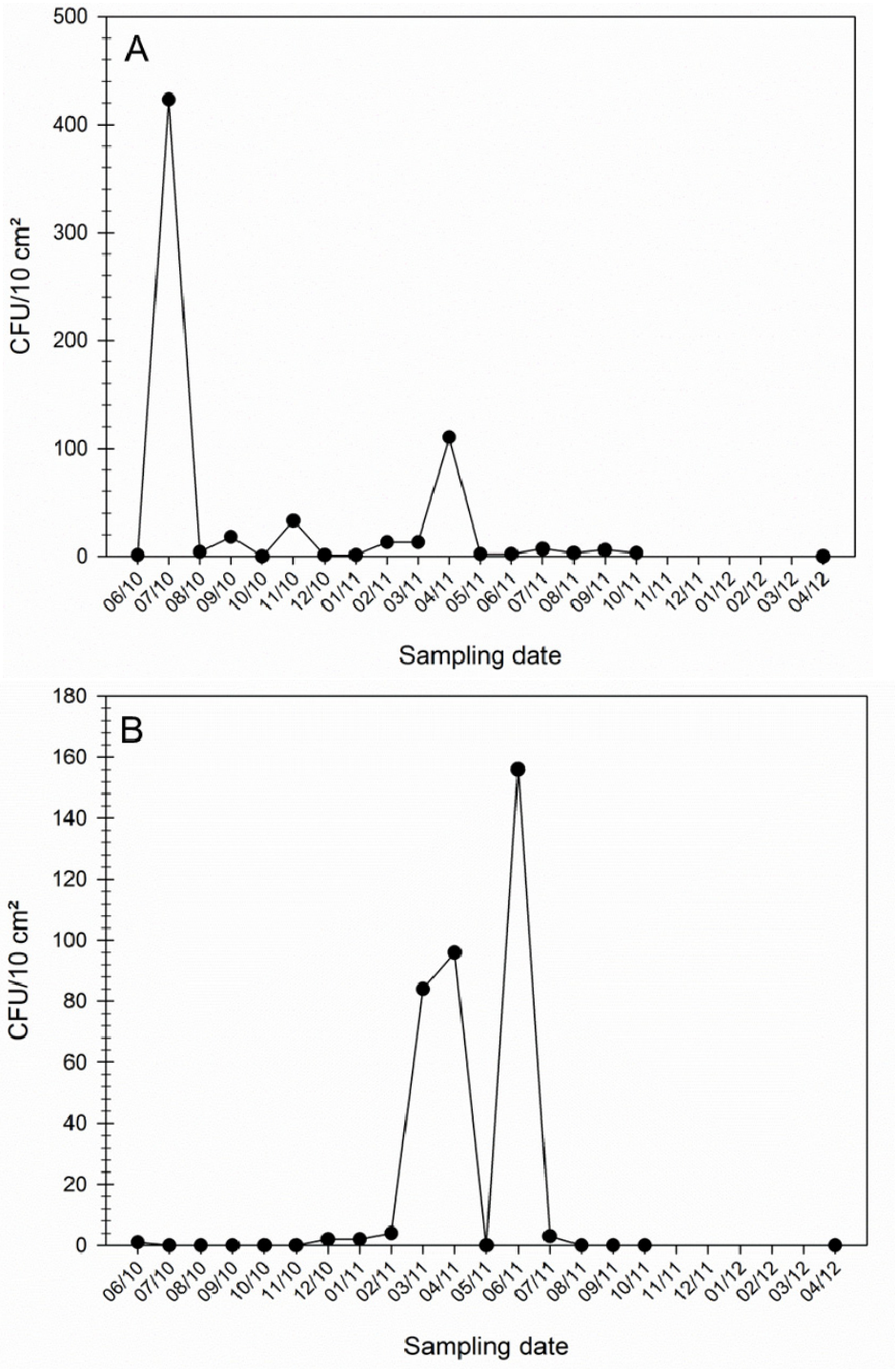

Fig. III.1.6.2.2 Microbial inventory of one surface over time: colony forming unit (CFU) counts per 10 cm² sampled surface on R2A after 72 h incubation at 32°C of one sampling site (location 4; table in community room) from the corresponding module EU-150 (habitable) of the MARS 500 facility. A: swab assay “vegetatives”; B: swab assay “bioburden”

Fig. III.1.6.2.2. exemplarily displays differences between the vegetative inventory and the corresponding bioburden cell counts of the table in the community room. More detailed results of the colony counts of all samples plated directly (“vegetatives”) and the corresponding percentage of obtained “bioburden” load are grouped per module (EU-250, EU-150, and EU-100) and listed in Table III.1.6.2.1.

Table III.1.6.2.1 Total microbial colony counts of "vegetative" microbial inventory and the corresponding percentage of the heat-shock resistant (80°C, 15 min) microbes (="bioburden") on R2A after 72 h incubation at 32°C according to 10 cm² sampled surface in different locations in Module 250 (utility), 150 (habitable) and 100 (medical)

Module	Utility module - Locations 2, 3, 9, 11 CFU/10m²								Habitable module - Locations 1, 4, 5, 6, 10 CFU/10m²										Medical module - Locations 7,8 CFU/10m²			
Sampling date	2		3		9		11		1		4		5		6		10		7		8	
	no HS	% HS	no HS	% HS	no HS	% HS	no HS	% HS	no HS	% HS	no HS	% HS	no HS	% HS	no HS	% HS	no HS	% HS	no HS	% HS	no HS	% HS
June 10	39	5	39	118	0	300	99	2	1,464	1	1	100	81	2	8	13	39	0	195	1	26	4
July 10	1,880	2	13	8	0	0	6	0	1,796	8	423	0	105	0	35	9	992	1	302	1	12	67
Aug 10	172	0	415	0	1	2,400	11	0	976	1	4	0	191	1	753	0	1,632	1	315	1	18	6
Sep 10	44	0	31	0	0	0	45	2	1,512	1	18	0	36	0	124	1	896	1	83	1	0	0
Oct 10	287	0	55	2	10	0	16	0	1,464	1	0	0	82	0	58	0	1,640	1	66	6	1	100
Nov 10	580	0	21	0	0	0	63	11	1,152	1	33	0	639	0	80	0	21	0	178	1	1	100
Dec 10	25	8	50	8	1	100	26	0	1,728	1	1	200	404	7	202	1	640	1	9	0	0	0
Jan 11	4	0	129	1	0	1,900	5	1,120	768	1	1	200	60	3	458	2	1104	1	247	1	4	125
Feb 11	300	1	151	1	3	100	86	7	29,760	1	13	30	1,127	1	91	2	536	1	63	8	7	157
March 11	158	0	39	226	1	3,000	13	439	3,928	2	13	646	185	437	783	3	1,346	1	16	13	2	100
Apr 11	84	19	499	21	1	300	14	14	1,088	1	110	87	1,732	1	546	14	10,880	1	7	29	2	250
May 11	660	81	663	1	0	100	1	1,300	656	2	2	0	500	1	340	1	705	20	8	0	4	275
June 11	9	144	60	20	2	100	29	21	4560	0	2	7,800	620	0	360	1	104	6	6	1,683	27	19
July 11	1,752	0	392	0	20	5	33	6	23,600	0	7	44	546	1	570	0	1,353	1	121	1	21	10
Aug 11	58	0	25	0	2	0	41	2	951	1	3	0	1,362	1	1,460	0	788	0	218	0	2	0
Sep 11	228	1	39	0	17	6	12	17	1,433	0	6	0	695	0	275	0	693	0	1	0	103	0
Oct 11	534	1	302	1	27	0	10	10	360	1	3	0	71	1	545	0	1,453	1	22	0	11	0
Apr 12	30	20	0	0	3	0	23	0	3,292	2	0	0	0	100	0	0	40	20	0	0	2	0
Mean value	380		162		5		30		4,472		36		469		372		1,381		103		14	

Summary:
The culturable amount of microbial contaminants was reduced after HS treatment. “Bioburden” measurements demonstrated a generally low, but very heterogeneous distribution of microorganisms on surfaces within the confined manned habitat. Furthermore, there was no correlation between the amount of “vegetative” CFU and the corresponding “bioburden” proportion.

III.1.7. DLR: Microbial Surface Contamination

A diverse set of surface samples were taken at the DLR, Germany, and analyzed according to the ESA standard procedure. Both, “vegetative” and “bioburden” enumerations were performed.
This procedure was applied to determine whether the obtained results regarding the amount of microbial inhabitants from the MARS 500 were enhanced compared to samples taken at DLR. These places represented locations with public access housing a microflora humans are confronted with every day. Additionally, samples were taken from laboratories (Table III.1.7.1).

TABLE III.1.7.1 LIST OF SURFACE SAMPLE LOCATIONS AT DLR GIVING THE CORRESPONDING TOTAL MICROBIAL COLONY FORMING UNIT (CFU) COUNTS OF THE “VEGETATIVE” INVENTORY AND THE TOTAL AMOUNT OF DETECTED HEAT-SHOCK (HS; 80°C, 15 MIN) RESISTANT MICROBES (“BIOBURDEN”) ON R2A AFTER 72 H INCUBATION AT 32°C ACCORDING TO 10 CM² SAMPLED SURFACE

Sample location		Swab no HS CFU/10 cm²	Swab HS CFU/10cm²	% reduction after HS
2	Restroom men I (ceramic)	552	1	100
3	Restroom men II (ceramic)	0	0	-
4	Restroom men III (ceramic)	0	0	100
5	Sink (inside) I (ceramic)	75	69	8
8	Sink (inside) II (ceramic)	35	18	49
18	Kitchen sink (metal)	10	6	40
28	Restroom women I (ceramic)	38	0	100
21	Restroom women II (ceramic)	301	387	-29
1	Lab bench I	4	2	50
9	Lab bench II	1	0	100
13	Lab bench III	1	1	0
6	Desk I	11	2	81
11	Desk II	0	0	100
17	Desk III	108	3	97
22	Desk IV	55	1	99
23	Dining table	147	0	100
19	Kitchen counter	128	3	98
7	Table in lab (metal)	4	0	100
20	Surface of incubator (metal)	47	26	45
26	Hand rail (metal)	12	1	92
30	Door (metal)	1	0	100

Sample location		Swab no HS CFU/10 cm²	Swab HS CFU/10cm²	% reduction after HS
29	Work bench (wood)	4	1	75
25	Table surface I (wood)	3	1	67
16	Table surface II (wood)	1018	34	97
12	Rack surface (wood)	54	13	76
14	Door (glass)	0	18	-80
27	Clipboard surface (glass)	10	4	60
10	Window sill I	180	135	25
15	Window sill II	19	0	100
24	Window sill III	7	0	100
Mean values (n=30)		94	24	68

Similar to the MARS 500 samples, huge variations were observed regarding intra- and inter-compared sampling sites. The amount of observed colonies per 10 cm² ranged from 0 to 1,018 for non-heat-shocked aliquots, and from 0 to 387 after HS treatment. The overall mean value of the “vegetative” proportion obtained during the MICHAm campaign revealed a 7-fold increase of microbial specimens inhabiting 10 cm² compared to the samples taken at DLR.

An overall mean reduction after HS of approximately 70 % was measured at DLR samples, indicating a slightly lower value compared to the percentage (85 %) obtained from MICHAm samples (section III.1.6.2.).

Summary:

The microbial load in the confined MARS 500 facility was slightly increased as against comparative surface samples from public places, where the exchange with external environment is not restricted.

III.1.8. Scanning Electron Microscopy of Swabs

Scanning electron microscopy was applied to survey the structure of both an unused sterile nylon-flocked swab and a swab that was used to sample a surface area. The aim was to determine its significant features, sample entrapment and structural changes after applying pressure and rotational forces to the head of the swab. Therefore, a combination of low-magnification and high-magnification imaging was performed (Fig. III.1.8.1).

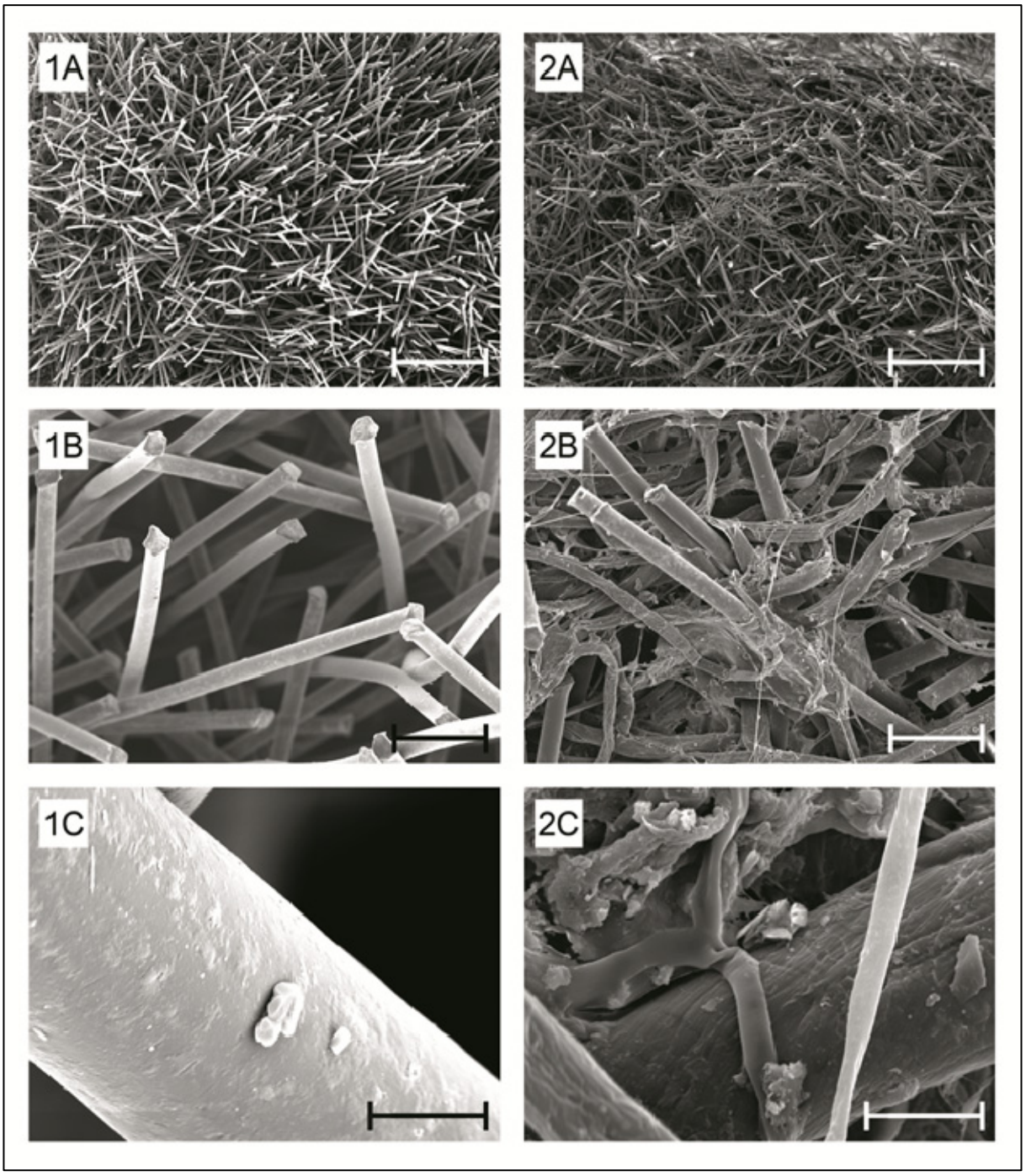

Fig. III.1.8.1 Magnification series of the head of a nylon-flocked swab prior to (1A-C) and after sampling a surface of 25 cm² (2A-C). 1A and 2A: 30x, scale=500 µm; 1B and 2B: 300x, scale=50 µm; 1C and 2C: 4000x; scale=5 µm

The image series taken of a sterile swab (1A to C) displays that the fibers are attached to the shaft of the swab in a structured manner. The single fibers are arranged perpendicularly to the shaft, resulting in a surface area for specimen uptake that is maximized. Furthermore, homogeneity is observed in size, i.e., fiber length and diameter. Unlike the almost slick filament, the forefront of each fiber shows no smooth cut itself. Picture 1C displays only few abiotic residues that are attached on the side of the filament and might originate from the production process.

The second series (2A to C) is taken of a swab that has been used for sampling a dusty wooden surface. The images depict that the swab absorbed the material present on the (sampled) surface very well (2B and C). Due to the flexibility of the fibers, they bend during the sampling procedure without breaking. To increase matter uptake, the swab is rotated, hence the fibers stuck partially together so that their configuration and integrity get changed. Picture 2B clearly shows the uptake of hairs, filaments, and dust particles creating a kind of network adhering also microbial cells. Due to the diverse features and conformation of the uptaken matter, the differentiation of abiotic material from microorganisms as rods or cocci is proven difficult.

Summary:
The nylon-flocked swab is an effective tool for matter uptake. However, only small areas can be sampled due to saturation when sampling fibrous particles, settled dust, and microbial specimens. Furthermore, integrity of fibers is lost after sampling to a certain grade.

III.1.9. MICHAm: Taxonomic Assignment of Surface Isolates

III.1.9.1. “Vegetatives”

After plating and incubation of the MICHAm samples, one to three isolates were picked from each growth positive plate in due consideration of selecting a broad variety of colony shape and color. Due to the enormous number of colonies obtained on only one medium type, merely a low percentage of isolates was further characterized. Obtained sequences were classified by Second Genome. In parallel, all purified isolates were kept in liquid media containing 10 % glycerin for long-term conservation at -80°C.
The representative sequences were submitted to Genbank. The accession numbers are found on the provided data CD (folder: Genbank Submission Data).
The eighteen sampling events that have taken place at eleven diverse locations in the three modules of the isolation facility resulted in the identification of five different phyla (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Thermi; Table III.1.9.1.1). In module EU-150 (habitable) and EU-250 (utility) all five phyla were present, whereas the isolates of samples obtained from module EU-100 (medical) revealed no signatures that were assigned to Actinobacteria and Firmicutes. As already detected for airborne germs, the vast majority of the identified isolates of all three modules were gram-positives, whereas less than one quarter (approximately 20 %) were gram-negative (Fig. III.1.9.1.1). This trend of distribution was nearly identical for all three modules.

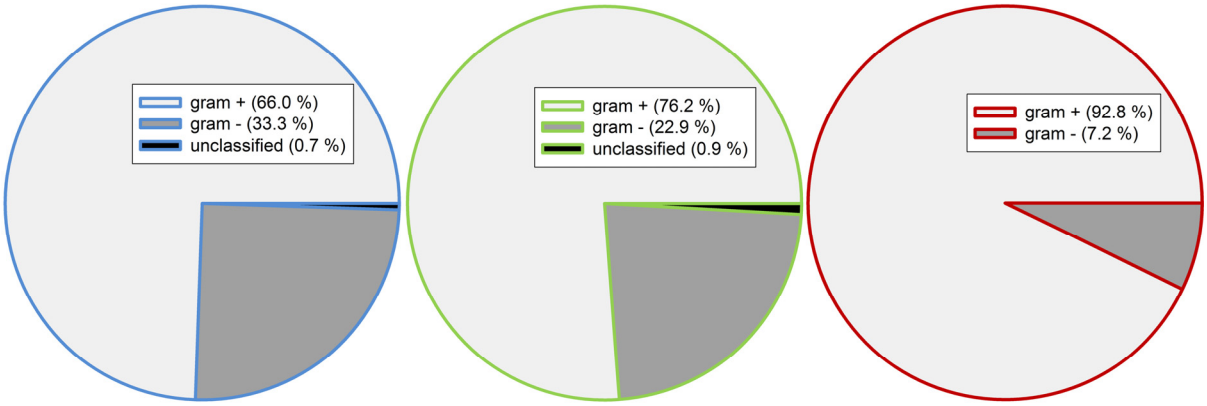

Fig. III.1.9.1.1 Percentage distribution of the "vegetative" isolates from surface samples concerning their gram affiliation originating from the utility module (EU-250=blue), the habitable module (EU-150=green) or the medical module (EU-100=red). Light grey=gram-positives; dark grey=gram-negatives; black=unclassified due to inconclusive classification

In all, 443 sequences were assigned to eight classes (three remained unclassified), 16 orders (five unclassified), 34 families (three unclassified), and 36 genera revealing a high overall diversity after cultivation under only one enrichment condition.

TAB. III.1.9.1.1 CLASSIFICATION OF SURFACE ISOLATES ("VEGETATIVES") OF THE MICHAM CAMPAIGN DISPLAYING ALL TAXONOMIC TAXA FROM PHYLA TO FAMILY; TOTAL NUMBER OF COLONIES AND ISOLATES PROCESSED, GREY AREAS ON FAMILY LEVEL REFER TO GRAM-POSITIVES. BOLD: THESE AFFILIATIONS WERE ALSO DETECTED BY AIR SAMPLING (SECTION III.1.5.). THE GIVEN NUMBERS REPRESENT THE ISOLATES OBTAINED FROM ALL SAMPLES OF ONE MODULE.

		Utility module	Habitable module	Medical module
Total number of colonies		10,388	121,114	2,100
Number of sequences processed		150 (1.4 %)	210 (0.2 %)	83 (4.0 %)
Phylum	**Actinobacteria**	21	25	7
	Bacteroidetes	1	2	-
	Firmicutes	77	136	70
	Proteobacteria	49	46	6
	Thermi	2	1	-
Class	**Actinobacteria**	20	23	7
	Bacilli	77	136	70
	α-Proteobacteria	28	27	3
	β-Proteobacteria	3	3	-
	γ-Proteobacteria	8	16	3
	Deinococci	2	1	-
	Flavobacterium	-	2	-
	Sphingobacterium	1	-	-
	unclassified	1	2	-
Order	**Actinomycetales**	20	23	7
	Bacillales	69	132	68
	Burkholderiales	3	2	-
	Caulobacterales	6	6	-
	Deinococcales	2	1	-

		Utility module	Habitable module	Medical module
	Enterobacteriales	-	4	-
	Flavobacteriales	-	2	-
	Lactobacillales	8	4	2
	Pseudomonadales	5	9	3
	Rhizobiales	12	10	1
	Rhodobacterales	8	5	2
	Rhodocyclales	-	1	-
	Rhodospirillales	2	4	-
	Sphingobacteriales	1	-	-
	Sphingomonadales	10	2	-
	Xanthomonadales	3	3	-
	unclassified	1	2	-
Family	***Acetobacteraceae***	2	4	-
	Aerococcaceae	2	4	1
	Aurantimonadaceae	-	2	-
	Bacillaceae	26	17	12
	Brevibacteriaceae	4	-	-
	Brucellaceae	-	3	-
	Burkholderiaceae	1	2	-
	Caulobacteraceae	6	6	-
	Comamonadaceae	1	-	-
	Corynebacteriaceae	2	1	1
	Deinococcaceae	2	1	-
	Enterobacteriaceae	-	4	-
	Enterococcaceae	4	-	-
	Flavobacteriaceae	-	2	-
	Gordoniaceae	1	1	-
	Methylobacteriaceae	12	3	1
	Microbacteriaceae	1	5	1
	Micrococcaceae	10	14	5
	Moraxellaceae	3	8	2
	Nocardiaceae	1	1	-
	Oxalobacteraceae	1	-	-
	Paenibacillaceae	3	-	-
	Planococcaceae	3	1	-
	Pseudomonadaceae	2	1	1
	Rhizobiaceae	-	2	-
	Rhodobacteraceae	8	5	2
	Rhodocyclaceae	-	1	-
	Sphingobacteriaceae	1	-	-
	Sphingomonadaceae	10	2	-
	Staphylococcaceae	37	114	56
	Streptococcaceae	2	-	1
	Streptomycetaceae	1	-	-
	Tsukamurellaceae	-	1	-
	Xanthomonadaceae	3	3	-
	unclassified	1	1	-

- **Genus level**

Overall, 36 different genera were detected. Ten of those were enriched from all three modules (Fig. III.1.9.1.2). Other than those observed from the airborne community structure,

the utility and the habitable module shared nine genera. Nevertheless, both modules revealed their specific bacterial signatures.

Some of the cultivated genera were capable of forming spores. The enriched isolates were members of the genera *Bacillus* and *Paenibacillus*. Additionally, different types of contaminants could be detected, such as human-associated germs, which can be found on and within the human body, as well as isolates that originated from the environment of the isolation facility. The latter-mentioned fraction represented microbes that survived the initial sterilization procedure (gasing with 3 % H_2O_2) or were carried in with clothes, food packages, soil of the greenhouses, or equipment needed for the diverse experiments.

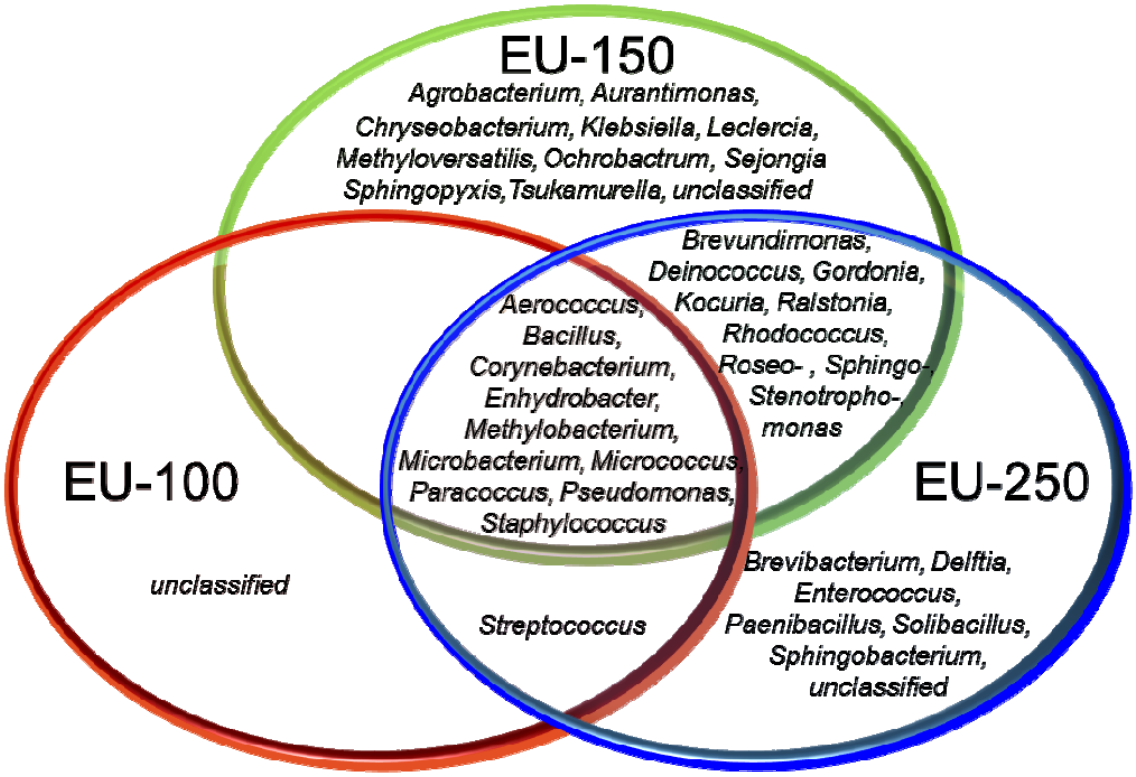

Fig. III.1.9.1.2 Cultivable "vegetative" bacterial genera distribution analysis. The figure highlights the distribution pattern of the bacterial genera within the MARS 500 isolation facility. The genera were grouped into three modules; habitable (EU-150=green), utiliy (EU-250=blue), and medical (EU-100=red)

- **Species level**

Overall, 47 different strains were identified on the species level, whereas 284 gene signatures were revealed as unclassified in case the sequence length of 700 to 1,000 bps was not enough to assign it to a certain species. Forty-four percent from EU-250 (utility), 35 % from EU-150 (habitable) and 30 % from EU-100 (medical) were unclassified. Nevertheless, the highest microbial diversity was observed in the utility module (34 different species), followed by the habitable module (30 different species), whereas only 15 species were detected in the medical module.

Based on these results, a core microbiome was identified. The following strains were enriched multiple times from all three modules: *Bacillus amyloliquefaciens*, *Bacillus safensis*, *Micrococcus luteus*, *Paracoccus yeei*, *Pseudomonas libanensis*, and the

Staphylococcus strains *S. aureus*, *S. cohnii*, *S. epidermidis*, *S. haemolyticus*, and *S. hominis*. As shown in Table III.1.9.1.1 and Fig. III.1.9.1.3, most of the isolates belonged to the genus *Staphylococcus*.

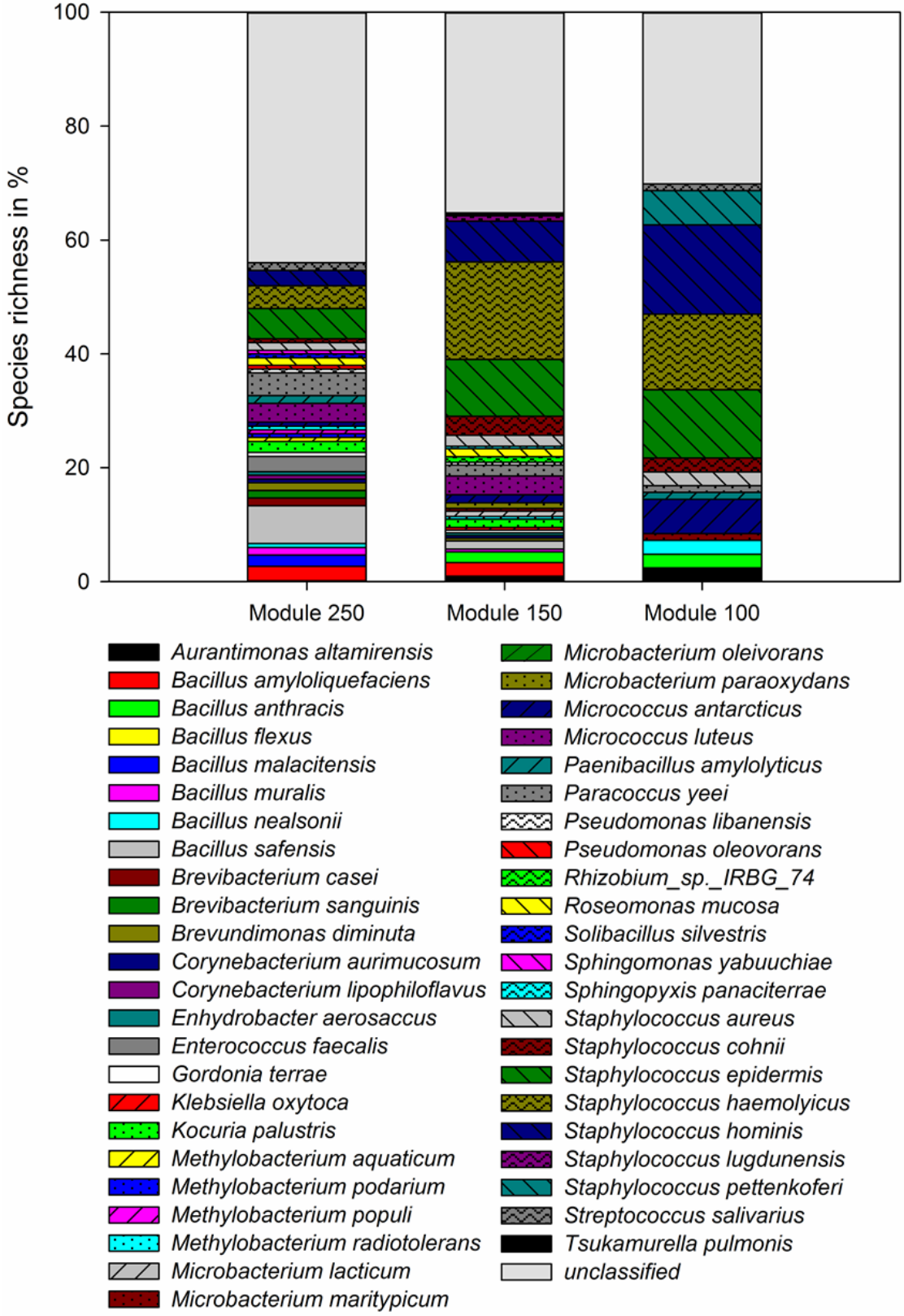

Fig. III.1.9.1.3 Cultivable microbial "vegetative" diversity from surface samples of the MARS 500 facility as detected via 16S rRNA gene sequence analysis. The distribution by percentage of the detected microbial diversity at the three modules (EU-250=utility, EU-150=habitable, EU-100=medical) is shown on species level.

Summary:
The phylogenetic analysis of the isolates that were enriched from the swab assay covering the “vegetatives”, showed an extraordinarily broad cultivable biodiversity. Both human-associated microorganisms (vast majority) as well as bacteria originated from the environment were detected. *Staphylococcus* and *Bacillus* spp. were found to be the dominant bacterial species on surfaces of the isolation facility during the whole timeframe of the long-term confinement. Furthermore, the differential representation obtained on the lowest classification levels suggested that each of the three sampled modules, though harboring a shared set of abundant microbes, has its own unique indoor microbiota.

III.1.9.2. “Bioburden”

In order to determine the “bioburden”, space agencies rely on a standard protocol (ECSS-Q-ST-70-55C, 2008) that measures the amount of cultivable, heat-shock surviving microorganisms per given area. As described above for determination of vegetative specimens, representative colonies were chosen to get an insight into the microbial diversity that is able to survive a HS treatment. The following Figure III.1.9.2.1 illustrates the distribution of identified strains per module indicating a high percentage of different *Bacillus* species in all three modules. The vast majority of 16S rRNA gene affiliations were those from *Bacillus safensis* followed by *B. amyloliquefaciens*. Additionally, one *Paenibacillus* (*campinasensis*) strain was identified. All species belonging to these two genera are spore-forming organisms and have to be considered as persistent contaminants. In all three modules, the spore-forming strains accounted for approximately 70 %, and around 20 % remained unclassified on the species level.
The remaining 10 % consisted of vegetative cells that were able to survive the HS treatment. *Micrococcus luteus* was enriched in all three modules, whereas the utility module also contained heat-resistant *Enhydrobacter aerosaccus* and *Staphylococcus epidermidis*. Besides spore-forming strains, the habitable module also housed *Sphingobacterium faecium*, and the two *Staphylococcus* strains *epidermidis* and *hominis*. *Paracoccus yeei* was observed in the medical module.

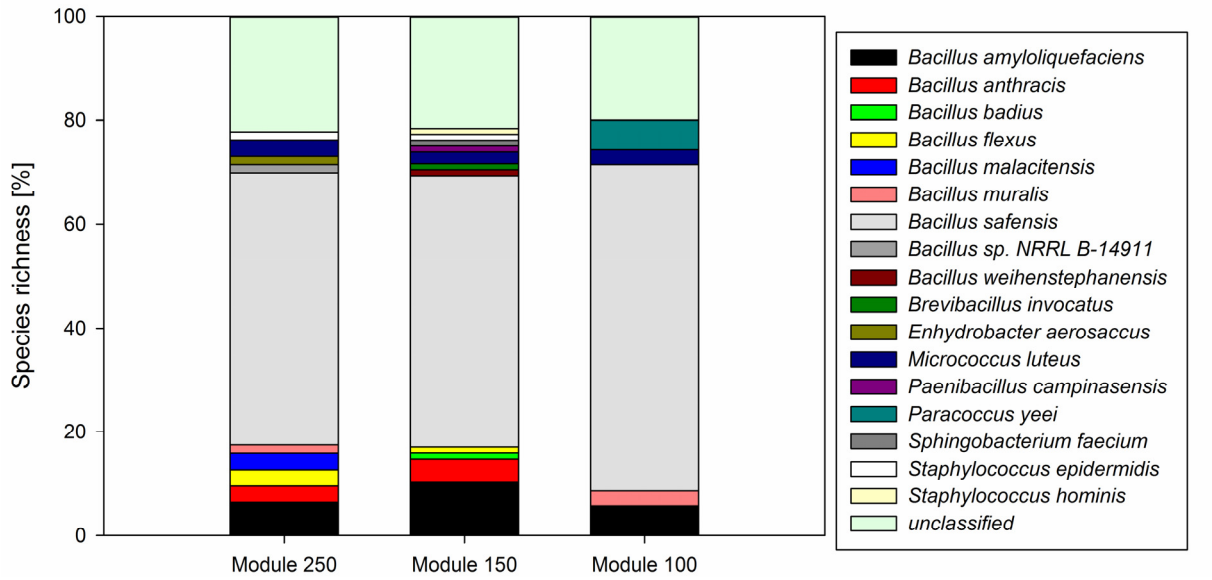

Fig. III.1.9.2.1 Cultivable microbial "bioburden" diversity from surface samples of the MARS 500 facility as detected via 16S rRNA gene sequence analysis. The distribution by percentage of the detected microbial diversity at the three modules (EU-250=utility, EU-150=habitable, EU-100=medical) is shown on species level.

Summary:
Not only genera that have the ability to produce spores as a protection mechanism survived the HS treatment (80°C; 15 min) but also vegetative cells revealed tolerance against this procedure. However, this fraction states the minority, whereas diverse bacilli (spore-formers) were counted among the majority.

III.1.10. MICHAm: Detection of Potentially Pathogenic Organisms

Potentially Pathogenic Organisms (PPOs) were identified according to the TRBA 466 document (Classification of prokaryotes (bacteria and archaea) into risk groups, 2010), which specifies microorganisms according to their pathogenicity into four groups. With increasing group number, the pathogenic potential of a strain raises, thus 1 indicates species that are harmless. Species belonging to groups 2 to 4 are of potential danger for humans, animals, or plants, and cause infectious diseases. The TRBA 450 document defines "Criteria for the classification of biological agents" (TRBA 450, 2000). Some species of group 1 are marked with +, i.e., in individual cases this species was detected or suspected as a pathogen, mainly in the context of immune depressed people. However, identification is often not reliable, so that they were not counted as PPOs in our context.

To initially gain an overview, all sequences that were assigned on the species level were implemented when looking for PPOs, whereas unclassified sequence affiliations were excluded.
The following organisms were identified as PPOs and are members of risk group 2. Some taxa are marked with (ht), indicating pathogenicity for humans and vertebrates, although generally no transfer is possible between two host groups.

Bacillus weihenstephanensis, *Brevibacterium sanguinis*, *Brevundimonas diminuta*, *Corynebacterium amycolatum* (ht), *Enterobacter hormaechi* (ht), *Enterococcus faecalis*, *Gordonia terrae*, *Klebsiella oxytoca* (ht), *Paracoccus yeei*, *Roseomonas mucosa*, *Sphingobacterium multivorum*, *Staphylococcus aureus* (ht), *Staphylococcus epidermidis* (ht), *Staphylococcus haemolyicus* (ht), *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus pettenkoferi*, *Streptococcus salivarius* and *Tsukamurella pulmonis*.
Bacillus anthracis was the only species that was assigned to risk group 3.
Solibacillus silvestris and *Sphingopyxis panaciterrae* could not be defined regarding their pathogenic potential, since they were not included in the list.

Summary:
Due to the enrichment of a mainly human-associated microbial community inhabiting the confined manned habitat, human-associated commensals and opportunistic pathogenic microorganisms were also present. They resided in the isolation facility as living organisms able to survive, multiply, and thrive and thus may affect human's health.

III.2. Evaluation of Air Quality During the Confinement

An artificial atmosphere of suitable composition is the most immediate need during confinement. Good air quality is one of the requirements decisive for the physiological state and performance of humans none more so than for confined manned habitats. The most important parameters for measuring air quality are concentrations of various gases (CO_2 and O_2 in particular), atmospheric relative humidity (RH), and temperature. In hermetically closed habitats, aeration by opening doors and windows is not possible to improve air quality. Therefore, an air control system was installed in each module to regulate the environmental parameters within a certain allowed range and thus maintain the optimal gas composition. Indoor temperature could be adjusted based on the provided guidelines by the marsonauts themselves regarding their well-being. Airborne microorganisms are distributed in the atmosphere from various sources such as dead skin cells, sneezes, and dust. For reduction of dust particles, aerosols, and airborne microorganisms, a five-step filter system (Fig. II.2.1.2) was installed in the air ventilation system.
Thanks to continuously acquired information (based on records at least every ten days) from the air control systems installed in each module of the MARS 500 complex, the air quality could be determined (Table III.2.1).

TABLE III.2.1 ENVIRONMENTAL INDOOR PARAMETERS FOR UTILITY (EU-250), HABITABLE (EU-150), AND MEDICAL (EU-100) MODULE

Parameters	O_2 partial pressure [%]			CO_2 partial pressure [%]			Relative humidity [%]			Temperature [°C]		
Limits	mean	min	max	mean	min	max	mean	min	max	mean	min	max
Reference		19.0	25.0		0.00	1.00		40.0	75.0		18.0	28.0
EU-250	20.7	20.5	20.9	0.30	0.09	0.62	41.3	**33.0**	56.3	22.0	**17.9**	25.8
EU-150	20.7	20.3	21.8	0.28	0.02	0.57	41.8	**36.5**	56.4	23.3	20.7	25.3
EU-100	20.8	20.5	21.0	0.23	0.01	0.56	39.1	**32.8**	48.8	23.1	20.5	25.8

Bold: values obtained below the reference

During the confinement the measured values for O_2 and CO_2 partial pressure and temperature ranged within the pre-specified limits (=reference) revealing only one exception for temperature in module EU-250, which can be neglected. However, the measured values for RH were below the allowed minimum.

In general, the modules varied only slightly from each other regarding the environmental parameters. The data acquired over 520 days was plotted exemplarily for module EU-150 in Fig. III.2.1, indicating an almost stable oxygen partial pressure at normal air condition. The remaining factors exhibited slight fluctuations during this period. Plots of the utility and medical module are stored on the data CD (folder: Environmental factors).

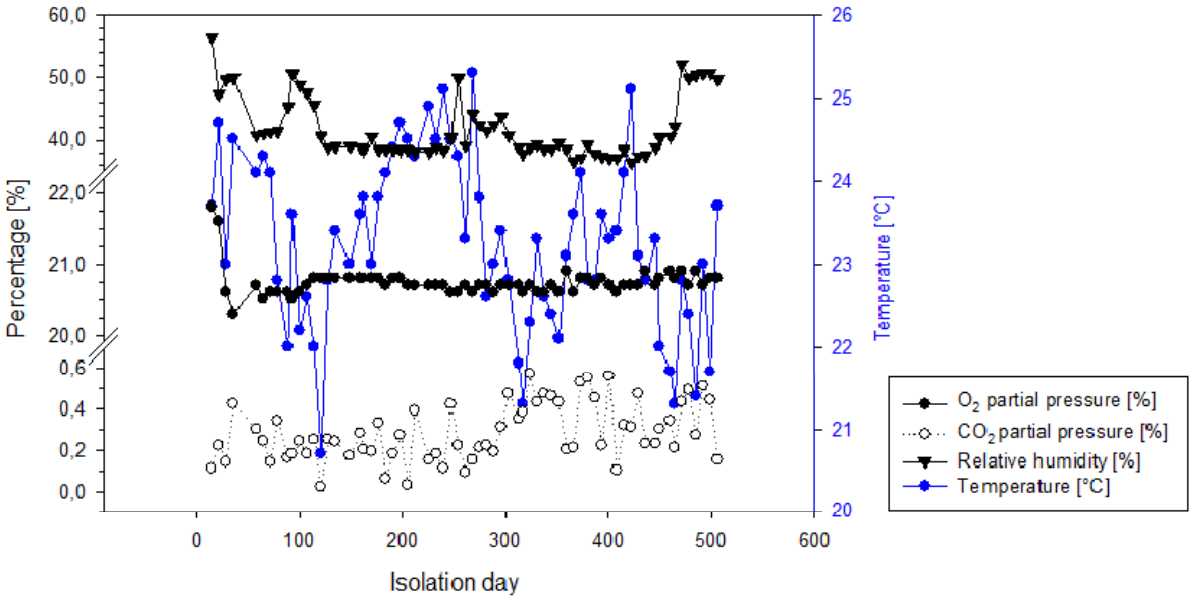

Fig. III.2.1 Prevalent environmental conditions shown exemplarily for module EU-150 (habitable) of the MARS 500 facility during the whole isolation experiment. Values for partial pressure of O_2, CO_2, RH, and temperature were taken approximately every 10 days.

To obtain more insights into the fluctuation, inter-module comparisons of each environmental factor were performed and exemplarily displayed for temperature (Fig. III.2.2; remaining factors: data CD [folder: Environmental Factors]). The recorded local minima and maxima of each module show similar curve shapes.

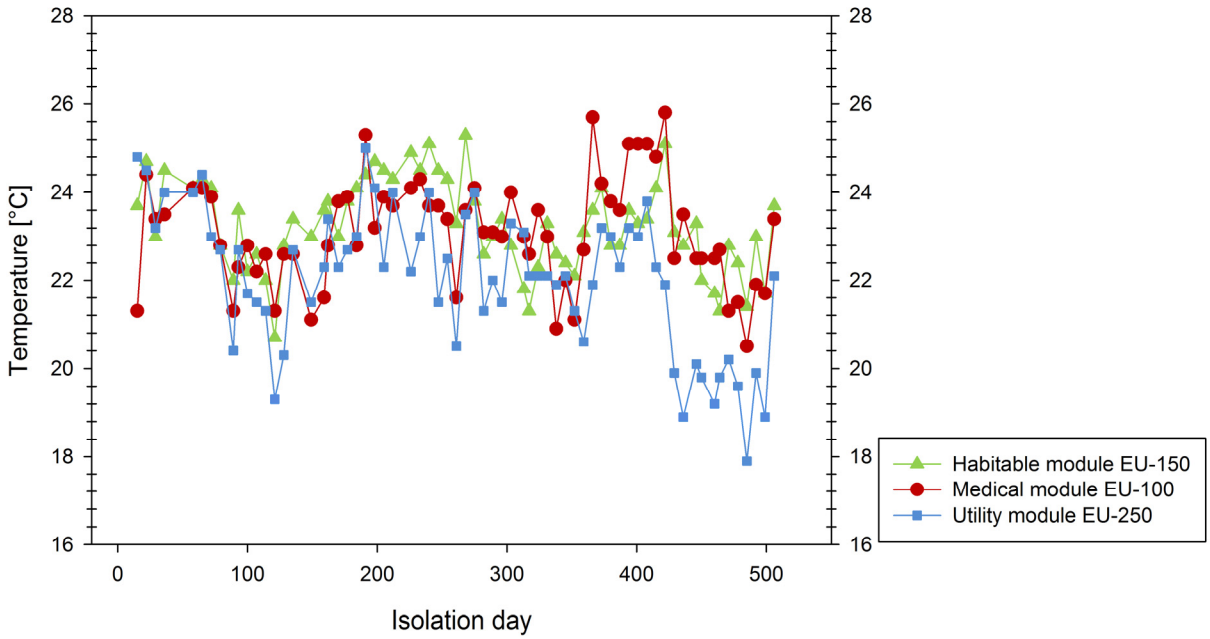

Fig. III.2.2 Temperature conditions of module EU-250, EU-150, and EU-100 from the MARS 500 facility during the whole isolation experiment. Values were taken approximately every 10 days.

Summary:
Monitoring of environmental parameters is important, since the prevailing conditions have an influence on microbial growth. Unfortunately, the points of measurement of environmental parameters seldom complied with the sampling dates. Though fluctuations in temperature and humidity were measured, the values are not indicative for the actual conditions at the sampling dates. Consequently, no extrapolation and further correlation of environmental factors with CFU data is achievable.

III.3. Molecular Approaches

Molecular approaches were the method of choice to further investigate the overall, i.e., cultivable, non-cultivable and dead microbial load being present in the MARS 500 facility.

III.3.1. Evaluation of Best-Suited DNA Extraction Method

The focus of the following experiment was to elucidate the efficiency of two DNA extraction methods. Furthermore, it should simultaneously evaluate the extraction efficiency of swabs. A commercially available kit (PeqGOLD) and a phenol-chloroform based method (XS-buffer) were tested.

- In a first series of tests, serial dilutions of a bacterial pure culture cell suspension with known concentration were subjected to the extraction process.
- In a second series of tests, swabs were spiked with 50 µl of the identical bacterial pure culture cell suspensions in order to simulate DNA extraction from the real sampling tool.

S. cohnii was chosen as the representative strain, since staphylococci are typical human contaminants that constituted the majority of cultivable species from MICHAm samples (section III.1.5.; section III.1.9.). Based on the results, the standard procedure for the following extraction experiments (including MICHAm samples) was defined.

A fresh overnight staphylococci culture was the starting suspension for direct extraction and parallel spiking of swabs. The total cell number of the initial overnight culture was approximately 8 x 10^7 cells per 50 µl, as determined via Thoma counting chamber.
In order to exclude artifacts originating from chemicals used to prepare the buffer, 2, 5, 10, and 20 µl of 2x XS-buffer were subjected to DNA concentration measurement. The results exhibited no detectable amount of DNA. The same could be shown for all blanks (H_2O blanks, swab blanks) carried along, indicating a sterile handling procedure, including sterility of vessels and prepared solutions.

