Dissertation

Microbes in restricted indoor environments
- with focus on the International Space Station and spacecraft associated clean rooms

Submitted by

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for the Academic Degree of

Doctor of Philosophy

(PhD)

at the

Medical University of Graz

Department of Internal Medicine

under the Supervision of

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2019

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

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Maximilian Mora
Graz, Februar 2019

Disclosures

Parts of the introduction are already published in, and contain verbatim text of:

Mora, M.*, Mahnert, A.*, Koskinen, K., Pausan, M. R., Oberauner-Wappis, L., Krause, R., Perras, A. K., Gorkiewicz, G., Berg, G. and Moissl-Eichinger, C. (2016) 'Microorganisms in confined habitats: Microbial monitoring and control of intensive care units, operating rooms, cleanrooms and the international space station', *Frontiers in Microbiology*, 7, p. 1573. doi: 10.3389/fmicb.2016.01573.

Parts of the materials & methods, results and discussion are already published in, and contain verbatim text of:

Mora, M.*, Perras, A. K,*, Alekhova, T. A., Wink, L., Krause, R., Aleksandrova, A., Novozhilova, T. and Moissl-Eichinger, C. (2016) 'Resilient microorganisms in dust samples of the International Space Station-survival of the adaptation specialists', *Microbio*me, 4(1), p. 65. doi: 10.1186/s40168-016-0217-7.

Mora, M., Wink, L., Kögler, I., Mahnert, A., Rettberg, P., Schwendner, P., Demets, R., Cockell, C., Alekhova, T., Klingl, A., Alexandrova, A. and Moissl-Eichinger, C. (2019) 'The International Space Station selects for microorganisms adapted to the extreme environment, but does not induce genomic and physiological changes relevant for human health', *bioRxiv*. doi: 10.1101/533752. → This is a preprint and currently submitted to a peer reviewed journal

*Authors contributed equally

All co-authors presented here gave their consent to re-use data from the publications within this thesis and contributed to the data shown in this thesis and the publications as follows:

Alekhova, Tatiana A. provided critical discussions throughout the whole project and provided and pre-processed the Russian dust-filter and vacuum cleaner samples samples and the Russian swab samples and provided the respective metadata.

Alexandrova, Alina provided and pre-processed the Russian dust-filter and vacuum cleaner samples and the Russian swab samples and performed phenotype-based taxonomic classification of fungi.

Berg, Gabriele provided critical discussions throughout the whole project.

Cockell, Charles provided critical discussions and the facilities for the cultivation of the radiation resistance assay.

Demets, René provided critical discussions throughout the whole project and helped in composing the ESR (Appendix 1) and establishing the EXTREMOPHILES flight project on board ISS.

Gorkiewicz, Gregor provided critical discussions while composing Mora, Mahnert *et al.* 2016.

Klingl, Andreas provided critical discussions on electron microscopy and performed scanning electron microscopy for Figures 4-7.

Koskinen, Kaisa provided critical discussions throughout the whole project and composed the ICU chapter in Mora, Mahnert *et. al.* 2016, and revised the thesis.

Kögler, Ines assisted in processing the samples from the EXTREMOPHILES flight project.

Krause, Robert provided critical discussions and supported the antibiotic resistance tests .

Mahnert, Alexander provided critical discussions and composed the clean room chapter and the figures in Mora, Mahnert *et. al.* 2016.

Moissl-Eichinger, Christine provided critical discussions and valuable help regarding all methods used throughout the whole project, revised the manuscripts of all publications and the thesis, and was as principaI investigator of the ARBEX and EXTREMOPHILES mainly responsible for the initial experiment design.

Novozhilova, Tatiana provided and pre-processed the Russian dust-filter and vacuum cleaner samples samples.

Oberauner-Wappis, Lisa composed the high security laboratories chapter in Mora, Mahnert *et al.* 2016.

Pausan, Manuela Raluca provided critical discussions throughout the whole project and composed the operating rooms chapter in Mora, Mahnert *et al.* 2016.

Perras, Alexandra K. provided critical discussions throughout the whole project and bioinformatical analyses in Mora, Perras *et al.* 2016.

Rettberg, Petra, provided critical discussions, the facilities for the irradiation experiment and performed the irradiation experiments with UV radiation and X rays.

Schwendner, Petra performed cultivation of microbes following the radiation resistance assay.

Wink, Lisa provided critical discussions and omnipresent practical assistance throughout the whole project.

Maximilian Mora was furthermore involved in following publications during this time which were not directly related to this thesis:

Mora, M., Veijalainen, A.-M., Heinonen-Tanski, H. (2018) 'Performic Acid Controls Better Clostridium tyrobutyricum Related Bacteria than Peracetic Acid', *Sustainability*, 10(11), 4116. doi: 10.3390/su10114116

Perras, A., Koskinen, K., Mora, M., Beck, M., Wink, L., & Moissl-Eichinger, C. (2018) 'Dead Or Alive? Molecular life-dead distinction in human stool samples reveals significantly different composition of the microbial community', *bioRxiv*, 2018, 343194. doi: 10.1101/343194

Koskinen, K., Pausan, M. R., Perras, A. K., Beck, M., Bang, C., Mora, M., Schilhabel, A., Schmitz, R. Moissl-Eichinger, C. (2017) 'First insights into the diverse human archaeome: Specific detection of archaea the gastrointestinal tract, lung, and nose and on skin', *MBio*, 8(6), e00824-17. doi: 10.1128/mBio.00824-17