The results of a preliminary test with XS-buffer method revealed measurable DNA amounts at the detection limit. Since gDNA concentration measurements obtained by Qubit® Fluorometer technology are more reliable compared to NanoDrop ND-2000c, this technique was applied for all further concentration estimations (data not shown). Thus, changes of the XS-buffer based extraction process were required, trying to minimize the loss of DNA which occurs when only 1 ml out of 2.5 ml was used for DNA extraction. The following changes were done to optimize the outcome.

- The XS-buffer was pre-warmed and the swab was directly put into the extraction buffer to avoid any dilutions and hence loss of gDNA.
- The swab was transferred in 1 ml buffer since this was determined to be the minimal required liquid, so that the head of the swab is completely dipped into the buffer.

Following extraction after adjustments, the gDNA yield was plotted according to the initial cell content per sample (cell suspension or spiked swab; Fig. III.3.1.1).

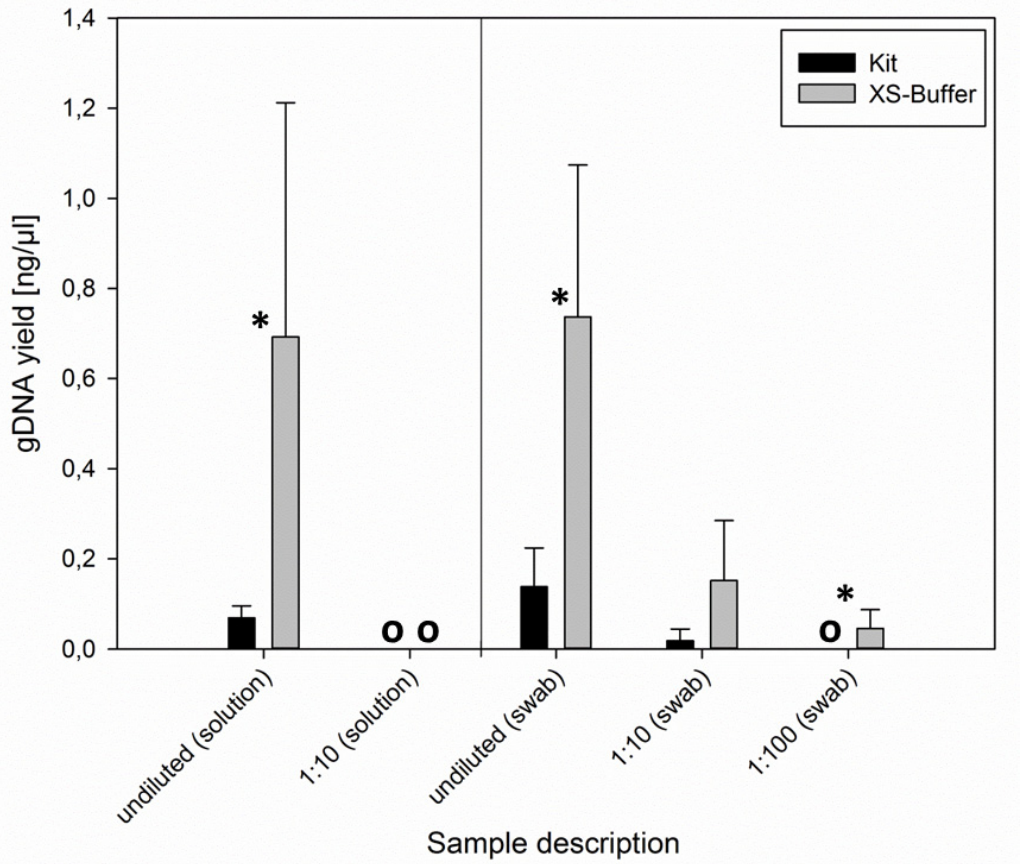

Fig. III.3.1.1 Comparison of gDNA extraction efficiencies using PeqGOLD Bacterial DNA Kit and phenol-chloroform based XS-buffer method. Either cell suspensions or spiked swabs containing different cell concentrations were used. The black columns indicate the obtained gDNA yield using the kit. The grey columns display the gDNA concentration after extraction with XS-buffer method. Columns represent mean (n=3) values with standard deviation. Asterisks denote significant differences between values obtained by both methods (p-value <0.05). **o**: no measurable gDNA amount

Comparisons of gDNA yields from extraction of the cell suspensions and values derived from swabs indicate almost identical extraction efficiencies for the undiluted cell suspension. Higher dilution factors from cell suspension samples revealed results worse than expected. For spiked swabs (cells can remain on the swab), higher gDNA yields were obtained compared to the cell suspensions that were directly pipetted into XS-buffer. While measurable DNA amounts were obtained from all tested swabs that were subjected to XS-buffer extraction, the direct extraction with the same method led only to measurable results in case of the undiluted sample. In four out of five extraction setups, higher gDNA concentrations were received with the XS-buffer method. Direct adding of cells in the extraction buffer minimized the possibility of cell loss and was therefore thought to result in at least identical if not higher values.

Summary:
The gDNA recovery of the cells from the swab was very effective compared to the DNA concentration values that were reached after extraction when the cells were directly pipetted into XS-buffer. Due to greater accuracy and reliability during the measurement, Qubit® assay was applied for future gDNA concentration estimations. Higher extraction efficiencies

were obtained by use of the classical chloroform-phenol based DNA extraction method with XS-buffer compared to a commercially available kit. Consequently, the following gDNA isolation procedures were performed with the XS-buffer method.

III.3.2. Optimization of DNA Isolation from Surface Samples

The main goal was to measure the effectivity of the removal and lysis process for bacterial cells from the swab head to fulfill the requirements of Second Genome for analysis: ≥ 200 ng of isolated DNA at a concentration of 25 ng/µl per sample. Therefore, the following experimental plan was setup:

Parallel surface samples (n=2) were taken from locations at the DLR that are comparable to sample sites in the MARS 500 facility. One swab sample was subjected to cultivation to estimate the number of vegetative CFU per sampled area (section III.1.7.). The second was used for determination of the corresponding gDNA amount per sample and was subjected to DNA isolation (XS-buffer).

Exemplary areas that were swabbed and subjected to both, cultivation and DNA extraction approach, were the surface of a lab bench, a desk, toilet bowls in restrooms, a hand rail, a kitchen counter, and wooden surfaces of a rack and a table.

As shown in Table III.3.2.1, a low overall amount of gDNA was obtained.

TABLE III.3.2.1 RESULTS OF GDNA EXTRACTION WITH XS-BUFFER METHOD AND CORRESPONDING COLONY FORMING UNIT (CFU) COUNTS OF SURFACE SAMPLES TAKEN AT DLR (SEE ALSO TABLE III.1.7.1)

Sample # and description		gDNA [ng/µl]	gDNA [ng/swab]	Swab assay[a] CFU per 10 cm²
1	Lab bench I	0.08	1.6	10
2	Restroom men I	0.21	4.2	1,375
3	Restroom men II	0.05	1.0	0
4	Restroom men III	0.08	1.6	0
6	Desk I	0.05	0.9	28
12	Rack surface (wood)	1.05	21.0	135
16	Table surface (wood)	1.32	26.4	2,545
17	Desk III	0.48	9.5	270
19	Kitchen counter	1.32	26.4	320
21	Restroom women II	0.42	8.4	752,5
26	Hand rail (metal)	0.52	10.3	30
B	Blank	OOR[b]	OOR[b]	nd[c]

[a]Cultivation assay “vegetatives”: plating on R2A medium, incubation for 72 h at 32°C

[b]OOR=out of range, due to too low DNA content

[c]nd=not determined

The values ranged between 1.0 and 26.4 ng per swab. In general, the amount of DNA that was extracted from one swab sample was less than the 200 ng (25 ng/µl) needed for amplification. The highest amount of gDNA was isolated from wooden surfaces specified by

a rough surface. A similar amount was found on the kitchen counter, whereas metal and ceramic surfaces revealed extremely low gDNA yields that did not exceed 10.3 ng per swab. These results were reliable since the blank sample was negative, indicating sterile extraction procedure. Furthermore, there was no correlation between CFU count per sampled area and extracted gDNA amount. For example, a gDNA yield of 26.4 ng per swab was received from samples that contained 128 CFU and 1,018 CFU. Contrary, CFU counts of 552 revealed only 4.2 ng per swab, whereas 54 CFU resulted in 21.0 ng per swab. This observation was also confirmed by Pearson correlation (p-value >0.05).

Summary:
No correlation between CFU counts and extracted gDNA amount could be observed. Genomic DNA extracted from surface samples taken at DLR indicated that the amount was too low for the PhyloChip analysis according to the statement of Second Genome. Since microrarray analysis was planned for MARS 500 samples, it had to be checked via PCR and subsequent amplicon yield measurement whether the required 500 ng can be obtained.

III.3.3. DLR: PCR from Surface Samples

In order to determine whether the isolated gDNA was sufficient to gain 500 ng of amplified product, gDNA was employed for 16S rRNA gene amplification. PCR was performed according to the standard of Second Genome using the TaKaRa ExTaq® PCR Kit.
For pre-tests, samples 2, 3, 6, 16, and 17 (refer to Table III.3.2.1) were selected as representative templates, since they contained various concentrations of gDNA (lowest and highest) and/or revealed different CFU counts, i.e., amount of gDNA and number of colonies did not correlate. Additionally, negative and positive controls were carried along during PCR reaction. The obtained amplicons were analyzed on an agarose gel (Fig. III.3.3.1). All samples (except the negative control) revealed a distinct band at 1.5 kb to be the 16S rRNA gene. Ideally, the gel would show a single band at 1.5 kb per lane, and that band constitutes the total PCR yield >500 ng. However, an additional faint band at 700 bp was visible, but it seemed to be less than 50 % of total product, so that the PCR product would be moved on (pers. comment: Christel Chehoud).

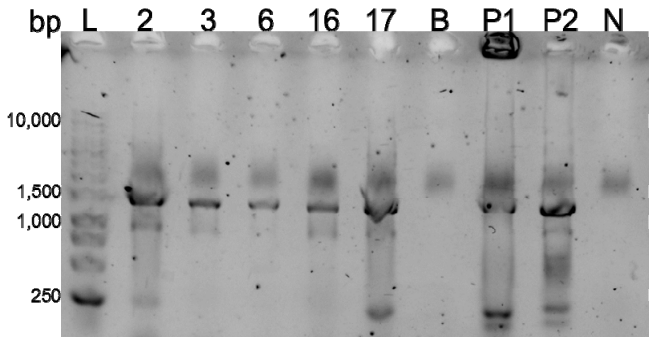

Fig. III.3.3.1 PCR results (TaKaRa ExTaq® protocol) visualized on an agarose gel of DNA extracted from swab samples taken at DLR using XS-buffer method. Lanes: L, 1 kb ladder; 2, restroom men I; 3, restroom men II; 6, desk I; 16, table surface (wood); 17, desk III; B, extraction blank (sterile swab); P1, positive control: colony PCR from *S. cohnii*; P2, positive control: extracted gDNA from *S. cohnii* cells; N, negative control: water template

To determine the amount of PCR product, the amplicons were purified by use of Qiagen MinElute PCR Purification Kit (final elution volume: 20 µl) and visualized on a gel for quality control. Afterwards, DNA concentration was measured (Table III.3.3.1). Estimation of DNA content before purification would not have given reliable results, due to the presence of primer dimers. Gel electrophoresis would have allowed at least a semi-quantitative estimation by comparing the marker intensity of specific bands with the respective PCR sample, but purification is more reliable and more precise.

The obtained results after purification revealed controversial results regarding gDNA input and PCR yield (e.g., sample # 3 and # 6; Table III.3.3.1). Higher gDNA input are expected to reveal higher amplicon amounts up to a certain limit, where it comes to inhibition reaction. However, it could be demonstrated that even a much lower gDNA input, as demanded from Second Genome, in the identical PCR reaction setup resulted in PCR yields higher than 500 ng in two out of five cases. Even template input of 0.9 ng led to almost 900 ng PCR amplification product indicating a 1,000-fold increase, whereas in the case of sample 16, only a 4-fold increase of the 2.6 ng input DNA was achieved. These findings pointed towards a high quality of the isolated DNA. Multiplication factor and output after amplification were not correlating with the initial gDNA template content. This was confirmed by Pearson correlation (p-value >0.05).

TABLE III.3.3.1 PCR YIELD USING TAKARA EXTAQ® PROTOCOL AFTER PURIFICATION WITH CORRESPONDING GDNA VALUES (XS-BUFFER)

Sample #	gDNA [ng/20µl]	gDNA template [ng/50 µl reaction]	PCR yield [ng/50µl]	Multiplication factor
2	4.2	0.42	890.0	2,119
3	1.0	0.09	43.6	484
6	0.9	0.09	4.4	49
16	26.4	2.64	10.7	4
17	9.5	0.90	894.0	993
Blank	OOR[a]	0	OOR[a]	0

[a]OOR=out of range, due to too low DNA content

Summary:

The amount of amplification product after applying the Second Genome conform PCR reaction setup demonstrated that even with up to 100-fold less template input, the demanded 500 ng per reaction can be obtained. Thus, samples from the MICHAm project were moved on for microarray-based analysis, even if a lower amount of gDNA was isolated from the swab samples than originally requested. To ensure an adequate amount of gDNA and maximize the gDNA content per extraction volume, all swab samples (MICHAm) taken from one module per sampling event should be pooled. However, further experiments were needed to optimize the pooling procedure.

III.3.4. Optimization of DNA Extraction from Swabs

Monitoring of the whole microbiome located in each complex of the MARS 500 facility was planned via DNA microarray technique. However, this approach requires a certain amount of DNA to be functional and reliable. Previous experiments (DNA extraction followed by 16S rRNA gene amplification and determination of the PCR yield) of swab samples taken at the DLR in the same manner as done for the MICHAm experiment indicated that the DNA amount, which can be isolated from one swab, might be too low for further down-stream analysis (section III.3.2.; section III.3.3.).

The following two different handling procedures were designed with the overall goal to increase the amount of extracted gDNA. Up to five swabs (maximum possible number of swabs that can be pooled per sampling event and module from the MICHAm experiment) were combined:

- either by pooling them directly in extraction XS-buffer
- or by pooling the individually extracted gDNAs of each swab afterwards.

Both pooling methods were applied simultaneously with parallel samples to isolate gDNA from either spiked swabs or from environmental swab samples. Following DNA extraction the final gDNA concentration was measured. The best-suited extraction procedure was then applied to the real MICHAm samples.

III.3.4.1. Environmental Swabs

Several environmental swab samples were taken at one time from a wooden table surface and from a smooth desk surface in an office at the DLR. To obtain numerous comparable swab samples (n=12) of one sampling location, 2 x 6 neighboring squares (5 x 5 cm²) with a maximum space of 1 cm in between were swabbed according to the scheme depicted in Fig. III.3.4.1.1. One swab of each of the two rows was subjected to cultivation (CFU determination “vegetatives”), whereas the remaining five samples were moved on to DNA extraction. CFUs were determined in order to get information about the amount of cells being present, and also to serve as the comparison for MICHAm samples.

DNA extraction single swabs	1	2	3	4	5	11 CFU
DNA extraction pooled swabs	6	7	8	9	10	12 CFU

Fig. III.3.4.1.1 Schematic sampling plan for taking parallel swab samples. Each square displays a sampling area of 5 x 5 cm². Number within each square refers to swab sample taken. Colors refer to further handling procedure. CFU corresponds to swabs subjected to cultivation assay “vegetatives”.

The experiment was performed twice, i.e., 24 swab samples were taken per sampling location. Four batches containing each five swabs were subjected to DNA extraction, whereas the remaining four swabs were analyzed regarding CFU amount in parallel. It has to be considered that these samples were treated the same way (parallel handling: same

day, same equipment at the same time), and should be comparable. The results of the DNA extractions of both environmental sampling locations (and both pooling procedures) are shown in Fig. III.3.4.1.2.

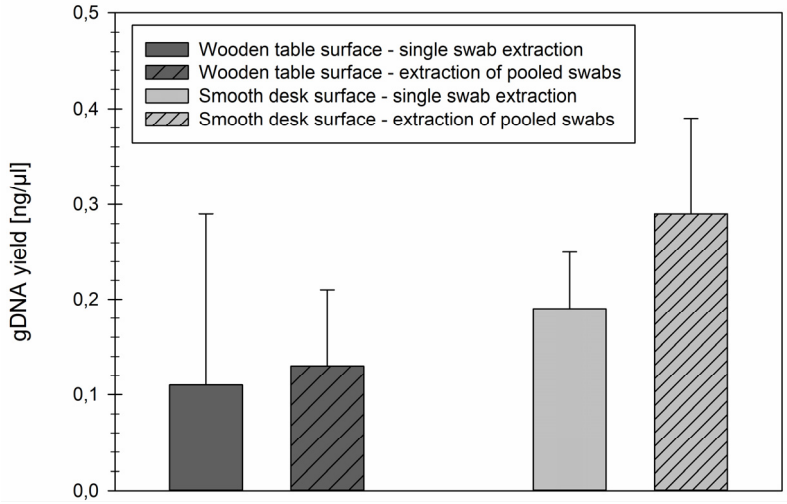

Fig. III.3.4.1.2 Effect of different pooling points of swabs during the gDNA extraction process on the obtained gDNA yield from parallel swab samples. The same procedure was performed at two different sampling locations. Monochrome columns display mean gDNA yield of a single extracted swab (n=20; gDNA dissolved in 20 µl H_2O); diagonally hatched columns represent average gDNA yield of four repeats from swabs (n=5; dissolved in 100 µl H_2O) being pooled in XS-buffer. Bars indicate standard deviation.

The average DNA yield of samples from the smooth desk surface is around 35 % higher for swabs pooled after extraction as compared to the single swabs, whereas the variation is lesser for the wooden surface samples. Nevertheless, the standard deviation in general is very high, especially for the single swab measurements of the wooden surface.
By plotting the gDNA content of the ten single swab extractions performed per location (Fig. III.3.4.1.3), the fluctuation of the isolated gDNA amounts became obvious.

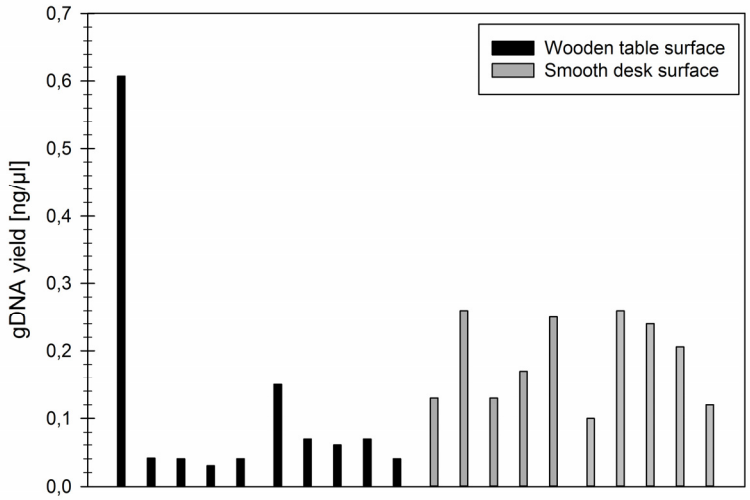

Fig. III.3.4.1.3 Effect of intra-sample variations regarding obtained gDNA yields after extraction (XS-buffer) from single swabs taken at ten neighboring 5 x 5 cm² squares from a wooden surface and from a smooth desk surface (n=1).

These results provide evidence of the high intra-sampling site variations regarding concentrations of extracted DNA of samples taken from nearly identical sampling locations and treated the same way.
This observation is also supported by the results of CFU determination from the single swabs, corresponding to one sample location, which revealed high variations (Table III.3.4.1.1).

TABLE III.3.4.1.1 CULTURABLE MICROBIAL CONTAMINATION MEASUREMENTS OF NEIGHBORING SURFACE SAMPLES (FIG. III.3.4.1.1) TAKEN AT TWO DISTINCT LOCATIONS AT DLR

Sample description	Sample #	"vegetatives"[a]		"bioburden"[b]	
		CFU/ml	Average per location	CFU/ml	Average per location
Wooden table surface	1	12	9.5	1	0.5
	2	22		0	
	3	2		0	
	4	2		1	
Smooth desk surface	1	7	40.5	4	3.25
	2	92		2	
	3	54		1	
	4	9		6	

[a]Cultivation assay "vegetatives": plating on R2A medium, incubation for 72 h at 32°C
[b]Cultivation assay "bioburden": heat-shock treatment (15 min at 80°C), plating on R2A medium, incubation for 72 h at 32°C

Summary:
Regarding the pooling point of swabs during the extraction process, no significant difference was observed in obtained gDNA yields of swabs taken at environmental surfaces. However, variations of both gDNA yields of neighboring sampling areas and CFU counts were monitored. It can be summarized that either the precision during handling was too low or samples are not comparable, even if they are taken next to each other. Therefore, the experiment was repeated with a defined cell concentration (section III.3.4.2.).

III.3.4.2. Spiked Swabs

Due to the fact that the above-described method did not yield significant results and in order to exclude the effect of possible natural variations in cell numbers per swabbed area, spiked swabs were analyzed. This approach should help to standardize the protocol and lead to reliable results.
Swabs were spiked with 50 µl of *B. safensis* cell/spore suspension containing 2 x 10^8 cells per 50 µl (determined by cell counts via Thoma cell counting chamber). Thereby, comparable samples with a defined amount of bacterial cells were generated to minimize bias being a result of different starting cell concentrations when sampling parallel surfaces.
The obtained gDNA yields of both extraction methods revealed no definite result due to the high standard deviation derived from high intra-sample variations (Fig. III.3.4.2.1). This

finding was also clarified by applying the student's t-test, which indicated no significant (p-value >0.05) difference regarding the gDNA content that was obtained by both pooling procedures.

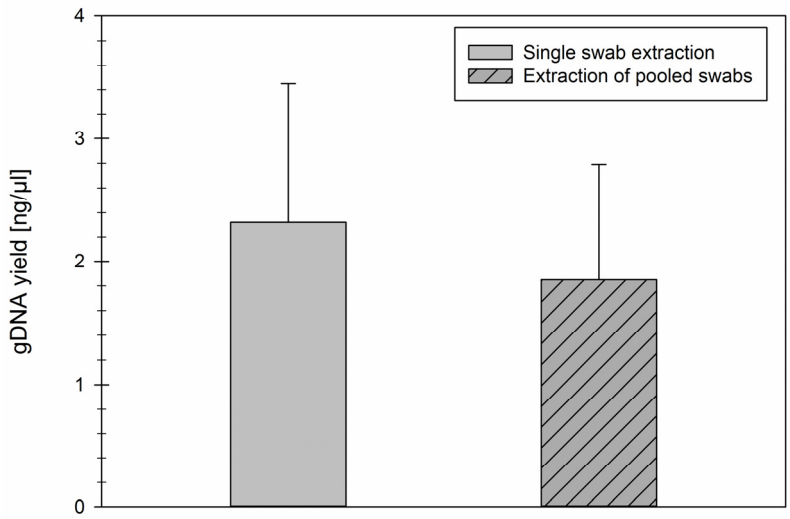

Fig. III.3.4.2.1 Effect of different pooling points of swabs during the gDNA extraction process on the obtained gDNA yield from swabs spiked with *B. safensis* cells; Monochrome column displays mean gDNA yield of single extracted swabs (n=20; gDNA dissolved in 20 µl H_2O); diagonally hatched column represents average gDNA yield of four repeats from swabs (n=5; dissolved in 100 µl H_2O) being pooled in XS-buffer. Bars indicate standard deviation.

Summary:
Results indicated that there is no significant difference in DNA yield between the two tested pooling methods, hence both methods can be used for MICHAm samples. It was decided, that the extraction process was carried out in only one reaction vessel per pool (either four or five swabs depending on the module, section III.3.7.1.).

III.3.5. PCR Yield - gDNA Input Relationship

Quantification of PCR yields was performed in order to investigate the (minimum) gDNA input that is required to receive 500 ng PCR product. Additionally, it should help to evaluate the optimum of the PCR reaction regarding the obtained amplicon product and the highest amplification efficiency.
Five swab samples were taken from a dusty surface in an office at the DLR, and gDNA was extracted the same way as for Second Genome samples (section II.8.1.3.). The only exception was that DNA was resuspended in 20 µl instead of 100 µl H_2O in order to get higher concentrations. Following DNA concentration measurement, PCR with Molzym™ 16S Basic Master Mix was performed in duplicate, since Second Genome changed their protocol. Therefore, different gDNA quantities (0.1, 0.5, 1.0, 1.5, 2.0, 1.5, 3.0, 4.5, 6.0, 9.0, 15.0, 20.0, and 60.0 ng) served as templates. PCR yield was measured after purification and plotted against gDNA input (Fig. III.3.5.1). Amplification of gDNA was detected for all samples, except for the negative control, indicating a pure Master Mix solution. Amplicon amounts of 500 ng can already be obtained with gDNA input of 1.5 ng. However, as can be

seen by the high standard deviation, it is not reliable and the mean value of both reactions was below 500 ng. Genomic DNA inputs between 1.5 ng and 6.0 ng resulted in PCR yields of 450 up to 620 ng per reaction, respectively. Amplification of 4.5 ng led to stable outputs of around 600 ng and reached a global maximum. Higher template amounts resulted in a steady decrease until the inhibition reaction (starting already with 15 ng input) occurred.

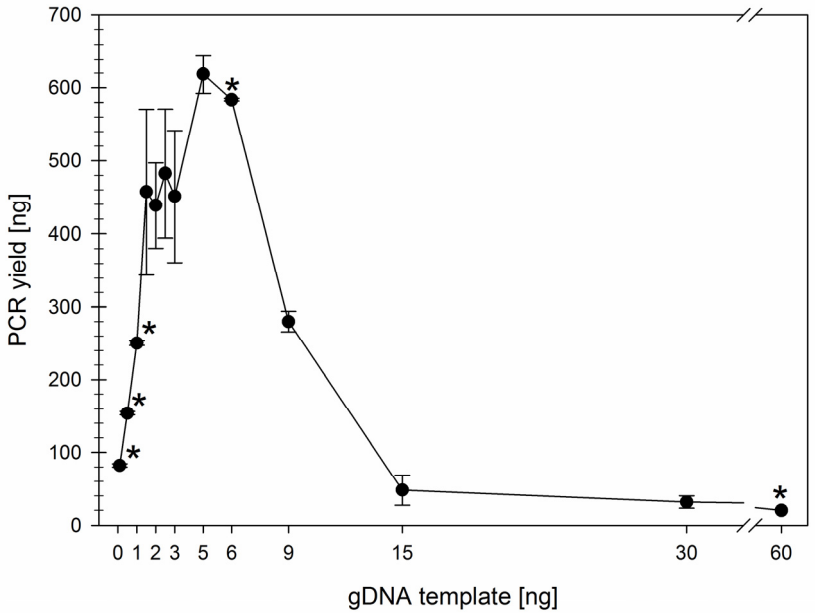

Fig. III.3.5.1 Relationship between gDNA template input and PCR yield of the amplified 16S rRNA gene using the Molzym™ 16S Basic Master Mix. Data are expressed as mean (n=2) values with standard deviation. Asterisks denote standard deviation too low to display.

In order to determine the efficiency rate of amplification, the multiplication factor (quotient of PCR yield [ng/25 µl] and gDNA input [ng/25 µl]) was plotted against the gDNA template (Fig. III.3.5.2). The plot revealed that the lower the gDNA template, the higher the amplification rate. Furthermore, the results suggested that gDNA input of 0.1 ng is sufficient to yield measurable amounts of PCR amplicons. This sample even revealed the highest efficiency with an 800-fold multiplication. Additionally, it was observed that PCR amplification of gDNA input above 9 ng is much less efficient than that of low initial biomass. Genomic DNA templates of 30 and 60 ng already showed inhibition reactions leading to multiplication factors of approximately 1.

To determine whether correct amplicons were generated, PCR products were run on an agarose gel to check the band's position. The desired length of approximately 1.5 kb was confirmed (data not shown).

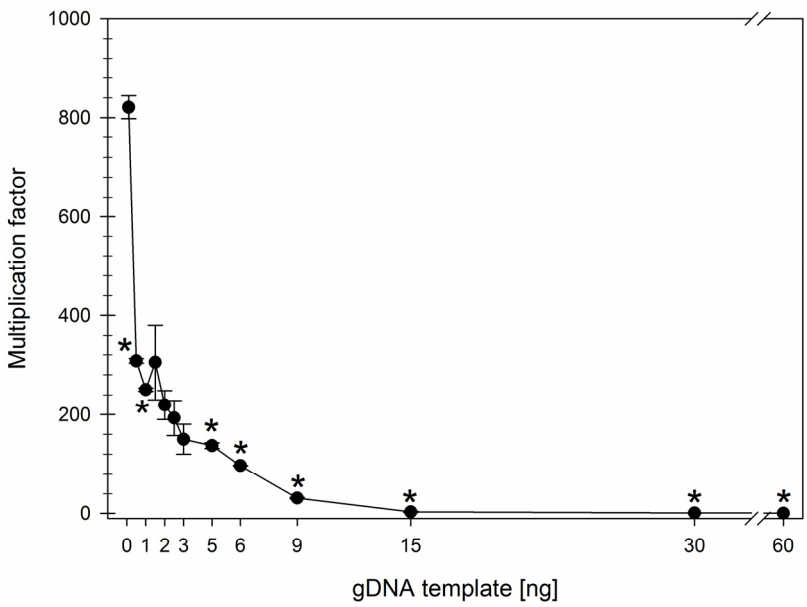

Fig. III.3.5.2 Relationship between gDNA template input and efficiency of PCR using the Molzym™ 16S Basic Master Mix. Data are expressed as mean (n=2) values with standard deviation. Asterisks denote standard deviation too low to display.

Summary:
The lower the gDNA input, the higher the PCR efficiency. It could be shown that template amounts between 1.5 ng and 6.0 ng per 25 µl reaction can result in the 500 ng PCR yield, necessary for subsequent PhyloChip analysis. The inhibition reaction occurred following the demand of Second Genome (2 µl gDNA template at 10 - 30 ng/µl per 25 µl reaction), indicating that their PCR setup is not optimized.

III.3.6. MICHAm: PhyloChip

PhyloChip of the third-generation (G3) is a reliable, high-throughput method that does scale well for large comparative studies without *a priori* knowledge of the microbial community structure, as is the case for environmental monitoring studies. This was the tool of choice to explore the following questions:

- How does the molecular analysis complement the cultivation assay?
- What kind of taxa can be detected in both methods?
- Can a core microbiome be identified from all analyzed samples?
- How similar are the biosignatures from two samples taken at the same time but at two different locations?
- Can a change in the microbiome be detected on the species level over time when comparing samples from the same location but from different sampling events?
- Do changes in microbial richness correlate?
- Does the relative abundance of potentially pathogenic taxa partition the two sampling locations into separate microbiomes?
- Is there a correlation of potentially pathogenic taxa with time?

- Do environmental factors, such as relative humidity, temperature, or partial pressures of oxygen and carbon dioxide, have an influence on the microflora?

The proposed set of PhyloChips for inter- and intra-comparison of samples was generated from pooled swabs of sampled surfaces in the habitable (EU-150) and the utility (EU-250) module during seven selected sampling events (Fig. III.3.6.1). Module EU-250 samples are a combination of four surface sites, and module EU-150 samples are a combination of five surface samples.

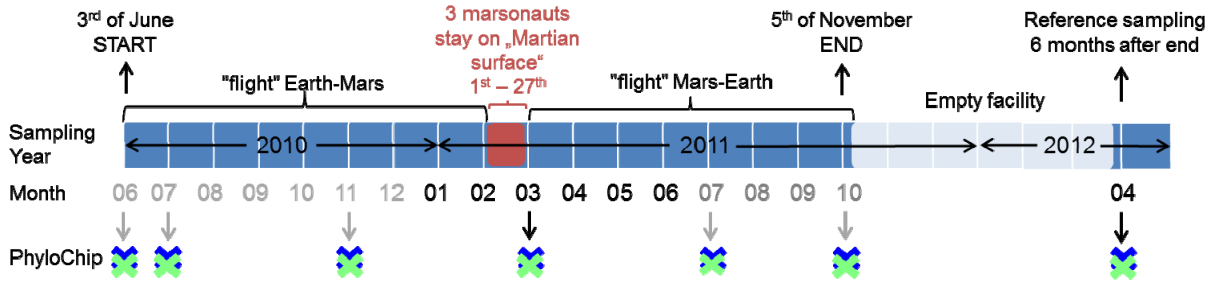

Fig. III.3.6.1 Schematic outline to display samples that were subjected to PhyloChip assay shown as a timeline. Green cross: five swabs were pooled from module EU-150 (habitable); blue cross: four swabs were pooled from module EU-250 (utility)

III.3.6.1. DNA Extraction of MARS 500 Samples

Once the DNA extraction method was optimized (section III.3.4.), the MARS 500 samples were moved on to DNA extraction, which achieved gDNA amounts indicated in Table III.3.6.1.1.

TABLE III.3.6.1.1 RESULTS OF GDNA EXTRACTIONS WITH XS-BUFFER METHOD FROM SURFACE SAMPLES TAKEN AT THE MARS 500 FACILITY. ALL SWABS PER MODULE AND SAMPLING DATE WERE POOLED DIRECTLY IN EXTRACTION BUFFER.

Sampling date (point in time)	gDNA yield [ng] in 100 µl[a]	
	EU-250 (n=4)	EU-150 (n=5)
06/2010 (1)	14	45
07/2010 (2)	18	62
11/2010 (3)	42	31
03/2011 (4)	55	41
07/2011 (5)	11	25
10/2011 (6)	5*	31
04/2012 (7)	37	51
SB (0)	OOR	OOR
EB	OOR	OOR

[a]2 µl of each extraction were measured using Qubit® dsDNA HS Assay

*5 µl were measured instead of 2 µl

SB=swab blank; EB=extraction blank; OOR=out of range, due to too low DNA content

In all, gDNA amount extracted from module EU-150 (habitable) ranged between 25 and 62 ng per 100 µl, whereas values from 5 to 55 ng per 100 µl were obtained for module EU-250 (utility). None of the negative samples contained any detectable gDNA. The amount of extracted gDNA per swab was lower in module EU-250 compared to module EU-150 with only two exceptions (at points 3 and 4 in time). Thus, the amount of DNA was assumed to be sufficient to get PCR amplification products (section III.3.3.; Fig. III.3.5.1). Only sample 10/2011 from module EU-250 might have caused problems, due to the low gDNA yield of 5 ng per 100 µl.

Summary:
The DNA extraction process was successful and did not introduce any contamination (negative blanks). In general, the retrieved gDNA yield was low. However, a higher amount of DNA was obtained from the habitable module compared to the utility module. To check the quality of gDNA, representative samples were moved on to PCR (section III.3.6.3.).

III.3.6.2. Comparison of gDNA Yield with Corresponding CFU Data

In order to determine, whether inferences about gDNA concentration (Table III.3.6.1.1) can be drawn from CFU data ("vegetatives" from surfaces, Table III.1.6.2.1), both values were plotted for each sampling point (Fig. III.3.6.2.1 for the utility module; Fig.III.3.6.2.2 for the habitable module). Furthermore, Pearson correlation was calculated for both modules to see, if a correlation or at least a trend could be observed, and if the results were statistically significant.

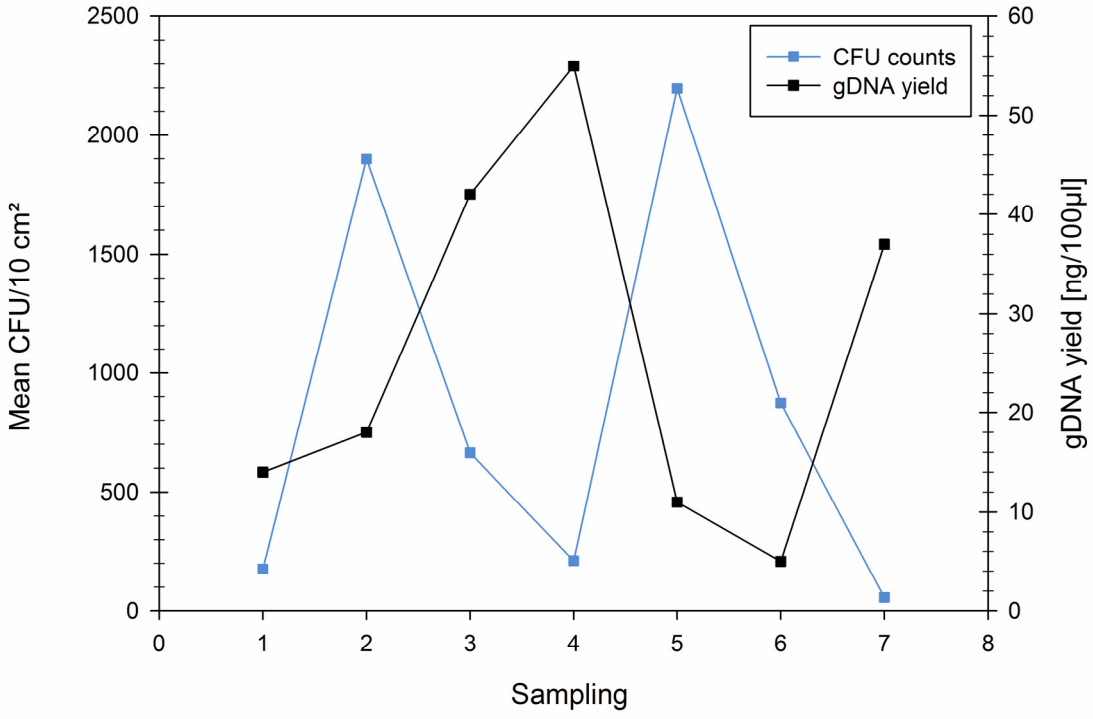

Fig. III.3.6.2.1 Microbial "vegetative" colony forming unit (CFU) counts per 10 cm² of surface samples from the utility module and corresponding gDNA yield per ng/100 µl from parallel samples. CFU data are expressed as mean (n=5) values. For reasons of clarity and due to the high variations, standard deviations are not displayed.

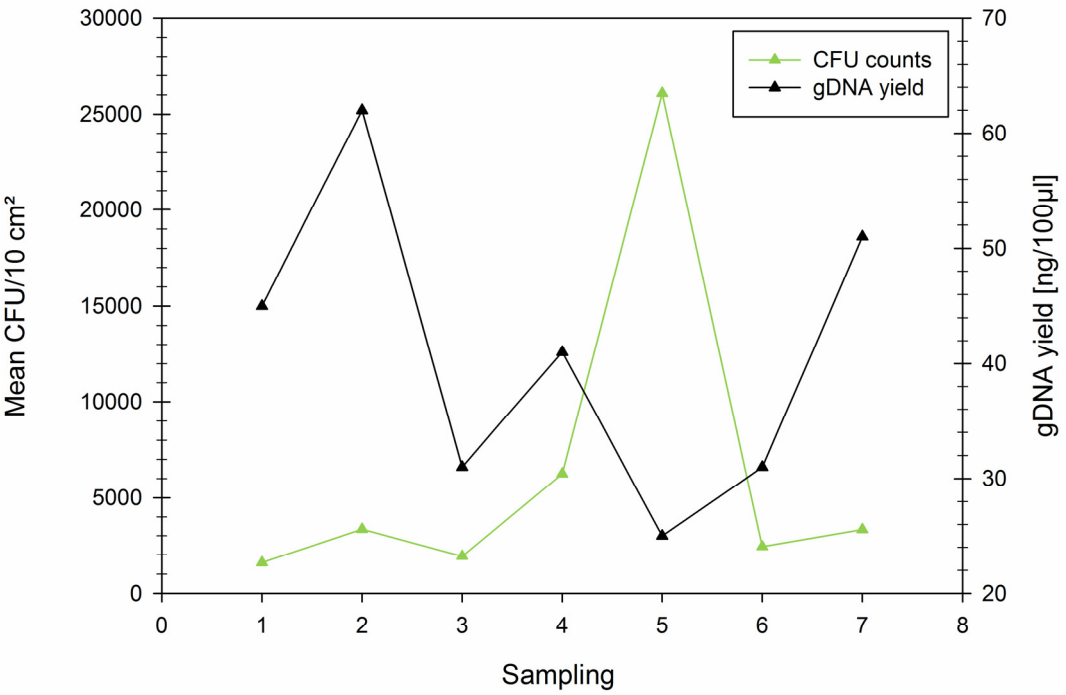

Fig. III.3.6.2.2 Microbial "vegetative" colony forming unit (CFU) counts per 10 cm² of surface samples from the habitable module and corresponding gDNA yield per ng/100 µl from parallel samples. CFU data are expressed as mean (n=4) values. For reasons of clarity and due to the high variations, standard deviations are not displayed.

Both plots indicate that local maxima and minima of gDNA yield and the corresponding CFU value per sampling point dissent from each other. No statistical correlation (p-value >0.05) was observed for MICHAm samples.

Summary:
As already detected for DLR surface samples, there was also no correlation between gDNA content and measured "vegetative" CFU count.

III.3.6.3. Quality Check of gDNA

Prior to shipment of the samples to Second Genome, the gDNA quality and PCR efficiency were checked. Therefore, the TaKaRa ExTaq® PCR Kit was used. At that time, I was informed by Second Genome that this is the appropriate kit, which they are applying to process the samples. During several teleconferences, all parties involved agreed upon the substitution of water by gDNA template to increase the input in case the concentration of gDNA is insufficient. Therefore, a 50 µl PCR reaction was setup by adding 4 µl DNA from the samples 06/2010, 07/2010, and 10/2011 of module EU-250 (utility; Table III.3.6.1.1). To minimize the loss of gDNA, only three representative samples were used. During the following PCR reaction, initial gDNA amounts of 1.3 ng (06/2010), 2.5 ng (07/2010), and 1.2 ng (10/2011) were amplified and the products were visualized on an agarose gel.

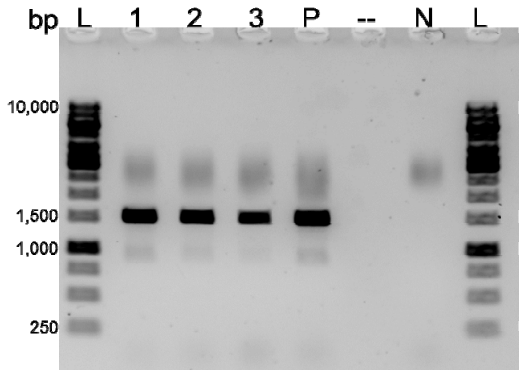

Fig. III.3.6.3.1 PCR results (TaKaRa ExTaq® protocol) visualized on an agarose gel of DNA extracted from swab samples taken at the utility module (EU-150) within the MARS 500 facility using XS-buffer method. Lanes: L, 1 kb ladder; 1, 06/2010; 2, 07/2010; 3, 10/2011; P, positive control: colony PCR from *S. cohnii*; --, empty lane; N, negative control: water template

As seen in Fig. III.3.6.3.1, amplification of the 1.5 kb 16S rDNA gene occurred in all tested samples, but not in the negative control. The clear and bright bands indicate a flawless amplification procedure with a high efficiency rate and thus point to high quality gDNA. In fact, the amount of amplicons from MICHAm samples was almost as high as for the positive control.

Summary:
Pooling (directly in XS-buffer) of swabs followed by gDNA extraction and amplification (TaKaRa ExTaq® protocol) resulted in high quality gDNA. The initial gDNA concentrations (1.2 to 2.5 ng per 4 µl) per reaction were low, but still sufficient to get visible PCR products (approximately 500 ng) of the expected size. Therefore, gDNA could be shipped to Second Genome for further processing.

III.3.6.4. Amplification of gDNA and Hybridization

Once the samples arrived at the laboratories of Second Genome, the gDNAs' concentrations were measured once again, followed by PCR amplification. All corresponding values are stated below in Table III.3.6.4.1.
It has to be noted that Second Genome changed the handling procedure arbitrarily as follows: The 50 µl (or 60 µl) delivered per sample were concentrated to 4 µl. This amount was split and inserted in two parallel PCR assays. Unfortunately, we were not informed about the concentration step, especially since it was shown beforehand that the requested amount of amplification product can be reached (section III.3.3.; section III.3.6.3.).
Due to the concentration step, Second Genome used gDNA amounts of approximately 1.5 to 15 ng as templates for PCR (Table III.3.6.4.1). The obtained results were very heterogeneous regarding gDNA input and PCR yield. After PCR, the mean yield (n=2) ranged from 47.5 to 301.0 ng. Based on these results, all samples revealed more than 100 ng for hybridization with one exception (EU-150-03/11), where only 95 ng were obtained but moved on regardless. In fact, PCR yield of both reactions from sample EU-150-04/12 exceeded the demanded 500 ng. Additionally, the efficiency rate was partially extremely low

(minimal multiplication factor of five) and did not exceed 72-fold increase. Samples with high gDNA content (e.g., EU-250-11/10, EU-250-03/11, EU-150-06/10, EU-150-04/12) exhibited the worst efficiency during the amplification reaction (5-fold increase to a maximum of 21).

TABLE III.3.6.4.1 RESULTS AFTER DNA EXTRACTION BY MYSELF, 16S RDNA AMPLIFICATION AND HYBRIDIZATION OF SAMPLES THAT WERE PREPARED FOR FURTHER PHYLOCHIP ANALYSIS. BLUE: SAMPLES FROM THE UTILITY MODULE; GREEN: SAMPLES FROM THE HABITABLE MODULE, MM/YY

Sample ID	Concentration gDNA [ng/µl]	Amount shipped total mass [ng]	gDNA template (calculated) per reaction	Mean PCR yield per reaction n=2 (ng)	Multiplication factor	Hybridized product (ng)
EU-250-06/10	0.135	6.75	3.24	126.5	39	253
EU-250-07/10	0.180	9.00	4.32	228.5	53	457
EU-250-11/10	0.419	20.95	10.06	210.0	21	420
EU-250-03/11	0.545	27.25	13.08	265.0	10	530
EU-250-07/11	0.112	5.60	2.68	107.5	40	215
EU-250-10/11	0.053	3.168	1.53	111.0	72	222
EU-250-04/12	0.366	18.30	8.78	106.5	12	213
EU-150-06/10	0.449	22.45	10.78	109.0	5	118
EU-150-07/10	0.620	31.00	14.88	166.0	11	332
EU-150-11/10	0.307	15.35	7.37	185.0	25	370
EU-150-03/11	0.405	20.25	9.72	47.5	5	95
EU-150-07/11	0.254	12.70	6.10	207.5	34	415
EU-150-10/11	0.309	15.45	7.42	205.0	28	410
EU-150-04/12	0.511	25.55	12.26	301.0	25	602
Blank	0	0	0	0	0	0

Summary:
In general, amplicon amounts between 100 and 500 ng were obtained with only two exceptions namely EU-150-03/11 (95 ng) and EU-150-04/12 (602 ng). PCR was not run under optimal conditions, as indicated by the general low multiplication factors, and thus low efficiency rates were observed. Nevertheless, all samples were subjected to the next steps (fragmentation, labeling and hybridization, section II.8.6.).

III.3.6.5. Community Characterization

The following section presents the overall results of the molecular microbial community regarding richness, diversity, and taxonomic composition of each sample. The more detailed analyses concerning microbial changes over time, influence of diverse metadata, identification of potentially pathogenic species, and the comparison of data derived from molecular approach with cultivation studies are provided in following sections (III.3.6.6 to III.3.6.12, Fig. III.3.6.5.1).

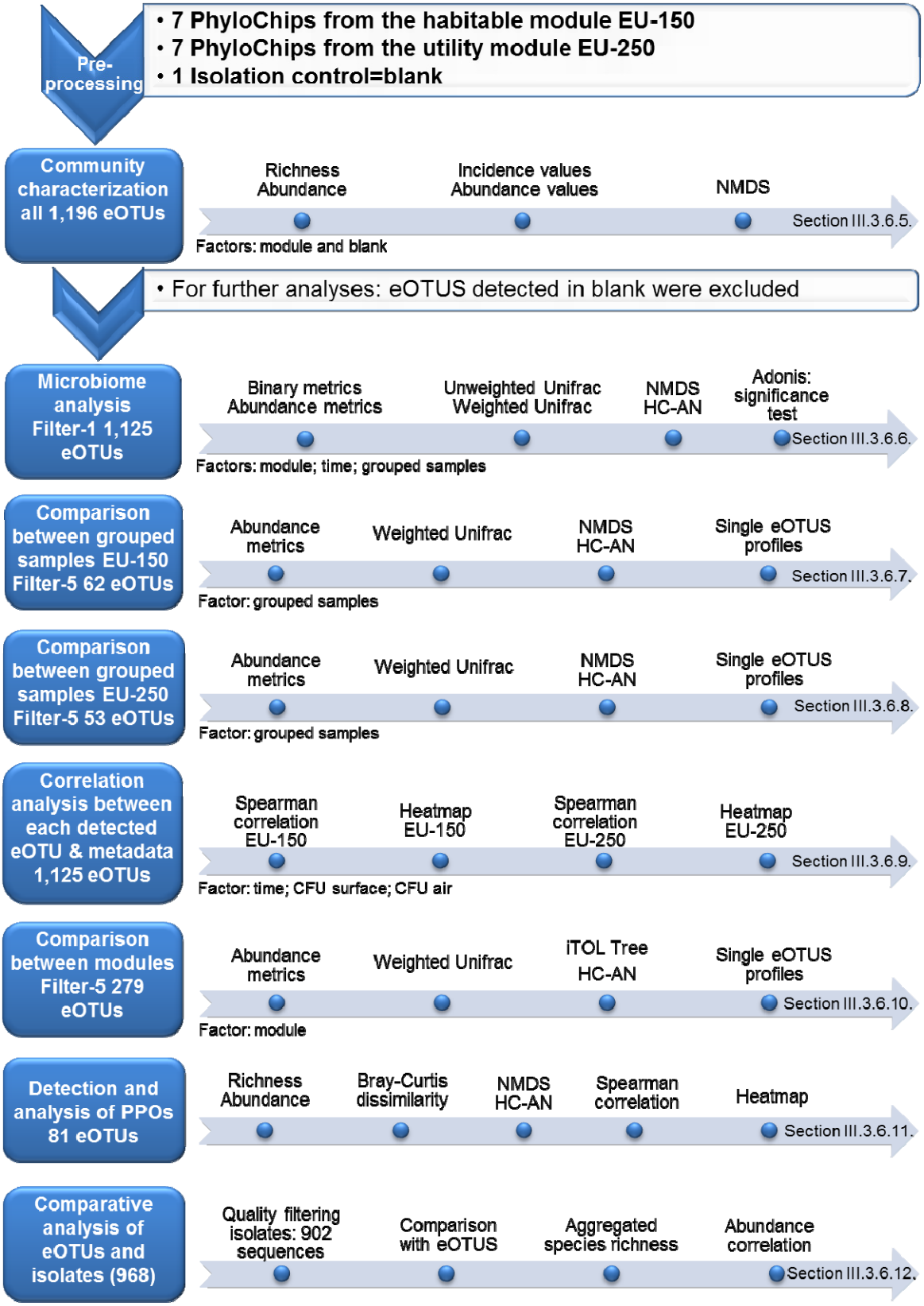

Fig. III.3.6.5.1 Schematic outline of the analytical strategy of PhyloChip data designed to identify the microbial community structure and its changes over time. eOTU=empirical operation taxonomic unit; HC-AN hierarchical clustering using average-neighbor method; iTOL=interactive Tree Of Life; NMDS=non-metric multidimensional scaling; PPO=potentially pathogenic organism

After PCR, hybridization of amplicons on the array, and rank normalization of the hybridization score[16] (HybScore) for each array, including the control array, 1196 empirical operational taxonomic units (eOTUs) were determined. The HybScore for an eOTU was calculated as mean fluorescence intensity of the perfectly matching probes, exclusive of the maximum and minimum (Probst *et al*., 2014). It has to be noted that the herein applied PCR setup, using primers 27F and 1492R, can also amplify archaeal 16S rRNA gene signatures but is not specific for this phylum. Applicable for all samples is the observation that no archaeal biosignatures were found.

The overall community analysis of both modules, displayed in Fig. III.3.6.5.2, revealed that approximately 95 % of all eOTUs are assigned to the four phyla: Proteobacteria (41 %, mainly γ-Proteobacteria, followed by α- and β-Proteobacteria); Firmicutes (34 %, two thirds represent Clostridia, remaining are bacilli and unclassified); Bacteroidetes (11 %, mainly Prevotella); and Actinobacteria (8 %, almost exclusively Corynebacteria). The distribution of those four phyla is almost identical on the module level, but revealed differences in more resolved taxonomic levels.

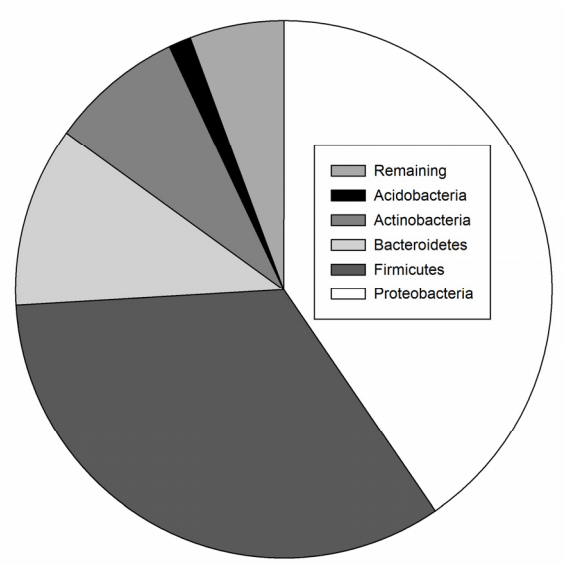

Fig. III.3.6.5.2 Percentage distribution of the overall selected eOTUs on phylum level. Pie slice assigned to "remaining" consists of 14 different phyla.

The bacterial taxon richness distribution on the genus level was plotted (Fig. III.3.6.5.3), indicating that the bacterial richness obtained from the isolation control sample (IsoCntrl=blank) is the lowest of all samples. Generally, quantitative predications, i.e., amount of gene copies per genus, were not possible due to the rank normalization of the original data. Furthermore, eOTUs were assigned as unclassified when the comparison of the individual probe set with the Greengenes databank revealed a sequence that is deposited there as unclassified. Only 32 different bacterial genera were found in the blank,

[16] Abundance metric used to compare relative changes in a taxon's population across samples. It is derived from the background-substracted fluorescence intensity of the multiple probes within a probe set (Second Genome).

whereas the bacterial genus richness for samples from the habitable module (EU-150) ranged from 101 to 139, and 130 to 171 for the utility module. In all, samples from the latter module exhibited in almost all cases a greater diversity than in module EU-150. The significance of the observed difference between both modules was statistically proven by application of a non-paired, heteroscedastic student's t-test that revealed a p-value smaller than 0.05. For both modules, fluctuations in the microbial community structure could be detected over time without showing a strict positive or negative trend.

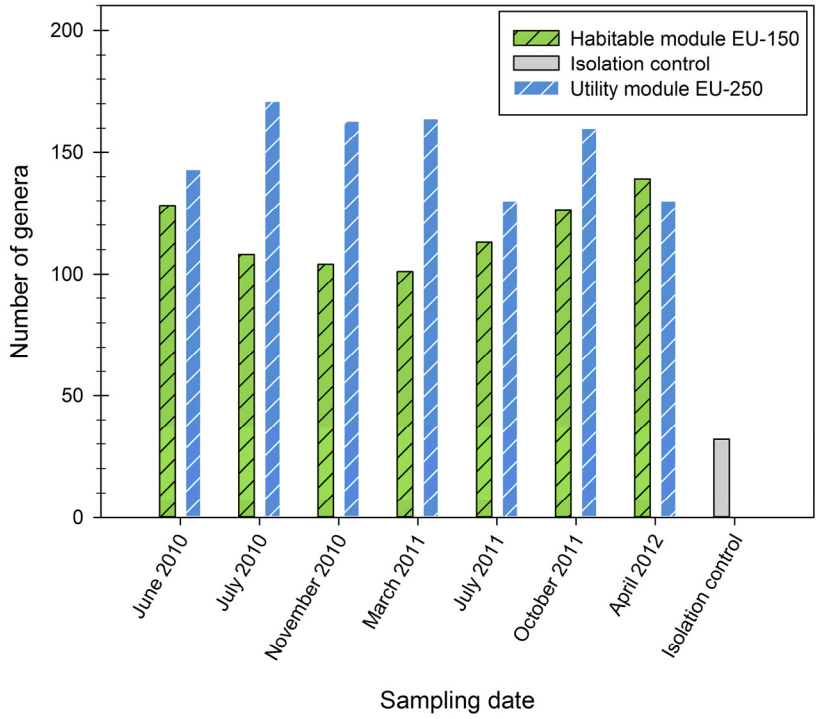

Fig. III.3.6.5.3 Bacterial taxon richness on genus level based on binary data (presence/absence) and taxa that are present in at least one sample.

In order to prove that the blank sample is a potential outlier sample, a NMDS (non-metric multidimensional scaling) analysis based on Bray-Curtis (quantitative similarity measure) distance between samples, given presence/absence as well as abundance of 1196 taxa present in at least one sample (stress value[17]: 0.1062 or 0.1298, respectively), was performed. The reasonable assumption was confirmed. In general, the eOTU determination for abundance plots and incidence plots were based on two different statistical scripts. It is important to remember that the selection procedure for eOTUs based on absence/presence values is more stringent and programmed in a way to minimize the identification of false negatives. Plots are not shown here, but can be viewed on the data CD (folder: PhyloChip: Second Genome Microbial Profiling Report, p 5f), and clearly displayed a separation of the blank PhyloChip from the other samples. Furthermore, only 71 eOTUs out of 1197 eOTUS were detected in the blank, which is a good indicator for sterile sample processing.

[17] The stress value is a measure of the quality of the configuration. The higher the value, the worse the configuration meets the monotonicity requirements.

Summation of HybScores (relative values) from eOTUs of each taxonomic family allowed the calculation of proportional abundance on the family level, and therefore comparisons between samples. The aggregated HybScores revealed that on the family level, richness patterns were not uniform across the samples. Empirical OTUs within the *Lachnospiraceae* and *Comamonadaceae* made up the major part (more than 15 %) of the overall HybScores. *Pseudomonadaceae*, *Ruminococcaceae*, *Corynebacteriaceae*, and *Rikenellaceae II* were also among the most abundant families. Cultivation results (section III.1.9.) led to the assumption that a high HybScore value would be found for *Staphylococcaceae*. However, even the top ten families based on PhyloChip did not reveal *Staphylococcaceae* signatures (data CD, [folder: PhyloChip: Second Genome Microbial Profiling report, p 3f]).

Summary:
Since no archaeal genera were detected, there is a major prevalence of bacterial gDNA in the samples. The small number of eOTUs in the IsoCntrl pointed explicitly towards an adequate handling during sampling and DNA extraction, as well as towards the cleanliness of the sampling tools and chemicals used. The assumption that microbial diversity will increase or decrease over time was not confirmed. Regarding the low abundance of *Staphylococcaceae*, further investigation and information about the probe design for identification of *Staphylococcaceae* on the PhyloChip are required.

III.3.6.6. Whole Microbiome Analysis

To investigate β-diversity[18], data from the whole microbiome was taken into account. Another goal was to reveal differences or commonalities between samples and modules.
As the previous analysis showed that the blank is an outlier sample, the taxa detected therein were removed from all data so that the following data analysis was based on 1125 eOTUs present. Abundance and binary metrics (NMDS) were run on the new filtered eOTU data (applying filter 1) instead of 1196. This was followed by weighted and unweighted UniFrac[19] analysis, as well as principal co-ordinates analysis (PCoA), hierarchical clustering using average neighbor method (HC-AN), and Adonis tests (section II.8.6.).
In a first step NMDS analysis based on abundance Bray-Curtis distance was recalculated (without taxa from IsoCntrl) and the result is displayed in Fig. III.3.6.6.1.
A separation of the microbiome of samples from module EU-150 versus EU-250 was noticed. This observation was verified by applying Adonis test, revealing a significant difference (p-value of 0.003[20]).

[18] ß-diversity: species diversity is determined by differentiation among sampling locations

[19] UniFrac is a widely used method to calculate the distance between grouped samples and provided the basis for following standard multivariate statistical methods (Lozupone and Knight, 2005).

[20] Adonis test and significance summary of all analyses carried out can be viewed on data CD (folder: PhyloChip: Second Genome Microbial Profiling Report, p 18).

This effect was even greater (Adonis test; p-value 0.002) when calculating NMDS on presence/absence of the 1125 taxa, and additionally displayed a tight grouping of samples from module EU-250 (utility).

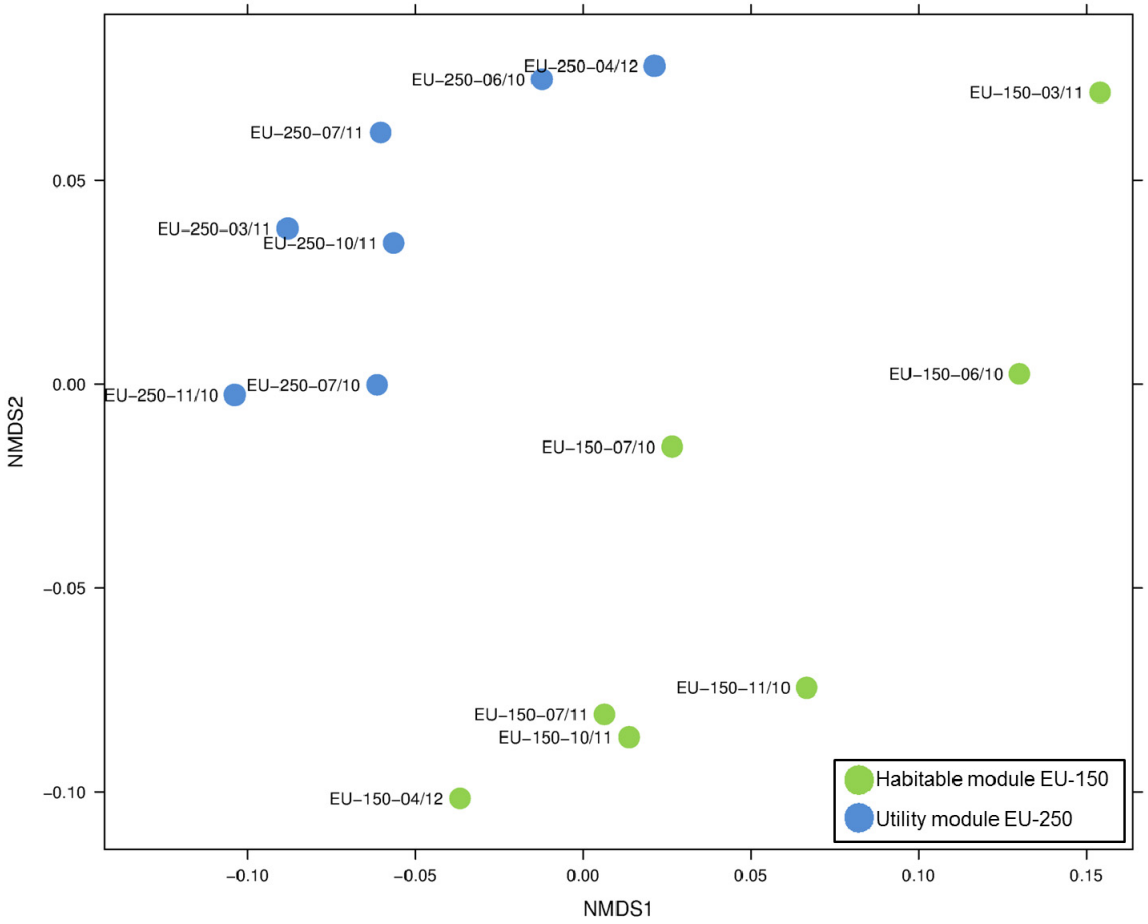

Fig. III.3.6.6.1 NMDS (non-metric multidimensional scaling) based on Bray-Curtis distance between samples given abundance of 1125 taxa present in at least one sample; stress=0.1417

Computation of a NMDS based on abundance Bray-Curtis distances between samples was performed to compare each single point in time (1 to 7) of both modules with each other. For example, the June of 2010 (time 1) sample of module EU-150 was compared to the corresponding sample of module EU-250. Additionally, significance calculation (Adonis test) yielded a p-value of 0.748, indicating that samples collected at different times in module EU-150 are not distinct from the ones of module EU-250 (data CD, [folder: PhyloChip: Second Genome Profiling Report, p 18). However, it has to be noted that with only two samples per group, an insufficient number of replicates were present in each group.

The same effect (no distinct separation) was detected for both modules, when samples of one module were grouped in early (June of 2010, July of 2010), mid (November of 2011, March of 2011, July of 2011), and late (October of 2011, April of 2012) and analyzed with NMDS. Identical stress values of 0.1417 were obtained for both modules (plots not shown here; please refer to data CD [folder: PhyloChip: Second Genome Profiling Report, p 10f]). Intra-module comparisons of the configuration of data points in the plot displayed no grouping of the samples referring either to early, mid, or late stage. P-values (0.302 for

module EU-150 and 0.186 for module EU-250, respectively) obtained using the Adonis test confirmed the findings by NMDS analysis.
No separation was observed when applying HC-AN analysis with regard to any of the above-mentioned categories, except for category module (data CD, [folder: PhyloChip: Second Genome Microbial Profiling Report, p 12 and p 17]).

Summary:
Multivariate ordination analysis and HC-AN, based on either abundance or incidence values revealed a significant separation of the microbiome of the habitable (EU-150) and the utility (EU-250) module. This result was confirmed by Adonis test (p-value <0.05), and therefore indicated that hitherto unknown factors influenced the bacterial community in each module so that further analysis is necessary. No distinct separation in each single module was found based on single points of time (1 to 7), or on early, mid, and late points consisting of pooled samples. A significant correlation was found between the amount of PCR product that was hybridized and the calculated Bray-Curtis distances based on abundances.

III.3.6.7. Comparison between Grouped Samples of the Habitable Module

To identify those eOTUs that are significantly increased or decreased within the grouped (early, mid, late) samples of the habitable module, the previously 1125 identified taxa were filtered. Therefore, filter 5 was applied, selecting for taxa that showed an increased/decreased abundance in one category (for example early) compared to an alternate category (mid and/or late). Significance of results was tested using parametric Welch test with the restriction that there were not sufficient samples (minimum n=3) per group in two out of the three categories (n=2 for early and late). However, this was due to the amount of available resources, which led to the above-mentioned design (Fig. III.3.6.1). In order to avoid the detection of false selection of eOTUs and to evaluate and correct the results if necessary, q-values were calculated by employing Benjamini-Hochberg procedure. After application of filter 5, 62 taxa remained for further calculation of abundance metrics (NMDS), Weighted UniFrac analysis, PCoA, and HC-AN evaluation.
NMDS and hierarchical clustering indicated a significant separation of microbiome communities due to formation of distinct clusters after applying filter 5 for sample categories early, mid, and late of module EU-150. The figures can be viewed in detail on the data CD, ([folder: PhyloChip: Second Genome Microbial Profiling Report, p 20f]). Additionally, a correlation of single eOTUs with the factor time was performed by comparisons of mean abundance rates (rank normalized hybridization values) of each eOTU per one time category samples with the two remaining time categories samples. P-values indicated the significance of the generated profiles (data CD, [folder: PhyloChip: Second Genome Microbial Profiling Report, p 22]).

Exemplary samples are shown for the eOTUs which generated the most distinct plots and the lowest p-values (680 *Halonella*, 63 R*oseomonas*, 531 *Microbacterium*, and 715

Acinetobacter johnsonni; Fig. III.3.6.7.1). The first three mentioned examples displayed a significant increase in late (yellow) samples as compared to early (green) and mid (blue).

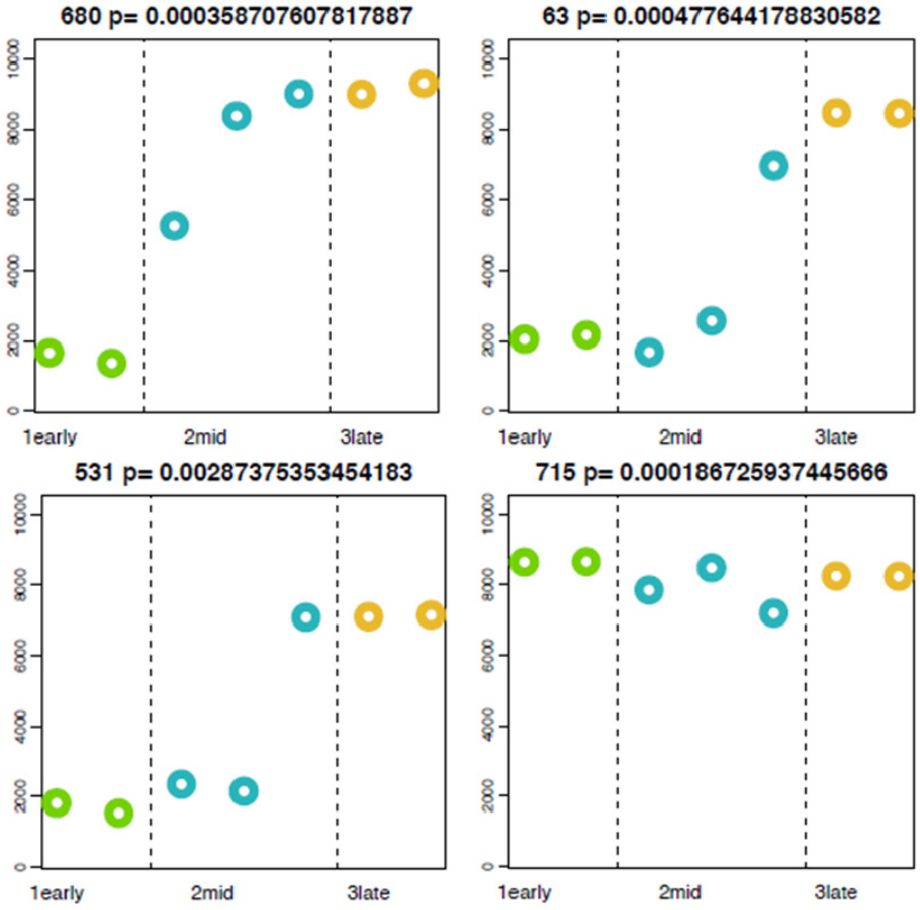

Fig III.3.6.7.1 Profiles of eOTUs with significant p-values (<0.05). Numbers shown at top left of each plot refer to eOTU-ID (680: *Halonella*, 63: *Roseomonas*, 531: *Microbacterium*, and 715: *Acinetobacter johnsonni*). P-values of each eOTU plot are unadjusted for multiple testing. The y-axis represents the HybScore. Samples are grouped, arranged and colored by category time along the x-axis in the following order: Green refers to EU-150-06/10 and EU-150-07/10, blue represents samples EU-150-11/10, EU-150-03/11 and EU-150-07/11, and yellow stands for samples EU-150-10/11 and EU-150-04/12.

Empirical OTU 715 (*Acinetobacter johnsonni*) indicated less fluctuation, but was present on a relatively high level (mean HybScore over time is approximately 8,200), whereas a steady decrease could be identified for eOTU 102 (*Burkholderia*, data not shown).

Summary:
62 taxa revealed a distinct microbiome based on abundance metric between samples of the habitable module that were grouped in early, mid and late. Furthermore, HC-AN analysis detected a clustering of microbiomes of early samples from mid and late samples. In general, no distinct trend was observed in abundances of eOTUs that belong to one particular taxon at certain times. The majority of the selected 62 eOTUs were assigned to Proteobacteria (25), followed by Firmicutes (15), Bacteroidetes (12), Actinobacteria (7), Thermi (2), and Planctomycetes (1).

III.3.6.8. Comparison between Grouped Samples of the Utility Module

The same analysis as described in the previous chapter was also performed for the second module (EU-250). Once applying filter 5 on Bray-Curtis distance analysis (overall data set), 53 eOTUs were selected for module EU-250 (utility).
Elaborate results of NMDS analysis and hierarchical clustering can be viewed on the data CD (folder: PhyloChip: Second Genome Microbial Profiling Report, p 25f). Both analyses indicated a significant separation of the 53 eOTUs from early, mid, and late time categories.

Representative eOTUs are displayed that generated the most distinct plots and the lowest p-values (1143 *Comamonadaceae*, 1227 *Enterobacteriaceae*; Fig. III.3.6.8.1). *Sphingomonas* (384) is an example for accumulation of gene signatures over time, whereas *Lachnospiraceae* (772) was present on a relatively high level and varied only between HybScores of 800 and 1,000.

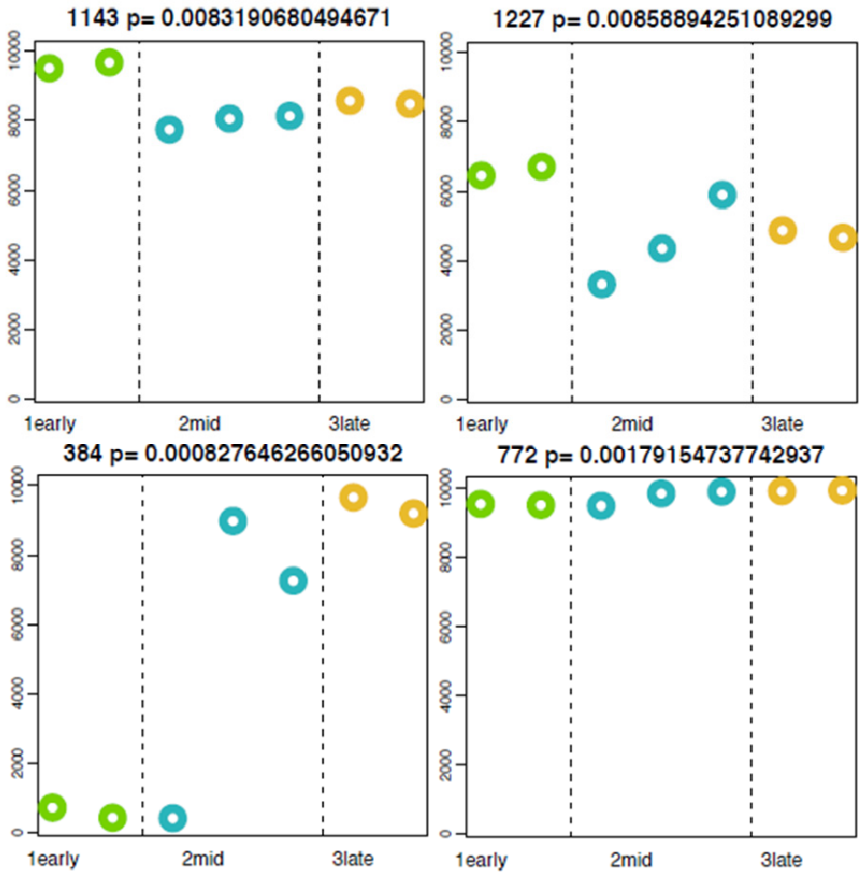

Fig. III.3.6.8.1 Profiles of eOTUs with significant p-values (<0.05). Numbers shown at top left of each plot refer to eOTU-ID (1143: unclassified *Comamonadaceae*, 1227: unclassified *Enterobacteriaceae*, 384: *Sphingomonas*, and 772: *Lachnospiraceae*). P-values of each eOTU plot are unadjusted for multiple testing. The y-axis represents the HybScore. Samples are grouped, arranged and colored by category time along the x-axis in the following order: Green refers to EU-150-06/10 and EU-150-07/10, blue represents samples EU-150-11/10, EU-150-03/11 and EU-150-07/11, and yellow stands for samples EU-150-10/11 and EU-150-04/12.

Summary:

As detected for the habitable module, there was no distinct trend observed for eOTUs that belong to one taxon regarding their abundance in the utility module at certain times.
In all, 53 taxa showed significant differences in their abundance from samples collected at different times. The majority of the selected 53 eOTUs were assigned to Proteobacteria (20), followed by Firmicutes (18), Actinobacteria (8), Bacteroidetes (2), Fusobacteria (4) and Planctomycetes (1). Clustering according to this factor was displayed by both methods, NMDS based on abundance and HC-AN.

III.3.6.9. Correlation between Each Detected eOTU per Module and Metadata

The MARS 500 habitat is a complex environment being influenced by a variety of different factors that may also affect the microbial community structure.
To investigate the effect of environmental factors like partial O_2 and CO_2 pressures, temperature, and relative humidity, a Spearman rank correlation was performed to look for those eOTUs that reveal significant correlation with each of the above-mentioned factors. The Spearman rank correlation is a non-parametric measure of statistical dependence between two variables. Since it is based on the allocation of ranks, it minimizes the effect of outliers. How well the relationship between two variables can be described is assessed by use of a monotonic function. Obtained values of +1 and -1 indicate a perfect Spearman correlation, with positive values referring to monotone increase and negative to decrease. No significant results were obtained for any metadata factors as can be looked up in Table III.3.6.6.1, indicating p-values >0.05. This observation may be accounted for time differences of up to five days between acquisition of environmental data and day of sampling, especially when considering the fluctuation that were detected between two sampling points over the whole period of time (Fig. III.1; Fig. III.2.1). Consequently, making predictions regarding the prevailing environmental values on the sampling day is nearly impossible.
Furthermore, Spearman rank correlations were applied to identify those eOTUs (out of 1125) that have a significant correlation with factor time in each module. Results of the correlation were displayed as a heatmap, depicting only those eOTUs of the habitable module that showed a significant correlation with the factor time (Fig. III.3.6.9.1). Empirical OTUs are arranged according to positive and negative correlation by p-value in an increasing manner. The Z-score is computed by taking a data point, subtracting the mean of distribution, and then dividing the whole term by the standard deviation. The probability of a score that occurs within the normal distribution can be calculated. Thus, the relative abundance values were transformed into Z-scores. A negative Z-score means the observed HybScore is below the mean.

- **Habitable module**

In all, 57 different eOTUs, of which many were unclassified at the species level, were found to correlate significantly with factor time (Fig III.3.6.9.1).

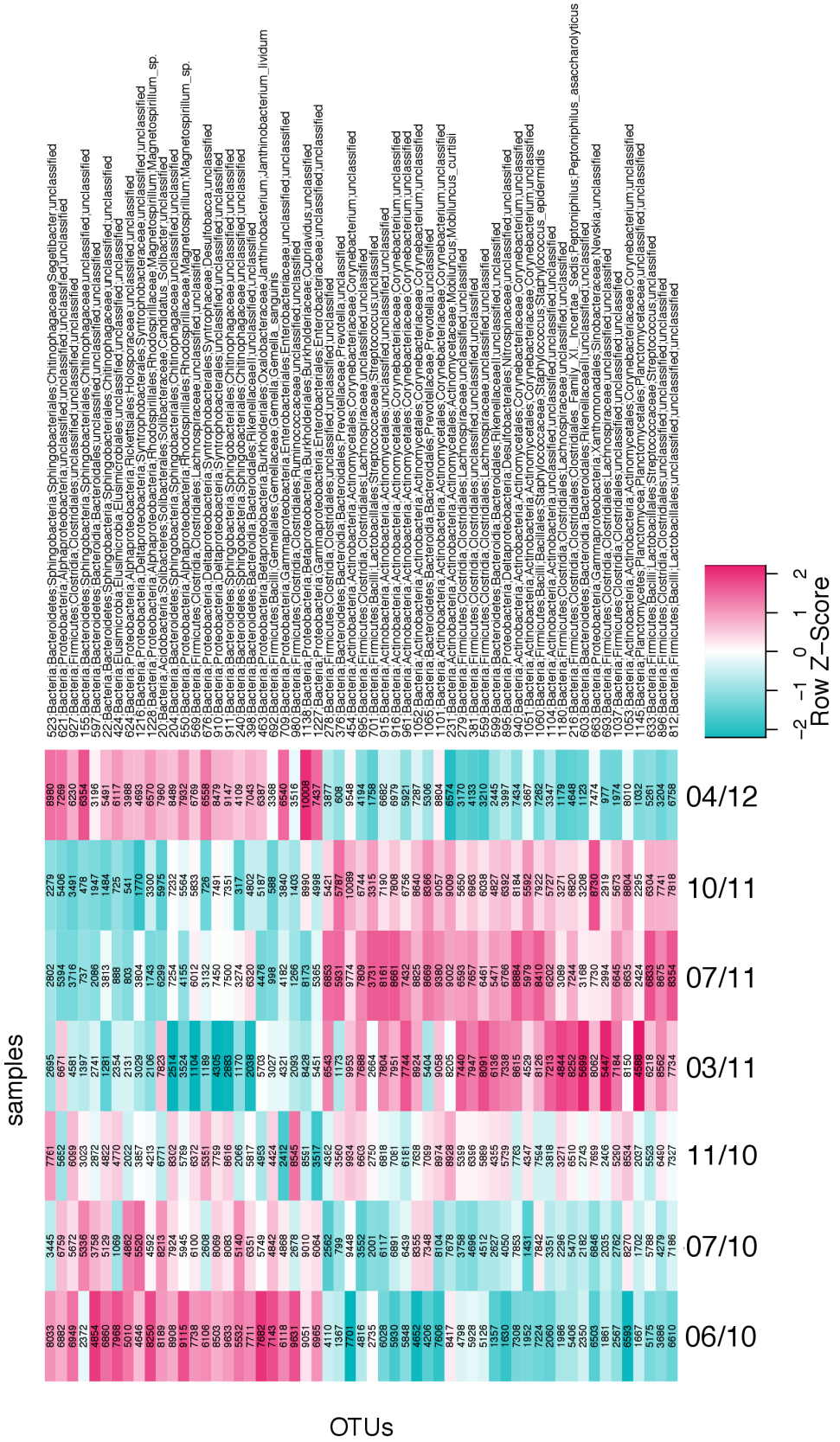

Fig III.3.6.9.1 Heatmap of eOTUs that showed a significant correlation (Spearman, p-value <0.05) with the factor time in module EU-150 (habitable). The eOTUs are ordered by positive (pink) and negative (cyan) correlation and by p-value in increasing manner. Z-score refers to the probability of a HybScore occurring within the normal distribution. A positive Z-score means the observed HybScore is above the mean and *vice versa*. Numbers indicate retrieved HybScores.

While 24 eOTUs decreased over time, 33 eOTUs increased with proceeding confinement. The 24 eOTUs that were less abundant in the end of the confinement compared to the beginning belonged mainly to Proteobacteria (11), followed by Bacteroidetes (8), Firmicutes (4), Elusimicrobia (1), and Planctomycetes (1). In contrast, out of the 33 eOTUs mostly Firmicutes (14), Actinobacteria (11), Bacteroidetes (4), and Proteobacteria (2) were detected. Additionally, one Planctomycetes was found. In the following comparisons, only taxa that were covered by more than one eOTU were taken into account.
All eOTUs assigned to α-, β-Proteobacteria and Sphingobacteria (only *Chitinophagaceae*) strikingly abated during the confinement, whereas Actinobacteria (merely *Corynebacteriaceae*) and Clostridia (including mainly *Lachnospiraceae*) revealed an accumulation over time. Bacilli and Bacteroidia displayed mixed responses.

- **Utility module**

Altogether, 38 taxa were identified which showed a significant abundance correlation with time. Thereof, 21 eOTUs revealed a negative correlation, whereas 17 gene signatures correlated positively (Fig. III.3.6.9.2).
A decline with increasing confinement duration was observed in 21 eOTUs that belonged to Firmicutes (13), Proteobacteria (4), Actinobacteria (3), and Bacteroidetes (1). However, after the facility was left unoccupied for six months, all of those eOTUs increased again and revealed greater HybScores in the post-confinement sampling. For 17 eOTUs a reverse trend was detected. Those were less abundant in the beginning, showed a peak between six to twelve months during the isolation, and a decrease in samples from the post-confinement sampling in April of 2012. This proportion consisted of mainly Proteobacteria (12) and Firmicutes (5).
Regarding eOTUs being representatives of Actinobacteria and Clostridia (*Lachnospiraceae* and *Clostridiales*), a reverse trend was observed compared to module EU-150, where representatives of these phyla increased as isolation duration proceeded. The same is true for α-Proteobacteria (*Sphingomonadaceae*). In contrast, ɣ-Proteobacteria (mainly *Pseudomonadaceae*) accumulated as time of confinement progressed.
It is important to mention that the eOTU assigned to no. 36, *Enterococcus faecalis*, also revealied a significant increase over time.

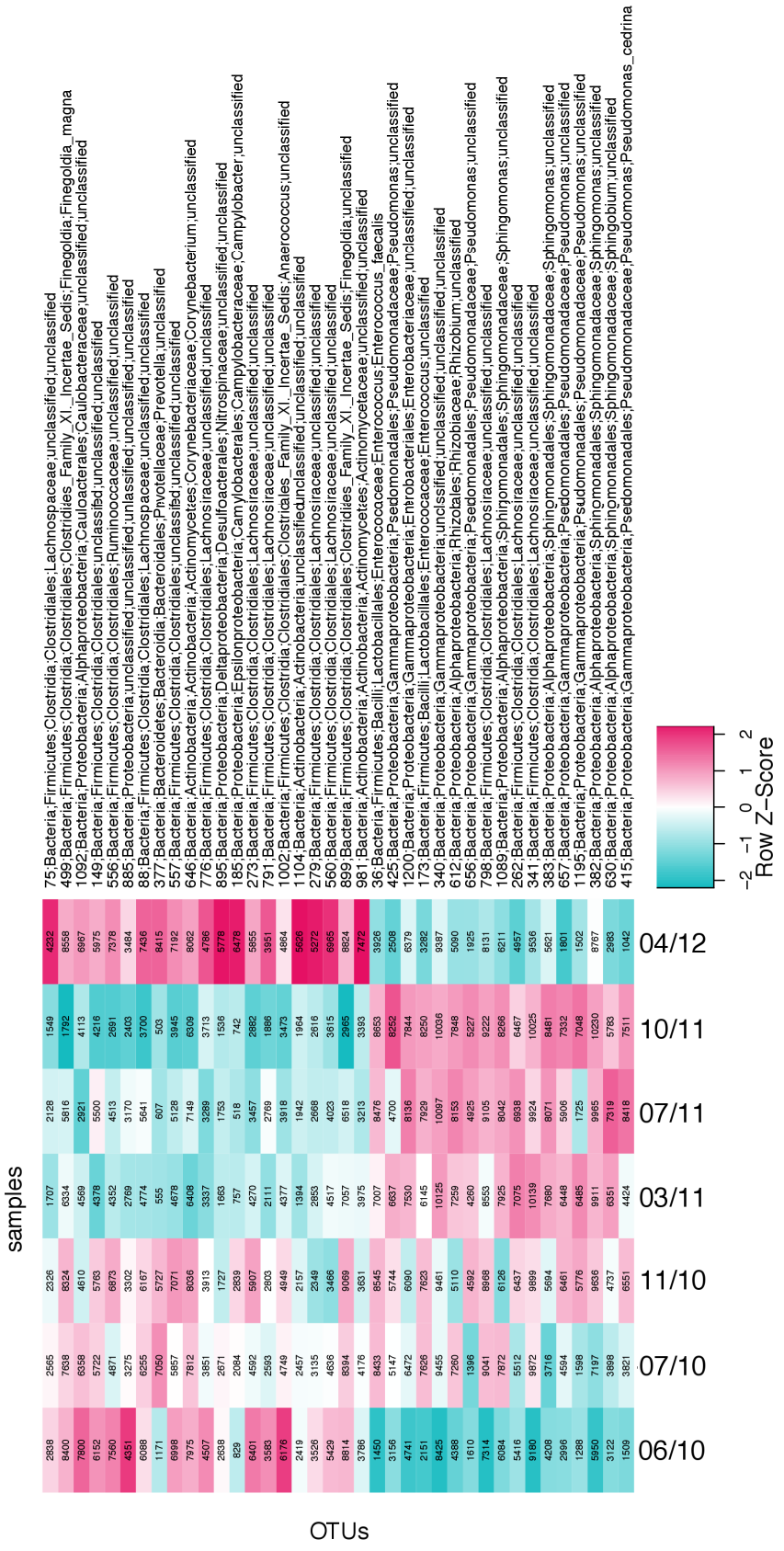

Fig. III.3.6.9.2 Heatmap of eOTUs that showed a significant correlation (Spearman, p-value <0.05) with the factor time in module EU-250 (utility). The eOTUs are ordered by positive (pink) and negative (cyan) correlation and by p-value in increasing manner. Z-score refers to the probability of a HybScore occurring within the normal distribution. A positive Z-score means the observed HybScore is above the mean and *vice versa*. Numbers indicate retrieved HybScores.

Summary:
In both modules only a small fraction of eOTUs, i.e., 56 in the habitable and 34 in the utility module, revealed a significant time correlation. The identified eOTUs mainly belong to Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes and revealed a reverse trend in both modules. Where in the habitable module the majority of representatives of the above-mentioned taxa were increased, they were decreased in the utility module.

III.3.6.10. Comparison between the Two Modules

Once the microbial communities inhabiting each of the modules were characterized in detail, the two modules' microbiomes were compared with each other. Therefore, a parametric Welch test was applied which provided a list of eOTUs that revealed a significant difference regarding increase or decrease of abundance in the respective samples.
After generating abundance metrics (NMDS) and utilizing Weighted UniFrac, PCoA as well as HC-AN tool, phylogenetic differences were investigated and visualized in an interactive Tree of Life (iTOL, Letunik and Bork, 2007).
On the basis of variations in abundance rates per eOTU, 279 taxa passed the filtering. NMDS, based on Bray-Curtis distance between samples in terms of abundance values, revealing significant abundance differences across at least one module, were calculated and plotted (Fig. III.3.6.10.1). The analysis revealed a very good stress value of 0.0553.

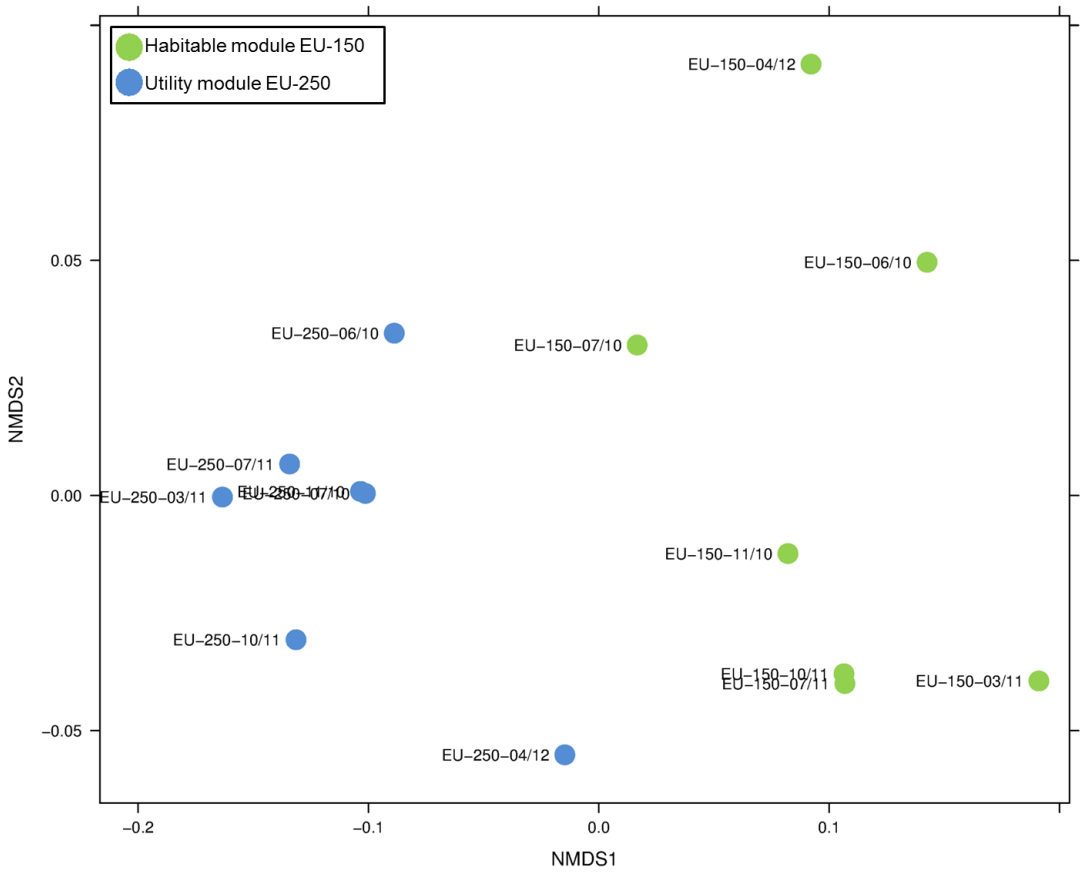

Fig. III.3.6.10.1 NMDS (non-metric multidimensional scaling) based on Bray-Curtis distance between samples given abundance of 279 taxa with significant abundance differences across at least one module; stress=0.0553

Observing the distances between the samples that belonged to one module in comparison with samples from the second module, an obvious separation of the modules' microbial communities could be detected. Significance tests on filtered data are not worthwhile, thus values for these plots were not calculated.
To strengthen this finding, an average linked hierarchical clustering analysis (Fig. III.3.6.10.2) was performed, based on the same data as used for NMDS. Branch lengths indicate the connectivity between samples.

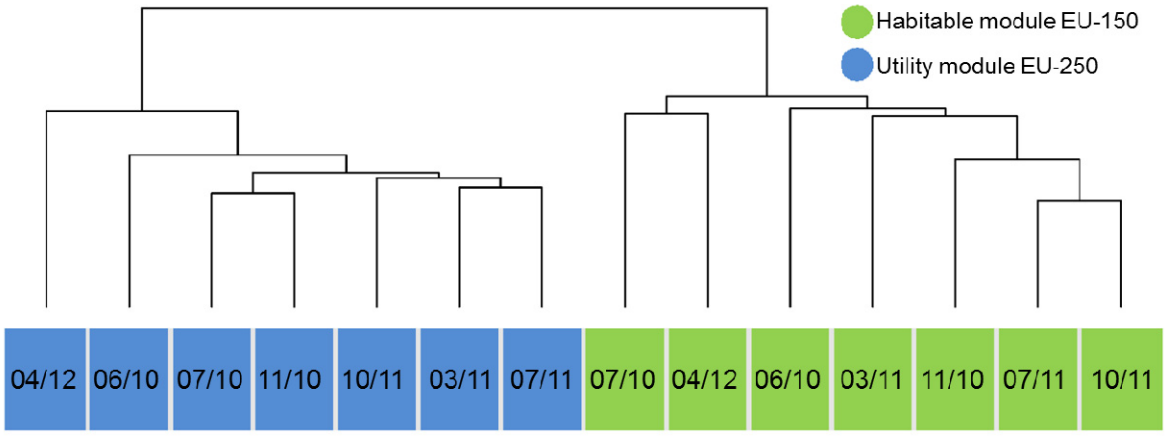

Fig. III.3.6.10.2 Hierarchical clustering (average linkage) based on Bray-Curtis distance between samples given abundance of 279 taxa with significant abundance differences across at least one module (EU-150=habitable; EU-250=utility).

Inclusion of 279 taxa swamped out the differences between samples of both modules due to calculation of clusters from similarity matrices, which revealed the formation of two distinct clusters. Each consisted of only samples from one category (module).
In order to get more insights in abundance differences of single eOTUs between the two modules, profiles were generated based on HybScores (data CD, [folder: PhyloChip: Second Genome Microbial Profiling Report, p 32]).
In comparison to the sum of all HybScores from the samples per module, 123 eOTUs were increased and the remaining were decreased in module EU-150. Referring to the phylum level, the percentage of Actinobacteria that was increased in module EU-150 was higher compared to module EU-250, whereas the number of Bacteroidetes, Proteobacteria and Fusobacteria was significantly increased in module EU-250. No difference was seen for Firmicutes.

Based on the above-mentioned table, only the eOTUs that could be assigned to the family level are explained in more detail. The twelve eOTUs (eight belong to Firmicutes and four to Proteobacteria) that exhibited the lowest p-values were significantly increased in module EU-250 over the whole timeframe. However, only five of them were identified on the family level, revealing four representatives of the *Lachnospiraceae* (eOTUs 348, 810, 700, 809) and one member of the *Hydrogenophilaceae* (eOTU 1133). With regard to *Staphylococcus*, only two eOTUs (952, 1170) showed a significant increase in module EU-250 compared to EU-150.

Additionally, on the genus level, eOTUs with the lowest p-values and the highest difference regarding sum of HybScores of each module were exemplarily selected. The following representatives showed a significant increase in the habitable module: eOTU 694, 216, and 492 were assigned to *Peptoniphilus*; 187 to *Actinobaculum*; 231 to *Mobiluncus*; 491 to *Clostridium*; and 1006 to *Bifidobacterium* (data CD, [folder: PhyloChip: Microbial Profiling Report, p 32]).

To display the changes of eOTUs that showed significant differences between the two modules with regard to their phylogenetic relationships, an iTOL tree was generated. Therefore, eOTUs were selected that are significantly different (p-value <0.05) in one of the modules from the overall microbiome consisting of 1125 eOTUs. The resulting 279 eOTUs were assigned to 69 families. One eOTU from each family was selected that revealed the greatest difference between the two modules. However, within 13 families, eOTUs were detected that showed both, significant increases and decreases in their relative abundances. Regarding these families, both eOTUs were picked as representatives (in total 82). All 16S rRNA genes were aligned and used to create a phylogenetic tree, which is shown below (Fig. III.3.6.10.3).