Perras AK, Daum B, Ziegler C, Takahashi LK, Ahmed M, Wanner G, Klingl A, Leitinger G, Kolb-Lenz D, Gribaldo S, Auerbach A, Mora M, Probst AJ, Bellack A, Moissl-Eichinger, C (2015) 'S-layers at second glance? Altiarchaeal grappling hooks (hami) resemble archaeal S-layer proteins in structure and sequence', *Front.Microbiol.* 6:543. doi: 10.3389/fmicb.2015.00543

Acknowledgements

First, I would like to thank my supervisor, **Prof. Christine Moissl-Eichinger** for the possibility to work on this fascinating topic and her constant support during my time in her workgroup. I really enjoyed my time(s) in the "CME workgroup" from the first internship as undergraduate student until the completion of this thesis!
I also would like to thank my Co-Supervisors **Prof. Gabriele Berg, Prof. Martin Grube** and **Prof. Reinhard Wirth (†)** for their support, critical discussions, and helpful input.
Special thanks go to **Prof. Reinhard Wirth (†)** for his guidance throughout my biology studies. His sudden and much too early departure fills me with grief and I would like to express my deepest condolences to his family.

I want to thank all involved people from ESA and BIOTESC for their help in making the EXTREMOPHILES flight project reality. Special thanks in this regard go to **René Demets** (ESA), **Lobke Zuijderduijn** (ESA), **Magdalena Herová** (BIOTESC), and **Jeannine Winkler** (BIOTESC).
I also want to thank **Jennifer Struble** (ESA) and **Frits de Jong** (ESA) for organizing the visit to the Columbus mockup and **Stéphanie Raffestin** (ESA) for sampling the ESA clean room in Kourou.

Special thanks go also to **Jack Fischer** (NASA) for taking the samples on board the ISS.

My sincere gratitude goes to all collaboration partners within this project:
Prof. Tatiana Alekhova, I really enjoyed our meetings and discussions and I also want to thank **Elena Alekhova** for her linguistic help during our first meetings.
Thanks to **Petra Rettberg**, **Stefan Leuko**, and **Simon Barczyk** from DLR for the irradiation of the samples and additional halophile assays, as well as to **Thomas Berger** (DLR) for providing radiation data from within the Columbus module.
I want to thank **Prof. Charles Cockell**, **Petra Schwendner** and **Anna Zolotariof** for cultivating the irradiated samples in Edinburgh.
I also want to express my thanks to **Kasthuri Venkateswaran** (NASA) and his workgroup for providing additional unidentified clean room isolates, and to **Prof. Robert Krause** and **Daniela Srienz** for their help with the antibiotic resistance assay.
Special thanks go to **Prof. Andreas Klingl** for taking the beautiful electron microscopy pictures during the first days of his summer vacation.

I also want to express my gratitude to the **Austrian "Forschungsförderungs-gesellschaft" FFG** for funding my position and the ARBEX project (Project number: 847977) as well as to the Medical University of Graz and the **PhD Program Molecular Medicine (MolMed)** for providing a high-quality educational environment.

Furthermore, I want to express my gratitude to the whole ZMF staff for their assistance during my time here: In particular I want to thank **Ingeborg Klymiuk** and the rest of the Core Facility Molecular Biology for their help with all the sequencing, **Marija Durdevic** and **Slave Trajanoski** for their bioinformatics support, **Gerhard Hasenberger** for listening to - and helping with - all EDV complaints, **Hannes Angerer** for keeping me safe, **Dominik Sudy** and **Christina Smodej** for handling all my weird (work related) packages.

Very special thanks go to all other current and former members of the "CME workgroup", keep up the good team spirit! It was a pleasure to work and dine with you! First and foremost, I want to express my warmest gratitude to **Kaisa Koskinen** for her unwavering support and her ability to motivate me and bring out the best of me at work and beyond. Special thanks go also to **Lisa Wink**, our local hero, for helping me in the lab throughout the project, and to **Alexandra Perras**, it was really a special time when the workgroup consisted only of Christine, you and me. I also want to thank all Bachelor students, whom I had the pleasure to supervise in the lab, for their assistance with my labwork: **Daniel Eckl**, for his help with the Kourou samples, **Stefanie Duller** for her help with the unidentified NASA isolates, and **Ines Kögler** for her help with the EXTREMOPHILES samples. I hope I was able to teach you something. **Alexander Probst**, **Manuela Pausan**, **Alexander Mahnert**, **Marcus Blohs**, **Christina Kumpitsch**, **Michael Beck**, **Michael Mauermeir**, and all others whom I did not mention now, I am really grateful that I met you and I think we became more than just colleagues, I really enjoyed working with you and our extracurricular activities.

Special thanks go also to **Patrick Ziegler** for being my beer and food buddy in Graz.

Last, but for sure not least, I would like to extend my utmost gratitude to my family and all my friends. Your constant support and emotional backup enabled me to reach this point. I want to thank my mother **Anna-Maria Mora** for her continuous advice and also for proofreading the thesis, and also my father, **Josef Baumann**, for his support and cutting the Aluminium chunks to a size suitable for the co-incubation experiments and electron microscopy. I also want to thank my brother **Benjamin Baumann** and his fiancé **Christine Lindenthal** for proofreading parts of the thesis, and both of my brothers Benjamin and **Ingmar Baumann** for helping me relax during fishing trips when I found the time for it. I also want to thank my grandparents, aunts, uncles, cousins, and friends for being there for me when needed and their support during my studies. I also want thank you all, and especially my grandfather **Josef Mora,** for your patience with regard to all the birthday parties and family get togethers I missed in the last years.

Abstract

Restricted indoor environments are extraordinary living and working areas which have only limited exchange with other environments. This work investigated the microbiome of two distinct restricted indoor environments: The Internationals Space Station (ISS) and spacecraft associated clean rooms (SACR) on Earth. The ISS is inhabited by the human crew and co-inhabiting microorganisms and is characterized by a variety of unique environmental features, such as microgravity and a higher background radiation than most Earth environments. SACR are highly particulate controlled built environments. Both environments have in common that they are anthropogenic and are challenging for microbial life due to rigorous cleaning protocols, mechanical ventilation with highly efficient particulate air filters, and a low nutrient availability and water activity. At the timepoint of sampling (2014-2017), the investigated SACR housed cargo transporters destined towards the ISS. ISS samples comprised wipe samples obtained during increments 51 and 52 (2017) and dust samples retrieved during ISS expedition 9 (2004) and 16 (2008). Samples from both environments were analyzed by microbial amplicon sequencing, cultivation, comparative physiological studies, and antibiotic resistance tests. ISS microbial isolates were additionally investigated via genome analysis and co-incubation experiments with ISS-relevant materials. These analyses were conducted with the goal to elucidate following questions: I) Can Archaea and extremophilic (or extremotolerant) microorganisms be cultivated and/or detected on board the ISS? II) Do microbes adapt to a life in space on board the ISS and if yes to which extent? III) Are Isolates from the ISS more resistant than respective ground control strains according to physiologic tests and are observed resistances reflected in the genomes of selected isolates? IV) Does the ISS indoor microbiome differ over time or in different locations within the ISS? V) Is the ISS microbial diversity (dis)similar from that in SACRs If and if yes to what extent? VI) Are ISS isolates obtained in this study able to form biofilms on ISS relevant material and/or are they able to degrade or corrode these materials? It was possible to assess the detectable as well as the cultivable microbial diversity on board the ISS, including Archaea and a variety of extremophilic/tolerant Bacteria, and to identify a core diversity which was found to be stable over years. The ISS microbial diversity was, however, only to a minor extent influenced by SACR microbes. While the ISS environment was found to select for microbial survival and adaption specialists, these hardy microbes were not found to be significantly more or less resistant with regard to ISS environmental factors than related microbes from terrestrial environments with regard to their limits of growth or their genomic content. ISS isolates did partly show a strong antibiotics-resistance potential, however, also here we could not observe a difference to respective terrestrial strain. Furthermore, some ISS isolates were observed to adhere to ISS-relevant materials, although it remained unclear to what extent these microbes were able to damage these materials. The data obtained in this work indicates no reason for concern with respect to crew health, but indicates a potential threat towards biofilm formation and material integrity in moist areas. This thesis expanded the available knowledge of the ISS indoor microbiome with regard to the presence of Archaea and extremophilic and extremotolerant microbes on board the ISS and provided a baseline of isolates, including a variety of isolates which have not been cultured from the ISS environment before, as well as sequencing data providing information regarding the spatial distribution and temporal succession of the ISS indoor microbiome. These results will contribute to the development of a safe microbial management plan for future long-term space missions and other confined indoor environments on Earth.

Zusammenfassung

Abgeschottete Innenräume sind besondere Ökosysteme mit nur wenig Kontakt zur Außenwelt. In dieser Arbeit wurde das Mikrobiom von zwei verschiedenen abgeschotteten Innenräumen untersucht: Die Internationale Raumstation (ISS) und Reinräume mit Raumfahrtbezug auf der Erde (SACR). Die ISS wird ständig von einer menschlichen Besatzung und begleitenden Mikroorganismen bewohnt und besitzt eine Reihe einzigartiger Umweltfaktoren, wie z.B Mikrogravitation und eine höhere Hintergrundstrahlung als auf der Erde. SACR sind Innenräume die möglichst frei von luftgetragenen Partikeln gehalten werden. Diese beiden Arten von begrenzten Innenräumen haben gemeinsam, dass sie künstliche Ökosysteme mit regelmäßiger intensiver Wartung sind. Oberflächenreinigung und Luftumwälzung durch hocheffiziente Feinstaubfilter, sowie eine daraus resultierende sehr geringe Menge an verfügbaren Nährstoffen und geringe Wasseraktivität machen es sehr schwer für Mikroben, darin zu überleben und Fuß zu fassen. Die Proben in den SACR wurden zwischen 2014 und 2017 genommen, als in diesen Raumtransporter beladen wurden die zur Versorgung für die ISS bestimmt waren. Die ISS Proben bestanden aus Wischproben welche während ISS Expeditionen 51 und 52 (2017) genommen wurden und aus Staubproben welche während den ISS Expeditionen 9 (2004) und 16 (2008) genommen wurden. Die Proben beider Innenräume wurden mittels mikrobieller Amplikonsequenzierung und verschiedenen Kultivierungsansätzen, gefolgt von physiologischen und Antiobiotikaresistenz-tests, untersucht. Ausgewählte mikrobielle Isolate von der ISS wurden zusätzlich zusammen mit ISS-relevanten Materialien inkubiert und Genom-sequenziert. Diese Experimente wurden mit dem Ziel durchgeführt, folgende Fragen zu beantworten: I) Ist es möglich Archäen und extremophile (oder extremotolerante) Mikroorganismen auf der ISS nachzuweisen und/oder zu kultivieren? II) Passen sich Mikroben den Umweltbedingungen auf der ISS and und falls ja, in welchem Ausmaß? III) Sind Isolate von der ISS allgemein resistenter als terrestrische Vertreter der gleichen Spezies und sind diese Resistenzen auf Genomebene nachweisbar? IV) Verändert sich die mikrobielle Diversität des ISS Innenraums über Zeit und/oder ist diese an verschiedenen Orten unterschiedlich? V) Gibt es Unterschiede oder Gemeinsamkeiten bezüglich der mikrobiellen Diversität auf der ISS und in SACR und in welchem Ausmaß? VI) Können Mikroben, die im Rahmen dieser Studie isoliert wurden, einen Biofilm auf ISS relevanten Materialien bilden und/oder diese Materialien beschädigen?