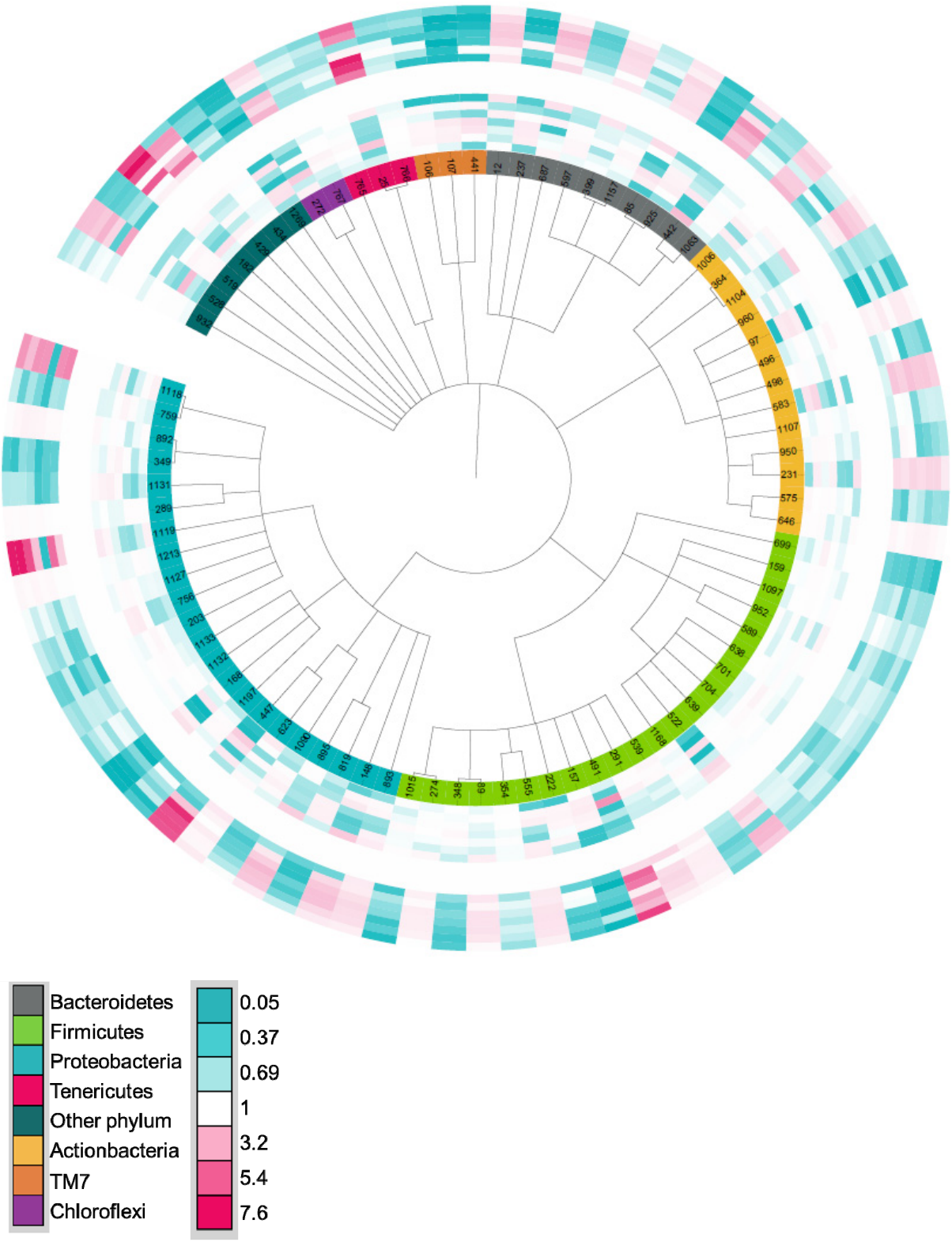

Fig. III.3.6.10.3 Interactive Tree Of Life (iTOL) tree based on 16S rRNA genes of 82 eOTUs that are significantly different (p-values <0.05) when comparing module EU-250 samples (inner rings) and module EU-150 samples (outer rings). The color saturation indicates the degree of difference from the mean EU-250 value; dark blue refers a rank normalized ratio of 0.05; white of 1.0 (steady state samples as reference); dark red of 7.6, respectively. The closer the samples of each ring are to the center of the tree, the earlier the time they were taken.

The selected eOTUs that are representatives of the phyla Chloroflexi, Tenericutes, Bacteroidetes, Actinobacteria, Firmicutes, and Proteobacteria exhibited mixed responses. Exclusively all eOTUs assigned to the candidate division TM7 group generally revealed a decrease in module EU-150.

Summary:
Statistical tools that are based on abundances using Bray-Curtis distance, revealed 279 taxa that are distinct influenced and separated based on the different modules (NMDS and HC-AN). Via iTOL tool, eOTUs thereof were displayed that were affiliated with Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes, Actinobacteria, and Chloroflexi, and exhibited mixed responses. Only eOTUs belonging to TM7 group showed a decrease in the habitable module.

III.3.6.11. Tracking of Specific Pathogens

To ensure the safety and health of the astronauts during long-term spaceflight, the diversity, distribution, and abundances of potentially pathogenic organisms (PPOs) were of special interest, especially when considering that the immune system of astronauts is depressed during spaceflight (Cogoli, 1993).
Consequently, eOTUs that were identified by PhyloChip analysis were compared with a reference catalogue based on the TRBA 466 document that lists an updated version of all pathogenic organisms known so far.
Following the identification of eOTUs as PPOs, statistical tools like NMDS, based on Bray-Curtis distances, and Spearman correlation analysis were applied. Furthermore, HC-AN clustering and heatmaps were compiled.

In all, 81 eOTUs were ascertained as 52 different PPOs species. The proportional distribution in the utility module (EU-250) ranged from 21 to 30, whereas a lower number of 11 to 23 PPOs was detected in the habitable module (EU-150; Fig. III.3.6.11.1). This observed tendency was also reflected when comparing the mean values of both modules, revealing 26 PPOs in module EU-250 versus 16 in module EU-150. By applying a heteroscedastic t-test, a highly significant difference (p-value <0.0002) was retrieved between PPO species richness of both modules.

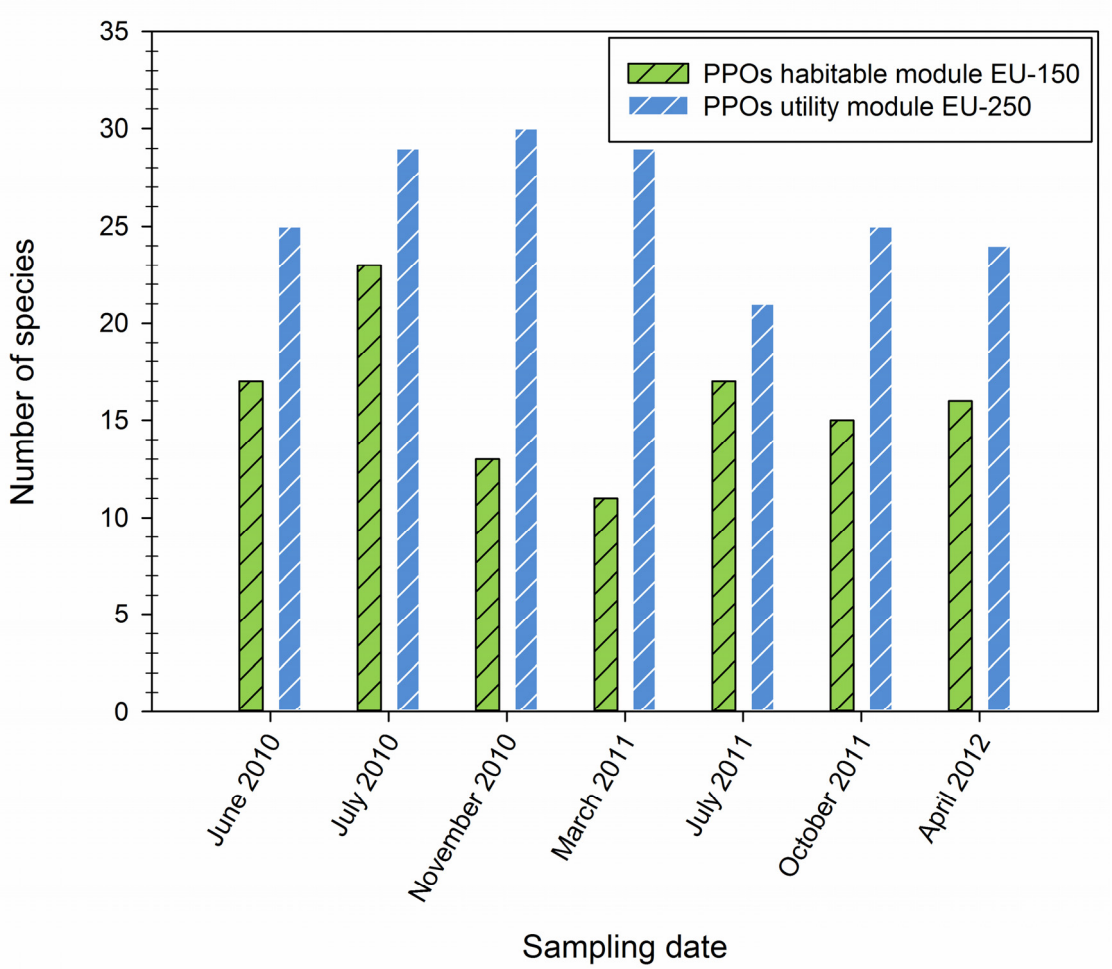

Fig. III.3.6.11.1 Potentially pathogenic organism (PPO) richness on species level grouped per module based on obtained eOTUs (taxonomic characterization from PhyloChip assay) and the reference catalogue of pathogenic organisms (TRBA 466 document ≥level 2).

NMDS analysis based on Bray-Curtis distance between samples given abundance and incidence values of 52 PPOs, and the corresponding HC-AN analyses (data CD, [folder: PhyloChip: Second Genome Microbial Profiling Report, p 50ff]) revealed nearly identical observations. Where the former displayed a trend, the latter outlined a distinct separation. Beyond that, the analyses indicated that samples from module EU-250 exhibit lower intra-group dissimilarities than samples from module EU-150. This result is based on the smaller number of data points from the utility module compared to the data points originating from the habitable module, which are distributed over a greater area.

Additionally, inter-sample relationships were graphically summarized by hierarchical clustering (Fig. III.3.6.11.2). Smaller branch lengths between samples indicate higher similarity between the microbial communities.
The dendrogram displays no entire separation of the microbiome, since one cluster contains all samples of module EU-250 (utility) and one outlier sample of module EU-150 (habitable). In the second cluster the remaining samples of the habitable module are grouped together.

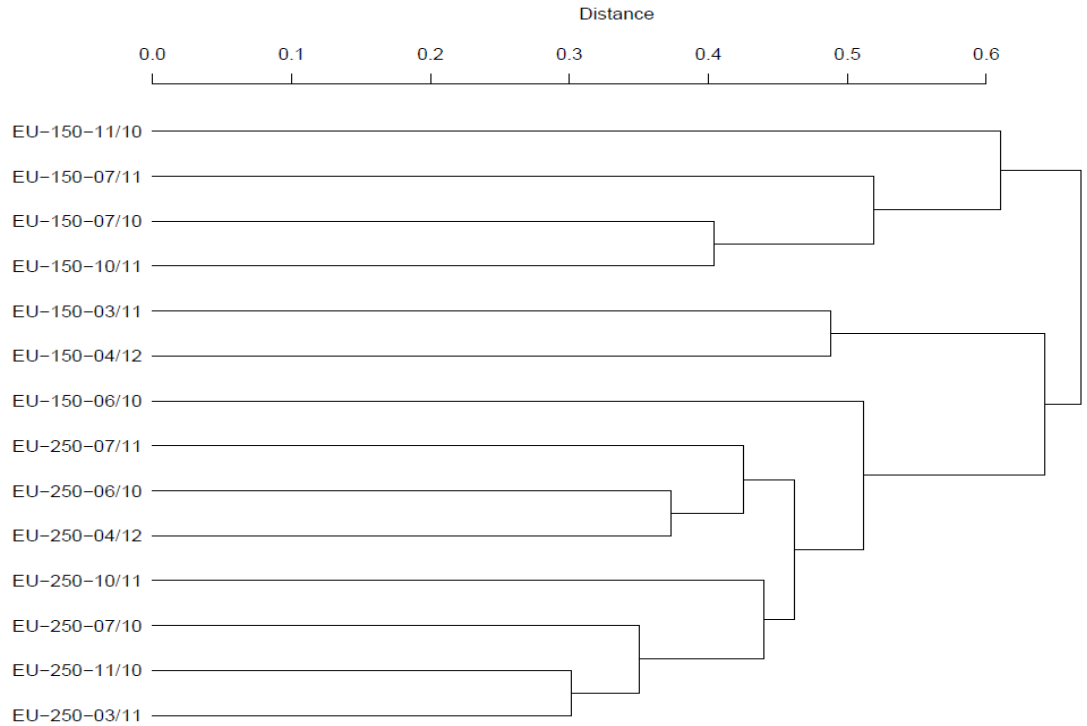

Fig. III.3.6.11.2 Hierarchical clustering based on abundance of identified potentially pathogenic organisms

Table III.3.6.11.1 shows a list with all identified PPOs belonging to the phyla Actinobacteria (27.5 %), Proteobacteria (17.6 %), Firmicutes (33.3 %), Bacteroidetes (17.6 %), Tenericutes (2 %), and Synergistetes (2 %). All PPOs that were exclusively detected in the utility or the habitable module are highlighted in blue or green, respectively.

TABLE III.3.6.11.1 LIST OF ALL IDENTIFIED POTENTIALLY PATHOGENIC ORGANISMS (PPOS); BLUE: PPOS THAT INHABITED ONLY THE UTILITY MODULE, GREEN: PPOS THAT WERE FOUND ONLY THE HABITABLE MODULE

Acinetobacter johnsonii	*Corynebacterium simulans*	*Prevotella melaninogenica*
Actinobaculum schaalii	*Corynebacterium urealyticum*	*Prevotella nanceiensis*
Actinomyces hyovaginalis	*Dialister invisus*	*Prevotella nigrescens*
Actinomyces neuii	*Dialister pneumosintes*	*Prevotella pallens*
Anaerococcus prevotii	*Enterococcus faecalis*	*Prevotella tannerae*
Avibacterium gallinarum	*Eubacterium yurii*	*Propionibacterium acnes*
Bacteroides ovatus	*Faecalibacterium prausnitzii*	*Propionibacterium granulosum*
Bacteroides ureolyticus	*Finegoldia magna*	*Rothia dentocariosa*
Bordetella hinzii	*Gemella sanguinis*	*Rothia mucilaginosa*
Brevundimonas diminuta	*Haemophilus parainfluenzae*	*Sphingobacterium multivorum*
Bulleidia extructa	*Jonquetella anthropi*	*Staphylococcus aureus*
Capnocytophaga sputigena	*Mobiluncus curtisii*	*Staphylococcus epidermidis*
Citrobacter rodentium	*Neisseria sicca*	*Staphylococcus hominis*
Collinsella aerofaciens	*Neisseria subflava*	*Streptococcus gordonii*
Corynebacterium freneyi	*Parvimonas micra*	*Streptococcus oralis*
Corynebacterium matruchotii	*Peptoniphilus asaccharolyticus*	*Streptococcus sinensis*
Corynebacterium renale	*Porphyromonas gingivalis*	*Veillonella parvula*

In all, *Peptoniphilus asaccharolyticus* was the only PPO detected in all samples. Four PPOs out of 52 were detected solely in module EU-150 and eight PPOs were retrieved only from samples from module EU-250.

- **PPO community of module EU-250**

The PPOs that were detected in all samples were *Peptoniphilus asaccharolyticus*, *Staphylococcus epidermidis*, *Acinetobacter johnsonii*, *Corynebacterium urealyticum*, *Haemophilus parainfluenza*, *Rothia dentocariosa*, *Gemella sanguinis*, *Propionibacterium acnes*, and *Veilonella parvula*. *Rothia mucilaginosa*, *Corynebacterium renale*, *Staphylococcus hominis*, *Bacteroides ovatus*, and *Citrobacter rodentium* were found at six out of seven analyzed sampling points in time. *Porphyromonas gingivalis*, *Finegoldia magna*, *Neisseria subflava*, *Prevotella pallens*, and *Streptococcus gordonii* were detected in 5/7 of all samples and *Corynebacterium freneyi*, *Dialister invisus*, and *Prevotella nanceiensis* in 4/7, respectively.

- **PPO community of module EU-150**

Out of 43 determined PPOs, two (*Staphylococcus epidermidis*, *Acinetobacter johnsonii*) were identified in six out of seven samples, five PPOs (*Corynebacterium urealyticum*, *Rothia mucilagionsa*, *Porphyromonas gingivalis*, *Anaerococcus prevotii, Mobiluncus curtisii*) were in 5/7 of analyzed samples, and five PPOs (*Haemophilus parainfluenzae*, *Rothia dentocariosa*, *Corynebacterium renale*, *Corynebacterium freneyi*, *Jonquetella anthropic*) were in 4/7, respectively.

Following identification, the proportional abundances calculated from aggregated HybScores of the detected PPOs across samples were under closer consideration (data CD, [folder: PhyloChip: Second Genome Microbial Profiling Report, p 49]). Altogether, the variation of the detected abundances per potentially pathogenic species displayed alterations, and is therefore heterogeneous. Furthermore, there was no recognizable, clear trend along the time-axis due to diverse fluctuations of abundances over time. However, six PPOs (four in module EU-150, and two in module EU-250) were identified to have a significant correlation (p-value <0.05) with time (Fig. III.3.6.11.3). Data were calculated using Spearman correlation.
Regarding the p-values and rho-factors, which both indicate statistical significance, four of the identified six eOTUs (*Mobiluncus curtisii*, *Staphylococcus epidermidis*, *Peptoniphilus asaccharolyticus,* and *Enterococcus faecalis*) correlate positively with the factor time. The rho-value indicates if the calculated p-value is significant.

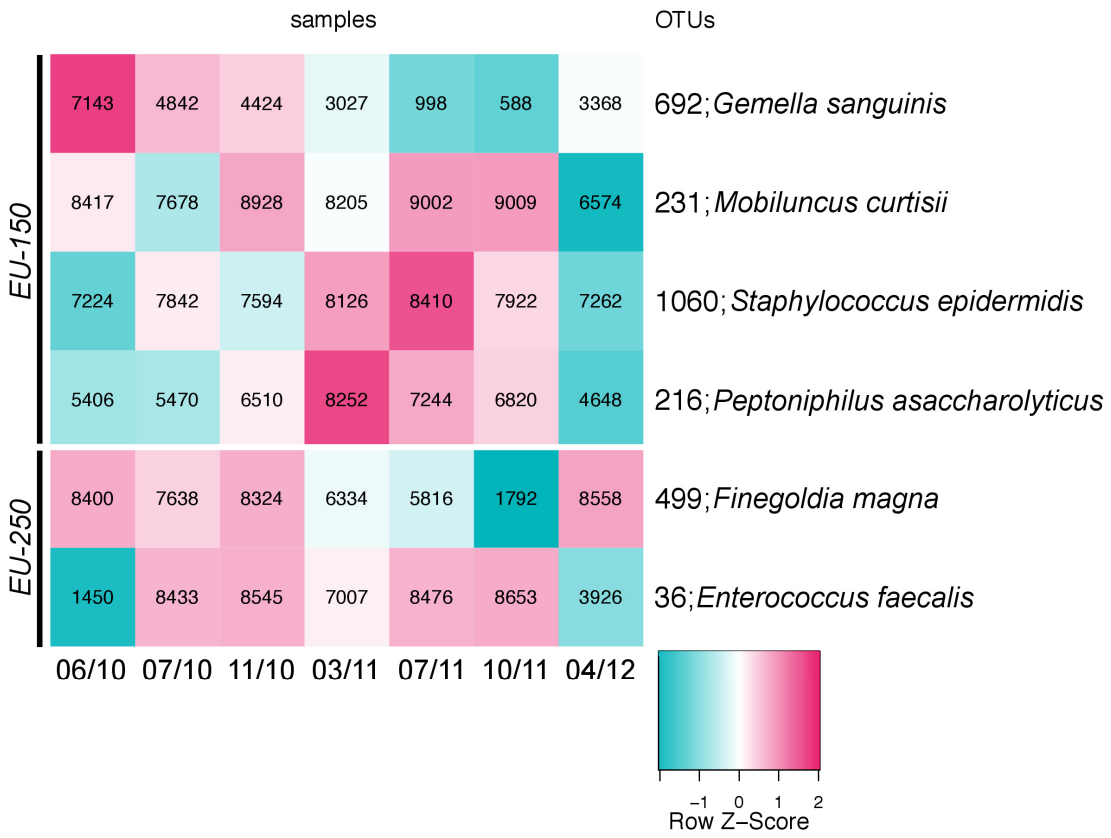

Fig. III.3.6.11.3 Heatmap of potentially pathogenic organisms (PPOs) that showed a significant correlation (Spearman, p-value <0.05) with the factor time in module EU-250 (utility) and EU-150 (habitable). Z-score refers to the probability of a HybScore occurring within the normal distribution. A positive Z-score means the observed HybScore is above the mean and *vice versa*. Numbers indicate retrieved HybScores.

Summary:
Altogether, both modules revealed 52 PPOs. The number of pathogenic species was significantly higher in the utility module compared to the habitable module, as found by ordination and HC-AN analysis. The proportional distribution was very heterogeneous, whereas samples from the utility module exhibited lower intra-group dissimilarities. Four of six PPOs correlated positively with time.

III.3.6.12. Comparative Analysis of eOTUs and Isolates

To identify similarities or dissimilarities between cultivation and PhyloChip assays as well as to emphasize advantages and disadvantages of each of the applied methodologies, all sequences obtained after enrichment (data CD, [folder: Isolate Sequences]) were compared with eOTUs detected via PhyloChip. Therefore, all 968 16S rRNA gene sequences received from air and surface samples were quality checked and re-classified using the same alignment pipeline as for PhyloChip data. It was essential that all sequences were classified on the same phylogenetic database (Greengenes). Those sequences that passed the quality filtering were compared with eOTUs obtained from the fourteen PhyloChips (excluding chip of IsoCntrl). More detailed analyses, i.e., abundance-based analysis and comparison with eOTUs, were performed with isolate data of non-heat-shocked samples

("vegetatives") from sampling points (June of 2010, July of 2010, November of 2010, March of 2011, July of 2011, October of 2011, and April of 2012) analogue to the samples that were subjected to PhyloChip assay.

902 sequences passed the quality filtering and were moved on to comparison results obtained from PhyloChip, whereas 66 sequences were qualitatively poor or too short and thus excluded from further analysis. The minimal length was set to 700 bp to obtain reliable classification results also on species level.

Differences and similarities of both methodologies, regarding the taxa that could be detected with either only one or both methods, were plotted on all taxonomic levels (Fig. III.3.6.12.1). Therefore, the entire set of classified taxa was considered. Additionally, sequences obtained by cultivation were not distinguished according to sampling date. Results after comparison displayed that the microarray technique complemented the cultivation approach on every taxonomic level from phylum to family, and even succeeded in detecting further taxa that would have been overlooked otherwise (additional 180 % ± 2 per phylum, class and order level, and 128 % on family level). Thus, a greater diversity was covered by PhyloChip. However, divergence was discovered on the genus and species level given the fact that cultivation revealed genera and species that were not retrieved by PhyloChip.

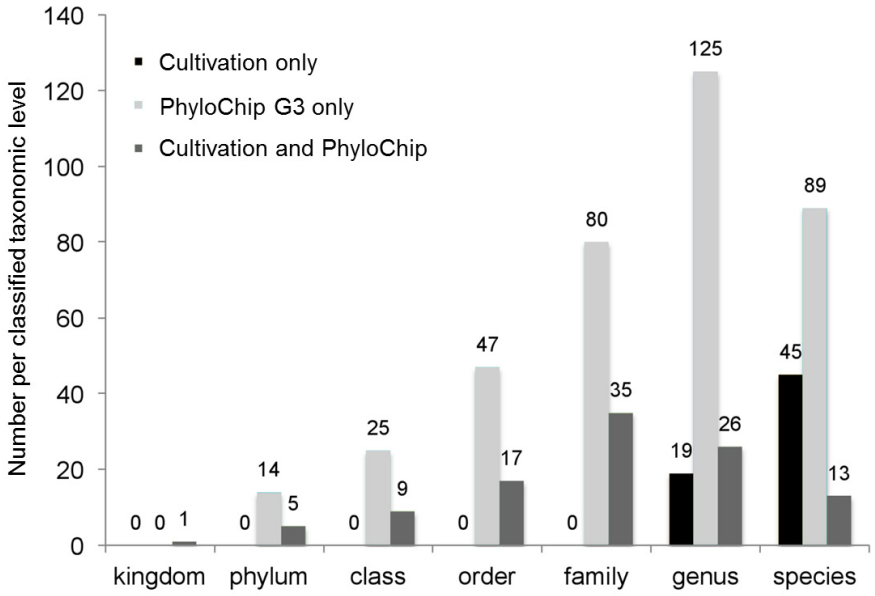

Fig. III.3.6.12.1 Comparison of number of classified taxonomic levels covered by each method. Entire isolation data of all sampling points in time are included.

Starting with the highest level, PhyloChip supplementally detected 14 phyla, 25 classes, 47 orders and 80 families. Drawbacks of this assay could be detected on more finely graduated levels, such as genus and species. 19 genera were detected only by cultivation. However, PhyloChip further revealed 125 genera that had not been identified during the enrichment approach. The greatest divergence was seen on the species level: Out of 902 isolate sequences, 45 were observed only by cultivation, whereas out of over 50,000 possibly detectable taxa that could be retrieved by PhyloChip, solely 89 eOTUS could be aligned on

the species level. Albeit, it has to be noted that unclassified taxonomic levels were not considered in this analysis. The majority of detected eOTUs were not classified at the species level, while only 276 isolate sequences out of 902 (less than one third) could not be aligned on the species level.

In order to break down the species richness of isolates depending on sampling time and their origin module, only those airborne isolates and “vegetatives” were considered that were enriched from the sampling dates that were congruent with those covered in microarrays (Fig. III.3.6.12.2). Spearman rank correlation of the number of different isolates with number of different eOTUs revealed a highly significant negative correlation (p=0.026, rho=-0.59). The species richness of isolates ranged between 12 and 32, whereas PhyloChip detected a greater diversity with minimal 104 and maximal 194 species per sample.

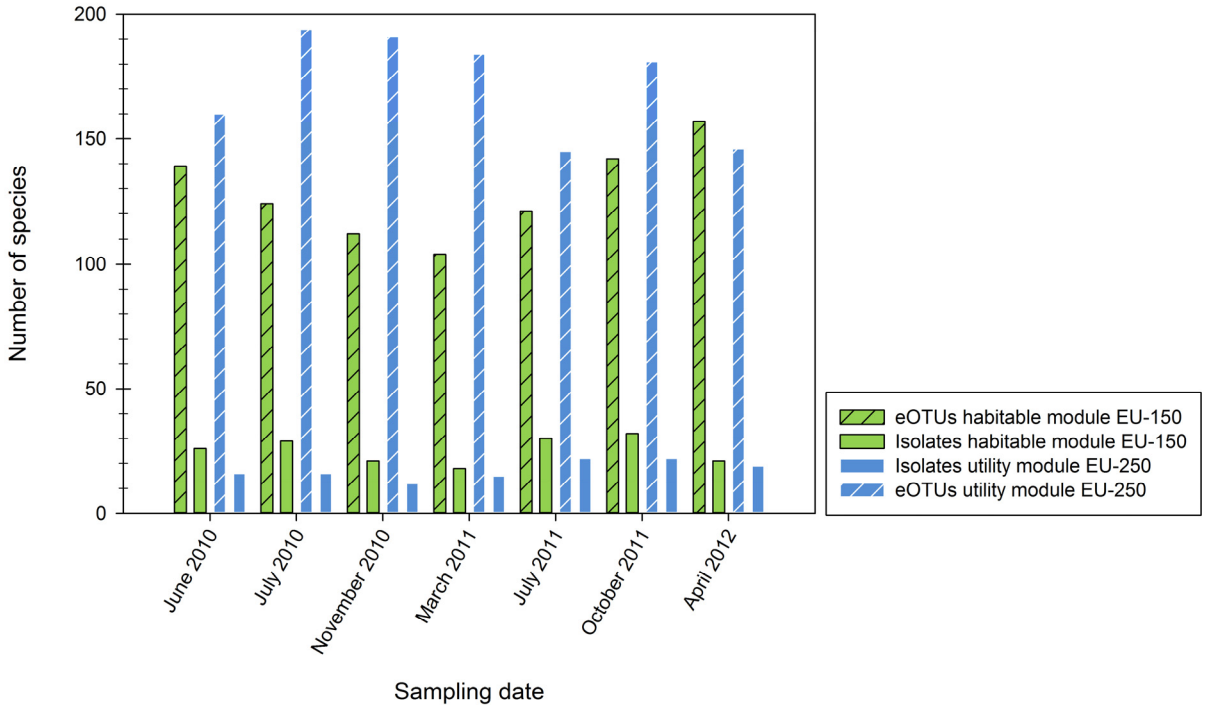

Fig. III.3.6.12.2 Species richness per module obtained by PhyloChip assay and enrichment approach (air and “vegetative” surface isolates) from sampling dates that were congruent with the samples that were subjected to PhyloChip.

The plot also displays that the number of isolates and the number of eOTUs behaved contradictory; where the former increased, the latter decreased, and *vice versa*. However, this finding is biased by probe design of the microarray and/or by enrichment conditions.

In order to obtain a relationship between aggregated HybScores with isolate counts on the genus level, a correlation analysis was performed. The analysis included 21 genera that were detected with both methods from samples of the seven analyzed points, and results are displayed in Fig. III.3.6.12.3. Solely the isolation abundances of *Rhodococcus*, *Methylobacterium,* and *Roseomonas* were found to significantly correlate with aggregated HybScores of the same genus detected on the PhyloChips.

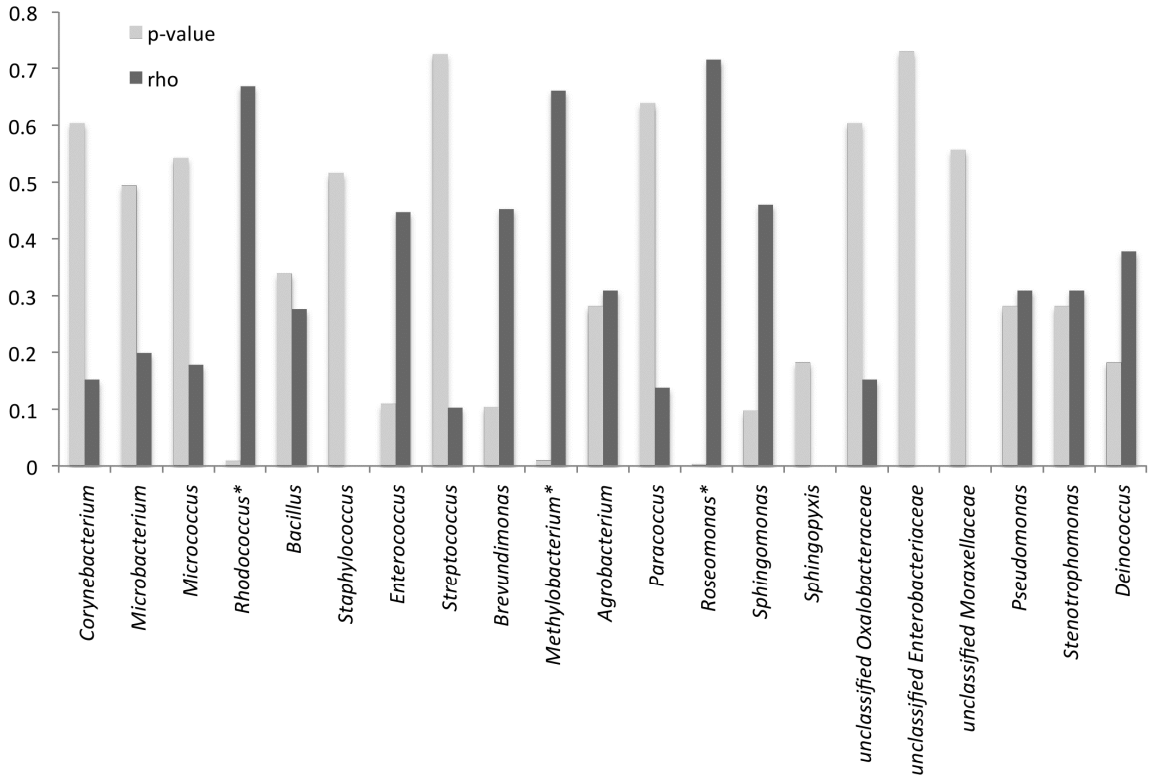

Fig. III.3.6.12.3 Correlation of aggregated HybScores with isolate counts on genus level including 21 genera. Only positive correlations are displayed. Asterisk denotes significant correlation (p-value <0.05).

As described earlier (section III.3.6.5.), there is a major difference between PhyloChip data and cultivation regarding the detection and identification of staphylococci on the species level. The majority of organisms identified after cultivation were assigned to diverse staphylococci, ranging from *S. aureus* to *S. cohnii*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunesis*, and *S. pettenkoferi* (Fig. III.1.5.3; Fig. III.1.9.1.3). The most abundant species enriched is *S. haemolyticus*. In contrast, PhyloChip revealed only nine eOTUs being assigned to the four *Staphylococcus* species *S. aureus*, *S. cohnii*, *S. epidermidis,* and *S. hominis* (Fig. IV.8.3.1). For the very same reason, cultivation data cannot be congruent on the species level with data from PhyloChip, but this does not necessarily affect higher taxonomic levels.

Summary:
Comparing both methods, PhyloChip analysis broadened the insight into the microbiome being present in the MARS 500 facility, since it revealed a greater proportion of classified taxonomic levels than the cultivation approach. However, as the plots display, the results were more biased on taxonomic levels with higher resolutions.
Diversity analysis revealed for both methods that the microbial community structure of the samples is very heterogeneous, indicating a continuous change over time in the microflora instead of reaching a steady state.
However, with increasing taxonomic resolution, the cultivation approach identified taxonomic affiliations that were not detected via PhyloChip and thus highlighted the importance of combination of several techniques.

IV. DISCUSSION

MARS 500 is the first ground-based full duration simulation of a manned flight to Mars. It was conducted from June of 2010 to November of 2011 by the Institute of Biomedical Problems in Moscow, Russia, together with the European Space Agency (ESA) and international partners. During this time, an international crew consisting of six members lived, worked and performed scientific experiments within this closed habitat. This isolation program constituted a unique opportunity to implement a research project called MIcrobial ecology of Confined Habitats and humAn health (MICHAm, modified) by Dr. Petra Rettberg.
The realization of such an experiment like MARS 500 was not only elaborate, complex, and costly, but also unique and inevitable. The complexity of the MARS 500 campaign with over 100 international sub-experiments being involved, and the availability of crew time set limits to design and operationality of each single project. Furthermore, subsequent changes of the existing experimental setup were practically impossible. Since a repetition of the whole isolation experiment or of sampling events was not realizable, samples received were handled with due skill, utmost care and diligence.
It was the aim of this work to contribute to the understanding of how microbial populations evolve on surfaces and in airborne biotopes during prolonged crew isolation in a confined mock-up spacecraft facility from start to the end. There has been an increasing concern over the potential effect of indoor contamination on human health, which was another point of interest. To meet the goals, the microbial inventory was studied with cultivation based approaches and on molecular level via DNA isolation and subsequent PhyloChip analysis.

IV.1. CONFINED HABITATS

Human exploration of extreme and isolated hostile environments (Arctic and Antarctic regions, space, deep sea, high altitude mountain regions, deserts) often requires special small-volume habitats to protect the crewmembers, house them or to deliver them to their destination. These missions are also often associated with long-term residence like a travel to Mars (Timmery *et al*., 2011). Since these habitats - including the MARS 500 facility - function as closed systems, inhabitants of such confinements experience unique stressors which could directly affect their health (e.g., stress, fatigue, indisposition), their performance, and thus the fulfillment of tasks and the mission success. One of the major challenges is the protection of human subjects (research crew, astronauts) from biological contaminants, which could cause infections and illnesses. However, microorganisms do not only pose a risk for humans, but may also have adverse effects on the integrity of materials and buildings. In contrary to open environments, confined habitats have restrictions on waste disposal, water and fresh air supply (section II.2.1. for MARS 500 facility), as well as on personal hygiene. All these present factors thus inevitably enable the building-up of a particular community of microorganisms (mainly microbial biofilms). Prominent examples for such confined residences which present exceptional work and living places are ISS, Mir,

and Antarctic Concordia Station (Van Houdt *et al*., 2009; Timmery *et al*., 2011). The unusual environmental conditions may result in bad air quality, water condensation or accumulation of biological residues (Van Houdt *et al*., 2009). The microbiological risks in space flight and confined habitats are summarized in the following Fig. IV.1.1.

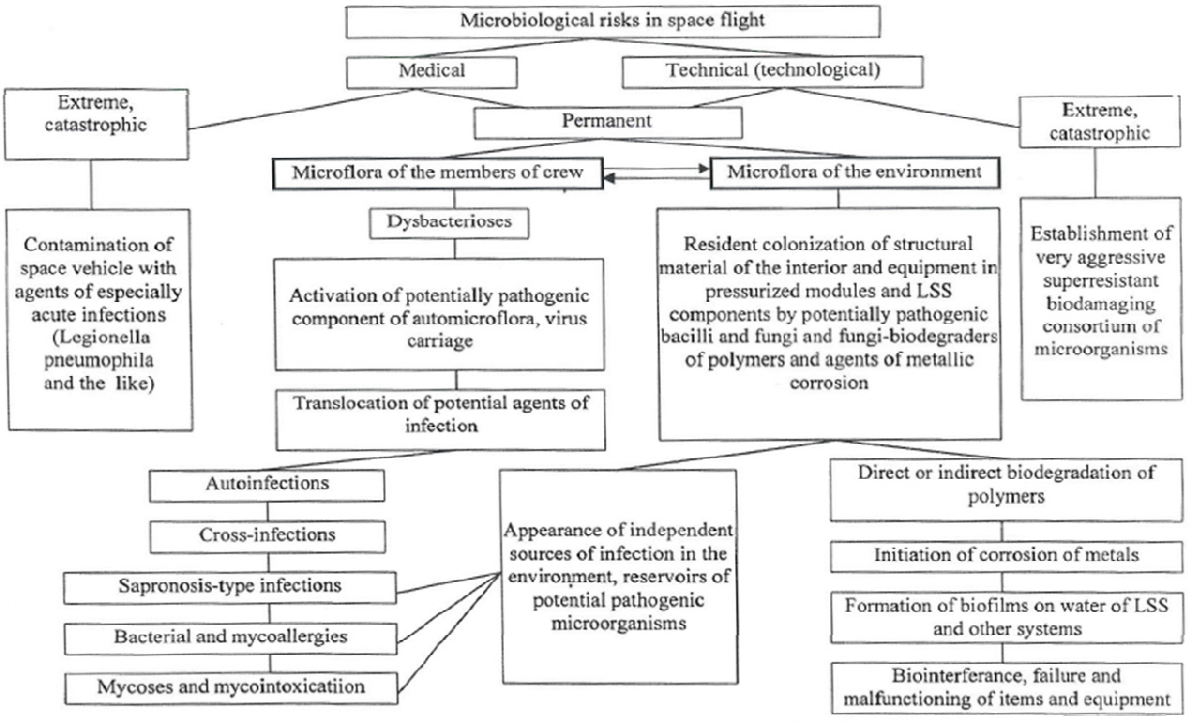

Fig. IV.1.1 Overview of potential microbiological risks in confined manned habitats. © ESA and State Research Center of Russian Federation - Institute for Biomedical Problems, 2000

Studies within the above-mentioned habitats were performed and literature revealed that it is still critical to monitor the microbial contamination in air as well as on surfaces, since every habitat and every inhabitant has its own features and own microbiota (section I.6.). Additionally, the microbial load is influenced by a plethora of different external and internal factors whose effects are so far unknown. Thus, it is almost impossible to make predictions about microbial contamination level, community structure or possible hotspots for the MARS 500 facility.

IV.2. Cultivation

The cultivation-dependent approach of microbial monitoring is limited by the fact that the overwhelming majority of microorganisms present in a habitat still cannot be cultivated under laboratory conditions (Amann *et al*., 1995). Important, however, is the continued isolation of bacterial representatives for several reasons. Enrichment offers the best opportunity to conduct detailed metabolic and functional studies. By obtaining pure cultures, whole genome sequencing is enabled which will accelerate the linkage to functionality. Additionally, cultivation of bacteria is inevitable for the determination of their pathogenic

potential, as this information is not located on the 16S rRNA gene region which is generally targeted by next-generation sequencing methods. All these aspects strengthen the necessity of cultivation studies.

Generally, the enumeration of bacteria is dependent on which agars and culture conditions are used. Bacteriological surveillance under special nutrient-poor culture conditions, e.g., on R2A have yielded higher cell counts compared to nutrient rich medium (Van der Linde *et al*., 1999). However, the results will always be biased since every medium is selective. Furthermore, overgrowing effects might occur rapidly on media with high nutrient content, and might lead to underestimations of the total microbial load. This can be observed not only due to swarming bacteria, also fungal growth can interfere with colony formation of bacterial organisms. Fungi build hyphae which results in an undifferentiated mycelium and hence expand on the agar plate. The described growth pattern, a kind of swarming, leads to a great coverage of the agar plate which in turn results in less space that is available as nutrient donor for microbial growth. To inhibit fungal growth, agents like benlate (25 µg/ml) and natamycin (40µg/ml) can be added to the medium as is described in Radosevich *et al.* (2002).

The MICHAm project presented herein, describes a comprehensive cultivation study over a long time that focused on the cultivation of either heterotrophic, aerobic microorganisms or spore-forming, heat-tolerant strains. Therefore, the corresponding ESA standard (section II.3.; section II.4.) was applied and cultivation was performed on R2A at 32°C.

IV.3. Airborne Microorganisms

To study the relevance of airborne microbial contamination in the confined manned MARS 500 facility, monthly air samples were taken at different sites in the utility, habitable, and medical module (section III.1.1.). Until the start of the study, little information was available about the microbial content of air, the influence of airborne microorganisms on occupants, and *vice versa,* especially with regard to longtime confinement.

Generally, airborne microorganisms are ubiquitous, highly diverse, and omnifarious and occur either as spores or vegetative cells. In air, microbes are often attached to dust particles or water droplets from sneezes and cough (Tringe *et al*., 2008). They are transported and further dispersed by air movements, whereas their concentration and diversity is dependent on different environmental factors (Cox, 1987).

Literature research resulted in a high number of publications that monitored microbial airborne contamination at diverse locations including homes (Frankel *et al*., 2012), offices (Gaüzère *et al*., 2013a), public buildings (Dacarro *et al*., 2003; Qian *et al*., 2012; Gaüzère *et al*., 2013b), public means of transport (trains [Wang *et al*., 2010] and airplanes [Osman *et al*., 2008; Korves *et al*., 2012]), and hospital facilities (Angenent *et al*., 2005; Poza, *et al*., 2012). The highly-controlled spacecraft-associated clean rooms (Rettberg *et al*., 2006; Venkateswaran *et al*., 2012a; Moissl-Eichinger *et al*., 2013), and space stations like Mir (Ilyin, 2000; Kawamura *et al*., 2001, Novikova, 2004) and ISS (Novikova *et al*., 2006) were also investigated. Additionally, the periodically confined Antarctic Base Concordia was

monitored (Van Houdt *et al.*, 2009). However, there remains a lack of environmental data in enclosed spaces.

IV.3.1. Indoor Air Pollution

Pollutants, i.e., gases, vapors, particles and microorganisms and their metabolites that are suspended in the air, settled, absorbed or attached to surfaces play a major role for human health in confined habitats. They may cause allergies, infections or asthma due to inhalation of spores, and metabolites attached to airborne particles may lead to unspecific complaints and symptoms. It is known that the sources for indoor pollutants cover a wide range. Furniture, rinsing water, cooking, consumer products, construction material, indoor greenhouses, and the occupants themselves are examples that generate and emit various airborne particle matter (Cox, 1987). The MARS 500 facility is lined with wooden side and rear wall panels and with furnishings and fitting. Furthermore, the floor is also made of wood and a carpet is unfurled under the dining table. However, the toilet and restroom area completely consist of stainless steel and are easy to rinse. In general, the whole facility is cozily equipped so that it is not completely imitating a real spacecraft (Fig. III.1.1.1ff; Fig. III.1.6.1ff). A larger deposition area of dust due to furniture leads to higher contamination levels that can be monitored by using air samplers (Cox, 1987).

As mentioned earlier (section I.3.1.), there is a great quantity of available air sampling devices which increases the option for choosing the most suitable tool. For the MICHAm project, the active sampling tool Sartorius AirPort MD8 was applied since this device was frequently used for microbial monitoring of spacecraft-related environments as described in the ESA standard (ECSS-Q-ST-70-01C, 2008). Furthermore, it is a portable unit that can be easily moved from one sampling point to another. Due to the initial sterilization procedure of the isolation facility (gasing with 3 % H_2O_2, pers. comment: Svetlana Poddubko), a sampling device had to be selected that can be sterilized, sanitized and disinfected. Additionally, when filtration based techniques are applied, it has to be ensured that the collection process is efficient and microorganisms survive the sampling procedure.

Due to the low amount of sampled air (500 l) during the MICHAm campaign, a relatively low-biomass was sampled and therefore, CFU counts might be not representative. Radosevich *et al.* (2002) developed a high-volume aerosol collection system that collects particulate matter of 1.4 x 10^6 l in a 24 h period. Their main prompting was to capture the airborne bacteria more efficiently by collecting large volumes and thus overcome low bioload problems. Furthermore, the longer sampling time was thought to provide more reliable results compared to the AirPort MD8 whose sampling duration of 17 min presents only a snapshot in time. However, during the MICHAm experiment this problem was overcome by repeated samplings at nine different locations over a long period of time, so that the sum of all samples gave a broad insight into the airborne microbial community structure. Additionally, it has to be considered that too long sampling might result in saturation of the filter or in a too dense growth of colonies on the agar plate, so that they cannot be differentiated any more from each other as it would be the case with colony counts above 300 per plate. This effect would also lead to underestimation of the microbial

content in the air but was excluded for MICHAm samples considering CFU counts between 0 and 269 per plate in 99 % of all cases (Table III.1.1.2). Further, 24 h sampling time per location is not feasible for occupied habitats since reliable results are only guaranteed if the airflow is not disturbed by inhabitants moving around and causing turbulences. Moreover, sampling with active samplers is usually noisy. Considering both aspects, a long-duration sampler would have not been applicable for monitoring in the MARS 500 facility where the marsonauts had to follow a tight schedule and consequently had to move within the facility. Due to the confinement they already lived in an exceptional situation where noise would have had an additional negative effect on their psychological situation. Thus, any adverse effect on their well-being should be avoided or at least minimized.

Lacey (1994) stated that the variety and amount of microbial specimens are also dependent on the type of indoor sampling. In the literature there are two different filters described that can be used for active filtration: Either gelatine filters that are directly put on the agar plates or polyester membrane filters from which the particles have to be extracted. It has to be considered that a certain number of microbes is inactivated by the impact on the nutrient (Pasquarella *et al*., 2000). However, gelatine filter are slightly moistened which results in the effect that even desiccation sensible germs survive the filtration process. The longer the filtration process the more the filter is drying out which results in lower survivability rates of samples cells (pers. comment: Dr. Petra Rettberg). Regarding the use of polyester membrane filters, the eluted filter concentrate can be diluted and several dilutions can be plated for viable cell amount estimation. With increasing sampling time, it has to be ensured that the CFU counts represent the true value of the air microbiota (Tringe *et al*., 2008). Therefore, additional tests have to be performed to prove that the sampled cells are arrested on the filters and do not actively grow. On the basis of the short sampling procedure of the AirPort MD8 and the mean doubling time of bacteria, active growth as a potential factor for bias has not to be considered. To give an example, the mean value of a *Staphylococcus aureus* strain is between 20 and 60 min (Petersen, 2003).

Airborne bacteria that stick to the above-mentioned gelatine filters can directly be cultivated on agar plates. Since the gelatine is water-soluble it can be can be dispersed in dilution fluid or directly put on nutrient media agar plates as it was done during the campaign described herein (section II.3.). Furthermore, after sampling they can be stored for a long time. Our findings revealed that storage of up to six months has no significant negative effect on viability (section III.1.2.; Fig. III.1.2.1). Applying this method has the benefit that high-biomass samples can be diluted by resolving, whereas when analyzing low-biomass samples no loss of cells occurs since the filter is directly transferred to the agar plate.

Conclusion:

In order to survey microbial indoor air pollution it was proven that the selected AirPort MD8 device was the best-suited tool with respect to the prevailing circumstances of the MARS 500 experiment. The adjusted sampling parameters as well as the choice of sampling frequency and depth led to the most reliable results concerning the enumeration of airborne microbial community.

IV.3.2. Microbial Limits of Maximal Allowed Contamination Levels

There are a number of published standards or guidelines available for diverse manned habitats to ensure high indoor environmental quality and thus minimize the direct risk for human's health. With regard to space-related environments, especially for long-term inhabitation, it is inevitable to provide a healthy housing and working area. In addition, this data is useful for assessing the effectiveness of cleaning and sanitization practices (Moldenhauer, 2005). All official regulations on the control of airborne microorganisms are primarily based on the count of CFU/m³ and do not specify the kind of active sampler to be used. For a summary on the generally valid standards of the diverse countries please refer to Pasquarella *et al*. (2000). In the study described herein, the investigation of the airborne community which is able to grow under ESA's ECSS-Q-ST-70-01C (2008) standard procedure, i.e., aerobic cultivation on R2A plates for 72 h at 32°C, revealed CFU counts between 0 and 716 per m³ (section III.1.1.). None of the 162 analyzed samples that were distributed over the three modules and over the whole time frame exceeded the limit that was set for the ISS and general indoor air quality. However, it has to be kept in mind that due to the weightlessness the distribution in space is different compared to the dispersion on Earth.

International valid standards have been designed to evaluate the limits for the maximal concentration of bacteria and fungi being allowed to be present in air onboard the ISS. The values are stated in the ISS Medical Operations Requirements Document (Duncan *et al*., 2008; ISS MORD, 2006) and constitute a trade-off between acceptable risk and realizable levels since a sterile environment is not achievable in a manned habitat. According to that document 1,000 bacterial CFU are allowed per m³ air. These threshold-levels are based on CFU counts on rich agar medium capturing the maximum amount of total viable aerobic and heterotrophic bacterial cells (ISS MORD, 2006; Pierson *et al*., 2012; Van Houdt *et al*., 2012) similar to the applied ESA standard. According to Verhoeff (1993) values for ISS are comparable when compared to the classification of the European Collaborative Action "Indoor Air Quality and its Impact on Man" for bacteria in homes and offices.

For the first time microbial airborne contamination was monitored onboard the Mir space station between 1986 and 2001. Standards for the Russian piloted space vehicles allow a maximal contamination by bacilli of 500 CFU/m³. Besides occasional increases due to human physical activity, 95 % of analyzed air samples contained less than 500 bacterial CFU/m³ (Ilyin, 2000; Kawamura *et al*., 2001; Novikova, 2004) and remained relatively stable. The highest values were reported for exercise machines in Kvant-2 and Kristall that ranged around 3.5 x 10³ CFU/m³. In the case of MICHAm, mean 102 CFU/m³ were obtained next to the treadmill with the highest value of 538 CFU/m³ (Table III.1.1.2). In a latter, comparable study, Novikova *et al.* (2006) analyzed airborne diversity from different sites of the US and Russian segments on the ISS. Air samples were collected regularly on a basis of every 90 days. Therefore, the authors used the US-supplied Microbial Air Sampler (MAS) Kit (NASA 2005b) and in the Russian segment the Ecosphaera Kit including SAS air sampling device on TSA and Czapek's medium. By not exceeding 710 CFU/m³, the reported values were well within the acceptable levels. The highest microbial load was

encountered in the toilet area, whereas the remaining sampling locations exhibited relatively low levels. Notwithstanding, in the MARS 500 facility the highest values were obtained in the individual compartment (260 CFU/m³) and in the community room (approximately 150 CFU/m³), whereas the mean airborne contamination level was relatively low outside the toilet (16 CFU/m³; section III.1.1.; Table III.1.1.2)

Conclusion:
Based on the guideline defined for in-flight ISS requirements, the marsonauts were never exposed to exceptional health hazard caused by the amount of present airborne microorganisms. But what is the daily normal airborne contamination level, each individual is exposed to at home or in public areas?

IV.3.3. Terrestrial Environments

In general, when comparing CFU values of diverse studies with each other, one has to keep in mind, that these data were retrieved with different methods including different air samplers and enrichment conditions. Therefore, each result is fraught with bias caused by various factors.
Dacarro *et al*. (2003) assessed the microbiological indoor air quality of eleven gyms in high schools and colleges in Pavia, Italy, during physical training lessons. Colony counts that were recovered per 1 m³ sampled air on R2A revealed ranges between 257 and 2,420 (mean 831±688) when central heating was on and 336 to 1,147 (mean 606±278) CFU after heating was turned off. Also modern buildings with forced ventilation system exhibited a mean value of 747±578 with maximum counts of 1,945 colonies per m³. These findings outvalue the maximum counts obtained from the MARS 500 facility (section III.3.1.). Furthermore, with a mean value of 86 CFU/m³, the marsonauts were approximately 10 times less exposed to microbial contaminants compared to students in scholastic environments during sports lessons. Observations in office buildings uncovered air quality values from 40 to 2,500 CFU/m³ and a mean of 280 CFU/m³ (Bouillard *et al*., 2005). Sessa *et al*. (2002) determined a mean of approximately 500 CFU/m³ in an office building, whereas in a public area the airborne cell concentration was significantly higher and varied between around 900 and 1,200 CFU/m³.
Microbial contamination has also been assessed in commercial airline cabin air during short and long-duration flights (Osman *et al*., 2008). This environment is characterized by the fact that the passengers breathe continuously recycled air, which is also true for the MARS 500 habitat. However, in the case of jet aircraft, the air is a mixture of external air brought in through the engines and cabin air. Heterotrophic plate counts ranged from 0 to 1,000 CFU/m³ for international and domestic flights indicating no significant difference regarding flight duration. Only less than 2 % of all analyzed samples retrieved 10^3 cells/m³. Prior to this study, it was reported by diverse groups that cultivation approaches from cabin air resulted in approximately 10 to 300 CFU/m³ (Wick and Irvine, 1995; Dechow *et al*., 1997). Comparing the values reported in literature, no significant increase could be detected during the MARS 500 experiment.

Furthermore, the group around Osman observed that the plate count fluctuated during each flight. Additionally, no particular trend was noticed so that these findings are similar to the observations made for the MICHAm samples (section III.1.1.).
The Antarctic base Concordia represents an occupied human habitat which is characterized by at least periodically confinement. Airborne microbial contamination level monitoring revealed in 90 % of all samples values lower than 10^3 CFU/m³, too. Interestingly, the contamination level increased during the confinement, but diminished after reopening of the base (Van Houdt *et al*., 2009). This trend was observed for the first time and could not be confirmed for the MARS 500 samples taken during the 520 days of isolation.
Besides Concordia, hospitals also represent highly controlled environments. Greene *et al*. (1962) displayed results from the airborne contamination at different locations of a hospital. Typical fluctuation curves, as were seen during the MARS 500 experiment (Fig. III.1.1.4), were illustrated from an operating room, laundry-chute closet and adjacent corridor. In a ward, the airborne contamination level varied from 160 to 440 CFU/m³ within a period of one year (Augustowska and Dutkiewicz, 2006).
Almost identical values were observed in homes contained (Bouillard *et al*., 2006). According to Sessa *et al*. (2002) bacterial load in occupied apartments were lower and ranged between 90 and 180 CFU/m³ in. Frankel *et al*. (2012) presented bacterial indoor values that peaked in spring (median 2,165 CFU/m³) and were lowest in summer (240 CFU/m³).
In sum, comparisons between CFU values are difficult to make since all of them were derived by applying different air sampling tools. Another fact that has to be considered is that enrichment was done under different cultivation conditions and on a diverse set of media whereas each medium is selective for a specific subset of microbes. An example is the study of Radosevich *et al*. (2002) that analyzed air samples of 1.4×10^6 l and revealed a mean CFU count of 22 per m³, which is relatively low, compared to the studies described above. However, the enrichment was done with King's B medium, usually applied to water samples and recommended for non-selective isolation, cultivation and pigment production of *Pseudomonas* species. In contrast to findings via cultivation, the microscopic motile cell estimation displayed values between 1.5×10^4 and 10^6 cells per m³, which indicates the measured bias due to enrichment conditions.
Generally, cultivation approaches to estimate the airborne contaminants have revealed numerous bacteria by application of a variety of sampling methods, but still represent underestimations due to the low percentage of cultivable microorganisms. Another concern is that bacteria might not be equally distributed in indoor air and can appear in clouds depending on the ventilation procedures and the behavior of the residents (Cox, 1987; Schleibinger *et al*., 2004). Finally, it has to be mentioned that the airborne microbiota is a system that underlies ongoing dynamic processes and is sensitive to changes by unknown factors.

Conclusion:
The enumeration of airborne microbes within the MARS 500 facility revealed that the overall microbial load in the air was moderate compared to other non-confined occupied rooms.

IV.3.4. Influence of Diverse External Factors

The influence of environmental factors on the composition of airborne microorganisms is poorly understood. These external impacts can range from indoor occupants, to micro-niche establishments due to nutrient availability, air filtration system, temperature, relative humidity (RH), or to concentration of diverse gases. Furthermore, also geographical locations, altitude, weather/season, and UV exposure might favor diverse kinds of species (Schleibinger *et al.,* 2004). However, the microbial community structure in the confined MARS 500 facility is not affected by the latter factors due to complete shading and isolation.
Although the microbial contamination is relatively well documented in all different kinds of environments and habitats, information on the factors that influence the observed spatial or temporal variations of microbial aerosols remain unclear.
Typical airborne microorganisms can tolerate low nutrient circumstances being prevalent in the air and still possess the ability to thrive and proliferate. Changes in the water content during dissemination via the airborne route appear permanent. Ultimately, the rate of desiccation and therefore the survivability is dependent on the RH of the air (Cox, 1989, Hatch and Dimmick, 1966). Not only air humidity but also moisture penetration of the construction material emerged from water condensation due to high water vapor in air leads to proliferation of microorganisms. This effect has been observed on the ISS where at the same time augmented accumulation of microorganisms was reported (Novikova *et al*., 2006). Fortunately, this was not observed by the marsonauts. Furthermore, each resident increases the air moisture by 1.0 to 1.5 kg/day by respiration, so that the atmospheric relative humidity values describe a very dynamic process. Continuous fluctuations were also noticed in the three modules of the MARS 500 facility where the measured values ranged from 32.8 % to 56.4 % (section III.2.; Table III.2.1).
Nevertheless, Cox (1989) stated that the availability of water is the major limiting factor for survivability and growth. In order to determine the survivability as a function of relative humidity (e.g., water activity in a cell) per se, experiments were conducted under inert atmospheric conditions. The results for *Escherichia coli* were surprising and revealed higher survival rates at lower RH rather than at high RH (Cox 1989). However, a general statement that is universally valid for all microorganisms cannot be found in the literature.
Besides RH, also atmospheric O_2 partial pressure might influence the bacterial community and survivability. For the first time Hess (1965) systematically investigated the role of oxygen on the survivability of desiccated *Serratia marcescens* and demonstrated its inactivation capacity. Later on the same baneful effect was detected for *E. coli* and *Klebsiella pneumonia*. However, further studies showed that not all bacterial strains are sensitive to oxygen such as *Francisella tularensis*. Again, no generally applicable trend regarding survivability and the presence of oxygen is available. Regarding the measured O_2 partial pressure in the MARS 500 facility, the values stayed on a relatively stable level around 20 % and therefore might not have positively or negatively influenced the living airborne community structure (Table III.2.1).
During the confinement, temperature values within the MARS 500 facility were continuously noted and revealed the highest inter- and intra-module fluctuations of all measured

environmental parameters. They ranged between approximately 18 and 26°C (Table III.2.1). Changes in this order of magnitude significantly affect the growth rate of certain bacterial species at least under laboratory conditions. However, unfortunately the sampling events were not conformal with dates when the environmental conditions were read. Due to the great fluctuations in all three modules statistical tests could not be applied (Fig. III.1; Fig. III.2.1; Fig. III.2.2). Van Houdt *et al*. (2009) also did not find any statistical correlation between the total airborne bacterial contamination in the periodically closed manned Concordia base and the prevailing temperature or relative humidity, but they assumed that some differences can be attributed to changes in these parameters.

The authors further reported that once these environmental conditions have been stabilized, an increase of the airborne bacterial contamination was observed. This accumulation was explained by human activity. Since the sources and origin of airborne bacteria are not well understood, Hospodsky *et al*. (2012) investigated the human occupancy as a source of indoor airborne bacteria. This group hypothesized that the effects originate from resuspension and direct shedding of skin cells. The study presented evidence that considerably increased bacterial amounts are present in indoor areas when being occupied compared to vacant state.

Based on these contradictory findings, we inquired the correlation between contamination level and the presence of marsonauts in the MARS 500 facility, for the simple reason that results indicated a significant increase in the habitable module over the whole time frame compared to the two other modules (Fig. III.1.1.6.; Fig. III.1.6.1.3). The locations in the habitable module included the community room, the dining area, as well as one individual compartment and represented areas where the marsonauts were supposed to spend the majority of their time (section II.2.1.; section III.1.1.; section III.1.6.1.). Therefore, we hypothesized that presence of human correlate positively with contamination level.

The team around the psychologist Dr. B. Johannes investigated group dynamics during the 520 days of isolation. For this purpose, all marsonauts were equipped with a wireless sensor measurement system that has been developed to record location of each crew member and the time he spent there. The marsonauts had to wear the unit twice a week on their body. Additional sensors were installed at different locations within the three modules. In cooperation with Dr. Johannes, sample locations and sensor points were grouped together, the mean occupancies per location were calculated and correlations were performed.

However, based on these data, no significant correlation was found for airborne as well as for surface contamination - neither for single sampling points nor for the complete module (data CD, [folder: Data Sharing Johannes]). The following conclusion can be drawn: Obviously, not only the time of human presence is meaningful, but also the action of each individual has to be taken into account. There might be a difference whether a subject is passively present by sitting, standing still or laying down or in an active way by moving around or doing sports activities. The latter was the case for one sample in the utility module where the air was monitored in the gym next to the treadmill (section III.1.1.) as mean values also showed increased values compared to other sampling sites in the same module. However, those locations like the area between the storage racks, fridges or

around the greenhouses were frequented less. Since the human occupancy cannot significantly be determined as the major source, the factors influencing the microbial contamination in the MARS 500 facility still remain unclear.

Another possible impact on the airborne microbial level could be emanated from the installation of air filtration system. Its purpose is to effectively retain airborne particles, microbial cells and aerosols, which is achieved by continuous filtering of air. Studies onboard the Mir revealed a decrease in microbial contamination after installing the Potok 150M in 1998, designed to remove aerosol particles and microorganisms from air (ESA and State Research Center of Russian Federation - Institute for Biomedical Problems, 2000). Compared to standard office buildings, the air in the above-mentioned facilities is exchanged more frequently per hour (Osman *et al*., 2008). Due to the high efficiency the effect of human occupancy might not be visible any more.

High-efficiency particulate air (HEPA) filters are also installed in clean rooms, onboard of aircraft, at the Bioechnikum in Regensburg (section II.2.3.) and also in the MARS 500 habitat (section II.2.1.). In general, clean rooms are highly controlled environments designed to provide an almost contamination-free workspace for sensitive fields of work such as pharmaceutical production or the assembly of space hardware with regard to planetary protection (DeVincenzi *et al*., 1996; Tweedie, 2005). Therefore, the indoor air is constantly filtered with a high air exchange rate through HEPA filters to remove particles. Furthermore, cleanliness is also maintained by strict compliance with regulations regarding cleaning procedures and schedules. Consequently, the facilities mentioned above are characterized by the fact that the exchange with the outer environment is limited as much as practicable. Microbial monitoring of spacecraft-associated clean rooms started with the early Apollo and Viking missions in the 60's and 70's (Puleo *et al*., 1973; Puleo *et al*., 1977). Rettberg *et al*. (2006) reported about airborne contamination in spacecraft assembly halls, where air quality is also controlled by use of HEPA filters. Heterotrophic plate counts revealed extremely low values with a maximum of 33 CFU/m³. Similar values were retrieved from samples taken at the Biotechnikum in Regensburg (section III.1.4.). Compared to indoor environments where air is not filtered, the observed concentration levels are lower. As a consequence of these consistent findings, it is highly recommended to install HEPA filtration systems to reduce the microbial load in confined manned habitats which in turn increase the quality of life.

Another aspect alongside the ventilation is the architecture that strongly influences microbial community structures (Kembel *et al*., 2012). In order to identify the critical locations in a certain habitat region, the BIOSMHARS (BIOcontamination Specific Modelling in Habitats Related to Space) project has been proposed. By developing, calibrating and validating a mathematical model, the transportation of bioaerosols in a closed environment and the concurrent spread of biocontamination were predicted (Berthier *et al*., 2012). Additionally, the mathematical model was tested by biological validation experiments and the following most important conclusions were drawn: Sedimentation of dispersed microorganisms can take up to two hours. Horizontal surfaces appear to be jeopardized by more excessive microbial cells compared to walls, which were least contaminated. The ceiling was essentially clean and air in the vicinity contained little if any microorganisms.

Conclusion:
The airborne concentration level is dependent on a plethora of different factors ranging from RH, oxygen content, temperature, ventilation, furniture, installation of air filtration systems, and presence of humans to the ongoing activities within the habitat. Due to the complexity, conception of causal relation was not possible. However, more important is that the general contamination level was investigated and possible hotspots (community room, individual area, and gym) were identified, so that in future countermeasures can be implemented to prevent high contamination levels.

IV.3.5. Molecular Methods

Our investigations regarding the airborne contamination were restricted to the cultivation of heterotrophic microorganisms (section II.3.4.). However, the airborne microbial population could also have been estimated by application of ATP assays or quantitative PCR assays (Osman *et al*., 2008). By the use of the latter method, one m³ of the air within a flight cabin was assigned to 10^6 to 10^7 gene copies. Hospodsky *et al*. (2012) retrieved 10^5 bacterial genome equivalents/m³ sampled air of university classrooms.
Furthermore, Gaüzère *et al.* (2013b) characterized the diversity and dynamics of airborne microorganisms in the heavily frequented Louvre-Museum over six months using high-throughput 454 sequencing. They detected similar variations in the bacterial concentration as observed during the MICHAm experiments (section III.1.1.). However, the contamination levels identified by molecular methods were more than 100 times higher than via cultivation-dependent approach. Average values revealed 10^3 *E. coli* genome equivalents per m³ air, which were one order of logarithm lower than the data obtained in previous studies from the Mona Lisa Room of the Louvre Museum and from the Decorative Arts Museum (Gaüzère *et al*., 2013a).

Conclusion:
The application of molecular tools to investigate the airborne microbial contamination revealed higher concentrations compared to cultivation approaches. This is not surprising since molecular methods detect gene signatures of both, dead and alive cells, whereas cultivation implicates only organisms that are alive (living) and able to thrive under the chosen conditions. Therefore, data obtained by cultivation are more biased. However, in order to gain insights into the features of bacteria, pure cultures are necessary and those can only be received by cultivation.

IV.3.6. Indoor versus Outdoor Microbial Values

Literature research revealed controversial results regarding the difference between outdoor airborne microbial level and the amount of indoor airborne contaminants. Studies can be found that significantly detected higher indoor than outdoor values (Dacarro *et al*., 2003; Frankel *et al*., 2012). These findings are consistent with the results of our study. The mean bacterial value of outdoor samples taken in Germany between August and October was

around one third lower compared to the values obtained from indoor occupied samples (section III.1.3.).
Zhu *et al*. (2003) stated that the concentration of airborne bacteria indoors follows that of outdoors, but with a time delay due to the lag associated with indoor-outdoor air exchange. One explanation for these observations might be given by Zhu *et al*. (2003). According to the three measurements per day, the concentrations of outdoor airborne bacteria is dependent on the sampling time and were highest in the morning, but declined in the afternoon and reached the lowest point in the evening. Contradictory, studies by Tringe *et al*. (2008) revealed that comparisons of air samples with each other and nearby environments suggested that the indoor airborne microbial community is not consisted of random transients from surrounding outdoor environments, but rather originate from indoor niches.
Furthermore, the influence of the four environmental factors temperature, RH, light intensity, and wind speed were also investigated. Data revealed that RH has the most pronounced influence on the outdoor bacterial concentration, with the number of bacteria increasing sharply on a day with high relative humidity (Cox and Christopher, 1995; Zhu *et al*., 2003).
Although temperature, RH, and air exchange rates were significantly associated with several indoor microbial levels, they could not fully explain the observed seasonal variations, when tested in a mixed statistical model. But in conclusion, the season significantly affects indoor microbial exposures (Frankel *et al*., 2012).

Conclusion:
As assumed, indoor contamination was higher when compared to outdoor microbial concentration levels. Literature has shown that various factors influence the amount and composition of indoor microbial airborne community. However, regarding the MARS 500 facility external factors as triggers can be excluded and therefore, it is proposed that indoor air contaminants consisted of microorganisms distributed by raised dust from indoor niches.

IV.4. Surface Contamination Level

Most of our time we spend indoors, either in public buildings or in private homes, where we are exposed to different amounts of microorganisms that live on nearly all surfaces (Dunn *et al*., 2013). Despite their ubiquity we have a limited understanding of the microbial abundance on diverse surface materials. As described above for airborne microorganisms, the settled cells are also subject to various environmental factors that are the impelling forces regarding contamination level on certain areas.
On surfaces, microorganisms can appear as single cells or form aggregates such as diplococci. Additionally, they can have the ability to form biofilms which is a universal survival strategy. The accumulation of microorganisms in a biofilm, where they adhere to each other and/or are associated to abiotic surfaces, is probably the dominant form of microbial life in nature (Costerton *et al*., 1995; Stoodley *et al*., 2002; Flemming and Wingender, 2010; Karunakaran *et al*., 2011). One essential property of microorganisms that

live in a biofilm compared to planktonic cells is that they are embedded in a matrix of extracellular polymeric substances (EPS) which are produced by the cells themselves. The EPS form a sort of protective layer that shield organisms from oxidizing or charged biocides, antibiotics, metallic cations, predation, desiccation, and UV radiation, but also provide mechanical stability, function as an external digestion system and form microhabitats (Flemming *et al*., 2007; Flemming and Wingender, 2010; Flemming, 2011). Ultimately, biofilms play a crucial role in the colonization and microbial degradation of housing materials by forming a reaction compartment close to the surface the cells are attached to. Even increased biofilm formation is demonstrated in microgravity (Lynch *et al*., 2006). When temperature and moisture conditions are favorable, even the dust on various surfaces is sufficient to provide nutrients for microbial growth (Pasanen *et al*., 1993; Pasanen *et al*., 1997; Korpi *et al*., 1997). With regard to risk assessment, it is therefore inevitable to also monitor surfaces besides airborne contamination levels.

At present, a number of established techniques exist to evaluate the level of microbial surface contamination (section I.3.2.). Though the direct surface contact technique (RODAC (Replicate Organism Direct Agar Contact) plates; Baldock, 1974) has the advantage of creating actual representations of the spatial distribution of the present microorganisms, the method can only be applied effectively on flat surfaces and will leave a residue on the tested object (Angelotti *et al*., 1964). This in turn might lead to accumulation of bacteria since the solid medium provides a great nutrient source. Due to these limitations, this technique cannot be applied for monitoring complex and uneven electronic parts or space flight hardware and is also not recommended for the MARS 500 facility (Kirschner and Puleo, 1979).

Besides direct surface contact techniques used for surveying the microbial contamination on surfaces, protocols for indirect samplings (e.g., swabbing) were established in the 1970's for monitoring program of Viking mission spacecraft which included only cultivation based approaches (Favero, 1971; Puleo *et al*., 1973). Generally, cotton swabs have been the preferred sampling device of NASA as it was demonstrated that they achieve a high recovery rate in case of bacterial endospores that are sampled from small areas (Buttner *et al*., 2001; Rose *et al*., 2004).

On the contrary, ESA used nylon-flocked swabs and specified this sampling device in their standards from beginning to nowawdays. Both tools are applied for assessing the microbial contamination of delicate parts of space hardware in particular (NASA, 2005a; ECSS-Q-ST-70-55 Working Group 2008; Stieglmeier *et al*., 2009; Stieglmeier *et al*., 2012; Venkateswaran, 2012a; Venkateswaran, 2012b; Schwendner *et al*., 2013). The main reason for modification of the ESA standard has been that cotton swabs possess a comparatively high DNA content due to their organic nature (section I.3.2.). This might lead to contamination and consequently to detection of false-positives and overestimation when DNA is extracted from the swab samples (Bruckner and Venkateswaran, 2007).

A more sophisticated issue is the estimation of the recovery of organisms from surfaces. However, a recent research communication has shown that nylon-flocked swabs are superior in recovery efficiency from both, smooth and rough surfaces (Probst *et al*., 2010a).

Since there were no speculations possible about the microbial content being present during the MARS 500 experiment, a sampling tool should be used that is accurate, precise, robust, and effective for all different kinds of surfaces in the isolation facility. Those are made of wood, steel, glass, and ceramics (section III.1.6.). Robustness and effectivity of sample uptake by help of nylon-flocked swabs were displayed by scanning electron micrographs (section III.1.8.) whereas cotton swabs showed a high grade of disintegrated fibers after sampling (Probst *et al*., 2010a).

Additional requirements that have to be fulfilled were that the sampling tool was easy-to-handle and pre-sterilized in sterile single containers since the marsonauts were not familiar with sampling procedures. In order to enable reliable procedures and to prevent bias by inappropriate utilization, an initial training was held about sterile sampling. In terms of user-friendly handling, nylon-flocked swabs also had one major advantage since they are provided with a predetermined breaking point in contrast to the wooden shaft of cotton swabs which require a cutter. Considering the high number of sampling events and sampling sites as well as the need for one sterile cutter per sample, cotton swabs would have been impractically.

One could ask why wipes were not considered. Wipes have the major advantage that greater areas can be sampled (section I.3.2.), which in turn leads to more biological uptake and hence higher accuracy of low-biomass samples. Since the sampled wipe is rinsed in buffer, even high-biomass habitats can be wiped and analyzed by preparing dilution series and plating them. However, the ESA standard defined that the wipes have to be pre-packed in single containers and a certain amount of water should be added before autoclaving. This procedure is necessary because wipes cannot be delivered in single packed DNA-free and moistening increases sample uptake. Since Falcon tubes are not completely tight after autoclaving for more than 520 days, the wipes might dry out. Therefore, it is also recommended by ESA to prepare the wipes freshly just before each sampling event. However, this procedure was not operable in the MARS 500 facility due to missing equipment like an autoclave and as a consequence this tool is also not practicable.

Although the sponge-based sampling tools such as BiSKits delivered good results in recovery efficiency, analyses also reported severe problems with respect to DNA contamination in the included buffer (Kwan *et al*., 2011; Moissl-Eichinger *et al*., 2013).

After reviewing the wide variety of types of equipment available and their advantages and disadvantages, the tool with the least risk and the most benefits was chosen (Angelotti *et al*., 1958; Bruckner and Venkateswaran, 2007).

Throughout the campaign, surface sampling events took place monthly without any incident concerning sampling procedure itself. Furthermore, each of the supplied tools was functional (pers. comment: Charles Romain). The frozen samples were divided in four batches and returned in good shape for further analysis which is summarized in Table IV.4.1.

TABLE IV.4.1 SUMMARY OF SURFACE SAMPLING AND PROCESSING STRATEGIES

Strategy	"Vegetatives"	"Bioburden"	Molecular analysis
Sampling tool	swab wet	swab wet	swab dry
Sample extraction	in H_2O	in H_2O	in XS-buffer
Heat-shock (HS)	-	80°C, 15 min	-
Cultivation	R2A, 32°C	R2A, 32°C	-
Target	culturable heterotrophs	culturable, heat-tolerant heterotrophs and spore-formers	gene signatures of living and dead cells

The analysis of the first batch of swabs (samples from June to November of 2010) that was subjected to both cultivation approaches returned useful data. The same was true for the third and fourth batch. Since a few overgrowth events occurred in the first batch, a further dilution step was included for the analysis of the remaining samples, so that reliable results could also be obtained for those sampled locations of the MARS 500 facility that exhibited high-biomass. However, when the plate count analysis of the second batch that consisted of samples taken from December of 2010 until June of 2011 were performed, some of the plates displayed different amounts of colonies with the same characteristic color and shape (Fig. III.1.6.4). These could be clearly recognized as a contaminating strain since witness plates were affected, too. Purification and subsequent sequencing identified the contaminant as *Bacillus atropheus*. Fortunately, all plates that were not treated with heat-shock were analyzable. The distinction whether the observed colony was a contaminant or not was more difficult to make for the heat-shocked samples, but colony counting was performed in all conscience. This might have led to overestimations, but with regard to risk assessment it would have been worse the other way round. It has to be noted that *B. atropheus* is one of the strains that are used for various resistance tests in the Astrobiology group. Although all cleaning and sterilization procedures of pipettes, clean and lab bench were performed preliminary to unpacking and processing of the swabs just as in case of the previous batch, contamination of plates was observed which in addition revealed an inhomogeneous occurrence pattern. However, the most important factor concerning crew health is that the MARS 500 facility could definitely be excluded as contamination source since *B. atropheus* was not found in air samples which were taken in parallel to surface samples. None of the efforts to determine the origin of the contamination source led to an unambiguous assignment (section III.1.6.).

Conclusion:
The above-mentioned examples proved that the decision to use nylon-flocked swabs for microbial monitoring of surfaces in the MARS 500 facility was the best choice. Despite the fact that a contamination event occurred, it can be summarized that the obtained results from surface monitoring of the closed manned MARS 500 facility represent valuable data helping to deepen our understanding about the abundance of microbial contaminants and their diversity.

IV.4.1. Manned Spacecraft

The first survey of bacterial contamination with emphasis on manned spacecraft was conducted onboard the Mir between the years 1987 and 1999. The Russian standard for piloted space vehicles sets the limit for maximal microbial contamination of the interior to 1,000 CFU/100 cm². In general, the surfaces contained 1.2 to 6.8 x 10^7 CFU/100 cm² revealing strong variation with approximately 34 % of the samples being above the threshold limit defined for Mir, which were one log lower compared to the limits on ISS that are valid at present (Ilyin, 2000). 25 % exceeded even the threshold of 10,000 CFU/100 cm² (Pierson, 2001; Novikova *et al*., 2006).

From 1998 to 2005 quantitative surface sampling was performed on the ISS. In the Russian segment, the Test Tube Kit for Microbiological Sampling was used to estimate heterotrophic cell counts. Therefore, a 10 cm by 10 cm surface area was swabbed. In the American segment, the heterotrophic cell count was obtained by use of the American Surface Sampler Kit. Analysis of 243 swab samples revealed strong variations of contamination levels ranging from 25 to 43,000 bacterial CFU/100 cm² (Novikova *et al*., 2006). As mentioned above (section IV.3.2.), stringent microbial limits have also been described for surfaces onboard the ISS and can be looked up in the ISS Medical Operations Requirements Document (ISS MORD, 2006; Duncan *et al*., 2008). Surface acceptability limits were set to 10,000 bacterial CFU/100 cm². Violation of the established limit was determined in approximately 40 % of the samples.

When comparing these values to the data obtained from the MICHAm samples one has to keep in mind that on Earth gravitational forces lead to continuous settlement of airborne bacteria (Cox, 1989). This does not apply to space environments with prevailing weightlessness conditions so that microbes exhibit a different settling behavior. Therefore, in case of a real space flight a lower surface contamination level can be expected compared to the earth-based simulation.

As assumed, the vegetative heterotrophic cell counts obtained from MICHAm samples lied in the range between 0 and 297,600 CFU/100 cm². The limits were outvalued in 14 % of all samplings (Table III.1.6.2.1). However, the limit of 10,000 CFU/100 cm² was exceeded only in samples from the habitable module and in case of two events that took place at the vanity basin in the utility modules. Having a closer look at the samples retrieved from the habitable module, nearly all (13 out of 18) samples from the external surface of the toilet bowl revealed values above the limit. Similar effects were observed for 8 out of 18 samples taken on the table surface in the individual compartment. On the desktop surface close to the keyboard in the main panel, three outlier events occurred and on the surface of the dining table only one outlier was detected. Since with 6,750 CFU/100 cm² the overall mean value was also below the limit, there was no ultimate alert situation. However, it has to be kept in mind that the toilet bowl is a hot spot of microbial accumulation and prevention is necessary for the future.

As the reported values and the Figures III.1.6.1.1, III.1.6.1.3, and III.1.6.1.4 indicate, high fluctuation and variations regarding the microbial inventory were observed over the whole timeframe as well as from sample location to sample location within the MARS 500 facility.

Already during the monitoring program of Mir, variations in frequency of emergence and amplitude of changes were observed in cultivation studies. Identical findings were retrieved with molecular methods. Morris *et al*. (2012) also reported about prevailing inhomogeneity of microbial contamination onboard the ISS by using a portable and rapid microbial detection unit, the Lab-On-a-Chip Application Development Portable Test System (LOCAD-PTS).

Conclusion:
The investigation of cultivable microbial load was restricted to enrichment of heterotrophic "vegetatives" and the corresponding "bioburden". A more thorough assessment would have included a broader media assortment, which in turn would have led to an enormous amount of samples which, for time reasons, would not have been possible to be analyzed in the frame of this work. Nevertheless, the use of the same medium for all sampling sites allowed a deeper sampling, tracking of changes over the whole time, and the comparison of the microbial load of all sampling sites and modules. The applied sampling and processing scheme facilitated the identification of hazardous sources where microbial contamination is originated. This is a key step in risk analysis and mitigation efforts.

IV.4.2. Terrestrial Environments

Compared to enumeration studies of air, less information is available from similar studies of surfaces. For example, Hewitt *et al*. (2012) investigated the bacterial diversity of offices in three metropolitan, but most of the publications mainly derive from spacecraft and their surrounding clean rooms.
A lot of effort was put in microbial monitoring of exchange-restricted environments like clean rooms and hospitals. The allowed limits for microbial residents on spacecraft and their surrounding clean rooms were defined by the U.N. Outer Space Treaty in 1976 (COSPAR 2002, U.N. Outer Space Treaty, 1967). Five distinct planetary protection categories and thereby "bioburden" (spores per m^2) restriction levels were established. Spore-forming strains have multi-resistance properties and are of special interest since once carried to planets of significant biological and chemical concerns, these microbes could affect the search for extraterrestrial life by surviving a spaceflight.
In order to assess the risk caused by spore-forming strains, ESA and NASA implemented a protocol in their standards that clearly selects for enrichment of spore-formers and heat-tolerant microbes. This is achieved by performance of a heat-shock treatment, where samples are heated to 80°C for 15 minutes. Innumerable cultivation assays from Viking and other spacecraft have proven the presence of a broad variety of spore-forming microorganisms on spacecraft related surfaces. Based on the first results from the 70's, current space agency protocols concentrate on the detection of aerobic, mesophilic bacterial spores for the estimation of the overall bioload. In general, the highest microbial contamination level observed in all analyzed clean room was detected on the ground support equipment. A lower amount was determined on the floor and on the spacecraft itself (Vaishampayan *et al*., 2013).

The data obtained from diverse monitored clean rooms (ISO 8 to ISO 5, without strict "bioburden" control) housing a spacecraft revealed "bioburden" values between 0 and approximately 10^4 CFU/100 cm², whereas values from "vegetatives" ranged between 0 and 10^5 CFU/100 cm² and were well within the acceptable limits. These observations clearly indicate a reduction after HS treatment that was confirmed by various studies, and seems to be typical for this artificial type of environment (Venkateswaran *et al*., 2001; La Duc *et al*., 2004; Rettberg *et al*., 2006; Moissl-Eichinger *et al*., 2013). The "bioburden" load in the MARS 500 facility revealed comparable results (mean reduction of 85 %) with the occurrence of occasional outliers that might be derived from the contamination problem reported earlier (section III.1.6.; section IV.4.). Consistent with our findings none of the studies reported a correlation between the amount of vegetative CFU and the corresponding "bioburden" proportion. NASA scientists stated however, that a reduction of about 90 % of the total bioload can be expected after HS treatment (pers. comment: Dr. Kasthuri Venkateswaran).

Furthermore, the highly-controlled facility is characterized by relatively low human activity, dress regulations and controlled entrance permission by passing air locks dependent on clean room class. The distribution of "vegetatives" and "bioburden" load within the clean room turned out to be highly heterogeneous (Stieglmeier *et al*., 2012) which is comparable to our findings (section III.1.6.2.).

For comparison, hospitals and food production facilities also represent highly controlled environments and are even more restricted regarding the allowed microbial load per certain area. The European Commission Decision laid down that cleaned and disinfected surfaces in establishments for the production and marketing of fresh meat are within an acceptable range when total viable counts are between 0 and 1,000 CFU/100 cm^2 (2001/471/EC). For hand contact surfaces in hospitals, the total viable count should be less than 250 CFU/100 cm^2 (Mulvey *et al*., 2011).

Since little information was available about the microbial load on surfaces, samples were taken at DLR that covered a broad range of characteristics. Locations were selected on the one hand based on comparability with MICHAm samples and on the other hand they were chosen that they reflected as many different surfaces as possible. Cleaning frequencies, human occupancy, surface materials, and ventilation rates varied all among the surveyed locations (section II.2.2.; section III.1.7.; Table III.1.7.1).

Comparisons of the overall mean values for "vegetatives" showed higher values in MICHAm samples compared to surface samples taken at DLR. This is especially true for the dining table the desktop surface, and the toilet bowl and their corresponding surfaces in the confined facility (Table III.1.7.1; Table III.1.6.2.1).

It can be assumed that the higher values that were obtained during the MARS 500 experiment originate in the lack of exchange with the exterior and the relatively high number of people present in one room. None of the monitored rooms at the DLR was occupied for a longer time by six individuals, but was in contrast even left untenanted during the weekends. Furthermore, the cleaning frequency of once per day was higher compared to the maintenance of the isolation facility including vacuuming procedures that were stipulated to take place every Saturday (pers. comment: marsonauts). These might be only

the most obvious criteria that could have had an impact on the microbial load, but there are more factors that might explain inter- and intragroup variations of sampling locations and modules.

Conclusion:
The biocontamination levels on surfaces throughout the confined manned habitat, when compared to other places, reflected the adequate maintenance of the MARS 500 facility.

IV.4.3. Factors Influencing the Bacterial Community on Surfaces

When comparing the results of all surface samples taken in the MARS 500 facility, one should consider that different materials were monitored (Fig. III.1.6.1; Fig. III.1.6.2; Fig. III.1.6.3). They present either porous or nonporous, smooth or rough surfaces. The characteristics and features of surfaces consisting of wood and metal differ completely. In turn, the sample uptake and recovery efficiency is affected as well as the rate of microbial proliferation thereon (Valentine *et al*., 2008). One example where enumeration of samples resulted in interesting observations is displayed in Table III.3.4.1.1. The wooden rough surface revealed 9.5 CFU/10 cm², whereas a smooth desk surface led to four times higher enumerations. Both samples were taken with the nylon-flocked swab. These findings indicate a more efficient matter uptake from smooth surfaces compared to rough ones. All surfaces within the utility module were made of stainless steel, whereas all monitored samples from the medical and habitable module had a wooden character. There was only one exception in the habitable module, namely location 1 which was the external surface of the toilet bowl. Since the observed vegetative microbial inventory level of the utility module lies between the ones obtained for the remaining two modules (Fig. III.1.6.1.2), no definite statement can be made regarding the effect of the surface character on the amount of attached microbes.
Another aspect that has to be taken into account is the orientation of the area that was sampled. Vertically and horizontally swabbed surfaces might reveal different results as was shown by Berthier *et al*. (2012). The scientists discovered a lower contamination on vertical surfaces compared to the horizontal ones. This might be explained due to the fact that on horizontal surfaces dust can accumulate and provide nutrient sources for microorganisms which results in an increased microbial proliferation might occur. During the MICHAm campaign, seven samples were taken from horizontal surfaces (locations 1 toilet bowl, 5 desktop, 6 dining table, 7 table surface insulator zone, 8 table surface around water plum, 10 table surface in individual compartment, and 11 rack in storage area of clothes), whereas four samples (locations 2 vanity basin, 3 shower cabin, 4 wooden wall in community cabin, and 9 greenhouse) were taken from the walls.
This effect can also be the reason for the relatively low measured microbiota level being present on location four in the habitable module. Sampling of this wooden wall revealed only a mean of 36 CFU/10 cm², whereas the enumeration of vegetative heterotrophs from the remaining locations in the habitable module lead to mean values between 372 and

4,472 CFU/10 cm² (Fig. III.1.6.1.2). However, this significant effect was not observed for the samples of the utility module.

Additionally, all influences derived from environmental factors like RH, temperature, CO_2 and O_2 content and variations caused by ventilation systems that were discussed in the context of airborne microbial community apply also to surfaces (section IV.3.4.).

Moreover, it is assumed the human itself provides the major contamination source in confined habitats that were sterilized before being occupied, as it was the case for the MARS 500 facility. Various studies unveiled the fact that personnel represent the most common source of contamination in rooms which are strictly controlled (La Duc *et al*., 2012; Stieglmeier *et al*., 2012; Schwendner *et al*., 2013). Reinmüller and Ljungqvist (2003) stated that each human releases about 10^9 skin cells of the epithelial layer per day. This number is influenced by activities of the personnel and time that is spent in a certain room. Not only the human itself provides sources for microbial contamination, also shoes and clothing are colonized by microorganisms which are brought into the indoor environment (Moldenhauer, 2005).

Studies of less controlled environments uncovered the same effect and revealed that the microbiological contamination level is generally increased in areas with intensive human activities compared to empty facilities (Maule *et al*., 2009; Morris *et al*., 2012; Dunn *et al*., 2013). These impacts include dining, hygiene, exercise and vacuuming which might lead to a reallocation and/or increase of nutrient availability. Furthermore, in the frame of this study and in collaboration with Dr. Johannes the sole investigation of the presence of humans in a certain area exhibited no significantly correlation (section IV.3.4.)

On normal human skin, the total aerobic bacterial counts range from more than 1 x 10^4 CFU/cm^2 on the forearm, 4 x 10^4 CFU/cm^2 on the abdomen, and 5 x 10^5 CFU/cm^2 in the axilla to 1 x 10^6 CFU/cm^2 on the scalp (Selwyn, 1980). It is estimated that an average person disperses as many as 10^6 to 10^7 particles per day (ca. 10 to 100 particles per second) by natural skin renewal and shedding (Noble, 1975). These fragments (dry skin, dandruff) function as carriers or rafts for microorganisms. The likelihood of airborne dispersion is also enhanced by perspiration. In addition, as saliva contains approximately 10^8 CFU/ml, coughing or speaking expels between 10^3 and 10^4 droplets (sneezing up to 10^6) and consequently a certain amount of viable bacteria (Wilson, 2005). The human body as potential contamination source can be determined by investigation of the microbial diversity.

Conclusion:

Similarily to the airborne concentration level, the surface microbial load is dependent on a myriad of different factors. With regard to the confined manned MARS 500 habitat, it is proposed that the human itself constituted the major potential contamination source releasing microbial cells by skin renewal and shedding, coughing and speaking.

IV.5. Elimination of Microbial Contamination

In general, the proliferation of pathogenic bacteria has to be obviated rigorously to guarantee the residents'/astronauts' health.
First of all, microbial growth can be prevented by a consequent elimination of dampness to keep the indoor RH at a constant low level since this is one of the limiting factors for growth and proliferation of microbes.
Furthermore, microbial contamination can be reduced by accurately cleaning with efficient detergents that should be readily available. However, the marsonauts reported that they ran out of cleaning agents during the isolation program. Conflicting opinions of the crew on motivation and performance of maintenance procedures posed another problem. These differences were unveiled by the group around Prof. Dr. Gro Sandal and are indicated in the plot on subjects' ratings of their satisfaction with the hygiene of other crew members (Fig. IV.5.1). Different perception might also lead to different effort while cleaning, which might have also caused inter-sample fluctuations since every team member was responsible for a certain area.

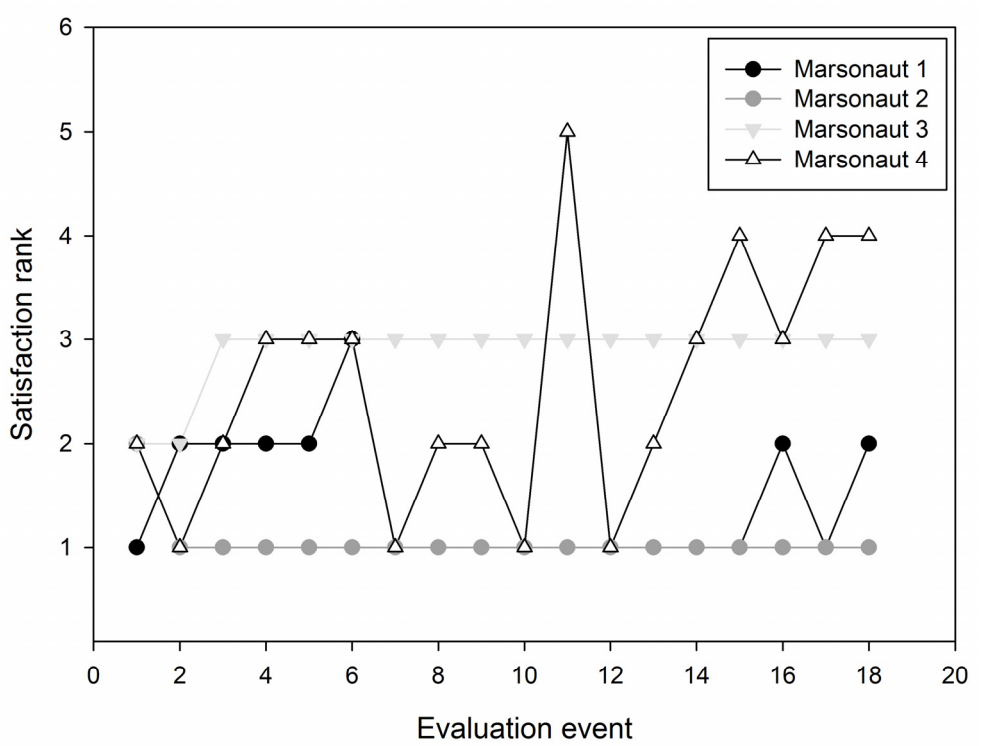

Fig. IV.5.1 Ratings of the crew members' satisfaction with the hygiene. A five point scale was used from 1 (very satisfied) to 5 (very unsatisfied) (data provided by Prof. Dr. Gro Sandal).

Generally, the following principle has to be kept in mind: "only a removed microorganism is a good microorganism" which implicates that only killing the cells is not sufficient (Schleibinger *et al.*, 2004). The emphasis has to be laid on the complete removal of biomass since dead cells provide nutrient sources for the remaining microbes.

Conclusion:
Prevention and reduction of microbial contamination is only possible when microbial hot spots have been identified in advance. Furthermore, the perception of cleanliness is bound to be subjective and therefore, the effort that is put into cleaning activities varies from individual to individual. Therefore, there is an urgent need to find a way to reduce contamination passively.

IV.6. Molecular Methods

Molecular studies have been initiated step-by-step to systematically and comprehensively characterize the microbial abundance and diversity that are associated with our homes, offices, public areas, spacecraft, and the human body itself.
However, each of the cultivation-independent techniques has their own pitfalls and biases (Von Wintzingerode *et al*., 1997; Head *et al*., 1998; DeLong and Pace, 2001).

IV.6.1. Determination of the Best-suited DNA Extraction Method

The first step includes extraction of the entire DNA from, in the case of MICHAm experiment, an environmental swab sample. The higher the efficiency of the extraction process and the better the quality of the isolated DNA, the more reliable and insightful are the results of the applied downstream methodology. Therefore, the choice of the optimal extraction method is critical. It is important that the lysis of the different microbial species within the given environment is maximized, even though all of them have specific cell walls. For example, gram-negative cells are easier to disrupt than gram-positives. In addition, spores are known to be harder to break up (Nicholson *et al*., 2000; Kwan *et al*., 2011). To overcome these drawbacks, additional steps can be included either on a chemical basis or by mechanical cell/spore disruption. However, recovery of high quality gDNA demands a balance between maximizing the yield with efficient cell disruption techniques and minimizing shear forces that will degrade DNA of more fragile cells (Radosevich *et al*., 2002). These requirements have to be fulfilled to avoid artifacts in PCR amplification and possible formation of chimeric PCR products (Liesack *et al*., 1991). Most of the techniques that are based on mechanical disruption, such as ultrasonic horn treatment and French Press, were not applicable because greater volumes with higher cell density are required and the probability of introducing contaminants is increased (Belgrader *et al*., 1999). In 2011, Kwan *et al*. demonstrated the maximized effectivity of DNA yield when a bead-beating step was applied prior to DNA extraction. This method is so far seen as the most practicable solution, but it also results in a loss of sample volume and is therefore not recommended for low-biomass samples, as observed for MICHAm, where even pooling of swabs was essential to obtain enough DNA (Schwendner *et al*., 2013). Furthermore, the application of chemicals to break up the cells has to be compiled with parallel removal of any possible inhibitors for further analysis.