Im Rahmen dieser Studie wurde die molekular nachweisbare und kultivierbare mikrobielle Diversität der ISS, inklusive Archäen und extremophiler/toleranter Bakterien, erfasst. Es konnte eine mikrobielle Kern-Gemeinschaft identifiziert werden, welche über Jahre stabil auf der ISS vorkam. Die mikrobielle Diversität auf der ISS hatte jedoch nur zu einem geringen Teil Überschneidungen mit der mikrobiellen Diversität in SACR. Die Umweltbedingungen auf der ISS begünstigen zwar offensichtlich mikrobielle Überlebens- und Anpassungskünstler, aber diese Mikroben waren nicht mehr oder weniger resistent als terrestrische nahverwandte Vertreter ihrer Spezies, weder mit Bezug auf die durchgeführten physiologischen Tests, noch auf Genomebene. Einige der ISS Isolate wiesen hohe Antibiotikaresistenzen auf, aber auch diese waren nicht höher als von vergleichbaren terrestrischen Mikroben. Des Weiteren konnte beobachtet werden, dass einige ISS Isolate sich an ISS relevante Materialien anheften konnten. Es war jedoch nicht möglich eindeutig zu beurteilen in welchem Ausmaß diese Mikroben die getesteten Materialien beschädigen konnten. Die hier präsentierten Daten erregen keinen Grund zur Besorgnis bezüglich einer gesundheitlichen Gefährdung der

Besatzung, aber sie weisen auf eine mögliche Bedrohung der Integrität von ISS relevanten Materialien in feuchtem Milieu hin. In dieser Arbeit wurden neue Erkenntnisse über das ISS Mikrobiom gewonnen, vor allem mit Bezug auf das Vorkommen von Archäen und extremophilen/toleranten Mikroben in diesem abgeschotteten Innenraum. Es konnte eine hohe Diversität an Mikroben kultiviert werden, inklusive einer Reihe von Arten welche zuvor noch nie von Proben aus diesem Lebensraum kultiviert wurden. Außerdem wurden Sequenzdaten erfasst welche Informationen über die räumliche Verteilung und zeitliche Veränderung der mikrobiellen Diversität auf der ISS liefern. Diese Ergebnisse bilden eine Basis für weiterführende Experimente und werden dazu beitragen einen Plan zu erstellen um potentielle mikrobielle Gefahren in zukünftigen Raumfahrtsmissionen längerer Dauer zu vermeiden. Diese Erkenntnisse werden voraussichtlich auch bei ähnlichen abgeschotteten Innenräumen auf der Erde anwendbar sein.

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List of Abbreviations

°C	degree Celsius
µg	microgram
µGy/day	microGray per day
µl	microlitre
µSv/day	microSievert per day
AAM	Autotrophic Allrounder Medium
AB	antibiotics
AFT	backwards orientation (within the ATV)
AHM	Autotrophic Homoacetogenics Medium
ANOVA	Analysis of Variance
ARBEX	ARchaea and Bacterial EXtremophiles on board the ISS
ARED	Advanced Resistive Exercise Device
ARG	Antibiotic Resistance Gene
ASM	Autotrophic Sulfate reducer Medium
ATU	Audio Terminal Unit
ATV	Automated Transfer Vehicle (ESA´s cargo transporter)
BIOTESC	Biotechnology Space Support Center of the Lucerne University of Applied Sciences and Arts, Switzerland
CRS	Commercial Resupply Service
CRV	Commercial Resupply Vehicle
CSA	Canadian Space Agency
cug	corresponding unique genera
DLR	German Aerospace Center – Deutsches Zentrum für Luft und Raumfahrt
DNA	DeoxyriboNucleic Acid
dsDNA	double stranded DNA
DSMZ	German Collection of Microorganisms and Cell Cultures
ENA	European Nucleotide Archive
ESA	European Space Agency
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FWD	forward orientation (within the ATV)
g	gram (weight) or g-force (centrifugal unit)

GESTIS	Information system on hazardous substances of the German Social Accident Insurance
Gy	Gray
h	hour
HEPA	High Efficiency Particulate Air
HS	Heat Shock
ILSRA	International Life Science Research Announcement
ISS	International Space Station
ITS	Internal Transcribed Spacer
J/m2	Jules per squaremetre
JAXA	Japan Aerospace Exploration Agency
JPL	NASA´s Jet Propulsion Laboratory at the California Institute of Technology, USA
K	Kourou (S5C Clean room)
k	kilo = thousand
kDa	kilo-Dalton
kHz	kilo-Hertz
LB	Lysogeny Broth
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis Effect Size evalutaion
LMG	Laboratory for Microbiology of the Faculty of Sciences of the Ghent University housing the Belgian Coordinated Collection of Microorganism
MDA	Multiple Displacement Amplification
mg	milligram
MIC	Minimal Inhibitory Concentration
min	minute
mL	millilitre
mm	millimetre
MS	Methanobrevibacter Smithii medium
mSv/a	milliSievert per year
NASA	National Aeronautics and Space Administration (USA)
NGS	Next Generation Sequencing
nm	nanometre
NMDS	Non-metric MultiDimensional Scaling