Based on these reasons, the extraction process had to be optimized. Therefore, tests were performed with a purchased, widely used DNA extraction kit and with the XS-buffer method, representing a classical phenol extraction protocol (section III.3.1.). Recent comparative studies for the purification of DNA from low-biomass samples based on total yield have shown that the XS-buffer method is more efficient than a kit (La Duc *et al*., 2009). However, this finding had to be proven for swab samples. Besides spiked swabs, cell solutions were also evaluated. Interestingly, DNA yields from staphylococci-spiked swabs were generally higher compared to the corresponding cell suspendions. This effect might originate in the production of external DNases by staphylococci (pers. comment: Prof. Dr. Reinhard Wirth). The degradation effect in liquid is higher since DNA molecules are more accessible for the enzymes when compared to swabs where the liquid is distributed over a larger surface area due to the plethora of fibers on the swab's head. The test further clearly demonstrated superior performance for DNA isolation with the XS-buffer method (Fig. III.3.1.1). As also reported by Willner *et al*. (2012), DNA extraction methods varied in their technical reproducibility, resulting in high standard deviations, which were higher for the XS-buffer extracted samples. The potential for revealing higher technical variation by use of phenol-based methods is more probable due to the greater number of handling procedures that have to be performed in comparison to the kit. The latter additionally uses pre-made buffers and column purifications that reduce the introduction of error. Since only one swab was available per sampling event from a specific location within the MARS 500 facility, it was more important to reach higher extraction efficiencies than reproducibility, especially with regard to low-biomass samples. Another aspect that had to be considered was the pooling method. Due to the fact that one swab was not efficient to gain enough DNA (section III.3.3.; section III.3.4.), several swabs had to be pooled. For the tested protocols, no significant difference was observed in obtained gDNA yields of spiked swabs and environmental swabs samples (Fig. III.3.4.1.2; Fig. III.3.4.2.1), hence both methods could have been used for MICHAm samples. However, for swabs that have been pooled in the beginning, the whole sample set is lost in case irregularities occur during processing (e.g., contamination, dropping or spilling of the substance). On the contrary, comparing the handling procedure itself, it is recognizable that time, effort, and materials input is much (up to five times) higher when pooling is done consequent upon extraction process. Based on this background and on the number of swabs (n=68) that were planned to be moved on to DNA extraction, it was decided to directly pool the swabs in XS-buffer. Thus, extraction was carried out in only one reaction vessel per pool (either four or five swabs depending on the module). The protocol was followed without occurrence of any incidents and resulted in a successful extraction process of all samples, revealing a low quantity but of high quality gDNA (section III.3.6.1.).

Furthermore, it was also of great need to reliably quantify the isolated DNA. The Qubit® 2.0 Fluorometer™ recommended by Second Genome is superior compared to NanoDrop® ND-2000, since it distinguishes between DNA and RNA. In addition, it accurately quantitates DNA as low as 10 pg/µl, whereas the use of UV absorbance measurements generally leads to significant overestimations and variations in the same sample. Therefore, Qubit 2.0

Fluorometer™ was applied for all MICHAm samples and PCR amplification products in order to quantify the isolated gDNA as precisely as possible.

IV.6.2. Recovered gDNA from Environmental Samples

As previously described in section III.3.2. for DLR surface samples (Table III.3.2.1) and in section III.3.6.2. for MICHAm (Fig. III.3.6.2.1f), there was no correlation between CFU counts and extracted DNA, due to heterogeneous distribution of cells on surfaces (section III.1.1.; section III.1.6.1.; Table III.3.4.1.1.). Indeed, great variations were also detected when comparing gDNA yields from samples that were from neighboring surfaces in close proximity. Radosevich *et al*. (2002) described an identical observation after cultivation and extraction of gDNA from taken environmental air samples in a parallel manner. The reported effect might be grounded on the fact that both the cultivation and lysis processes are biased. Moreover, DNA isolation from an environmental sample might also lead to overestimation bias since the samples also contain fungal DNA, which cannot be distinguished from bacterial DNA by the DNA measurement technologies.
Therfore, it is not possible to predict the amount of DNA based on cultivation-dependent data by enumeration of CFU.

IV.6.3. PCR Setup

Once the extraction process had been optimized and high quality DNA was obtained, different downstream analyses were available. PCR was used as an initial step to multiply the target molecules. Therefore, universal primers were applied to cover a broad range of detectable gene signatures. However, depending on the species' 16S rDNA sequence, these primers bind with different affinities to their DNA target (Forney *et al*., 2004). Thus, during amplification of the isolated DNA, a taxa bias will be introduced in environmental samples possessing heterogeneous 16S rRNA gene signatures. This effect is also observed but minimized when degenerated primers are used, as was done for MICHAm samples (Baker *et al*., 2003). Since broad range 16S rDNA PCR is a highly sensitive method, the chance of obtaining false positive results due to contamination of the working equipment needs to be addressed carefully. To identify potential error due to contamination, negative controls were carried along that revealed an aseptic procedure (Fig. III.3.3.1; section III.3.6.5.). Though it is theoretically possible to detect rare species by amplifying genes from the few original copies, the binding of the primers is not an actively guided process, thus heavily influenced by the starting copy numbers and affinity of each 16S rDNA sequence (Farrelly *et al*., 1995). Contrary to this, the PCR reaction can be inhibited by adding too much DNA template in the PCR reaction setup. The results displayed in section III.3.5. demonstrated a great dependency on the efficiency of initial added DNA amount. Inhibition occurred at gDNA input of 9 ng in the reaction setup used for PhyloChip assay. However, Second Genome demands higher input amounts of 10 to 30 ng. When PCR yields of MICHAm samples were compared to results from environmental samples, the following curve was obtained after PCR with Molzym™ 16S Basic Master Mix, which

displays a relatively low efficiency for amplification performed by Second Genome (Fig. IV.5.3.1).

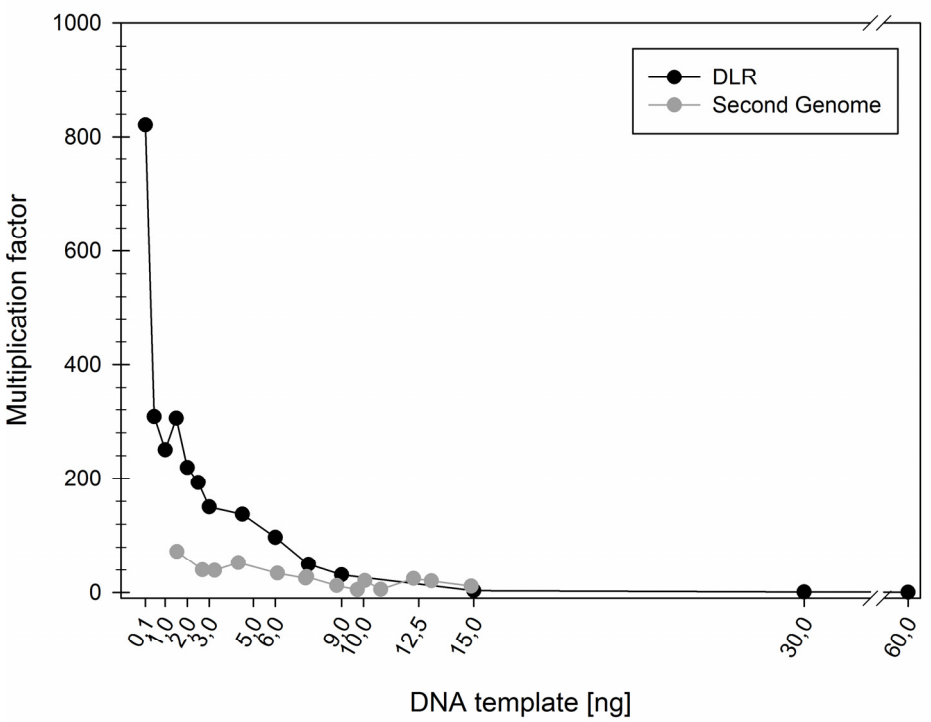

Fig. IV.5.3.1 Comparison of relationship between gDNA template input and efficiency of the PCR using the Molzym™ 16S Basic Master Mix. gDNA isolated from environmental swab samples taken at DLR (Fig. III.3.5.1.1) and from the MICHAm samples served as templates (Table III.3.5.4.1). DLR samples are expressed as mean (n=2) values, n=1 for MICHAm samples processed by Second Genome

This was very surprising since representative samples taken at DLR that were tested in advance (Fig. III.3.3.1; Table III.3.3.1) demonstrated that even with up to 100-fold less template input, the demanded 500 ng per reaction can be reached. Besides the pre-test with environmental samples, PCR assay was performed with selected MICHAm samples and more adequate results were received with the TaKaRa ExTaq® protocol Second Genome wanted to use initially (Fig. III.3.6.3.1), compared to the performance of the ultimately applied Molzym™ 16S Basic Master Mix. All the above-mentioned variations might lead to biased amplification and inaccurate identification of the present bacterial species. However, even though lower values were obtained and different amounts of PCR product were hybridized (Table III.3.6.4.1), the data read out from each single PhyloChip are comparable when the measured fluorescence intensities are rank-normalized, as done. In order to check the quality of the rank-normalization, the results of the distribution of the assigned ranks were displayed in a Box-Whisker-Plot (data CD, [folder: PhyloChip: Second Genome Supplementary]), which revealed no outlier samples and a similar distribution for all arrays. Therefore, data of all PhyloChips are comparable among themselves.
These facts have to be kept in mind when analyzing a microbial community based on PhyloChip data that are derived from fluorescence intensities of PCR-amplified gDNA when bound to the corresponding probe.

Conclusion:
The data presented herein clarified the importance of accurate planning of experiments and the need to perform pre-tests in order to choose the best-suited method for sample processing. This is based on the fact that microbial contamination present in a certain habitat and its development over time cannot be predicted. The same is true for the prognosis of the DNA amount after extraction that can be expected since the yield did not correlate with the obtained colony forming unit counts.

IV.7. Microbial Community Analysis Platforms

For assessing the microbial community that is present in an environmental sample, the construction of clone libraries is an approved method that is economical and relatively fast, but semi-quantitative (section I.4.2.1.). The recovery rate from complex microbial communities is lower compared to sparse communities which lead to bias. In addition, the detected diversity is shifted towards high-abundance gene copies. This tool is not applicable for the MICHAm samples since we aimed to obtain insights into changes of the microbial community structure. This means that at least 14 clone libraries of a highly diverse community would have been needed. Furthermore, high-density universal 16S rRNA microarray analysis reveals a broader diversity (70-fold more bacterial taxa) than typical clone library when sampling the environment (DeSantis *et al.*, 2007, La Duc *et al.*, 2009).
Genetic fingerprinting methods such as T-RLFP (section I.4.2.2.) and ARISA (section I.4.2.3.) generate a profile of microbial communities either on sequence polymorphisms or length polymorphisms, and also allow simultaneous analyses of multiple samples (Marsh, 1999; Rastogi and Sani, 2011). However, the resolution of inhomogeneous and complex community structures is not as good as microarray technology, which was the tool of choice.
The knowledge about ubiquity and complexity of microbial communities has been continuously improved (Hugenholtz, 2002; Wilson *et al.*, 2002; Breitkopf *et al.*, 2005; Vaishampayan *et al.*, 2010). The latest advances in high-throughput sequencing supplemented culture-based approaches (Segata *et al.*, 2013) and allowed a new and deeper understanding of microbial community structures. One possibility is the application of 454 pyrotag sequencing (section I.4.2.4.). However, the formation of homopolymers leads to more probable sequencing errors when compared to Sanger Sequencing assigning sequences on the species level (DeSantis *et al.*, 2007). Although sequencing approaches provide a deeper investigation of a microbial community regarding the amount of analyzed amplicons than cloning, less phylogenetic resolution is observed due to the short sequence length that allows only for classification on the family and/or genus level. Illumina enables the analysis of a greater part of the “rare-diversity” since more reads are generated when compared to 454 sequencing (Caporaso *et al.*, 2012; section I.4.2.5.). However, due to the short reads via Illumina MiSeq of about 100 bp, which is similar to 454, an identical taxonomic resolution is obtained. Illumina HiSeq is preferred over the above-mentioned since identification on the genus level is possible.

The advent of phylogenetic DNA microarray technologies has dramatically increased the resolution and accuracy of detection of distinct microbial lineages in mixed microbial assemblages (Vaishampayan *et al*., 2013). The second-generation (G2) PhyloChip was developed by DeSantis *et al*. (2006) and has been applied to monitor urban aerosols (Brodie *et al*., 2007), clean room environments (Probst *et al*., 2010b), human clinical samples (Maldonado-Contreras *et al*., 2011; Saulnier *et al*., 2011), and soil communities (He *et al*., 2012). According to present knowledge of sequences, the PhyloChip G2 probes were designed to complement only the sense strand (Probst *et al*., 2013a). Contrary to G2, PhyloChip of the third-generation (G3) complements the sense as well as the antisense strands, contains a greater diversity of probes, and therefore leads to greater sensitivity and possibly detectable diversity with detection limit of bacteria that represent as little as 0.01 % of community (Brodie *et al*., 2007, section I.4.2.6.). The presence and relative abundance of more than 50,000 bacterial OTUs can be determined by applying PhyloChip G3, which was firstly described by Hazen *et al*. (2010). Generally, it allows not only the deepest sequencing for routine 16S rRNA amplicon analysis, but also a parallel screening of several samples. One downfall is that new microorganisms cannot be detected due to the probe design and the coverage of the microbial diversity that is limited to the number of probes being present on a chip. Consequently, the high-density PhyloChip G3 is a powerful and sensitive tool to provide insights into the microbial composition of known lineages and its changes over time. This is especially true for the detection of shifts in minority populations. Additionally, the data can also be custom analyzed by selection of probe sequences that are complementing the 16S rRNA genes of specific strains or taxa, which is beneficial since we intended to track specific potentially pathogenic organisms and their abundance shifts over time. Convinced by the afore-mentioned aspects, the PhyloChip was the appropriate tool to comprehensively analyze the MICHAm samples based on the questions that should be addressed.

However, it has to be mentioned that one short-coming of all herein presented DNA-based molecular methods is that these results may not accurately reflect the living/active members of a community, since DNA signatures of dead cells and/or “naked” DNA are also taken into account. DeAngelis *et al*. (2011) therefore suggested the extraction of the less stable rRNA, which can be directly used for hybridization or subsequent reverse-transcription to cDNA. Using this method, DeAngelis and Fierstone (2012) demonstrated different microbial profiles compared to the ones generated from genomic DNA.

Another possibility is to mask free DNA molecules derived from dead organisms prior to DNA extraction with an intercalator called propidium monoazide (PMA). Since PMA cannot enter cells with an intact cell membrane (Nocker *et al*., 2007), only DNA molecules of living cells are accessible for DNA polymerase during PCR amplification reaction. Vaishampayan *et al*. (2013) found significantly different microbial community structures in low-biomass clean room samples depending on whether PMA was added or not. The effect is more pronounced the higher the content of dead cells since a greater amount of free DNA is present.

Conclusion:
Real-time quantitative PCR or PCR with specific primers, construction of several clone libraries, terminal restriction fragment length polymorphism (T-RLFP), next-gen sequencing or DNA microarray-based hybridization technique constitute several state-of-the-art techniques that are in line for microbial community analysis. Considering assets and drawbacks from the afore-mentioned, PhyloChip G3 assay was the best choice.

IV.8. Microbial Diversity

To date, approximately 7,000 bacterial and archaeal species have been enriched and validly described[21]. First hints that cultivation approaches deliver only a minor fraction of the existing microbial diversity were given in "the great plate count anomaly" (Staley and Konopka, 1985). Comparative sequence analysis and whole genome approaches mentioned above (section IV.7.) confirmed that the diversity is much greater than imagined, and unveiled new insight into the evolutionary history. This led to the description of 30 cultivable and 40 candidate phyla. The latter contain only members which have not been cultivated so far (McDonald *et al*., 2012). The fact that today the majority of the known bacterial phyla cannot be cultured in the laboratory stresses the importance of complementary culture-independent methods when characterizing bacterial communities in depth.
Literature reveals a large number of phylogenetic studies regarding indoor microbial community structure, since estimation of contamination levels only does not necessarily reflect the real microbiological risks (Van Houdt *et al*., 2009). These approaches unveiled a high prokaryotic diversity that consists of different bacterial and archaeal phyla. In the study described herein, the main focus was laid on the detection of bacterial gene signatures.

IV.8.1. Isolated Microorganisms

The cultivation approach was based on one enrichment condition to target aerobic, vegetative heterotrophs and "bioburden" in air and on surfaces. The findings presented from the MARS 500 facility resulted in the observation that the airborne community was mainly formed by Firmicutes, Actinobacteria, and Proteobacteria. Of these, the Firmicutes represented the overwhelming majority within all three modules. This result is in accordance with studies from the Concordia base (Van Houdt *et al*., 2009). Mainly staphylococci and, in lower abundance, bacilli were observed in all three habitats, which is consistent with findings in periodically confined habitats, such as airplanes (Osman *et al*., 2008) and Concordia base (Van Houdt *et al*., 2009). Space-related environments, such as onboard the ISS (Castro *et al*., 2004) and manned Russian space vehicles (Ilyin, 2000), also revealed identical microbial structures.
Most of the detected airborne genera are ubiquitously (soil, water, plants, food, human) distributed (section III.1.5.). However, the microbial community structure of each monitored

[21] List of Prokaryotic names with Standing in Nomenclature: LPSN www.bacterio.net

module showed slight variation, and *Staphylococcus* was the only microbial genus that was observed everywhere. In addition, the habitable module and the utility module shared the incidence of airborne *Sphingomonas*, but also exhibited their own flora consisting of at least five different genera, including unclassified species (Fig. III.1.5.2). This finding hinted at the prevalence of different microbial community structures, which was confirmed by surface sample analysis and PhyloChip assay (Fig. III.1.9.1.2; section III.3.6.5.). The high number of organisms that could not be annotated on the highest resolved taxonomic level might originate in sequence lengths of only 700 to 1000 bps that were used for alignment. Ideally, the whole 1.5 kb 16S rRNA gene sequence would have given more accurate results. This was not implicitly required since molecular methods complemented the findings. However, if more detailed information would have been desired, the isolate sequence of interest could have been further investigated by sequencing the whole 16S rRNA gene or other diverse gene regions that help to exactly identify the species.

The distinct prevalence of gram-positive bacteria in the air (~95 %; Fig. III.1.5.1) is also in accordance with the above-mentioned studies of the ISS and Concordia. Gram-positive genera are known as a characteristic part of the airborne microbial community, in contrast to gram-negative bacteria, which are normally not found in the air or only to a small degree (Cox, 1989). This is due to the fact that they are less protected against the hostile conditions prevalent in airborne environments than gram-positives. *Micrococcus luteus* is a gram-positive airborne representative that is also part of the skin flora. This strain reveals yellow colonies when grown on R2A, TSA, or on blood agar. The embedding of carotinoids in the cell wall that results in yellow pigmentation is a feature for better protection against permanent outdoor UV radiation, and leads to longer survival. Many airborne bacteria possess this protective feature and form colored colonies (Schleibinger *et al*., 2004). However, the higher survival rate does not include that gram-positives are multiplying in their airborne state. The augmented detection of gram-negatives in indoor air is a good indicator for high RH in the building (Schleibinger *et al*., 2004), but the latter was not true for the isolation facility, since an overwhelming presence of gram-positives was detected in air and on surfaces.

Generally, it is anticipated that microorganisms are ventilated from outside into indoor areas, but that was impossible for the MARS 500 facility. The hatch was instantly closed after the crew entered the previously sterilized mock-up spacecraft, thus from that point, microbial community was no longer influenced by the external microbial load. Once airborne germs have settled, they start to colonize surfaces and reproduce if appropriate conditions are encountered, or at least remain in a viable but nonculturable (VBNC) or a resting (spore) state. On that account, the surface sediment could reflect a fraction of the airborne contaminants (Dunn *et al*., 2013). A similar distribution regarding the occurrence of gram-positives and gram-negatives in air as well as on surfaces (Fig. III.1.5.1 air samples; Fig.III.1.9.1.1 surface samples) gave the first hint to corroborate this hypothesis. Regarding the diversity, the study described herein revealed a substantially lower number of identified taxa in the air than on sampled surfaces (section III.1.9.). An identical trend was observed by Tringe *et al.* (2008) and on space vehicles (Ilyin, 2000). The studies from the MARS 500 facility suggested a broader diversity in surface samples and covered the information

obtained by air sampling. Variations were recognized exclusively on the species level. *Corynebacterium amycolatum*, *Enterobacter hormaechei*, *Kocuria marina*, and *Pseudomonas geniculata* could only be enriched from the air. In fact, 100 % overlapping of the air sample community with surface isolates on the genus level was discovered which is even more significant when compared to the observations by Tringe (Table III.1.9.1.1). However, this slight difference in ascertained overlap might have been evolved in consequence of the confinement, where no exchange with the exterior is possible. This implies that the contamination sources are less manifold and the MARS 500 community is less influenced by outdoor airborne germs, which differ from indoor airborne microbiota (Osman *et al*., 2008). On this account, our study validated the common presumption that the airborne microbes are a random assortment of aerosolized cells from nearby environments, such as dust and water bodies, since the air environment is inadequate to sustain growth (Cox and Christopher, 1995; Tringe *et al*., 2008).

Lacey (1994) stated that the amount and spectrum of indoor bacteria are strongly influenced by a broad range of factors like occupants, plants, and ventilation systems. Furthermore, pets, geographical location, and seasons are also mentioned to impact the microbial flora. However, the latter are neglectable regarding the MICHAm experiment due to confinement, initial sterilization, and the absence of pets.

Despite variations in the module-specific microbial structure, a core microbiota consisting of members belonging to ten different genera (*Aerococcus*, *Bacillus*, *Corynebacterium*, *Enhydrobacter*, *Methylobacterium*, *Microbacterium*, *Micrococcus*, *Paracoccus*, *Pseudomonas*, and *Staphylococcus*) was identified within the isolation facility (Fig. IIII.1.9.1.2). Most of the above-mentioned genera are typical representatives that are known to be associated with humans, such as staphylococci and *Corynebacterium* spp., which can be found in respiratory, gastro-intestinal, and genito-urinary tracts, as well as on skin. *Enterobacter* spp. inhabits the respiratory and gastrointestinal tract. *Micrococcus luteus* can be enriched from skin, whereas *Pseudomonas* spp. exists mainly in the gastrointestinal tract. In contrast, bacilli cannot be assigned to a certain habitat. They are known as typical soil bacteria, but are also part of the normal human microbial flora of the gastrointestinal tract and of the skin (Murray, 2010). The same is true for *Aerococcus* that is found in the upper respiratory tract or on skin but also in air, dust, and vegetation. *A. viridans*, for example, can also rarely cause bacteremia (Uh *et al*., 2002). Slow-growing *Methylobacterium* is a facultatively methylotroph and can easily be distinguished from other colonies on agar plates due to its pink pigmentation. Some strains are suggested to be part of the natural human foot and mouth flora, but are mostly found in soils and on the leaves of plants (Lidstrom and Chistoserdova, 2002; Anesti *et al*., 2004; Anesti *et al*., 2005). Up to now, only one species is assigned to the genus *Enhydrobacter*, namely *E. aerosaccus*, a facultatively anaerobic heterotroph (Staley *et al*., 1987). Paracocci are considered environmental strains and were frequently reported to be part of the soil community, although *P. yeei* also exhibits a low pathogenic potential (Funke *et al*., 2004).

Results of the phylogenetic data indicated that human-associated microbes (either as members of the normal flora, commensals, or as clinical specimens causing nosocomial infections) were dominant, whereas environmental strains were less prevalent on surfaces

and in the air. This is not surprising since Hospodsky *et al*. (2012) stated that human occupancy is the major source for indoor airborne bacteria in occupied buildings. Similar findings were published for clean rooms. Even though, depending on the clean room class, personnel had to wear special suits, and items that were brought into the clean room had to pass air locks to minimize the import of microbial contaminants, humans still represent the major contamination source (Stieglmeier *et al*., 2009; Stieglmeier *et al*., 2012; Moissl-Eichinger *et al*., 2012; Moissl-Eichinger *et al*., 2013; Schwendner *et al*., 2013). This statement is based on the ubiquitous distribution of staphylococci and bacilli that are typical human-associated microorganisms within these habitats. However, as commonly accepted, the human microbiome represents a complex interplay of an extremely diverse community with all different kinds of (micro-)niches influenced by external and internal factors. Grice *et al*. (2008) analyzed samples from the inner elbow of five healthy humans by creating a clone library, and found 16S rRNA sequences that closely matched *Staphylococcus epidermidis* and *Propionibacterium acnes*. However, these two strains represented only less than 5 % of the analyzed microbiota. This is contrary to the commonly held notion that *S. epidermidis* is the dominant aerobic bacteria residing on the skin (Marples, 1965).

It is reasonable to question the absence of *Propionibacterium* in the cultivation approach since members of this genus are known to be part of the human skin flora. Furthermore, Flores *et al*. (2011) demonstrated the major prevalence of *Propionibacteriaceae* on surfaces routinely touched with our hands, especially with regard to restroom surfaces, which have also been monitored in the MARS 500 facility. It has to be mentioned that *Propionibacterium* is a slow growing gram-positive anaerobic bacterium. By use of only aerobic enrichment conditions, cells of this taxa are not targeted, and consequently not detected within the MARS 500 facility by the cultivation approach. However, PhyloChip data revealed at least five eOTUs that are assigned to this bacterial lineage. Previous studies from clean rooms showed that by use of alternative media, isolation of *Propionibacteria* is feasible (Moissl-Eichinger *et al*., 2013). A large study of the airborne microflora in apartments, multi-family buildings, homes, and offices in Poland revealed in addition to the two mentioned genera, *Staphylococcus* and *Propionibacterium*, *Micrococcus* and *Kocuria* (Górney and Dutkiewicz, 2002), which were also detected in air and on surfaces of the MARS 500 facility, albeit to a lower extent. Strains belonging to these two genera were often found in the oral cavity, skin of palm, and forearm of healthy humans (Szczerba, 2003). A study by Aydogdu *et al*. (2005) performed in primary schools reflected a microbial community structure that is also consistent with the results obtained from the MARS 500 facility. The bacterial community was dominated throughout the year by the afore-mentioned genera, such as *Staphylococcus*, *Acinetobacter*, *Corynebacterium*, and *Pseudomonas*. In addition, great variation in the diversity was reported, which is also relevant for MICHAm samples.

Moreover, *Sphingomonas* spp., which was mainly enriched out of the utility module, has also already been isolated from the air and dust (Busse *et al*., 2003). Generally, this genus is widely distributed in water habitats, and has been isolated from water samples taken in the humidity condensate recovery system onboard the ISS (Castro *et al*., 2004).

As reported above, another focus was laid on the “bioburden” diversity, which was obtained after HS treatment that selects for spores and heat-tolerant vegetative cells. This fraction is

of eminent importance regarding planetary protection consideration due to its plethora of different properties that protects it from harsh environmental conditions, such as UV radiation, desiccation, heat, and low nutrient levels (Cano and Borucki, 1995; La Duc *et al*., 2007). Since spores and heat-tolerant organisms are very resistant, they are often found in normal air. Typical spore-forming genera are *Bacillus*, *Paenibacillus* - isolates of those two genera were enriched from the MARS 500 facility - and *Clostridium*. In particular, representatives of the genus *Bacillus* have been regarded as one of the most harmful and resistant spore-forming microorganisms for planetary protection related issues. Nowadays, this way of thinking is more and more extended to also consider paenibacilli. Spore-forming strains are hard to kill due to their multi-resistance properties, which allow them to survive, e.g., HS treatment (see MICHAm sample processing; section II.4.4.2.) and application of chemical disinfectants (cleaning of MARS 500 facility).

M. luteus, *S. epidermidis*, *S. hominis*, and *P. yeei* are examples of vegetative cells that also survived the HS treatment. Those strains have previously been described as heat-tolerant after HS treatment of clean room samples (Moissl-Eichinger *et al*., 2013). Besides those, some vegetative cells of *Brevibacillus*, *Enhydrobacter*, and *Sphingobacterium*, which were retrieved from the MARS 500 facility, also survived incubation at 80°C for 15 min. However, the vast majority of the "bioburden" was constituted of the spore-forming genus *Bacillus*. Some examples are: *B. safensis*, revealing the highest abundance; *B. anthracis*, which is a class-3 human pathogen and the etiological agent of anthrax (Spencer, 2003); as well as *B. licheniformes*, *B. subtilis*, and *B. pumilus* that are associated with food toxi-infections (Salkinoja-Salonen *et al*., 1999; From *et al*., 2007; Stenfors Arnesen *et al*., 2008; Fig. III.1.9.2.1).

Although alignment characterized most *Bacillus* gene signatures on the species level, these data represent only the first indications regarding their taxonomic position. Based on their horizontal gene transfer ability, bacilli can display a broad variety of plasmids in terms of number, size, or genetic determinants. In order to reliably characterize each *Bacillus*, the whole 16S rDNA as well as additional genes, especially the ones on plasmids, have to be taken into account (Timmery *et al*., 2011). However, in the frame of the study presented herein, this would go too far, especially when considering that in the case of Concordia base and the ISS, no specific virulence feature was observed (production of toxins or unusual antibiotic resistance) for any of the 43 bacilli, although genetic transfers can contribute to its capacity to spread (Timmery *et al*., 2011). Differences in the abundance of bacilli gene signatures were detected when cultivation data were compared with observed eOTUs obtained from microarrays.

Conclusion:

Phylogenetic classification of the enriched isolates revealed mainly human-associated microorganisms and environmental strains to a lower extent. This fact indicated that of the variety of potential contamination sources in a confined manned habitat, e.g., equipment, green houses, food supplies, and housing structures, none is more persistent and pervasive than the human being.

IV.8.2. Molecular Microbial Community

Once the fluorescence intensities of each array were received, the OTU selection followed. One has to keep in mind that the analysis methods for OTU discovery have changed in the course of microarray studies done with G3. Hazen *et al*. (2010), for example, analyzed the community by selecting reference-based OTUs (rOTU) with a tool called “PhyCA”, whereas the study presented herein is based on the final determination of empirical OTUs (eOTU) by use of a supplementary tool called “Sinfonietta” (Probst *et al*., 2014). This was applied when fluorescence intensities have been matched with the probes.

The eOTU selection process for analysis of PhyloChip data obtained from the MICHAm samples was chosen based on the following reasons: The great advantage is the option that probes, probe-pairs, probe-quartets, and/or probesets can be evaluated, which allows higher flexibility in data analysis compared to the pre-defined sets of probes for rOTU determination. Additionally, a greater confidence was achieved in sequence-specific detection and phylogenetic classification, since a larger number of probes were utilized. These aspects lead to a more straight-forward selection of outliers. Furthermore, the new “Sinfonietta” analysis could even have been used to track potentially novel species which were previously not included in the database (Probst *et al*., 2013a). However, this can only be achieved when cultivation data of this novel species are available, so that the already existing probes can be picked and arranged accordingly. The data presented herein are based on probesets, selected by also considering probe-pairs and -quartets, which supplementally allow presence/absence calling of eOTUs beyond interpretation of abundance values. PhyloChip evaluation considers both data sets, and is therefore superior to high-throughput methodologies and to most of the other molecular tools where only incident values can be evaluated. The advantage by analysis of binary data is that microbial richness can be compared.

By applying both selection methods on the same sample set, only subtle differences in the microbial community structures were unveiled for MICHAm samples. However, the higher the resolution of the phylogenetic level, the more refined the detected differences.

Once the eOTUs were defined, a variety of data analysis tools, such as NMDS, UniFrac, PCoA, Welch test, iTOL, and hierarchical clustering were available that can be employed to PhyloChip data. By applying various mathematical fundamentals, different inferences can be drawn from abundance values of eOTUs concerning overall microbial diversity, shifts in abundance of single eOTUs, potential outliers, or influence of metadata on the community structure. For example: Not all factors that may have influences on the microbial community structure can be displayed and evaluated by ordination analysis or hierarchical clustering, such as impacts derived from metadata like temperature or RH.

The microbial community structure from the MARS 500 facility as set out above is only based on bacterial gene signatures, since archaeal gene signatures were not obtained, despite the existing eventuality that is derived from PCR setup and probe design (section III.3.6.5.). However, there are first hints about the presence of archaea since Archaea-directed PCR of MICHAm samples revealed positive results (data not shown).

The overall community analysis of both (habitable and utility) modules revealed that approximately 95 % of all eOTUs are assigned to the four phyla Proteobacteria (41 %, mainly γ-Proteobacteria, followed by α- and β-Proteobacteria), Firmicutes (34 %, two thirds represent *Clostridia*, remaining are bacilli and unclassified genera), Bacteroidetes (11 %, mainly *Prevotella* being also present in oral cavity (Costello *et al*., 2009; Grice and Segre, 2011), and Actinobacteria (8 %, almost exclusively Corynebacteria; Fig. III.3.6.5.2; data CD, [folder: PhyloChip: Second Genome Supplementary]). The distribution of these phyla was almost identical on the module level, but revealed differences when comparing more resolved taxonomic levels which will be discussed later on. A similar trend was seen in commercial aircraft high-efficiency particulate air filters after PhyloChip G2 analysis (Korves *et al*., 2012). However, these four phyla were represented in the each sample at a maximum rate of 75 % and exhibited a higher diversity (41 phyla-level groups) compared to the microbial community of the MARS 500 facility that consisted of 19 phyla. These major lineages were also found in a study published by Osman *et al*. (2008). The abundance of Firmicutes, and to a lesser extent Actinobacteria, resembles findings from other indoor settings mainly retrieved from occupied houses (Noris *et al*., 2011), hospitals (Kembel *et al*., 2012), and dust within houses (Taubel *et al*., 2009) and offices (Rintala *et al.*, 2008). A study that aimed to identify household bacterial communities also stated that these four phyla are predominant, although a difference was observed regarding the abundance of Firmicutes (more frequent in the toilet) and Proteobacteria (more frequent in the refrigerator) depending on the monitored area (Jeon *et al*., 2013).

Acidobacteria, Chloroflexi, Deinococcus-Thermus, and Verrucomicrobia were found to a lesser extent in the MARS 500 facility, as they comprised a maximum of 2 % of the whole community. Since Kembel *et al*. (2012) detected those lineages in outdoor air, this fraction may constitute remnants of the initial microbial community. This would be possible because of the free DNA residues that were not removed when the facility was admittedly sterilized before isolation started, so that they can be targeted by molecular methods.

Analysis of the aggregated HybScores of all selected eOTUs from MICHAm samples revealed a pattern of the most abundant families that are typical members of the human microbiome. This included *Lachnospiraceae*, *Pseudomonadaceae*, *Ruminococcaceae*, *Corynebacteriaceae*, *Comamonadaceae*, and *Rikenellaceae* (section III.3.6.5.; Fig. IV.8.3.2).

The *Lachnospiraceae* and *Ruminococcaceae* are known as two of the most frequent families from the order Clostridiales found in the mammalian gut environment, where they represent active members and are associated with the maintenance of gut (Biddle *et al*., 2013). These obligate anaerobic gram-positive bacteria account for roughly 50 % and 30 % of phylotypes, respectively (Turnbaugh *et al*., 2008). Therefore, they are a good indicator to predict fecal contamination, and thus human health risk (Myers *et al*., 2007; Newton *et al*., 2011). 196 eOTUs assigned to *Lachnospiraceae* were obtained. Some of them were identified on the species level and revealed strains like *Eubacterium rectale*, *Roseburia faecis*, *Dorea formicigenerans*, and *Coprococcus catus*. Incidence values from the MICHAm samples exhibited almost identical occurrences of *Lachnospiraceae* within the habitable and utility module. The observed mixed responses and adverse trend with time of some eOTUS

that were assigned to *Lachnospiraceae* might result from the effort that was put into cleaning and maintenance procedure (section IV.5.).

Also, members of the family *Rikenellaceae* have been found to be major constituents of the human gastrointestinal microbiota (Nagai *et al*., 2010). The prevalence of these three microbial families can be explained by the fact that in both modules either the shower cabin or the external surface of a toilet bowl was monitored.

Bacterial families that are associated with normal human skin include besides *Staphylococcaceae* also C*orynebacteriaceae* (Grice and Segre, 2011). Human skin microbes were expected to be found ubiquitously distributed all over the facility since they can easily be spread either by shedding or direct contact with surfaces where they remain. Therefore, it is not surprising that these eOTUs that are assigned to *Corynebacteriaceae* and contemporaneously showed a significant correlation with time in the habitable module, increased with prolonged confinement (Fig. III.3.6.9.1). The habitable module represented the area of highest activities. Based on this fact, the accumulation over time is reasonable.

Microorganisms of the *Pseudomonadaceae* family, especially those of the genus *Pseudomonas*, are described as opportunist pathogens and colonize the human oral cavity, and dorsum of the human tongue (Conti *et al*., 2009). *Comamonadaceae*, a water-associated family, is also highly abundant in saline samples and is in general associated with the respiratory tract (Charlson *et al*., 2011). Furthermore, it includes genera that are known as animal and human pathogens. Some of these genera can also degrade aromatic compounds and are important for the biodegradation of toxic wastes (Kersters *et al*., 2006). The distribution of bacteria belonging to this family might occur by aerial transportation as they may be released while speaking or coughing. Therefore, their prevalence is also not astonishing.

The incidence of eOTUs that were assigned to the candidate TM7 group as part of the microbial community of the MARS 500 facility also have to be mentioned, even if they represent only a minor fraction (section III.3.6.10.). As previously stated, candidate phyla were only detected by sequencing, and up to now, cultivation efforts were not successful. Affiliations that are grouped within this major lineage were detected in samples from diverse environments, such as activated sludges (Bond *et al*., 1995; Godon *et al*., 1997), water-treatment plant sludge (Hugenholtz *et al*., 2001), human saliva (Lazarevic *et al*., 2010; Dewhirst *et al*., 2010), and human skin (Dinis *et al*., 2011). The latter two can be declared as the most probable source for the occurrence of candidate TM7 gene signatures in MICHAm samples.

These results clearly indicate that humans are important dispersal vectors for microbes that colonize a built environment, in particular the MARS 500 facility (Klevens *et al*., 2007; Kembel *et al*., 2012; Qian *et al*., 2012; Hewitt *et al*., 2012). But are there additional factors that had an impact on the bacterial composition?

In conformity with trends that were already observed from cultivation efforts, PhyloChip analysis uncovered a distinct separation of the microbiome being present in the habitable and utility module (section III.3.6.6.; Table III.3.6.6.1). None of the metadata factors, including temperature, RH and CO_2 and O_2 concentration in the air revealed a significant influence. However, the issues that came along with these factors (section IV.3.4.) should

be kept in mind, so that no general statement about their impact can be made. Ultimately, the significant difference in the community structure exhibited by each module might be explained by the fact that analyzed samples of the habitable module originated mainly from wooden surfaces, yet those within the utility module were taken from stainless steel. As previously discussed (section IV.4.3.), the features of the monitored surfaces can have an influence on the contamination level, so it would not be surprising if they would also have an influence on microbe diversity.

Despite observed fluctuations regarding taxa and species composition (Fig. III.3.6.5.3) in each module, no significant correlation between microbial diversity and duration of confinement could be detected (section III.3.6.5.). An increase or decrease with time was expected since there was no exchange with the external environment, which was supposed to be essential for maintaining the balance. In addition, due to food preparation a continuous supply of nutrients in the form of crumbs was released to the habitat, so that microorganisms could proliferate. The findings of the present thesis clearly indicated that under confined conditions, the community structure is still a very dynamic system which adapts to the prevailing habitat.

Although no general trend regarding the microbial diversity and its change was observed, single eOTUs of both modules exhibited a significant change in abundance values correlated with factor time or when being grouped in early, mid, and late phase (section III.3.6.7.ff). In both modules, the majority of these were assigned to Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. However, they represent only a small fraction of approximately 5 % of the overall detected eOTUs. Additionally, all of those eOTUs showed mixed responses, but yet altogether a reverse trend in both modules, so that no general statement is possible. This can be the result of a lot of different influences during confinement, like changes in the training sessions, which might have affected the microbial community of the utility module. Van Houdt *et al*. (2009) stated that the higher concentration of Proteobacteria, which was noticed in air samples from the so-called “noisy” part of the Concordia base, might have been a result of the handling with fresh products and vegetables. This might also apply for some eOTUs obtained from the MICHAm samples since the marsonauts grew vegetables, like tomatoes in the greenhouse within the utility module, whereas food preparation and dinners took place in the habitable module. Furthermore, at about half-time isolation, changes in the diet were made based on physiological investigations that were part of Russian and European experiments. Therefore, not only the food debris residing on surfaces or the floor, but also the gut microbiome adapted accordingly (pers. comment: Prof. Dr. Francesco Canganella).

It is important to mention that the eOTU that is assigned to *Enterococcus faecalis*, a representative of human gut microflora, also revealed a significant increase over time (Fig. III.3.6.9.2.; Fig. III.3.6.11.2). Generally, enterococci are used as an additional parameter of fecal pollution (LeChevallier *et al*., 1987). However, the accumulation of *E. faecalis* in the MARS 500 facility might not be the result of pollution, even though isolates were obtained exclusively in the utility module from samples taken above the vanity basin and in the shower cabin. Both locations represent wet areas, thus *E. faecalis* was already supposed to be mainly detected there. Moreover, the presence of this bacterium might have been

caused by the fact that inactivated *E. faecalis* cultures were added to the food as supplements in order to test the effect on the human gut flora. This investigation was planned and performed under the supervision of Prof. Dr. Canganella as another part of the MICHAm experiment.
An identical trend, i.e., increase over time, was observed in the habitable module for one eOTU that was assigned to *Bifidobacterium*, a probiotic microorganism that is mainly found in dairy foods (section III.3.6.10.). It has to be noted that *Bifidobacterium* was also part of the microbial food supplement that was administered to the marsonauts during the campaign to strengthen their intestinal microflora and their immune system. Therefore, it is not surprising that a greater amount of 16S rRNA gene signatures was mainly found in the habitable module, where samples were taken from the dining table.

Conclusion:
Molecular analysis of MICHAm samples confirmed the qualitative evidences that were obtained by cultivation and additionally allowed quantitative statements. The study demonstrated that the PhyloChip approach provided a way to deeply characterize the microbial community in each of the investigated module (e.g., habitable and utility). It even covered species, which could not have been enriched so far, as well as those where only trace amounts of genetic signatures were present.

IV.8.3. Divergences

As expected, some discrepancies were revealed that originated in the application of both culture-based and culture-independent methods (section III.3.6.12.). Differences in the composition of the major lineages - PhyloChip detected mainly anaerobic taxa - have emerged, which can be explained by the fact that with the applied enrichment assay, those could not be tracked. Furthermore, molecular analysis does not distinguish between alive and dead microbes. Therefore, it is not surprising that PhyloChip analysis exhibited a more comprehensive insight into the existing microbiome. In addition, shifts regarding the abundance of certain taxa were displayed when compared to the cultivation approach (Fig. III.3.6.12.2). Only *Rhodococcus*, *Methylobacterium*, and *Roseomonas* revealed a positive correlation of aggregated HybScores with isolate counts (Fig. III.3.6.12.3). On the one hand, this implies that the isolated microorganisms might not be the most frequent in their habitat nor the most important ones concerning their function. This bias can be explained by the fact that the minority of microorganisms are cultivable (approximately 1 % when applying all different kinds of known media). On the other hand, regarding molecular techniques, distortion of results could originate in the applied DNA extraction method, in PCR primer selection, or in amplification itself. The probe design was also an important factor concerning comparability of results. Last but not least, not all microorganisms can be targeted by a microarray tool. Some of them are hard to lyse, possess external DNases, cannot be targeted or distinguished by universal PCR setup, or remain undetected due to probe design. This was shown by the fact that with increasing taxonomic resolution, the number of taxa that were only detected via cultivation were also enhanced (Fig. III.3.6.12.1).

The findings on the species level clearly indicated that spore-forming strains belonging to *Paenibacillus* have not been detected by molecular methods. Some bacilli gene signatures were obtained by PhyloChip, but those represented only a minor fraction and were not classified on the species level. The low detection of strains that have the ability to form spores, and are therefore hard to lyse is not surprising and has also been reported in former studies (Schwendner *et al*., 2013).

In addition, the question arose whether the PhyloChip would even be capable of detecting the remaining cultivated genera and species or if these sequences were not covered due to the probe design. Having a look at the genera that were not tagged by PhyloChip uncovered *Aurantimonas*, *Brevibacillus*, *Chryseobacterium*, *Cohnella*, *Delftia*, *Enhydrobacter*, *Enterobacter*, *Gordonia*, *Klebsiella*, *Kocuria*, *Leclercia*, *Methyloversatilis*, *Ochrobactrum*, *Paenibacillus*, *Sejongia*, *Solibacillus*, *Sporosarcina*, *Streptomyces*, and *Tsukamurella* (data CD, [folder: PhyloChip: Second Genome Supplementary]). According to Second Genome, the probes on the PhyloChip are designed in a way that allows the detection of all of the above-mentioned genera. This clearly indicates that the differences originate in the applied DNA extraction methods and/or in the PCR reaction setup.

The most surprising finding when analyzing the PhyloChip data was the low detection rate of staphylococci. For example, when microbial diversity of occupied rooms was investigated via clone library, findings indicated a majority of *Staphylococcus*, *Enterobacteria*, *Corynebacteria*, and *Propionibacteria*, which all are associated with human skin, hair, nostrils, and/or oral cavities (Qian *et al*., 2012). Based on the retrieved cultivation data of the MARS 500 facility, a high abundance of species from this genus was expected when considering the proportional abundance values for staphylococci (Fig. IV.8.3.1). Van Houdt *et al*. (2009) even stated that the presence of *Staphylococcus* spp. increased from the start of the campaign and reached a maximum level in the middle of the confinement period within the Concordia base.

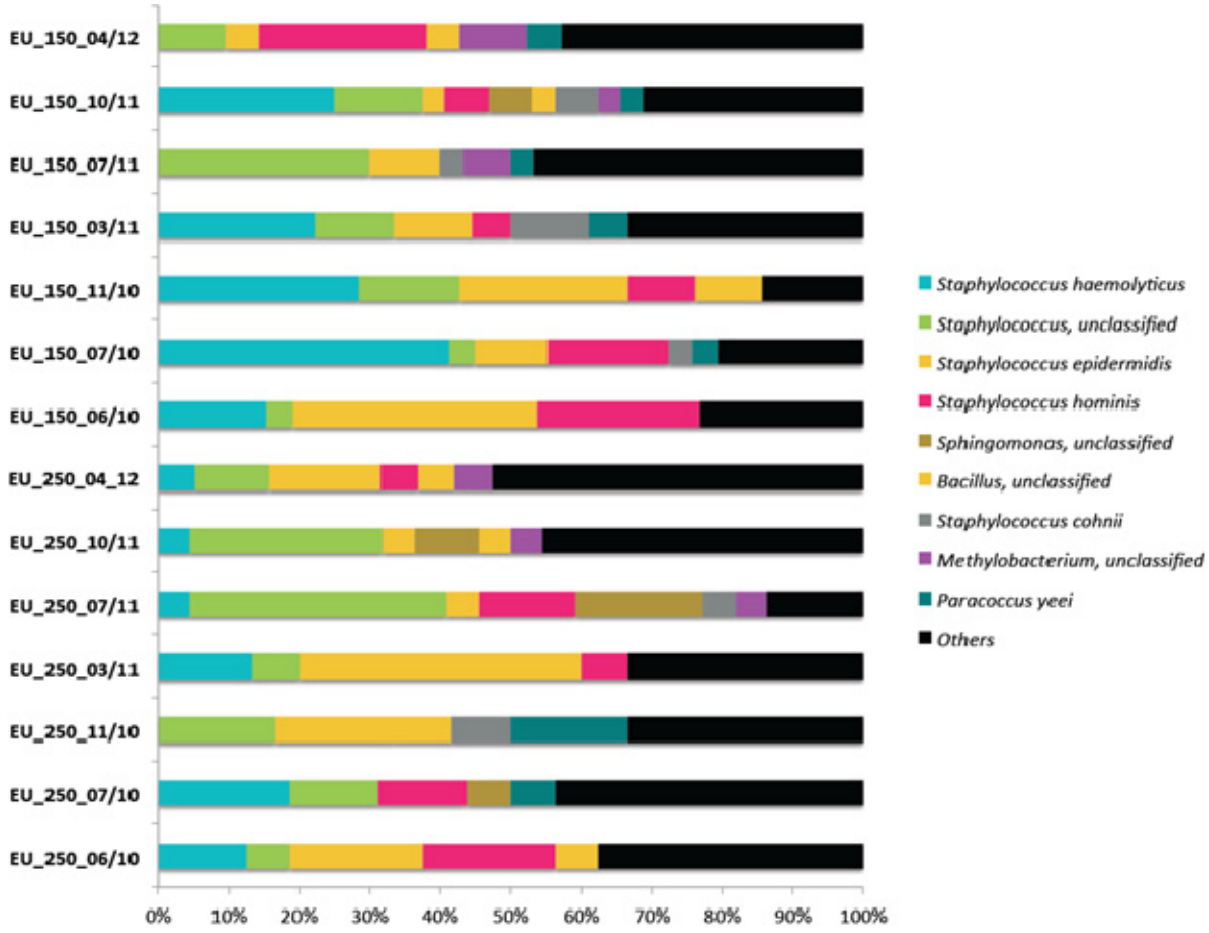

Fig. IV.8.3.1 Proportional abundance of isolates on species level across selected points in time. Nine species with the largest number of isolates are displayed.