Orbital ATK	US aerospace company - since 2017 Northrop Grumman Innovation Systems
PBA	Portable Breathing Apparatus
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PFE	Portable Fire Extinguisher
PK/PD	PharmacoKinetic/PharmacoDynamic
PMA	Propidium MonoAzide
R2A	Reasoner´s 2 A medium
RGSH	Return Grid Sensor Housing (Air intake of ISS Air circulation system)
Roscosmos	Roscosmos State Corporation for Space Activities (Russia)
rRNA	ribosomal RiboNucleic Acid
RSV	Ribosomal Sequence Variant
s	second
SACR	Spacecraft Associated Clean Room(s)
SDS	Sodium Dodecyl Sulfate
sp.	species
spp.	species pluralis (multiple species)
SpX	Space X Dragon cargo transporter flight
SSC	Station Support Computer
TE	tris(hydroxymethyl)aminomethane - Ethylenediaminetetraacetic acid (TRIS-EDTA) buffer
TSA	Tryptic Soy Agar
US	United States (of America)
UV-C	Ultraviolet C
w/v	weight per volume
WGA	Whole Genome Amplification
WHC	Waste and Hygiene Compartment
XSB	Xanthogenate-SDS-Buffer
YE	Yeast Extract
YPD	Yeast extract Peptone dextrose Agar

I Introduction

I.1 The driving factors of the built indoor microbiome

Humans constantly coexist with microorganisms. Microorganisms of all three domains of life, Archaea, Bacteria and Eucarya are found in the human microbiome as well as in the microbiome of animals and plants (Cho and Blaser, 2012; Hoffmann *et al.*, 2013; Koskinen *et al.*, 2017; Moissl-Eichinger *et al.*, 2018) and every individual harbors his or her own individual microbiome (Meadow *et al.*, 2015). A host needs its microbiome to function properly and survive and vice versa. Together with the host they build one single ecological entity, the so-called holobiont, which also has one shared, co-evolving hologenome comprised of the host genome and the genomic information of all associated microbes (Rosenberg and Zilber-Rosenberg, 2011).

The microbiome of an individual is always in a state of dynamic exchange with its surroundings. It has been calculated that one person emits up to 3.7×10^7 bacterial and 7.3×10^6 fungal genome copies per hour (Qian *et al.*, 2012) and an individual can constantly acquire new microbes by inhalation, ingestion or contact with surfaces or other individuals. A longitudinal study examining animal and human occupants as well as surfaces in homes could follow the transition of certain microbial signatures from one occupant to home surfaces and afterwards to another occupant (Lax *et al.*, 2014). As people in industrialized countries spend on average around 90% of their time indoors (Klepeis *et al.*, 2001), the interplay between the human microbiome and the built indoor microbiome has become increasingly important. The built indoor microbiome at large is defined as collection of all microbial matter from anthropogenic built environments. Different built environments which are encountered in daily life and have already been studied include for example living homes and offices (Lax *et al.*, 2014; Chase *et al.*, 2016), public buildings, as e.g. hospitals and retail stores (Oberauner *et al.*, 2013; Hoisington *et al.*, 2015), and public traffic vehicles (Kang *et al.*, 2018; Zhao *et al.*, 2018).

All these built environments differ from each other with regard to microbial sources (surroundings, ventilation, infiltration, and indoor emissions) and sinks (deposition, exfiltration, ventilation, and decomposition) which define their individual microbial composition (Gilbert and Stephens, 2018). It is understood, that increasing urbanization changes the indoor microbiome (Ruiz-Calderon *et al.*, 2016). A decrease of outdoor environmental microbial sources leads to a higher proportion of purely human associated microorganisms in the indoor microbiome by indoor emissions. This is further intensified by the fact that indoor environments are in general rather hostile to microbes from outdoor environments, due to the nature of most indoor surfaces (chemical treatment and used materials), low nutrient availability, and a low water activity which decreases microbial metabolism (Adams *et al.*, 2016; Gilbert and Stephens, 2018). When this hostility to microbial life within buildings increases even more, e.g. in health care facilities, the influence of the human associated microbiome on the indoor microbiome becomes even more prominent (Oberauner *et al.*, 2013; Lax *et al.*, 2014). The human microbiome contains many opportunistic pathogens which are normally not dangerous, but can easily infect patients

who already have an impaired immune system. Additionally, opportunistic pathogens may activate genes related with increased virulence, or acquire such genes via horizontal gene transfer, in the course of a stress response triggered by the hostile hospital environment (Gilbert and Stephens, 2018). This combination of increased microbial virulence and impaired host immune systems is reflected by the rise of hospital acquired infections (HAI). The frequency of HAI as well as the occurrence of multi-resistant bacteria (against antibiotics) is constantly increasing and one of the bigger challenges in managing modern health care environments (Dennesen *et al.* 1998; Hu *et al.*, 2015).