In all, out of 416 *Staphylococcus* strains that have complementary probes on the PhyloChip, 19 eOTUS were identified that belonged to the genus *Staphylococcus*. Nine of them were identified on the species level revealing *S. epidermidis*, *S. hominis*, *S. cohnii*, and *S. aureus*. *S. haemolyticus*, *S. lugdunensis*, and *S. petterkoferi* were only retrieved by cultivation, although probes on the PhyloChip would have covered all of them. This discrepancy might be due to the fact that staphylococci are hard to distinguish on the species level when shorter reads/fragments of the whole 16S rRNA gene are aligned (Mendoza *et al*., 1998).
However, only a low aggregated HybScore was detected for *Staphylococcaceae* for all MICHAm samples, indicating a weak response of the chip to this family (Fig. IV.8.3.1). With regard to aggregated HybScores, one should consider the overall diversity of OTUs that are called present in the samples and on the chip in general, and in addition, the known diversity of that particular family (pers. comment: Alexander Probst).

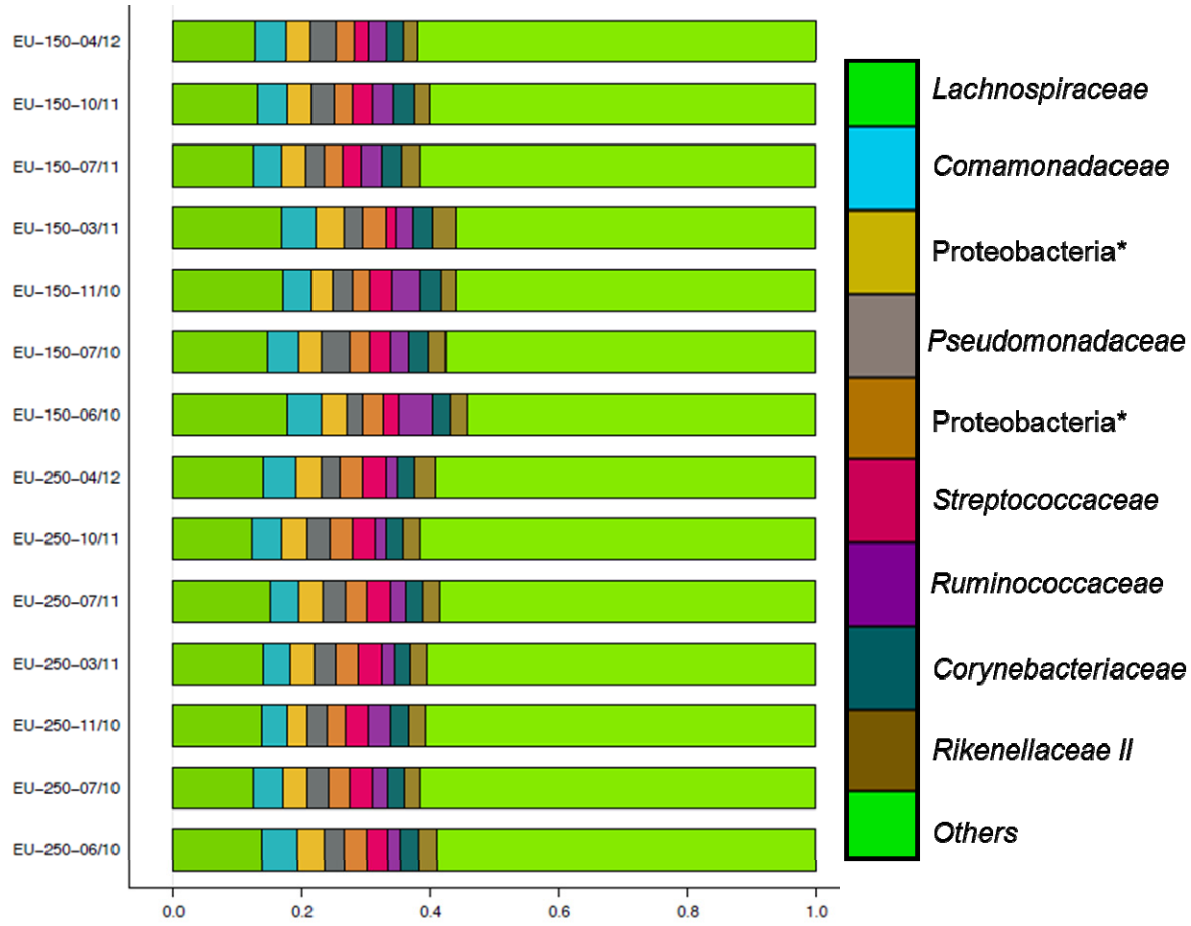

Fig. IV.8.3.1 Proportional abundance based on aggregated HybScores on family level across selected points in time. Nine families with the largest sum of HybScores from the OTUs within each family are displayed. *Staphylococcaceae* represent in all analyzed PhyloChip arrays less than 1 % of the overall diversity. Asterisk denotes unclassified Proteobacteria. IsoCntl=isolation control=blank

A possible explanation might be given by the fact that *Staphylococcus* possesses external DNases, which partially degrade DNA and consequently make it difficult to be trackable by molecular methods. Additionally, for any reason non-*Staphylococcus* species might have had a better amplification performance during the PCR run. However, there are lots of studies that prove the contrary. For example Gaüzère *et al*. (2013b) investigated the airborne microbial community in the Louvre Museum by use of 454-pyrosequencing and stated that on the genus level, the majority of gene sequences were affiliated with *Staphylococcus* sp., besides *Paracoccus* spp., *Acinetobacter* sp., *Pseudomonas* sp., *Enhydrobacter* sp., *Sphingomonas* sp., and S*treptococcus* sp.. Additionally, clone libraries established from clean room samples, as mentioned above, also mainly detected staphylococci (Moissl-Eichinger *et al*., 2013).

Conclusion:

In all, one can say that by applying both methodologies, a comprehensive insight into the microbial community structure was obtained. Microbial communities fluctuated based on their response to a diverse set of locally present stimuli, since all of the three modules exhibited different microbial community structures. However, the diversity was strongly

influenced by human's natural microflora. The combination of cultivation and molecular methods counterbalanced the advantages and disadvantages of each tool, resulting in a representative analysis.

IV.9. TACKLING THE PATHOGENS

Considering the emerging health risks that are associated with potentially pathogenic organisms (PPOs), it is inevitable to investigate this microbial fraction within manned habitats, especially when they are confined.

Based on the findings from the cultivation approach, several viable (opportunistic) pathogens were identified that were ubiquitously distributed over surfaces and in air of all three modules (section III.1.10.). Molecular analysis generally revealed a very heterogeneous distribution. The number of detected pathogenic species was significantly higher in the utility module when compared to the habitable module (Fig. III.3.6.11.1). These two modules do not only differ in the amount of PPOs, but also in their phylogenetic structure of detectable species as was shown. These findings are consistent with the data retrieved from the overall microbial load (Fig. III.3.6.5.3). Pathogen-containing lineages were to be expected given that the microbial community was strongly influenced by human-associated microbes, and therefore contains common human commensals. Furthermore, potential pathogens that have been detected by molecular methods may not have been viable since there is no distinction between infectious and non-infectious particles. Ilyin (2000) even reported an accumulation of pathogenic bacteria within the first weeks of confinement onboard the Mir. This was also the reason why samples from the first two sampling events were subjected each to PhyloChip.

Bacterial interactions with humans can have both, beneficial and detrimental effects. Humans can influence microbe activity, and microbes can affect human health through biostimulation, biocontrol, and infection, but the majority of them are in most cases not harmful. These activities involve complex transmission and signaling pathways, as well as interactions between the human microbiome and their surrounding microbial community in air and on surfaces and humans themselves, which are enhanced in confined habitats. However, once sanitary effects occur, very often the susceptibility of the individual increases (Schleibinger *et al*., 2004), and PPOs may therefore cause adverse health effects, like infections. For example, 1,3-ß-glucan, a polymer of D-glucopyranose, is a constituent of the cell wall of some bacterial genera which demonstrates toxic properties. These can include immunosuppressive and/or inflammatory effects, and can cause airway eosinophilia (Schäppi *et al*., 2008). Furthermore, when humans are exposed to stress and extreme environmental conditions as they would experience during a space flight or Antarctic expedition, the immune system is negatively affected, and susceptibility to infection is increased (Mehta *et al*., 2000; Aponte *et al*., 2006).

At the same time bacteria demonstrate enhanced virulence (Wilson *et al*., 2007; Rosenzweig *et al*., 2010) and less susceptibility to various classes of antimicrobial agents (Tixador *et al*., 1985; Lapchine *et al*., 1986) as a result of establishing a thicker cell wall in

microgravity environments (Sieradzki *et al*., 2003). Up to now, among 742 astronauts, which were passengers of 106 space shuttle flights, 29 infectious disease incidents were reported, and among viral and fungal triggers also bacteria were accountable for urinary tract and subcutaneous skin infections (Mermel, 2013).

Onboard the Mir space station, most strains from air, condensation water and surfaces of the inner wall were thought to contain opportunistic pathogens or environmental bacteria. For closer investigations, PCR methods (up to 380 different primers) were applied to survey pathogenic bacteria. However, this procedure was only performed for condensation water samples and revealed exclusively *Mycobacterium avium* at a concentration of approximately 10^4 cells/ml water (Kawamura *et al*., 2001).

Noteworthy is also the occurrence of potential opportunistic pathogens of the *Enterobacteriaceae* family *(Enterobacter*, *Escherichia*, *Proteus*, *Serratia*) within the MARS 500 facility (section III.1.10; section III.3.6.12), especially with regard to the work of Schiwon *et al*. (2013) that unveiled that ISS isolates of *Staphylococcus* and *Enterococcus* encoded more resistance genes and possessed higher gene transfer capacities than isolates that were obtained from the Concordia station. Fortunately, gene signatures that were assigned to *Enterobacteriaceae* were detected only to a low extent within the MARS 500 facility, and thus the corresponding bacteria revealed no severe risk. PhyloChip unveiled 26 eOTUs that were annotated mainly as unclassified *Enterobacteriaceae*. The remaining ones were assigned to *Citrobacter sp*., *Escherichia sp*., *Pantoea sp*., and *Citrobacter rodentium*. Cultivation approaches exhibited *Klebsiella* and *Leclercia* on surfaces, and *Enterobacter hormaechei* that was isolated out of one MICHAm air sample. Infection with a potentially pathogenic strain of *Klebsiella oxytoca*, which was identified only once among all sequenced MICHAm isolates, might lead to antibiotic-associated diarrhea (Beaugerie and Petit, 2004).

Campylobacter spp. is another obligate pathogen that is transmitted by ingestion of fecally contaminated water and can cause gastrointestinal diseases (Ivanova *et al*., 2010). Gene signatures of these bacteria were found in both modules, but the distribution and incidence values were very heterogeneous (data CD, [folder: PhyloChip: Second Genome Supplementary]). Since the abundance of *Campylobacter* did not increase over time (eOTU 185; Fig. III.3.6.9.2), it can be concluded that there was no accumulation of cells within the water supply system. In contrast, *Legionella* spp., an opportunistic pathogenic bacterium, was detected in the majority of samples from both modules.

Furthermore, *Brevundimonas diminuta* was also isolated. Strains belonging to this species have already been enriched from the Mir space station and from clinical settings, where they have been implicated in opportunistic infections (Abraham *et al*., 1999; Kawamura *et al*., 2001; Han and Andrade, 2005).

Staphylococcus aureus, which frequently colonizes the skin or nose of humans, was not only detected in all three modules of the MARS 500 facility, but also in other manned habitats, such as Concordia and Mir (Ilyin, 2000; Van Houdt *et al*., 2009). It was enriched from various surfaces and also from air samples. Under normal conditions *S. aureus* is not harmful and suppressed by the presence of its commensals, *S. hominis* and *S. epidermidis*. However, it can also cause a range of illnesses, such as toxic shock syndrome and

staphylococcal scalded skin syndrome (Chiller *et al*., 2001). Even transmission of microbial specimens among crew members was reported, especially in the case of *S. aureus* (Taylor, 1974; Pierson *et al*., 1996). Ilyin (2000) claimed an increased incidence of *S. aureus* with time, which is partly consistent with data from MARS 500. There, in the case of *S. epidermidis*, only one eOTU exhibited a significant increase in the habitable module, whereas the remaining eOTUs classified as staphylococci showed no increase (Fig. III.3.6.11.3).

In general, six PPOs (four in the habitable and two in the utility module) were identified to have a significant correlation with time (Fig. III.3.6.11.3).

Further studies show that we are permanently confronted with PPOs in our daily life. Tringe *et al.* (2008) investigated the indoor air of two shopping centers in Singapore by establishing clone libraries. The analysis revealed bacteria, including potentially opportunistic pathogens. This fraction was represented by nosocomial strains which are commonly isolated from human-inhabited environments, such as hospitals (Poza *et al*., 2012).

Our data took into account only potentially pathogenic bacteria that were identified based on the 16S rRNA gene information. However, this gene sequence does not provide any information about the pathogenicity of a strain. Moreover, the biological role or even the viability status of microorganisms that were detected by molecular methods remains unclear (Fredericks and Relman, 1996). Due to the difficulty of controlling dissemination of airborne pathogens, transmission of infectious agents cannot always be avoided. Roth and James (1988) described a phenomen in which resident microbial cells are able to develop pathogenic features. Additionally, there are indications for pathogenic bacteria being able to persist in a VBNC status in a biofilm population (Flemming and Wingender, 2010). *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Salmonella typhi,* and *Vibrio vulnificus* are examples of bacterial pathogens that are known to enter the VBNC status (Oliver, 2010).

In order to improve risk assessment, different approaches or closer investigation of the identified PPOs are necessary. There are several sensitive molecular techniques described for the detection and quantification of human pathogenic microorganisms. In addition, for various strains and species, protocols are available that allow quantification of the microbial load, while for others only type-specific methods can be applied. Chakravorty *et al*. (2007) suggest an approach for diagnosis of pathogenic bacteria that is based on considerable sequence diversity in hypervariable regions of the 16S rRNA genes. Results show that V1 is best suited to differentiate among *S. aureus* and coagulase negative *Staphylococcus* sp., whereas *Enterobacteriaceae* are hardly to distinguish by targeting hypervariable regions. The application of endotoxin tests would be another possibility to overcome the above-mentioned drawbacks. Endotoxins are produced by gram-negative bacteria and are integrated within the outer membrane as endogenous toxic components of the lipopolysaccharides (LPS). Despite differences in their structure when originating from different strains, all these toxins cause similar effects on the host when infused or inhaled. Since they are liberated during cell growth and death, endotoxins pose a potential risk for human health. Especially when proliferating, gram-negative bacteria accumulate in closed water recycling systems, for example, and high concentrations of endotoxins can occur

(Roman and Mittelmann, 2013). Fortunately, the number of identified gram-negative bacteria seemed to be low compared to the amount of identified gram-positive strains in the MARS 500 facility (Fig. III.1.5.1; Fig. III.1.9.1.1).
Even if potential pathogens are characterized by low levels of metabolic activity, there are indications that they are still able to cause infections (Wingender, 2011). Therefore, continuous cleaning with disinfecting agents is important. However, it is more feasible to prevent the pathogenic contamination of surfaces, water, or food than that of air (Gaüzère *et al*., 2013b). Additionally, it has to be considered that relatively low levels of total microbial contaminants were detected throughout the campaign, thus health problems caused by microorganisms seem to be extremely unlikely, especially when considering that the amount of PPOs is the driving factor whether an infection occurs or not. Moreover, to date, serious infections during space travel have been limited to mostly superficial skin infections (Mermel, 2013). One has to consider that Van Houdt *et al*. (2009) hypothesized that certain niches, such as food processing areas or toilets, were colonized by a higher number of PPOs, which might lead to an outbreak of microbial-related diseases when contacted. This might also be true for the MARS 500 facility, when taking into account that the highest microbial contaminations were found on surfaces similar to those mentioned above, which therefore presented hot spots of microbial accumulation.
However, there is one important study that underlines our assumption that the marsonauts were at no point exposed to an increased health risk. Roda *et al*. (2013) continuously monitored the health status of the crewmembers during the MARS 500 isolation experiment. By the use of non-invasive panel tests for gastrointestinal motility investigation, such as via periodic blood biochemical function tests and clinical examinations, the researchers found that no significant pathology or physiological alteration appeared.
Although no clinical symptoms associated with microbial infections were reported during this isolation program, it cannot be completely excluded for all following studies.

Conclusion:
The identification of certain potentially pathogenic organisms illustrates the necessity to monitor periodically the microbial population in isolated manned environments. Serendipitously, since most of the afore-mentioned PPO's are allied with pathogenity group 2, and no infections were reported, there was no urgent risk situation during the whole confinement.
For future sampling campaigns it is proposed that one focus should be led on the identification of the pathogenic potential of certain microorganisms.

IV.10. Potential Biodegraders of Material

In addition to pathogenicity, microorganisms can have the potential to destroy polymers and/or corrode metals directly or indirectly (Iverson, 1967; Velikanov *et al*., 1978; Andreyuk *et al*., 1980; Gu, 2007). These capacities can lead to deterioration of specific hardware and sustainable systems (Pierson *et al*., 2012; Van Houdt *et al*., 2012). Furthermore, Novikova

(2004) reported several cases of equipment failures and their negative impact on operations onboard the ISS (e.g., deterioration of mechanic strength, alteration of dielectric or other properties). Active biodegraders of various materials have already been found onboard the Mir. Common initiators of metal corrosion are *Acinetobacter* sp., *Alcaligenes* sp., *Flavobacterium* sp., *Hydrogenophaga* sp., *E. coli*, *Micrococcus* sp., *Pseudomonas flourescens*, *P. putida,* and *P. stuzeri*. *P. aeruginosa*, *P.* sp., and *Actinomyces* sp., which are examples of polymer degraders, and can make up 10 % of the microbial community present. An even higher percentage of bacteria (22.5 %) fall into the category of metal biodegraders. *K. pneumonia* and *Bacillus* sp. even have the ability to degrade both metals and polymers (ESA and State Research Center of Russian Federation - Institute for Biomedical Problems, 2000). In terms of the isolation program in Moscow, the following cultivable representative strains were enriched: *Pseudomonas* sp.; *Micrococcus* sp.; and *Bacillus* sp. (section III.19.). The MARS 500 facility also contained gene signatures of *Acinetobacter* sp., *Flavobacterium* sp., *Escherichia* sp., and *Actinomyces* sp., identified by PhyloChip (data CD [folder: PhyloChip: Second Genome Supplementary]].
Although the occurrence of biocorrosion was not reported by the marsonauts, the identification of possible biodegrading microorganisms clearly indicates that cleaning and disinfection is critical in order to minimize possible risks caused by them.

Conclusion:
The detection of potential biodegraders either by isolation or molecular approach offer evidence that microbial monitoring throughout the confined habitat is needed to maintain a desired high-level sanitary and low-level microbial status to prevent the possibility of material biodestruction.

V. CONCLUSION AND OUTLOOK

The evaluation of the microbial community structure, and thus the potential risk for human's health in confined habitats, requires a solid understanding of the microorganisms' origin, the knowledge of the routes, frequencies, and means of spreading of microbial cells. The study of the microbiome in a confined habitat, where exchange with the exterior is completely prevented, presented a unique opportunity to unveil bacterial behavior, proliferation, and survivability over a long time of isolation.

Taken together, this is the first microbiome study from a confined facility, where people lived for 520 days without leaving the facility that has successfully applied cultivation and molecular based methods to examine the microbial "bioburden" facing whole microbial community structure and its changes over time.

In particular, concerning the spread of microorganisms and their further development in a closed manned habitat, it is important to pinpoint critical locations of a certain habitat, which were mainly observed in the habitable module.

The relatively low biocontamination level, especially in the air, reflected the adequate maintenance of the facility. Nevertheless, it has been shown that it is not enough to perform only one sampling at one selected location per confined habitat, since results varied from place to place, from surface to surface, and from time to time in terms of quantity and composition of bacterial contaminants. These "outlier values" might have originated in a combination of diverse environmental factors, less cleaning, and/or higher human activity. Ultimately, the observed fluctuations in microbial concentrations and contamination events suggest the urgent need for continuous diligence and monitoring.

However, compared to molecular monitoring tools, the standard survey strategy included cultivation and enumeration approaches that encompassed only a minor fraction of the microbial inhabitants actually present on the sampled sites. In addition, determination of absolute numbers is not enough to evaluate the associated risk for human's health, since these numbers do not comprise any information about potentially pathogenic microorganisms.

With regard to the pathogenic potential of microorganisms, literature unveiled that in space the bacteria demonstrate enhanced virulence, so that the possible risk is probably underestimated in earth-based monitoring programs.

Bridging the two methodologies – an overview and fast results via cultivation, based on ESA standard, and state-of-the-art molecular techniques that provide a more realistic and comprehensive estimation of the bacterial species present in manned habitats - led to meaningful success, as was demonstrated within this thesis. Phylogenetic data clearly identified the inhabitants as a major contamination source.

Additionally, the improvement of already existing methods and assays should always be updated, thus the progress in development and knowledge as well as the continuous advances in accuracy of detection limits is very fast.

In sum, having samples analyzed can also help to provide evidence of the scope and severity of a microbial contamination problem, as well as aid in assessing human exposure to potentially pathogenic microorganisms. The obtained results provided essential baseline

data for human health risk assessment and for defining thresholds to ensure safety for astronauts during future long-term missions.

Based on the data published herein, none of the marsonauts was exposed to an enhanced health risk due to microorganisms; nevertheless, in the future, it should always be acted according to the following policy:

An ounce of prevention is better than a pound of cure.

Some future trends are presented that might lead to improved prevention regarding microbial contamination in (confined) manned habitats. The accumulated data about the bacterial load offer evidence that control of microbial levels is needed throughout the spacecraft and space journey. But before prevention can be undertaken, information about the prevalent microbial community structure is essential.
Therefore, it is recommended for future long-term missions to develop a fast, reliable, portable, and cultivation-independent system, which allows onboard detection, enumeration, and identification of microorganisms to minimize delay in case of potential risk situations.
In addition, based on the background that commonly applied sterilization conditions, such as dry heat or chemical disinfectants that do not harm the spacecraft and its hardware, are not able to kill most bacterial spores and vegetatives, it is necessary to find ways how to prevent and counteract contamination.
Since the total inactivation of microorganisms and/or the inhibition of microbial biofilm formation is almost not achievable within a manned habitat, cleaning and maintenance is necessary to avoid microbial accumulation. To reduce crew time that is spent and efforts that are made to facilitate the prevention of microbial contamination, surfaces can be coated with antimicrobial agents. The findings presented herein provide basic knowledge and give hints at possible microbial hot spots. This information is critical in order to make predictions about where to install antimicrobials most effectively in future spaceships to mitigate propagation of microbes.
In the future, more endeavors will be required regarding the development and evaluation of antimicrobial surfaces. Due to the lack of standards, little is known about their impact on humans and microbes as well as about their effectivity. Alternatively, since a lot of microbial species are sensitive to UV radiation, the development of a hand-held UV-light unit might be useful to kill at least this fraction. Another detrimental effect for microorganisms is caused by exposure to plasma. By use of a portable cold atmospheric plasma source, the viability of vegetative microbial cells but also of spores can be reduced. Ultimately, the combination of many different methods that are based on different mechanisms of action should be aimed at reducing the viability, and thus survivability of microorganisms to a minimum.
The range of microbes isolated from the MARS 500 facility included environmental (non-pathogenic) organisms, opportunistic, as well as true human pathogens, but no archaeal gene signatures since they were not included in the investigation.

The latest findings of screening surveys of human skin unveiled the presence of archaea and provided evidence that they were thus far underestimated (Probst *et al.*, 2013b). Therefore, it is proposed that archaeal gene signatures should be taken adequately into account during future studies, despite the fact that no pathogenic archaeal strains are known to date. Since there are some remaining MICHAm samples, an experimental draft should be prepared for further molecular processing to get an impression about the archaeal community structure in a manned confined habitat.

Moreover, water-recycling systems of confined habitats exhibit a particularly critical environment that provides conditions for the formation of biofilms, which might result in contaminated water (Flemming and Wingender, 2010). This in turn can lead to water-related illness and outbreak within the crew. Therefore, when also considering the strength of occurrence of obligate water-related microbes, the sampling protocol should be extended in order to include the detection of pathogens in water.

Ultimately, there is an urgent need to establish a protocol for rapid verification, whether a potentially pathogenic organism exhibits infectious features or not. In addition to the effect of accumulation of pathogens, the microbes can also adapt resistances against potential stressors, such as antibiotics, radiation, and chemicals. It is also important to mention the possible impact of silver ions on microbes since they were used for water purification of the recycling system. In order to evaluate the unpredictable jeopardy caused by mutated microbes, it is recommended to screen the obtained isolates for whether they have acquired diverse resistance characteristics.

All these efforts will be necessary to provide a safe environment, to minimize human health risk, and to maximize the likelihood of success of prolonged human space travel in future.

VI. REFERENCES

Abraham W.R., C. Strompl, H. Meyer, S. Lindholst, E.R. Moore, R. Christ, M. Vancanneyt, B.J. Tindall, A. Bennasar, J. Smit, and M. Tesar (1999) Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov with *Maricaulis maris* (Poindexter) comb. nov. as the type species, and emended description of the genera *Brevundimonas* and *Caulobacter*, *International journal of systematic bacteriology* 3:1053-1073

Amann R.I., W. Ludwig, and K.H. Schleifer (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation, *Microbiology reviews* 59:143-169

Andreyuk E.I., V.I. Bily, E.Z. Koval, I.A. Kozlova (1980) Microbial corrosion and its agents, Kiev: Naukova dumka p 288

Anesti V., J. Vohra, S. Goonetilleka, I.R. McDonald, B. Straubler, E. Stackebrandt, D.P. Kelly, and A.P. Wood (2004) Molecular detection and isolation of facultatively methylotrophic bacteria, including *Methylobacterium podarium* sp. nov., from the human foot microflora, *Environmental microbiology* 6:820-830

Anesti V., I.R. McDonald, M. Ramaswamy, W.G. Wade, D.P. Kelly, and A.P. Wood (2005) Isolation and molecular detection of methylotrophic bacteria occurring in the human mouth, *Environmental microbiology* 7:1227-1238

Angelotti R., M.J. Foter, K.A. Busch, and K.H. Lewis (1958) A comparative evaluation of methods for determining the bacterial contamination on surfaces, *Food research* 23:175-185

Angelotti R., M.J. Foter, K.A. Busch, and K.H. Lewis (1964) Comparative evaluation of the cotton swab and Rodac methods for recovery of *Bacillus subtilis* spore contamination from stainless steel surfaces, *Health laboratory science* 1:289-296

Angenent L.T., S.T. Kelley, A.S. Amand, N.R. Pace, and M.T. Hernandez (2005) Molecular identification of potential pathogens in water and air of a hospital therapy pool, *Proceedings of the National Academy of Sciences of the United States of America* 102:4860-4865

Aponte V.M., D.S. Finch, and D.M. Klaus (2006) Considerations for non-invasive in-flight monitoring of astronaut immune status with potential use of MEMS and NEMS devices, *Life sciences* 79:1317-1333

Archuleta M.M. (1995) Aqueous foam: Toxicology evaluation and hazard review, Sandia National Laboratories, Livermore, CA

Armstrong N. (1970) First on the moon: A voyage with Neil Armstrong, Michael Collins [and] Edwin E. Aldrin, Jr., Little, Brown (Boston)

Augustowska M., and J. Dutkiewicz (2006) Variability of airborne microflora in a hospital ward within a period of one year, *Annals of agricultural and environmental medicine* 13:99-106

Aydogdu H., A. Asan, M.T. Otkun, and M. Ture (2005) Monitoring of fungi and bacteria in the indoor air of primary schools in Edirne city, Turkey, *Indoor and built environment* 14:411-425

Baer W. (2012) Physiologische Bakterienflora: Regulation und Wirkungen, iatrogene Störungen und Probiotika, In: Medizinische Mikrobiologie und Infektiologie, Springer Verlage pp 26-32

Baker G.C., J.J. Smith, and D.A. Cowan (2003) Review and re-analysis of domain-specific 16S primers, *Journal of microbiological methods* 55:541-555

Baldock J.D. (1974) Microbiological monitoring of the food plant methods to assess bacterial contamination on surfaces, *Journal of milk and food technology*, 37:361-368

Beaugerie L., and J.C. Petit (2004) Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea, *Best practice & research. Clinical gastroenterology* 18:337-352

Becker K., D. Harmsen, A. Mellmann, C. Meier, P. Schumann, G. Peters, and C. von Eiff (2004) Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species, *Journal of clinical microbiology* 42:4988-4995

Belgrader P., D. Hansford, G.T.A. Kovacs, K. Venkateswaran, R. Mariella, F. Milanovich, S. Nasarabadi, M. Okuzimi, F. Pourahmadie, and M.A. Northrup (1999) A minisonicator to rapidly disrupt bacterial spore for DNA analysis, *Analytical chemistry* 71:4232-4236

Bellack A. (2011) Cell architecture and flagella of hyperthermophilic Archaea, Dissertation, Universität Regensburg

Benjamini Y., and Y. Hochberg (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing, *Journal of the royal statistical society. Series B (Methodological)* 57:289-300

Berthier A., N. Leys, R. Van Houdt, A. Tikhomirov, N. Novikova, V. Ilyin, I. Kulmala, M. Lehtimäki, E. Kokkonen, P. Pasanen, and H. Abaibou (2012) BIOSMHARS: Biocontamination specific modelling in habitats related to space, In: Let's Embrace Space, Luxembourg, Luxembourg, Publications Office of the European Union pp 278-284

Biddle A., L. Stewart, J. Blanchard, and S. Leschine (2013) Untangling the genetic basis of fibrolytic pecialization by *Lachnospiraceae* and *Ruminococcaceae* in diverse gut communities, *Diversity* 5:627-640

Bik E.M., P.B. Eckburg, S.R. Gill, K.E. Nelson, E.A. Pudom, F. Francois, G. Perez-Perez, M.J. Blaser, and D.A. Relman (2006) Molecular analysis of the bacterial microbiota in the human stomach, *Proceedings of the National Academy of Sciences of the United States of America* 103:732-737

Blankenburg M., L. Haberland, H.D. Elvers, C. Tannert, and B. Jandrig (2009) High-throughput omics technologies: potential tools for the investigation of influences of EMF on biological systems, *Current Genomics* 10:86-92

Bond P.L., P. Hugenholtz, J. Keller, and L.L. Blackall (1995) Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors, *Applied and environmental microbiology* 61:1910-1916

Bouillard L.A., O. Michel, M. Dramaix, and M.J. Devleeschouwer (2005) Bacterial contamination of indoor air, surfaces, and settled dust and related dust endotoxin concentraions in healthy office buildings, *Annals of agricultural and environmental medicine* 12:187-192

Bouillard L.A., M.J. Devleeschouwer, and O. Michel (2006) Characteristics of the home bacterial contamination and endotoxin related release, *Journal de pharmacie de Belgique* 61:63-66

Breitkopf C., D. Hammel, H.H. Scheld, G. Peters, and K. Becker (2005) Impact of a molecular approach to improve the microbiological diagnosis of infective heart valve endocarditis, *Circulation* 111:1415-1421

Brodie E.L., T.Z. DeSantis, J.P. Parker, I.X. Zubietta, Y.M. Piceno, and G.L. Andersen (2007) Urban aerosols harbor diverse and dynamic bacterial populations, *Proceedings of the National Academy of Sciences of the United States of America* 104:299-304

Bruckner J., and K. Venkateswaran (2007) Overview of methodologies to sample and assess microbial burden in low biomass environment, *Japanese journal of food microbiology* 24:61-70

Busse H.J., E.B. Denner, S. Buczolits, M. Salkinoja-Salonen, A. Bennasar, and P. Kämpfer (2003) *Sphingomonas aurantiaca* sp. nov., *Sphingomonas aerolata* sp. nov. and *Sphingomonas faeni* sp. nov., air- and dustborne and Antarctic, orange-pigmented, psychrotolerant bacteria, and emended description of the genus *Sphingomonas*, *International journal of systematic and evolutionary microbiology* 53:1253-1260

Buttner M.P., P. Cruz-Perez, and L.D. Stetzenbach (2001) Enhanced detection of surface-associated bacteria in indoor environments by quantitative PCR, *Applied and environmental microbiology* 67:2564-2570

Buttner M.P., P. Cruz, L.D. Stetzenbach, A.K. Klima-Comba, V.L. Stevens, and P.A. Emanuel (2004) Evaluation of the Biological Sampling Kit (BiSKit) for large-area surface sampling, *Applied and environmental microbiology* 70:7040-7045

Cano R. J., and M. Borucki (1995) Revival and identification of bacterial spores in 25 to 40 million year old Dominican amber, *Science* 268:1060-1064

Capone K.A., S.E. Dowd, G.N. Stamatas, and J. Nikolovski (2011) Diversity of the human skin microbiome early in life, *Journal of investigative dermatology* 131:2023-2032

Caporaso J.G., C.L. Lauber, W.A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S.M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J.A. Gilbert, G. Smith, and R. Knight (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms, *The ISME journal* 6:1621-1624

Cases I., and V. de Lorenzo (2002) The grammar of (micro)biological diversity, *Environmental microbiology* 4:623-627

Castro V.A., A.N. Thrasher, M. Healy, C.M. Ott, and D.L. Pierson (2004) Microbial characterization during the early habitation of the International Space Station, *Microbial ecology* 47:119-126

Chakravorty S., D. Helb, M. Burday, N. Connell, and D. Alland (2007) A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria, *Journal of microbiological methods* 69:330-339

Charlson E.S., K. Bittinger, A.R. Haas, A.S. Fitzgerald, I. Frank, A. Yadav, F.D. Bushman, and R.G. Collman (2011) Topographical continuity of bacterial populations in the healthy human respiratory tract, *American journal of respiratory and critical care medicine* 184:957-963

Chiller K., B.A. Selkin, and G.J. Murakawa (2001) Skin microflora and bacterial infections of the skin, *The journal of investigative dermatology* 6:170-174

Choi B.K., C. Wyss, and U.B. Gobel (1996) Phylogenetic analysis of pathogen-related oral spirochetes, *Journal of clinical microbiology* 34:1922-1923

Claesson M.J., O. O'Sullivan, Q. Wang, J. Nikkilä, J.R. Marchesi, H. Smidt, W.M. de Vos, R.P. Ross, and P.W. O'Toole (2009) Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine, *PLOS One* 4:e6669

Clement B.G., L.E. Kehl, K.L. DeBord, and C.L. Kitts (1998) Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities, *Journal of microbiological methods* 31:135-142

Cogoli A. (1993) Space flight and the immune system, *Science* 11:496-503

Conti S., S.S. dos Santos, C.Y. Koga-Ito, and A.O. Jorge (2009) *Enterobacteriaceae* and *Pseudomonadaceae* on the dorsum of the human tongue, *Journal of applied oral science* 17:357-380

Cooper M., M.T. La Duc, A. Probst, P. Vaishampayan, C. Stam, J.N. Benardini, Y.M. Piceno, G.L. Andersen, and K. Venkateswaran (2011) Comparison of innovative molecular approaches and standard assays for assessment of surface cleanliness, *Applied and environmental microbiology* 77:5438-5444

COSPAR planetary protection policy (October 2002) COSPAR/IAU Workshop on Planetary Protection, Committee on Space Research (COSPAR), International Council for Science, Paris, France http://cosparhq.cnes.fr/Scistr/Pppolicy.htm

Costerton J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber, and H.M. Lappin-Scott (1995) Microbial biofilms, *Annual review of microbiology* 49:711-745

Costello E.K., C.L. Lauber, M. Hamady, N. Fierer, J.I. Gordon, and R. Knight (2009) Bacterial community variation in human body habitats across space and time, *Science* 326:1694-1697

Courtney K.C., L.D. Bainard, B.A. Dikes, A.M. Koch, H. Maherali, J.N. Klironomos, and M.M. Hart (2012) Determining a minimum detection threshold in terminal restriction fragment length polymorphism analysis, *Journal of microbiological methods* 88:14-18

Cox C.S. (1987) The aerobiological pathway of microorganisms, John Wiley & Sons Ltd, Chichester

Cox C.S. (1989) Airborne bacteria and viruses, *Science progress* 73:459-499

Cox C.S., and M. Christopher (1995) Bioaerosols Handbook, Lewis, Boca Raton, Florida

Curtis T.P., and W.T. Sloan (2004) Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology, *Current opinion in microbiology* 7:221-226

Dacarro C., A.M. Picco, P. Grisoli, and M. Rodolfi (2003) Determination of aerial microbiological contamination in scholastic sports environments, *Journal of applied microbiology* 95:904-912

Danovaro R., G.M. Luna, A. Dell'Anno, and B. Pietrangeli (2006) Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments, *Applied and environmental microbiology* 72:5982-5989

DeAngelis K.M., C.H. Wu, H.R. Beller, E.L. Brodie, R. Chakraborty, T.Z. DeSantis, J.L. Fortney, T.C. Hazen, S.R. Osman, M.E. Singer, L.M. Rom, and G.L. Andersen (2011) PCR amplification-independent methods for detection of microbial communities by the high-density microarray PhyloChip, *Applied and environmental microbiology* 77:6313-6322

DeAngelis K.M., and M.K. Firestone (2012) Phylogenetic clustering of soil microbial communities by 16S rRNA but not 16S rRNA genes, *Applied and environmental microbiology* 78:2459-2461

Dechow M., H. Sohn, and J. Steinhanses (1997) Concentrations of selected contaminants in cabin air of airbus aircrafts, *Chemosphere* 35:21-31

DeLong E.F., and N.J. Pace (2001) Environmental diversity of Bacteria and Archaea, *Systematic Biology* 50:470-478

DeSantis T.Z., C.E. Stone, S.R. Murray, J.P. Moberg, and G.L. Andersen (2005) Rapid quantification and taxonomic classification of environmental DNA from both prokaryotic and eukaryotic origins using a microarray, *FEMS Microbiology Letters* 245:271-278

DeSantis T.Z., P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G.L. Andersen (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB, *Applied and environmental microbiology* 72:5069-5072

DeSantis T.Z., E.L. Brodie, J.P. Moberg, I.X. Zubieta, Y.M. Piceno, and G.L. Andersen (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment, *Microbial ecology* 53:371-383

DeVincenzi D.L., P. Stabekis, and J. Barengoltz (1996) Refinement of planetary protection policy for Mars missions, *Advances in space research* 18:311-316

Dewhirst F.E., T. Chen, J. Izard, B.J. Paster, A.C.R. Tanner, W.-H. Yu, A. Lakshmanan, and W.G. Wade (2010) The human oral microbiome, *Journal of Bacteriology* 192:5002-5017

Dinis J.M., D.E. Barton, J. Ghadiri, D. Surendar, K. Reddy, F. Velasquez, C.L. Chaffee, M.C.W. Lee, H. Gavrilova, H. Ozuna, S.A. Smits, and C.C. Ouverney (2011) In search of an uncultured human-associated TM7 bacterium in the environment in Yang, Ching-Hong, *PLoS One* 6:e21280

Dunbar J., L.O. Ticknor, and C.R. Kuske (2000) Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis, *Applied and environmental microbiology* 66:2943-2950

Duncan J.M., V.V. Bogomolov, F. Castrucci, Y. Koike, J.M. Comtois, and A.E. Sargsyan (2008) Organization and management of the International Space Station (ISS) multilateral medical operations, *Acta Astronautica* 63:1137-1147

Dunn R.R., N. Fierer, J.B. Henley, J.W. Leff, and H.L. Menninger (2013) Home life: factors structuring the bacterial diversity found within and between homes, *PLoS One* 8:e64133

Eckburg P.B., E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, and D.A. Relman (2005) Diversity of the human intestinal microbial flora, *Science* 308:1635-1638

ECSS-Q-ST-70-55 Working Group (2008) Microbial examination of flight hardware and clean rooms, ECSS-Q-ST-70-55C, *European Cooperation for Space Standardization, ESA Requirements and Standards Devision*, European Space Agency, ESTEC, Noordwijk, the Netherlands

ESA and State Research Center of Russian Federation - Institute for Biomedical Problems (2000) Survey of the microbial contamination knowledge on board of manned Russian space vehicles, Contract No. 14254/00/F/TB, Moscow, Russia

Falkow S. (1988) Molecular Koch's postulates applied to microbial pathogenicity, *Reviews of infectious diseases* 10:274-276

Farrelly V., F.A. Rainey, and E. Stackebrandt (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species, *Applied and environmental microbiology* 61:2798-2801

Favero M.S. (1971) Microbiologic assay of space hardware, *Environmental biology and medicine* 1:27-36

Fisher M.M., and E.W. Triplett (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities, *Applied and environmental microbiology* 65:4630-4636

Flemming H.-C., T.R. Neu, and D.J. Wozniak (2007) The EPS matrix: the "house of biofilm cells", *Journal of bacteriology* 189:7945-7947

Flemming H.-C., and J. Wingender (2010) The biofilm matrix, *Nature Reviews Microbiology* 8:623-633

Flemming H.-C. (2011) The perfect slime, *Colloid Surface B: Biointerfaces* 86:251-259

Flores G.E., S.T. Bates, D. Knights, C.L. Lauber, J. Stombaugh, R. Knight, and N. Fierer (2011) Microbial biogeography of public restroom surfaces, *PLoS One* 6:e28132

Forney L.J., X. Zhou, and C.J. Brown (2004) Molecular microbial ecology: land of the one-eyed king, *Current opinion in microbiology* 7:210-220

Frankel M., G. Bekö, M. Timm, S. Gustavsen, E.W. Hansen, and A.M. Madsen (2012) Seasonal variations of indoor microbial exposures and their relation to temperature, relative humidity, and air exchange rate, *Applied and environmental microbiology* 78:8289-8297

Fredericks D.N., and D.A. Relman (1996) Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates, *Clinical microbiology reviews* 9:18-33

Fritz J.V., M.S. Desai, P. Shah, J.G. Schneider, and P. Wilmes (2013) From meta-omics to causality: experimental models for human microbiome research, *Microbiome* doi:10.1186/2049-2618-1-14

From C., V. Hormazabal, and P.E. Granum (2007) Food poisoning associated with pumilacidin-producing *Bacillus pumilus* in rice, *International journal of food microbiology* 115:319-324

Funke G., R. Frodl, and H. Sommer (2004) First comprehensively documented case of *Paracoccus yeei* infection in a human, *Journal of clinical microbiology* 42:3366-3368

Gagarin Y.A., and V.I. Lebedev (1969) Survival in space, F.A. Praeger, New York, USA

Gao Z., C.H. Zeng, Z. Pei, and M.J. Blaser (2007) Molecular analysis of human forearm superficial skin bacterial biota, *Proceedings of the National Academy of Sciences of the United States of America* 104:2927-2932

Gaüzère G., M. Moletta-Denat, F. Bousta, S. Moularat, G. Orial, S. Ritoux, J.-J. Godon, and E. Robine (2013a) Reliable procedure for molecular analysis of airborne microflora in three indoor environments: an office and two different museum contexts, *CLEAN - Soil, Air, Water* 41:226-234

Gaüzère G., M. Moletta-Denat, H. Blanquart, S. Ferreira, S. Moularat, J.-J. Godon, and E. Robine (2013b) Stability of airborne microbes in the Louvre Museum over time, *Indoor Air* doi:10.1111/ina.12053

Gevers D., F.M. Cohan, J.G. Lawrence, B.G. Spratt, T. Coenye, E.J. Feil, E. Stackebrandt, Y. Van de Peer, P. Vandamme, F.L. Thompson, and J. Swings (2005) Opinion: Re-evaluating prokaryotic species, *Nature reviews, Microbiology* 3:733-739

Gill S.R., M. Pop, R.T. Deboy, P.J. Turnbaugh, B.S. Samuel, J.I. Gordon, D.A. Relman, C.M. Fraser-Liggett, and K.E. Nelson (2006) Metagenomic analysis of the human distal gut microbiome, *Science* 312:1355-1359

Gobet A., Boetius A., and A. Ramette (2013) Ecological coherence of diversity patterns derived from classical fingerprinting and Next Generation Sequencing techniques, *Environmental microbiology* doi:10.1111/1462-2920.12308

Godon J.J., E. Zumstein, P. Dabert, F. Habouzit, and R. Moletta (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis, *Applied and environmental microbiology* 63:2802-1813

Gonzales N., J. Romero, and R.T. Espejo (2003) Comprehensive detection of bacterial populations by PCR amplification of the 16S-23S rRNA spacer region, *Journal of microbiological methods* 55:91-97

Górny R.L., and J. Dutkiewicz (2002) Bacterial and fungal aerosols in indoor environment in Central and Eastern European countries, *Annals of agricultural and environmental medicine* 9:17-23

Greene V.W., D. Vesley, R.G. Bond, and G.S. Michaelsen (1962) Microbiological contamination of hospital air I. Quantitative studies, *Applied microbiology* 10:561-566

Grice E.A., H.H. Kong, G. Renaud, A.C. Young, NISC Comparative Sequencing program, G.C. Bouffard, R.W. Blakesley, T.G. Wolfsberg, M.L. Turner, and J.A. Segre (2008) A diversity profile of the human skin microbiota, *Genome research* 18:1043-1050

Grice E.A., H.H. Kong, S.Conlan, C.B. Deming, J.Davis, A.C. Young, NISC Comparative Sequencing Program, G.G. Bouffard, R.W. Blakesley, P.R. Murray, E.D. Green, M.L. Turner, and J.A. Segre (2009) Topographical and temporal diversity of the human skin microbiome, *Science* 324:1190-1192

Grice E.A., and J.A. Segre (2011) The skin microbiome, *Nature reviews, Microbiology* 9:244-253

Gu J.-D. (2007) Microbial colonization of polymeric material for space application and mechanisms of biodeterioration: A review, *International biodeterioration and biodegradation* 59:170-179

Han X.Y., and R.A. Andrade (2005) *Brevundimonas diminuta* infections and its resistance to fluoroquinolones, *The Journal of antimicrobial chemotherapy* 55:853-859

Hatch M.T., and R.L. Dimmick (1966) Physiological responses of airborne bacteria to shifts in relative humidity, *Bacteriological reviews* 30:597-602

Hazen T.C., E.A. Dubinsky, T.Z. DeSantis, G.L. Andersen, Y.M. Piceno, N. Singh, J.K. Jansson, A. Probst, S.E. Borglin, J.L. Fortney, W.T. Stringfellow, M. Bill, M.S. Conrad, L.M. Tom, K.L. Chavarria, T.R. Alusi, R. Lamendella, D.C. Joyner, C. Spier, J. Baelum, M. Auer, M.L. Zemla, R. Chakraborty, E.L. Sonnenthal, P. D'haeseleer, H.-Y.N. Holman, S. Osman, Z. Lu, J.D. Van Nostrand, Y. Deng, J. Zhou, and O.U. Mason (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria, *Science* 330:204-208

He Z., Y. Piceno, Y. Deng, M. Xu, Z. Lu, T.Z. DeSantis, G.L. Andersen, S.E. Hobbie, P.B. Reich, and J. Zhou (2012) The phylogenetic composition and structure of soil microbial communities shifts in response to elevated carbon dioxide, *The ISME journal* 6:259-272

Head I.M., J.R. Saunders, and R.W. Pickup (1998) Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms, *Microbial ecology* 35:1-21

Heesemann J. (2012) Pathogenität und Virulenz, In: Medizinische Mikrobiologie und Infektiologie, Springer Verlage pp 11-24