Another special case of built indoor environments with an even higher hostility to microbial life are industrial clean rooms. Clean rooms are highly specific environments with a very intense particulate control regime, used for various purposes such as manufacturing of pharmaceutics, electronics, and food as well as spacecraft assembly. Spacecraft associated clean rooms and their housed spacecraft have been particularly studied regarding their microbial load to prevent a microbial contamination of extraterrestrial targets (Stieglmeier *et al.*, 2012; Moissl-Eichinger *et al.*, 2013; Vaishampayan *et al.*, 2013). Clean room maintenance includes highly efficient air filtration, surface sterilization, and artificial management of temperature and humidity (Mahnert *et al.*, 2015; Mora, Mahnert, *et al.*, 2016). Furthermore, clean room staff has to wear specific clothing to minimize the shedding of human associated microorganisms. Nevertheless, also in this case the human microbiome has been identified as the major contamination source. However, the proportion of hardy microorganisms and survival specialists like bacterial spore formers is higher than in less controlled indoor environments (Vaishampayan *et al.*, 2013; Mahnert *et al.*, 2015; Weinmaier *et al.*, 2015).

Paradoxically, intense maintenance regimes seem to select exactly for those microorganisms which are least desired in controlled indoor environments. This is the case for health care facilities, as well as for industrial clean rooms. Nevertheless, the overall microbial load is strongly reduced by increasing biocontrol.

Implications of the indoor microbiome on human health

As mentioned before, the human microbiome has co-evolved together with humans as a holobiont. This co-evolution was - and still is - also strongly influenced by the surrounding environmental microbiome. Consequently, it is not surprising that a loss or shift of microbial diversity within the immediate surrounding microbiome can also have its implications on human health. Especially in combination with antibiotic treatment, this can lead to the total extinction of certain microbial species within the human microbiome and cause or exacerbate a variety of diseases (Cho and Blaser, 2012). In particular the onset of allergies and asthma is proven to be strongly connected with urbanization (Ege *et al.*, 2011; Martikainen *et al.*, 2018). Furthermore, it has been shown in mice that early life exposure to dust from farm homes has a protective effect against asthma development, whereas dust from urban homes lacks this protective effect (Stein *et al.*, 2016). These positive health effects are in general correlated with a

higher microbial richness and diversity of microorganisms within rural homes. This considerably shifted the scientific perception of microorganisms from being purely negative (i.e. pathogenic) to being potentially beneficial (i.e. protective or preventive). Consequently, it has been (and still is) investigated how beneficial microorganisms can be artificially introduced into the built indoor microbiome in urbanized areas. It has already been shown that this is possible to a certain extent by introducing pets (Fujimura *et al.*, 2014) or plants (Mahnert *et al.* 2015) in built environments. Even a simple thorough ventilation with outdoor air can already enhance the microbial diversity in built environments (Kembel *et al.*, 2014; Meadow *et al.*, 2014), although the efficacy thereof is dependent on the degree of urbanization in the immediate surrounding of the building. These are cost effective and easily applicable methods to enrich microbial diversity in built indoor environments and should definitely be considered for indoor environments with healthy occupants, such as homes and offices. However, introducing complete additional microbial ecosystems (plants, pets, outdoor environment) has the disadvantage that the introduced microorganisms can only be poorly controlled. More research is needed in this regard to avoid the possible introduction of harmful microorganisms. Up to date it remains elusive which microorganisms in detail - or combinations thereof - are actually responsible for promoting human health in built environments, while health adverse microorganisms are well documented (Mensah-Attipoe *et al.*, 2017). Until the identity of beneficial environmental microorganisms is elucidated, especially in health care facilities maximal microbial control (often resulting in sterility) is still considered the safest approach.

Spacecraft as a special case of built environment

The most isolated built indoor environments with a constant human occupancy are operating spacecraft or a hypothetical artificial colony on another planet. The first manned missions to Mars might be launched already in the year 2023 (Musk, 2017), although experience shows that such high endeavor missions tend to be postponed. Nevertheless, human space exploration beyond the boundaries of Earth and Moon is a declared goal of NASA, ESA, Roscosmos and other space agencies. To ensure the safety of any future space travelers, it is crucial to know which microorganisms can be expected in such an environment and how they adapt to this special setting. Furthermore, it is of interest how a healthy microbiome can be maintained under these circumstances. To a certain extent this information can be derived from other indoor microbiome studies and also be specifically investigated in simulations in isolated habitats on Earth (Mayer *et al.*, 2016; Schwendner *et al.*, 2017), but not all environmental factors of a space habitat can be taken into consideration in terrestrial experiments. The best built environment up to date to investigate the development of a microbial community under real space conditions is the international space station.

I.2 The International Space Station (Mora, Mahnert *et al.* 2016)

The International Space Station (ISS) is one of the biggest and most complex international scientific projects in history. It circles our planet in low Earth orbit, about 400 km above ground. It is a joint venture of the five space agencies of USA (NASA), Europe (ESA), Russia (Roscosmos; Russian Federal Space Agency), Canada (CSA; Canadian Space Agency), and Japan (JAXA; Japanese Aerospace Exploration Agency) and it is organized in modules. The first module, namely the Russian Zarya module, was launched in 1998 and since 30th October 2000 the ISS has been constantly inhabited by humans. While the ISS kept growing by the addition of new modules over the years, also the crew size increased from initially three crew members to six international astronauts and cosmonauts who are now routinely inhabiting the ISS. Naturally, the presence of humans also imposes the presence of their associated microorganisms in this confined habitat. Besides the arrival of new crew members roughly every six months and about one cargo transporter per month, which delivers supplies and scientific equipment for experiments, the ISS is cut off from any other biological environment. Therefore, the ISS composes one of the most confined man-made and inhabited environments to date. In addition to its confinement, the ISS represents a very unusual microbial biotope. Higher radiation levels than on Earth, low nutrient levels due to reduced introduction of new material, constant temperature (approx. 22°C), stable humidity (approx. 36-60%) and microgravity characterise the ISS habitat and make it a unique and extreme-situated indoor environment (Coil *et al.*, 2016).

Microbial safety measures and risk factors

The microbiology on the ISS has been under surveillance since its first inhabitation. Standardised monitoring of surface and air samples on board the ISS as well as more detailed post-flight investigations thereof have been conducted (Pierson, 2001; Castro *et al.*, 2004; Alekhova *et al.*, 2005, 2015; Novikova *et al.*, 2006; Vesper *et al.*, 2008; Satoh *et al.*, 2011; Venkateswaran *et al.*, 2014; Checinska *et al.*, 2015; Yamaguchi *et al.,* 2016; Ichijo *et al.*, 2016; Lang *et al.*, 2017). Moreover, cleanliness of the ISS water supplies has been investigated (La Duc *et al.*, 2004; Bruce *et al.*, 2005). The greater part of the first microbial investigations were mainly based on cultivation of bacteria and fungi on commercial high-nutrient media and under moderate conditions (Castro *et al.*, 2004; Novikova *et al.*, 2006; Van Houdt *et al*., 2012). Since Roscosmos observed serious problems due to microbial contaminations during operation of the space station Mir, all involved space agencies agreed on preventive measures to protect spacecraft, cargo, and crew from harmful microorganisms (Novikova, 2004; Ott *et al.*, 2014).

For example, the air regeneration system is equipped with HEPA or equivalent filters (POTOK 150MK in Russian modules) to remove airborne microorganisms and particles ≥0.3µm. In order to avoid higher levels of microbial contamination, a rigorous housekeeping program is in place that includes weekly cleaning, biweekly disinfection and standard monitoring of ISS air and surfaces for viable bacterial and fungal contaminants every 90 days. The used disinfection agents are either based on a quaternary

ammonium compound which is supplied by the US or on the combination of a quaternary ammonium compound with hydrogen peroxide, which is supplied by Russia (Pierson, 2001; Castro *et al.*, 2004; Novikova *et al.*, 2006; Van Houdt *et al.*, 2012). Monitoring of the microbial community on board the ISS is highly important to evaluate material integrity of the spacecraft and to assess risk factors to the health of crew members. It is known that the human microbiome changes and the human immune system is compromised under spaceflight conditions, which may facilitate infections by (opportunistic) pathogens which are already present in the human body before launching to space (Voorhies and Lorenzi, 2016). For example, a significant decrease of lymphocytes was observed and also the activity of innate and adaptive immune response was reduced compared to terrestrial controls (Sonnenfeld and Shearer, 2002; Aponte *et al.,* 2006). Additionally, it has been shown that the virulence of most microorganisms is affected by microgravity. For some species virulence is enhanced in space conditions, such as *Salmonella typhimurium* (Wilson *et al.*, 2007), and for some other species virulence is reduced, such as *Listeria monocytogenes* or *Enterococcus faecalis* (Hammond *et al.*, 2013). It is also debated that the efficacy of antibiotics and other medications might decrease under spaceflight conditions (Taylor, 2015). In contradiction to these studies, a study investigating the growth behavior of non-pathogenic (terrestrial) bacteria on board the ISS found that the actual growth behavior of most bacteria does not change much in the ISS environment, given that they have enough nutrients (Coil *et al.*, 2016). Even the integrity of the spacecraft itself can be compromised by microorganisms. So-called technophilic microorganisms, in particular fungi, are known to be able to corrode alloys and polymers used in spacecraft assembly (Alekhova *et al.*, 2005). These technophilic microorganisms caused major problems on the former Russian space station Mir (Novikova *et al.*, 2001; Novikova, 2004).

The International Space Station microbiome and its origin

The main fungal genera detected on board the ISS by cultivation are *Aspergillus* and *Penicillium* (Alekhova *et al.*, 2005; Novikova *et al.*, 2006; Venkateswaran *et al.*, 2014). These fungi were also found in higher abundance using different molecular approaches; however, Satoh et al. 2011 did not find any *Penicillium* in the Japanese Kibo module one year after its installation, but detected a predominance of skin-associated *Malassezia* (Satoh *et al.*, 2011).

The main bacterial phyla detected on board the ISS in air and on surfaces, by either cultivation or molecular methods, were Firmicutes and Actinobacteria. In cultivation-based assays, *Bacillus* and *Staphylococcus* species were the most detected Firmicutes, whereas signatures of *Staphylococcu*s utterly dominate the Firmicutes-affiliated signatures detected by molecular methods in one study (Venkateswaran *et al.*, 2014) but were far less abundant in another (Lang *et al.*, 2017). The most probable reason for this observed discrepancy might be the disability of standard DNA isolation protocols to open spores adequately (Venkateswaran *et al.*, 2014).

This finding emphasizes that cultivation approaches – although generally not able to record the whole diversity of a given environment (also stated above) – are still necessary for regular monitoring

procedures. However, the ability of state-of-the-art culture-independent molecular methods to assess the total microbial diversity present in a given environment is a powerful tool which enables researchers to elucidate the microbial community structure within the ISS beyond the standard cultivation assays. Next generation sequencing is nowadays also facilitating the microbiome analysis of the ISS. For instance, vacuum cleaner dust and filter debris collected from HEPA filters within the US American part of the ISS were analyzed in detail and their microbial inventory was also compared to the microbial inventory from spacecraft assembly clean rooms (Checinska *et al.*, 2015). There are several current projects besides the work presented here which aim to broaden the knowledge about the ISS microbiome, including NASA's "Microbial Observatory" project (Venkateswaran, La Duc and Horneck, 2014; Checinska *et al.*, 2015; Be *et al.*, 2017; Romsdahl *et al.*, 2018; Singh *et al.*, 2018; Urbaniak *et al.*, 2018) and JAXA's "Microbe" experiment series (Satoh *et al.*, 2011; Ott *et al.*, 2014; Ichijo *et al.*, 2016; Yamaguchi *et al.* 2016).