Heppener M. (2008) Spaceward ho! The future of humans in space, *EMBO reports* doi: 10.1038/embor.2008.98

Hendrickx L., H. De Wever, V. Hermans, F. Mastroleo, N. Morin, A. Wilmotte, P. Jannssen, and M. Mergeay (2006) Microbial ecology of the closed artificial ecosystem MELiSSA (Micro-ecological life support system alternative): reinventing and compartmentalizing the Earth's food and oxygen regeneration system for long-haul space exploration missions, *Research in microbiology* 157:77-86

Hess G.E. (1965) Effects of oxygen on aerosolized *Serratia marcescens*, *Applied microbiology* 13:781-878

Hewitt K.M., C.P. Gerba, S.L. Maxwell, and S.T. Kelley (2012) Office space bacterial abundance and diversity in three metropolitan areas, *PLoS One* 7:e37849

Hodges L.R., L.J. Rose, A. Peterson, J. Noble-Wang, and M.J. Arduino (2006) Evaluation of a macrofoam swab protocol for the recovery of *Bacillus anthracis* spores from a steel surface*, Applied and environmental microbiology* 72:4429-4430

Hodges L.R., L.J. Rose, H. O'Connell, and M.J. Arduino (2010) National validation study of a swab protocol for the recovery of *Bacillus anthracis* spores from surfaces, *Journal of microbiological methods* 81:141-146

Horneck G., Klaus D.M., and R.L. Mancinelli (2010) Space microbiology, *Microbiology and molecular biology reviews* 74:121-156

Hospodsky D., J. Qian, W.W. Nazaroff, N. Yamamoto, K. Bibby, H. Rismani-Yazdi, and J. Peccia (2012) Human occupancy as a source of indoor airborne bacteria, *PLoS One* 7:e34867

Hugenholtz P., G.W. Tyson, R.I. Webb, A.M. Wagner, and L.L. Blackall (2001) Investigation of Candidate Division TM7, a recently recognized major lineage of the domain bacteria with no known pure-culture representatives, *Applied and environmental microbiology* 67:411-419

Hugenholtz P. (2002) Exploring prokaryotic diversity in the genomic era, *Genome Biology* 3:reviews0003.0001-0003.0008

Hugenholtz P., and G.W. Tyson (2008) Metagenomics, *Microbiology* 455:481-483

Hughes J.B., J.J. Hellmann, T.H. Ricketts, and B.J.M. Bohannan (2001) Counting the uncountable: Statistical approaches to estimating microbial diversity, *Applied and environmental microbiology* 67:4399-4406

Ilyin V.K. (2000) Survey of the microbial contamination knowledge onboard manned Russian space vehicles, ESTEC contract 14254/00/F/TB (Moscow)

Ilyin V.K. (2005) Microbiological status of cosmonauts during orbital spaceflights on Salyut and Mir orbital space stations, *Acta Astronautica* 56:839-850

Ishii N., and M. Tomita (2009) Multi-omics Data-driven Systems Biology of *E. coli*, In: Systems Biology and Biotechnology of *Escherichia coli*, Section D, Springer, Netherlands Book pp 41-57

ISS MORD (2006) SSP 50260 Revision C: ISS Medical Operations Requirement Document, Houston, Texas, USA

Ivanova K., M. Marina, P. Petrov, and T. Kantardjiev (2010) Campylobacteriosis and other bacterial gastrointestinal diseases in Sofia, Bulgaria for the period 1987-2008, *Eurosurveillance* 15/Issue 4 Article 3

Iverson W.P. (1967) Microbial corrosion of metals, *Advances in applied microbiology* 32:1-31

Jahn U., M. Gallenberger, W. Paper, B. Junglas, W. Eisenreich, K.O. Stetter, R. Rachel, and H. Huber (2008) *Nanoarchaeum equitans* and *Ignicoccus hospitalis*: New Insights into a unique, intimate association of two Archaea, *Journal of bacteriology* 190:1743-1750

Jeon Y.-S., J. Chun, and B.S. Kim (2013) Identification of household bacterial community and analysis of species shared with human microbiome, *Current microbiology* 67:557-563

Jones R.T., M.S. Robeson, C.L. Lauber, M. Hamady, R. Knight, and N. Fierer (2009) A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses, *The ISME Journal* 3:442-453

Kacena M.A., G.A. Merell, B. Manfredi, E.E. Smith, D.M. Klaus, and P. Todd (1999) Bacterial growth in spaceflight: logistic growth curve parameters for *Escherichia coli and Bacillus subtilis*, *Applied microbiology and biotechnology* 51:229-234

Karunakaran E., J. Mukherjee, B. Ramalingam, and C.A. Biggs (2011) „Biofilmology“: a multidisciplinary review of the study of microbial biofilms, *Applied microbiology and biotechnology* 90:1869-1881

Kawamura Y., Y. Li, H. Liu, X. Huang, Z. Li, and T. Ezaki (2001) Bacterial population in Russian space station “Mir”, *Microbiology and immunology* 45:819-828

Kearney J.N., D. Harnby, G. Gowland, and K.T. Holland (1984) The follicular distribution and abundance of resident bacteria on human skin, *Journal of general microbiology* 130:797-801

Kelly L.C., C.S. Cockell, A. Herrera-Belaroussi, Y. Piceno, G. Andersen, T. DeSantis, E. Brodie, T. Thorsteinsson, V. Marteinsson, F. Poly, and Xavier LeRoux (2011) Bacterial diversity of terrestrial crystalline volcanic rocks, Iceland, *Microbial ecology* 62:69-79

Kembel S.W., E. Jones, J. Kline, D. Northcutt, J. Stenson, A.M. Womack, B.J. Bohannan, G.Z. Brown, and J.L. Green (2012) Architectural design influences the diversity and structure of the built environment microbiome, *The ISME Journal* 6:1469-1479

Kent A.D., A.C. Yannarell, J.A. Rusak, E.W. Triplett, and K.D. McMahon (2007) Synchrony in aquatic microbial community dynamics, *The ISME Journal* 1:38-47

Kersters K., P. de Vos, M. Gillis, J. Swings, P. Vandamme, and E. Stackebrandt (2006) Introduction to the Proteobacteria, *Prokaryotes* 5:3-37

Kirschner L.E., and J.R. Puleo (1979) Wipe-rinse technique for quantitating microbial contamination on large surfaces, *Applied and environmental microbiology* 38:466-470

Klaus D., S. Simske, P. Todd, and L. Stodieck (1997) Investigation of spaceflight effects on *Escherichia coli* and a proposed model of underlying physical mechanisms, *Microbiology* 143:449-455

Klaus D.M., M.R. Benoit, E.S. Nelson, and T.G. Hammong (2004) Extracellular mass transport considerations for space flight research concerning suspended and adherent *in vitro* cell cultures, *Journal of gravitational physiology* 11:17-28

Klevens R.M., J.R. Edwards, C.L. Richards Jr., T.C. Horan, R.P. Gaynes, D.A. Pollock, and D.M. Cardo (2007) Estimating health care-associated infections and deaths in US hospitals, 2002*, Public health reports* 122:118-123

Koch R. (1891) Ueber bakteriologische Forschung, Verhandlung des X Internationalen Medizinischen Congress, Berlin, 1890

Koch R. (1942) The aetiology of tuberculosis (translation of Die Aetiologie der Tuberculose [1882]), In: Source book of medical history, Dover Publications, Inc., New York, pp 392-406

Korpi A., A.-L. Pasanen, P. Pasanen, and P. Kalliokoski (1997) Microbial growth and metabolism in house dust, *International biodeterioration and biodegradation* 40:19-27

Korves T.M., Y.M. Piceno, L.M. Tom, T.Z. DeSantis, B.W. Jones, G.L. Anderson, and G.M. Hwang (2012) Bacterial communities in commercial aircraft high-efficiency particulate air (HEPA) filters assessed by PhyloChip analysis, *Indoor air* 23:50-61

Kwan K., M. Cooper, M.T. La Duc, P. Vaishampayan, C. Stam, J.N. Benardini, G. Scalzi, C. Moissl-Eichinger, and K. Venkateswaran (2011) Evaluation of procedures for the collection, processing, and analysis of biomolecules from low-biomass surfaces, *Applied and environmental microbiology* 77:2943-2953

Lacey J. (1994) Indoor aerobiology and health, In: Building mycology: Management of deca and health in buildings, E & FN Spon, Champamn and Hall, London pp 72-115

La Duc M.T., R. Kern, and K. Venkateswaran (2004) Microbial monitoring of spacecraft and associated environments, *Microbial ecology* 47:150-158

La Duc M.T., A. Dekas, S. Osman, C. Moissl, D. Newcombe, and K. Venkateswaran (2007) Isolation and characterization of bacteria capable of tolerating the extreme conditions of clean room environments. *Applied and environmental microbiology* 73:2600-2611

La Duc M.T., S. Osman, and K. Venkateswaran (2009) Comparative analysis of methods for the purification of DNA from low-biomass samples based on total yield and conserved microbial diversity, *Journal of rapid methods and automation in microbiology* 17:350-368

La Duc M.T., P. Vaishampayan, H.R. Nilsson, T. Torok, and K. Venkateswaran (2012) Pyrosequencing-derived bacterial, archaeal, and fungal diversity of spacecraft hardware destined for Mars, *Applied and environmental microbiology* 78:5912-5922

Lapchine L., N. Moatti, G. Gasset, G. Richoilley, J. Templier, and R. Tixador (1986) Antibiotic activity in space, *Drugs under experimental and clinical research* 12:933-938

Lazarevic V., K. Whiteson, D. Hernandez, P. Francois, and J. Schrenzel (2010) Study of inter- and intra-individual variations in the salivary microbiota, *BMC Genomics* doi:10.1186/1471-2164-11-523

LeChevallier M.M.W., T.M. Babcock, and R.G. Lee (1987) Examination and characterization of distribution system biofilms, *Applied and environmental microbiology* 53:2714-2724

Lee S.H., H.-J. Lee, S.-J. Kim, H.M. Lee, H. Kang, and Y.P. Kim (2010) Identification of airborne bacterial and fungal community structures in an urban area by T-RFLP analysis and quantitative real-time PCR, *The Science of the total environment* 408:1349-1357

Leininger S., T. Urich, M. Schloter, L. Schwark, J.Qi, G.W. Nicol, J.I. Prosser, S.C. Schuster and C. Schleper (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils, *Nature* 442:806-809

Letunik I., and P. Bork (2007) Interactive Tree of Life (iTOL): an online tool for phylogenetic tree display and annotation, *Bioinformatics* 23:127-128

Ley R.E., P.J. Turnbaugh, S. Klein, and J.I. Gordon (2006) Microbial ecology: Human gut microbes associated with obesity, *Nature* 444:1022-1023

Li H., and R. Durbin (2011) Inference of human population history from individual whole-genome sequences, *Nature* 475:493-496

Lidstrom M.E., and L. Chistoserdova (2002) Plants in the pink: Cytokinin production by *Methylobacterium*, *Journal of bacteriology* doi:10.1128/JB.184.7.1818.2002

Liesack W., H. Weyland, and E. Stackebrandt (1991) Potential risks of gene amplification by PCR as determined by 16S rRNA analysis of a mixed-culture of strict barophilic bacteria, *Microbial ecology* 21:191-198

Loisel P., J. Harmand, O. Zemb, E. Latrille, C. Lobry, J. Delgenes, and J.-J. Godon (2006) Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity, *Environmental microbiology* 8:720-731

Lozupone C., and R. Knight (2005) UniFrac: A new phylogenetic method for comparing microbial communities, *Applied and environmental microbiology* 71:8228-8235

Lynch S.V., K. Mukundakrishnan, M.R. Benoit, P.S. Ayyaswamy, and A. Matin (2006) *Escherichia coli* biofilms formed under low-shear modeled microgravity in a ground-based system, *Applied and environmental microbiology* 72:7701-7710

Madhusudhan K.T. (2009) Studies on physical and microbiological characteristics of selected clinical swabs edited by Young, T. N., Clean Earth Technologies, LLC, Guilford, ME

Mackelprang R., M.P. Waldrop, K.M. DeAngelis, M.M. David, K.L. Chavarria, S.J. Blazewicz, E.M. Rubin, and J.K. Jansson (2011) Metagenomic analysis of a permafrost microbial community reveals a rapid response to thaw, *Nature* 480:368-371

Maldonado-Contreras A., K.C. Goldfarb, F. Godoy-Vitorino, U. Karaoz, M. Contreras, M.J. Blaser, E.L. Brodie, and M.G. Dominguez-Bello (2011) Structure of the human gastric bacterial community in relation to *Helicobacter pylori* status, *The ISME journal* 5:574-579

Manned Exploration Requirements and Considerations (1971) Advanced Studies Office, Engineering and Development Directorate, NASA Manned Spacecraft Center, Houston, Texas, February 1971, pp 1-8

Maron P.-A., D.P.H. Lejon, E. Carvalho, K. Bizet, P. Lemanceau, L. Ranjard, and C. Mougel (2005) Assessing genetic structure and diversity of airborne bacterial communities by DNA fingerprinting and 16S rDNA clone library, *Atmospheric environment* 39:3687-3695

Marples M.J. (1965) The ecology of skin, Charles C Thomas, Bannerstone House, Springfield, Illinois

Marsh T.L. (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products, *Current opinion in microbiology* 2:323-327

Maule J., N. Wainwright, A. Steele, L. Monaco, H. Morris, D. Gunter, M. Damon, and M. Wells (2009) Rapid culture-independent microbial analysis aboard the International Space Station (ISS), *Astrobiology* 9:759-775

McCabe K.M., Y.H. Zhang, B.L. Huang, E.A. Wagar, and E.R. McCabe (1999) Bacterial species identification after DNA amplification with a universal primer pair, *Molecular genetics and metabolism* 66:205-211

McDonald D., M.N. Price, J. Goodrich, E.P. Nawrocki, T.Z. DeSantis, A. Probst, G.L. Andersen, R. Knight, and P. Hugenholtz (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea, *The ISME journal* 6:610-618

McNeil L.K., C. Reich, R.K. Aziz, D. Bartels, M. Cohoon, T. Disz, R.A. Edwards, S. Gerdes, K. Hwang, M. Kubal, G.R. Margayan, F. Meyer, W. Mihalo, G.J. Olsen, R. Olsen, A. Osterman, D. Paarmann, T. Paczian, B. Parrello, G.D. Pusch, D.A. Rodionov, X. Shi, O. Vassieva, V. Vonstein, O. Zagnitko, F. Xia, J. Zinner, R. Overbeek, and R. Stevens (2006) The National Microbial Pathogen Database Resource (NMPDR): A genomics platform based on subsystem annotation, *Nucleic acids research* 35:345-353

Medini D., D. Serrute, J. Parkhill, D.A. Relman, C. Donati, R. Moxon, S. Falkow, and R. Rappioli (2008) Microbiology in the post-genomic era, *Nature reviews* doi:10.1038/nrmicro1901

Mehta S.K., D.L. Pierson, H. Cooley, R. Dubow, and D. Lugg (2000) Epstein-Barr virus reactivation associated with diminished cell-mediated immunity in antarctic expeditioners, *Journal of medical virology* 61:235-240

Mendoza M., H. Meugnier, M. Bes, J. Etienne, and J. Freney (1998) Identification of *Staphylococcus species* by 16S-23S rDNA intergenic spacer PCR analysis, *International journal of systematic bacteriology* 48:1049-1055

Mermel L.A. (2013) Infection prevention and control during prolonged human space travel, *Clinical infectious diseases* 56:123-130

Moeseneder M.M., C. Winter, J.M. Arrieta, and G.J. Herndl (2001) Terminal-restriction fragment length polymorphism (T-RFLP) screening of a marine archaeal clone library to determine the different phylotypes, *Journal of microbiologcial methods* 2:159-172

Mohapatra B.R., and M.T. La Duc (2013) Detecting the dormant: a review of recent advantages in molecular techniques for assessing the viability of bacterial endospores, *Applied microbiology and biotechnology* 97:7963-1975

Moissl C., N. Hosoya, J. Bruckner, T. Stuecker, M. Roman, and K. Venkateswaren (2007) Molecular microbial community structure of the regenerative enclosed life support module simulator air system, *International journal of systematic and evolutionary microbiology* 6:131-145

Moissl-Eichinger C., P. Rettberg, and R. Pukall (2012) The first collection of spacecraft-associated microorganisms: a public source for extremotolerant microorganisms from spacecraft assembly clean rooms, *Astrobiology* 12:1024-1034

Moissl-Eichinger C., R. Pukall, A.J. Probst, M. Stieglmeier, P. Schwendner, M. Mora, S. Barczyk, M. Bohmeier, and P. Rettberg (2013) Lessons learned from the microbial analysis of the Herschel spacecraft during assembly, intergration and testing operations, *Astrobiology* 12:1125-1139

Moldenhauer J. (2005) Environmental monitoring: A comprehensive handbook Volume 1, Davis Healthcare International Publishing, LLC

Morey-Holton E.R., E.L. Hill, and K.A. Souza (2007) Animals and spaceflight: From survival to understanding, *Journal of musculoskeletal & neuronal interactions* 7:17-25

Morris H.C., M. Damon, J. Maule, L.A. Monaco, and N. Wainwright (2012) Rapid culture-independent microbial analysis aboard the International Space Station (ISS) stage two: Quantifying three microbial biomarkers, *Astrobiology* 12:830-840

Mulvey D., P. Redding, C. Robertson, C. Woodall, P. Kingsmore, D. Bedwell, and S.J. Dancer (2011) Finding a benchmark for monitoring hospital cleanliness, *The Journal of hospital infection* 77:25-30

Murray P.R. (2010) Human microbiota, In: Topley and Wilson's Microbiology and Microbial Infections, John Wiley Sons, Ltd.

Myers D.N., D.M. Stoeckel, R.N. Bushon, D.S. Francy, and A.M.G. Brady (2007) U.S. Geological Survey, Biological indicators: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9

Nagai F., M. Morotomi, Y. Watanabe, H. Sakon, and R. Tanaka (2010) *Alistipes indistinctus* sp. nov. and *Odoribacter laneus* sp. nov., common members of the human intestinal microbiota isolated from faeces, *International journal of systematic and evolutionary microbiology* 60:1296-1302

NASA (2005a) MR050L, Microbial Analysis of ISS Surfaces Using the Surface Sampler Kit (SSK), Houston

NASA (2005b) MR052L, Microbial Analysis of ISS Air Using the Microbial Air Sampler (MAS), Houston

NASA (2010) Handbook for the microbiological examination of space hardware, NASA-HDBK-6022, National Aeronautics and Space Administration, Washington, D.C., USA

Newton R.J., A.D. Kent, E.W. Triplett, and K.D. McMahon (2006) Microbial community dynamics in a humic lake: differential persistence of common freshwater phylotypes, *Environmental microbiology* 8:956-970

Newton R.J., J.L. Van de Walle, M.A. Borchardt, M.H. Gorelick and S.L. McLLellan (2011) *Lachnospiraceae* and Bacteroidales alternative fecal indicators reveal chronic human sewage contamination in an urban harbor, *Applied and environmental microbiology* 77:6972-6981

Nicholson W.L., N. Munakata, G. Horneck, H.J. Melosh, and P. Setlow (2000) resistance of Bacillus endopsores to extreme terrestrial and extraterrestrial environments, *Microbiology and molecular biology reviews* 64:548-572

Noble W.C. (1975) Dispersal of skin microorganisms, *The British journal of dermatology* 93:477-485

Nocker A., P. Sossa-Fernandez, M.D. Burr, and A.K. Camper (2007) Use of propidium monoazide for live/dead distinction in microbial ecology, *Applied and environmental microbiology* 73:5111-5117

Noris F., J.A. Siegel, K.A. Kinney (2011) Evaluation of HVAC filters as a sampling mechanism for indoor microbial communities, *Atmospheric environment* 45:338-346

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

Novikova N., P. De Boever, S. Poddubko, E. Deshevaya, N. Polikarpov, N. Rakova, I. Coninx, and M. Mergeay (2006) Survey of environmental biocontamination on board to International Space Station, *Research in Microbiology* 17:5-12

Oliver J.D. (2005) The viable but nonculturable state in bacteria, *The Journal of microbiology* 43:93-100

Oliver J.D. (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria, *FEMS microbiology reviews* 34:415-425

Osman S., M.T. La Duc, A. Dekas, D. Newcombe, and K. Venkateswaran (2008) Microbial burden and diversity of commercial airline cabin air during short and long durations of travel, *The ISME journal* 2:482-497

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

Øvreas L., F.L. Daae, V. Torsvik, and F. Rodriguez-Valera (2003) Characterization of microbial diversity in hypersaline environments by melting profiles and reassociation kinetics in combination with terminal restriction fragment length polymorphism (T-RFLP), *Microbial ecology* 46:291-301

Pasanen P., A.-L. Pasanen, and M. Jantunen (1993) Water condensation promotes fungal growth in ventilation ducts, *Indoor air* 3:106-112

Pasanen P., A. Korpi, P. Kalliokoski, and A.-L. Pasanen (1997) Growth and volatile metabolite production of *Aspergillus versicolor* in house dust, *Environment international* 23:425-432

Pasquarella C., O. Pitzurra, and A. Savino (2000) The index of microbial air contamination, *Journal of hospital infection*, 46:241-256

Petersen E.E. (2003) Infektionen in Gynäkologie und Geburtshilfe, Lehrbuch und Atlas, Thieme, Stuttgart

Pierson D.L. M. Chidambaram, J.D. Head, L Mallary, S.K. Mishra, B. Sharma, and G.M. Weinstock (1996) Epidemiology of *Staphylococcus aureus* in the Space Shuttle, *FEMS immunology and medical microbiology* 16:273-281

Pierson D.L. (2001) Microbial contamination of spacecraft, *Gravitational and space biology* 14:1-6

Pierson D.l., D.J. Botkin, R.J. Bruce, V.A. Castro, M.J. Smith, C.M. Oubre, and C.M. Ott (2012) Microbial monitoring of the International Space Station, In: Environmental Monitoring: A comprehensive Handbook, River Grove, IL., pp1-27

Podar M., C.B. Abulencia, M. Walcher, D. Hutchison, K. Zengler, J.A. Garcia, T. Holland, D. Cotton, L. Hauser, and M. Keller (2007) Targeted access to the genomes of low abundance organisms in complex microbial communities, *Applied and environmental microbiology* 73:3205-3214

Poza M., C. Gayoso, M.J. Gomez, S. Rumbo-Feal, M. Tomas, J. Aranda, A. Fernandez, and G. Bou (2012) Exploring bacterial diversity in hospital environments by GS-FLX Titanium pyrosequencing, *PLoS One* doi:10.1371/journal.pone.0044105

Probst A., R. Facius, R. Wirth, and C. Moissl-Eichinger (2010a) Validation of a nylon-flocked-swab protocol for efficient recovery of bacterial spores from smooth and rough surfaces, *Applied and environmental microbiology* 76:5148-5158

Probst A., P. Vaishampayan, S. Osman, C. Moissl-Eichinger, G.L. Andersen, and K. Venkateswaran (2010b) Diversity of anaerobic microbes in spacecraft assembly clean rooms, *Applied and environmental microbiology* 76:2837-2845

Probst A.J., H.Y. Holman, T.Z. DeSantis, G.L. Andersen, G. Birarda, H.A. Bechtel, Y.M. Piceno, M. Sonnleitner, K. Venkateswaran, and C. Moissl-Eichinger (2013a) Tackling the minority: sulfate-reducing bacteria in an archaea-dominated subsurface biofilm, *The ISME journal* 7:635-651

Probst A.J., A.K. Auerbach, and C. Moissl-Eichinger (2013b) Archaea on human skin, *PLoS One* 8:e65388

Probst A.J., P.Y. Lum, B. John, E.A. Dubinsky, Y.M. Piceno, L.M. Tom, G.L. Andersen, Z. He, and T.Z. DeSantis (2014) Microarray of 16S rRNA gene probes for quantifying population differences across microbiome samples, In: Microarrays: Current Technology, Innovations and Applications, Horizon Scientific Press and Caister Academic Press, accepted

Puleo J.R., G.S. Oxborrow, N.D. Fields, C.M. Herring, and L.S. Smith (1973) Microbiological profiles of four Apollo spacecraft, *Applied microbiology* 26:838-845

Puleo J.R., N.D. Fields, S.L. Bergstrom, G.S. Oxborrow, P.D. Stabekis, and R. Koukol (1977) Microbiological profiles of the Viking spacecraft, *Applied and environmental microbiology* 33:379-384

Qian J., D. Hospodsy, N. Yamamoto, W.W. Nazaroff, and J. Peccia (2012) Size-resolved emission rates of airborne bacteria and fungi in an occupied class room, *Indoor air* 22:339-351

Qin J., R. Li, J. Raes, M. Arumugam, K.S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D.R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J.-M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H.B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, S. Li, M. Jian, Y. Zhou, Y. Li, X. Zhang, S. Li, N. Qin, H. Yang, J. Wang, S. Brunak, J. Doré, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, MetaHIT Consortium, P. Bork, S.D. Ehrlich, and J. Wang (2010) A human gut microbial gene catalogue established by metagenomic sequencing, *Nature* 464:59-65

Radosevich J.L., W.J. Wilson, J.H. Shinn, T.Z. DeSantis, and G.L. Anderson (2002) Development of a high-volume aerosol collection system for the identification of air-borne micro-organisms, *Letters in applied microbiology* 34:162-167

Ranjard L., E. Brothier, and S. Nazaret (2000) Sequencing bands of ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking, *Applied and environmental microbiology* 66:5334-5339

Ranjard L., F. Poly, J.-C. Lata, C. Mougel, J. Thioulouse, and S. Nazaret (2001) Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: Biological and methodological variability, *Applied and environmental microbiology* 67:4479-4487

Rastogi G., and R.K. Sani (2011) Molecular techniques to assess microbial community structure, function and dynamics in the environment, In: Microbes and microbial technology: Agricultural and Environmental Applications, Springer Science+Business Media, LLC pp 29-57

Reinmüller B., and B. Ljungqvist (2003) Modern cleanroom clothing systems: People as a source of contamination, *PDA journal of pharmaceutical science and technology* 57:114-125

Rettberg P., D. Fritze, S. Verbarg, J. Nellen, G. Horneck, E. Stackebrandt, and G. Kminek (2006) Determination of the microbial diversity of spacecraft assembly, testing and launch facilities: First results of the ESA project MiDiv, *Advances in Space Research* 38:1260-1265

Rintala H., M. Pitkäranta, and M. Täubel (2008) Microbial communities associated with house dust, *Advances in applied microbiology* 78:75-120

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

Roda A., M. Mirasoli, M. Guardigli, P. Simoni, D. Fest, B. Afonin, and G. Vasilyeva (2013) Non-invasive panel tests for gastrointestinal motility monitoring within the MARS-500 Project, *World journal of gastroenterology* 19:2208-2216

Roman M.C., N.E. Weir, M.E. Wilson, and B.H. Pyle (2006) Microbial characterization of internal active thermal control system (IATCS) hardware surfaces after five years of operation in the International Space Station, SAE technical paper 2006-01-2157 SAE, Warrendale, PA, USA

Roman M.C., and M.W. Mittelmann (2013) Microbiological tests performed during the design of the International Space Station environmental control and life support systems. Part1, Bulk Phase, American Institute of Aeronautics pp 1-21

Rose L., B. Jensen, A. Peterson, S.N. Banerjee, and M.J. Srduino (2004) Swab materials and *Bacillus anthracis* spore recovery from nonporous surfaces, *Emerging infectious diseases* 10:1023-1029

Rosenzweig J.A., O. Abogunde, K. Thomas, A. Lawal, Y.-U. Nguyen, A. Sodipe, and O. Jejelowo (2010) Spaceflight and modeled microgravity effects on microbial growth and virulence, *Applied microbiology and biotechnology* 85:885-891

Roth R.R., and W.D. James (1988) Microbial ecology of the skin, *Annual review of microbiology* 42:441-464

Rykova M.P., E.N. Antropova, I.M. Larina, and B.V. Morukov (2008) Humoral and cellular immunity in cosmonauts after the ISS missions, *Acta Astronautica* 63:697-705

Saiki R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, and H. A. Erlich (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, *Science* 230:1350-1354

Saiki R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, *Science* 239:487-491

Salkinoja-Salonen M.S., R. Vuorio, M.A. Andersson, P. Kämpfer, M.C. Andersson, T. Honkanen-Buzalski, and A.C. Scoging (1999) Toxigenic strains of *Bacillus licheniformis* related to food poisoning, *Applied and environmental microbiology* 65:4637-4645

Salo S., A. Laine, T. Alanko, A. Sjöberg, and G. Wirtanen (2000) Validation of the microbiological methods hygicult dipslide, contact plate and swabbing in surface hygiene control: A Nordic collaborative study, *Journal of AOAC international* 83:1357-1365

Sanderson W.T., M.J. Hein, L. Taylor, B.D. Curwin, G.M. Kinnes, T.A. Seitz, T. Popovic, H.T. Holmes, M.E. Kellum, S.K. McAllister, D.N. Whaley, E.A. Tupin, T. Walker, J.A. Freed, D.S. Small, B. Klusaritz, and J.H. Bridges (2002) Surface sampling methods for *Bacillus anthracis* spore contamination, *Emerging infectious diseases* 8:1145-1151

Saulnier D.M., K. Riehle, T.A. Mistretta, M.A. Diaz, D. Mandal, S. Raza, E.M. Weidler, X. Qin, C. Coarfa, A. Milosavljevic, J.F. Petrosina, S. Highlander, R. Gibbs, S.V. Lynch, R.J. Shulman, and J. Versalovic (2011) Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome, *Gastroenterology* 141:1782-1791

Schäppi M, C. Deffert, L. Fiette, G. Gavazzi, F. Herrmann, D. Belli, and K.H. Krause (2008) Branched fungal beta-glucan causes hyperinflammation and necrosis in phagocyte NADPH oxidate-deficient mice, *The journal of pathology* 214:434-444

Schiwon K., K. Arends, K.M. Rogowski, S. Fürch, K. Prescha, T. Sakinc, R. Van Houdt, G. Werner, and E. Grohmann (2013) Comparison of antibiotic resistance, biofilm formation and conjugative transfer of *Staphylococcus* and *Enterococcus* isolates from International Space Station and Antarctic Research Station Concordia, *Microbial ecology* 65:638-651

Schleibinger H., R. Keller, and H. Rüden (2004) Indoor air pollution by microorganisms and their metabolites, In: The Handbook of Environmental Chemistry Vol. 4, Part F, Spinger-Verlag Berlin Heidelberg, pp 149-177

Schlüter A., T. Bekel, N.N. Diaz, M. Dondrup, R. Eichenlaub, K.-H. Gartemann, I. Krahn, L. Krause, H. Krömeke, O. Kruse, J.H. Mussgnug, H. Neuweger, K. Niehaus, A. Prühler, K.J. Runte, R. Szczepanowski, A. Tauch, A. Tilker, P. Viehöver, and A. Goesmann (2008) The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology, *Journal of biotechnology* 136:77-90

Schmidt T.M., E.F. DeLong, and P.R. Pace (1991) Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing, *Journal of bacteriology* 173:4371-4378

Schwendner P., C. Moissl-Eichinger, S. Barczyk, M. Bohmeier, R. Pukall, and P. Rettberg (2013) Insights into the microbial diversity and bioburden in a South-American spacecraft assembly clean room, *Astrobiology* 13:1140-1154

Segata N., D. Boernigen, T.L. Tickle, X.C. Morgan, W.S. Garrett, and C. Huttenhower (2013) Computational meta'omics for microbial community studies, *Molecular systems biology* doi:10.1038/msb.2013.22

Sekiguchi H., M. Watanabe, T. Nakahara, B. Xu, and H. Uchiyama (2002) Succession of bacterial community structure along the Changjiang river determined by denaturing gradient gel electrophoresis and clone library analysis, *Applied and environmental microbiology* 68:5142-5150

Selwyn S. (1980) Microbiology and ecology of human skin, *Practitioner* 224:1059-1062

Sessa R., P.M. Di, G. Schiavoni, I. Santino, A. Altieri, S. Pinelli, and P.M. Del (2002) Microbiological indoor air quality in healthy buildings, *The new microbiologica* 25:51-56

Shepard A.B., and S. Slayton (1994) Moon shot: the inside story of America's race to the moon, Turner Publishing, Atlanta, Georgia, USA

Sieradzki K., and A. Tomasz (2003) Alterations of cell wall structure and metabolism accompany reduces susceptibility to vancomycin in an isogenic series of clinical isolates of *Staphylococcus aureus*, *Journal of bacteriology* 185:7103-7110

Smalla K., M. Oros-Sichler, A. Milling, H. Heuer, S. Baumgarte, R. Becker, G. Neuber, S. Kropf, A. Ulrich, and C.C. Tebbe (2007) Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragment: Do the different methods provide similar results?, *Journal of microbiological methods* 69:470-479

Sogin M.L., H.G. Morrison, J.A. Huber, D.M. Welch, S.M. Huse, P.R. Neal, J.M. Arrieta, and G.J. Herndl (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere", *Proceedings of the National Academy of Sciences of the United States of America* 103:12115-12120

Spencer R.C. (2003) *Bacillus anthracis*, *Journal of clinical pathogolgy* 56:182-187

Staley J.T., and A. Konopka (1985) Measurements of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats, *Annual review of microbiology* 39:321-346

Staley J.T., R.L. Irgens, and D.J. Brenner (1987) *Enhydrobacter aerosaccus* gen. nov., sp. nov., a gas-vacuolated, facultatively anaerobic, heterotrophic rod, *International journal of systematic bacteriology* 37:289-291

Stenfors Arnesen L.P., A. Fagerlund, and P.E. Granum (2008) From soil to gut: *Bacillus cereus* and its food poisoning toxins, *FEMS microbiology reviews* 32:579-606

Stieglmeier M., R. Wirth, G. Kminek, and C. Moissl-Eichinger (2009) Cultivation of anaerobic and facultatively anaerobic bacteria from spacecraft-associated clean rooms, *Applied and environmental microbiology* 75:3484-3491

Stieglmeier M., P. Rettberg, S. Barczyk, M. Bohmeier, R. Pukall, R. Wirth, and C. Moissl-Eichinger (2012) Abundance and diversity of microbial inhabitants in European spacecraft-associated clean rooms, *Astrobiology* 12:572-585

Stohr K., B. Hafner, O. Nolte, J. Wolfrum, M. Sauer, and D.P. Herten (2005) Species-specific identification of mycobacterial 16S rRNA PCR amplicons using smart probes, *Analytical chemistry* 77:7195-7203

Stoodley P., K. Sauer, D.G. Davies, and J.W. Costerton (2002) Biofilms as complex differentiated communities, *Annual review of microbiology* 56:187-209

Szczerba I. (2003) Occurrence and number of bacteria from the *Micrococcus*, *Kocuria*, *Nesterenkonia*, *Kytococcus* and *Dermacoccus* genera on skin and mucous membranes in humans, *Medycyna doświadczalna i mikrobiologia* 55:67-74

Täubel M., H. Rintala, M. Pitkäranta, L. Paulin, S. Laitinen, J. Pekkanen, A. Hyvärinen, and A. Nevalainen (2009) The occupant as a source of house dust bacteria, *The Journal of allergy and clinical immunology 124:834-840*

Taylor G.R. (1974) Recovery of medically important microorganisms from Apollo astronauts, *Aerospace medicine* 45:824-828

The Committee on Metagenomics (2007) The new science of Metagenomics: Revealing the secrets of our microbial planet, The National Academies Press, Washington DC, USA

Tillett B., and A. Neilan (2000) Xanthogenate nucleic acid isolation from cultured and environmental Cyanobacteria, *Journal of Phycology* 26:251-258

Timmery S., X. Hu, and J. Mahillon (2011) Characterization of bacilli isolated from the confined environments of the Antarctic Concordia Station and the International Space Station, *Astrobiology* 11:323-334

TRBA 450, Technical Rule for Biological Agents 450 (2000) Criteria for the classification of biological agents, with the amendment and addition: Bundesarbeitsblatt 04/2002 and 10/2001 amendment and addition: Bundesarbeitsblat 11-2004

TRBA 466, Technical Rule for Biological Agents 466 (2010) Classification of prokaryotes (bacteria and archaea) into risk groups, Gemeinsames Ministerialblatt (GMBI), No. 68-80 of 06.12.2010, pp 1428-1667; Appendix April 2012, GMBl, No. 15-20 of 25.04.2012, p 380

Tringe S.G., C. von Mering, A. Kobayashi, A.A. Salamov, K. Chen, H.W. Chang, M. Podar, J.M. Short, E.J. Mathur, J.C. Detter, P. Bork, P. Hugenholtz, and E.M. Rubin (2005) Comparative metagenomics of microbial communities, *Science* 308:554-557

Tringe S.G., T. Zhang, X. Liu, Y. Yu, W.H. Lee, J. Yap, F. Yao, S.T. Suan, S.K. Ing, M. Haynes, F. Rohwer, C.L. Wei, P. Tan, J. Bristow, E.M. Rubin, and Y. Ruan (2008) The airborne metagenome in an indoor urban environment, *PLoS One* 3:e1862

Tixador R., G. Richoilley, G. Gasset, J. Templier, J.C. Bes, N. Moatti, and L. Lapchine (1985) Study of minimal inhibitory concentration of antibiotics on bacteria cultivated *in vitro* in space (Cytos 2 experiment), *Aviation, space, and environmental medicine* 56:748-751

Turnbaugh P.J., R.E. Ley, M. Hamady, C.M. Fraser-Liggett, R. Knight, and J.I. Gordon (2007) The Human Microbiome Project, *Nature* 449:804-810

Turnbaugh P.J., M. Hamady, T. Yatsunenko, B.L. Cantarel, A. Duncan, R.E. Ley, M.L. Sogin, W.J. Jones, B.A. Roe, and J.P. Affourtit (2008) A core gut microbiome in obese and lean twins, *Nature* 457:480-484

Tweedie A. (2005) Updated guidance for clean rooms reviewed, *Health estate* 59:25-30

Uh Y., J.S. Son, I.H. Jang, K.J. Yoon, and S.K. Hong (2002) Penicillin-resistant *Aerococcus viridans* bacteremia associated with granulacytopenia, *Journal of Korean medical science* 17:113-115

U.N. Outer Space Treaty (1967) London/Washington

Vaishampayan P., S. Osman, G. Andersen, and K. Venkateswaran (2010) High-density 16S microarray and clone library-based microbial community composition of the Phoenix spacecraft assembly clean room, *Astrobiology* 10:499-508

Vaishampayan P., A.J. Probst, M.T. La Duc, E. Bargoma, J.N. Benardini, G.L. Andersen, and K. Venkateswaran (2013) New perspectives on viable microbial communities in low-biomass cleanroom environments, *The ISME Journal* 7:312-324

Valentine N.B., M.G. Butcher, Y.-F. Su, K.H. Jarman, M. Matzke, B.-J. Webb-Roberton, E.A. Panisko, B.A.B Seiders, and K.L. Wahl (2008) Evaluation of sampling tools for environmental sampling of bacterial endospores from porous and nonporous surfaces, *Journal of applied microbiology* 105:1107-1113

Van de Peer Y., S. Chapelle, and R. de Wachter (1996) A quantitative map of nucleotide substitution rates in bacterial rRNA, *Nucleic acids research* 24:3381-3391

Van der Linde K., B.T. Lim, J.M. Roneel, L.P. Antonissen and G.M. de Jong (1999) Improved bacteriological surveillance of haemodialysis fluids: a comparison between Tryptic soy agar and Reasoner's 2A media, *Nephrology dialysis transplantation* 14:2433-2437

Van Houdt R., P. DeBoever, I. Coninx, C. LeCalvez, R. Dicasillati, J. Mahillon, M. Mergeay, and N. Leys (2009) Evaluation of the airborne bacterial population in the periodically confined Antarctic base Concordia, *Microbial ecology* 57:640-648

Van Houdt R., K. Mijnendonckx, and N. Leys (2012) Microbial contamination monitoring and control during human space missions, *Planetary and space science* 60:115-120

Velikanov L.L., O.A. Panova, and V.A. Timonin (1987) Influence of some microorganisms on electro-chemical and corrosive behavior of structural materials, In: Biological damages to structural and industrial materials, Kiev, p 37

Venkateswaran K., M. Satomi, S. Chung, R. Kern, R.C. Koukol, C. Basic, and D. White (2001) Molecular microbial diversity of a spacecraft assembly facility, *Systematic and applied microbiology* 24:311-320

Venkateswaran K., M.T. La Duc, and P. Vaishampayan (2012a) Genetic Inventory Task: Final Report, Volume 1, JPL Publication 12-12, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA.

Venkateswaran K., M.T. La Duc, and P. Vaishampayan (2012b) Genetic Inventory Task: Final Report, Volume 2, JPL Publication 12-12, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA.

Venter J.C., K. Remington, J.F. Heidelberg, A.L. Halpern, D. Rusch, J.A. Eisen, D. Wu, I. Paulsen, K.E. Nelson, W. Nelson, D.E. Fouts, S. Levy, A.H. Knap, M.W. Lomans, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parson, H. Baden-Tillson, Y.H. Rogers, and H.O. Smith (2004) Environmental genome shotgun sequencing of the Sargasso Sea, *Science* 304:66-74

Verhoeff A. (1993) Biological particles in indoor environments: In European Collaborative Action, Indoor Air Quality and Its Impact on Man, COST Project 613, Report N. 12, EUR 14988 EN1993, Luxembourg

Von Wintzingerode F., U.B. Gobel, and E. Stackebrandt (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis, *FEMS microbiology reviews* 21:213-229

Wang Q., G.M. Garrity, J.M. Tiedje, and J.R. Cole (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy, *Applied and Environmental Microbiology* 73:5261-5267

Wang Y.F., C.-H. Wang, and K.-L. Hsu (2010) Size and seasonal distributions of airborne bioaerosols in commuting trains, *Atmospheric environment* 44:4331-4338

Wick Jr. R.L., and L.A. Irvine (1995) The microbiological composition of airliner cabin air, *Aviation, space, and environmental medicine* 66:220-224

Wick L.M., M. Quadroni, and T. Egli (2001) Short- and long-term changes in proteome composition and kinetic properties in a culture of *Escherichia coli* during transition from glucose-excess to glucose-limited growth conditions in continuous culture and *vice versa, Environmental microbiology* 3:588-599

Willner D., J. Daly, D. Whiley, K. Grimwood, C.E. Wainwright, and P. Hugenholtz (2012) Comparison of DNA extraction methods for microbial community profiling with an application to pediatric bronchoalveolar lavage samples, *PLoS One* doi:10.1371/journal.pone.0034605

Wilson J.W., C.M. Ott, K. Höner zu Bentrup, R. Ramamurthy, L. Quick, S. Porwollik, P. Cheng, M. McClelland, G. Tsaprailis, T. Radabaugh, A. Hunt, D. Fernandez, E. Richter, M. Shah, M. Kilcoyne, L. Joshi, M. Nelman-Gonzalez, S. Hing, M. Parra, P. Dumars, K. Norwood, R. Bober, J. Devich, A. Ruggles, C. Goulart, M. Rupert, L. Stodieck, P. Stafford, L. Catella, M.J. Schurr, K. Buchanan, L. Morici, J. McCracken, P. Allen, C. Baker-Coleman, T. Hammond, J. Vogel, R. Nelson, D.L. Pierson, H.M. Stefanyshyn-Piper, and C.A. Nickerson (2007) Spaceflight alters bacterial gene expression and virulence and reveals a role for global regulater Hfq, *Proceedings of the National Academy of Sciences of the United States of America*104:16299-16304

Wilson K.H., W.J. Wilson, J.L. Radosevich, T.Z. DeSantis, V.S. Viswanathan, T.A. Kuczmarski, and G.L. Andersen (2002) High-density microarray of small-subunit ribosomal DNA probes, *Applied and environmental microbiology* 68:2535-2541

Wilson M. (2005) Microbial inhabitants of humans: their ecology and role in health and disease, Cambridge University Press, New York, NY, USA

Wingender J. (2011) Hygienically relevant microorganisms in biofilms of man-made water systems, In: Biofilm Perspectives, Springer International, Heidelberg, New York

Wright E.S., L.S. Yilmaz, and D.R. Noquera (2012) DECIPHER, A search-based approach to chimera identification for 16S rRNA sequences, *Applied and environmental microbiology* 78:717-722

Xu J., M.A. Mahowald, R.E. Ley, C.A. Lozupone, M. Hamady, E.C. Martens, B. Henrissat, P.M. Coutinho, P. Minx, P. Latreille, H. Cordum, A. Van Brunt, K. Kim, R.S. Fulton, L.A. Fulton, S.W. Clifton, R.K. Wilson, R.D. Knight, and J.I. Gordon (2007) Evolution of symbiotic bacteria in the distal human intestine, *PLoS biology* 5:e156

Zhang W., F. Li, and L. Nie (2010) Integrating multiple 'omics' analysis for microbial biology: application and methodologies, *Microbiology* 156:287-301

Zhu H., P.E. Phelan, T. Duan, G.B. Raupp, H.J.S. Fernando, and F. Che (2003) Experimental study of indoor and outdoor airborne bacterial concentrations in Tempe, Arizona, USA, *Aerobiologia* 19:201-211

VII. ACKNOWLEDGMENTS

I would like to express my gratitude to Prof. Dr. Reinhard Wirth for his supervision, continuous support and coming all the way to Cologne for several TAC meetings throughout the years. The critical discussions together with Prof. Dr. Reinhard Rachel and Dr. Harald Huber, completing the Regensburger trio, were very fruitful and improved the quality of my thesis. Vergelt's Gott an die Regensburger Fraktion.

I am also thankful to Prof. Dr. Bettina Siebers for her encouragement and her willingness to be available as a second referee.

Many thanks to Dr. Günther Reitz, who gave me the opportunity to carry out the thesis in his department and in particular to Dr. Petra Rettberg, who continuously supported and supervised my work on this fascinating and unique topic. I am also grateful that I got the possibility to present my work at conferences, to attend summer schools in order to establish a scientific network, and to supervise two interns (Dominique Haag, Marc Schulte). Dominque and Marc - you did a great job.

I additionally thank all colleagues of the Radiation Biology Department for a great working climate. Specifically, I would like to thank my office mates Dr. Stefan Leuko (and the yodeling hat), Marko Wassmann, Dr. Anja Bauermeister, Dagmar Koschnitzki, Dr. Kristina Beblo-Vranesevic, and Claudia Hahn and my "coffee break" mates Dr. Birgitt Ritter and Dr. Tina Koch. I thank Dr. Ralf Möller for providing the opportunity to get involved in his research proposals as a scientific team member. I am grateful to Britta Rowehl and Dr. Daniel Matthiä for giving me a ride to the volleyball training every Tuesday, Thomas Urlings for the IT-support and Simon Barczyk for the help regarding the MICHAm samples and orders. Schee woars!

Furthermore, without the help of the marsonauts this thesis would not have been possible. You (Charles Romain) and the others did a great job, which made it easy to work with the samples you took. Thanks a lot!

Finally, I would like to acknowledge the SpaceLife graduate program (Helmholtz Space Life Sciences Research School) for funding the thesis and providing insights into a diverse field of space-related topics. The organization of workshops substantially helped to get an overview on the current status of my thesis. As part of SpaceLife I am furthermore grateful to my mentor Prof. Dr. Rupert Gerzer, Dr. Christine Hellweg, and Anna-Maria Trautmann.

The "Regensburg International Graduate School of Life Sciences" (RIGeL) from the University of Regensburg is also acknowledged.

I deeply appreciate the collarboration with Dr. Bernd Johannes and Prof. Dr. Gro Sandal for data sharing and Ganna Aleshcheva for the translation work (Russian - German).

I am deeply grateful to my dear friend in Regensburg (Dr. Christina Sarbu) for taking your time to proof-read my thesis and your support also beyond science. Wos hätt I bloß gmacht ohne Di?!

I furthermore thank Kelly Kwan (native speaker), Dr. Stefan Leuko, and Dr. Parag Vaishampayan for proof-reading activities. That was absolutely awesome.

I am deeply indebted to André for continuous support in every respect and always believing in me.

Mein allergrößter Dank geht an meine Familie - ohne Euch wäre dieser Schritt nicht realisierbar gewesen. Danke für die bedingungslose Unterstützung trotz der 500 km Entfernung. Ia seids de Bestn!

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

STATEMENT OF CANDIDATE

Herewith I declare that this thesis is the result of my independent work. All sources and auxiliary materials used by me in this thesis are cited completely.
I also declare that the intellectual content of this thesis is the product of my own work, except to the extent of that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Cologne (Köln), January of 2014

VIII. SUPPLEMENTARY - DATA CD

The supplementary data being located on the CD data are organized according to the following folder structure. Each folder contains a word file with figure and table captions. In addition, the data CD contains a pdf file of the work (folder: Main Document).