All studies which investigated the ISS microbiome agree in one major aspect: the crew members act as the main source for the ISS microbial community, since most of the detected microorganisms are human associated. As discussed in the previous chapter (I.1.1), this is in accordance with studies regarding other built indoor environments, because the only constant microbial source for the ISS indoor microbiome is represented by the human crew. The crew on board ISS wears normal clothing, which does not impede the dispersal of human associated microorganisms off the respiratory tract or skin. The only studies which did not report a dominance of microorganisms of a presumable anthropogenic origin were studies conducted on the water supplies of the ISS, which is reasonable since these should normally not come in extensive physical contact with humans. Most of the organisms in the ISS water supplies were Gram-negative Proteobacteria, such as *Methylobacterium*, *Sphingomonas*, *Ralstonia* and *Pseudomonas* (La Duc *et al.*, 2004; Bruce *et al.*, 2005). Besides the human body, the other possible contamination source in this secluded habitat is the cargo delivered to the ISS including food, general equipment and material for scientific experiments. Cargo is always subjected to adapted cleaning procedures inside spacecraft assembly clean rooms before upload and should be at least "visibly clean" before being sent to the ISS (Pierson, 2001; Mora, Mahnert, *et al.*, 2016). As discussed previously, many human associated fungal and bacterial species are known to be opportunistic pathogens capable of infecting individuals with a (severely) compromised immune system, the human immune system is proven to be compromised in space, and the virulence of some (opportunistic) pathogens could even become enhanced under space-flight conditions. Additionally, if left uncontrolled in a confined environment where environmental strains are not present, which would normally compete with human associated microorganisms under such conditions, human associated microorganisms can easily proliferate quickly and thereby pose a health hazard, as has been shown in artificial closed ecosystems on Earth (Sun *et al.*, 2016). However, to date, there have been only few infections reported on board the ISS and the contamination limits were exceeded only in a few cases. Appropriate countermeasures have always succeeded in a timely manner (Van Houdt *et al.* 2012; Crucian *et al.*, 2016).

The most recent publications in the course of NASA's "Microbial Observatory" project are focused on the detection of antimicrobial resistance genes on board the ISS and evaluated the potential risk these genes might represent in a closed spacecraft environment (Urbaniak *et al.*, 2018). Singh *et al.* 2018 assessed the succession and persistence of microbial communities and the associated antimicrobial resistance and virulence properties based on metagenomic reads obtained from samples of three flights. Overall, 46 microbial species were found, including eight biosafety level 2 species, to be persistent on the ISS over a timespan of roughly one and a half years (Singh *et al.*, 2018). The authors inferred an increase of antimicrobial resistance and virulence genes over time, bearing an alarming message that these factors seem to become an increasing proportion of the ISS microbiome. However, a pangenomics based study could not reveal potentially health-threatening differences when comparing *Bacillus* and *Staphylococcus* pangenomes from ISS and Earth (Blaustein *et al.*, 2019).

I.3 Hypotheses & objectives

This thesis is based on the ARBEX project (ARchaea and Bacterial EXtremophiles on board the ISS), which was originally approved and accepted by the European Space Agency (ESA) during the International Life Science Research Announcement (ILSRA) in 2009. At this point, research regarding the ISS microbiome was mainly based on standard cultivation methods. Consequently, 16S rRNA amplicon based next generation sequencing studies on the ISS microbiome were nonexistent. The final decision by ESA to include the ARBEX project in one of the upcoming ISS flights was made in 2014 and the operation name for the flight project was changed to EXTREMOPHILES in the following preparations.

The two main hypotheses of the ARBEX project were:

1) Archaea and extremophilic (or extremotolerant) microorganisms can be cultivated and/or detected on board the ISS.
2) Microbes adapt to a life in space on board the ISS. Isolates from the ISS are more resistant than respective ground control strains.

These hypotheses were tackled by three major approaches:

a) Presence and distribution of Archaea and bacterial extremophiles: Isolation and characterisation of Archaea and extremophilic (or extremotolerant) Bacteria by sampling of selected locations inside the ISS.
b) Adaptation and evolution of the ISS microbiome: Assessment of temporal changes in the ISS microbiome over the longest feasible timespan without crew exchange, and comparison of recent samples with 10-12 years old dust samples. Investigation of the ISS microbiome with regard to a potential spatial separation and/or specialization on board the ISS.
c) Similarities and differences between the ISS microbiome and terrestrial environments: Comparison of the microbial population on board ISS with that of spacecraft associated clean rooms and visiting vehicles as well as with other terrestrial close relatives.

In the course of this thesis, these approaches were also extended to the non-extremophilic proportion of the ISS microbiome and a special focus was put on investigating the antibiotics-resistance potential of ISS isolates. Furthermore, the potential abilities of ISS isolates to form biofilms on ISS relevant material and to degrade or corrode these materials was examined.

Samples from both environments were analyzed by amplicon and metagenomics sequencing, cultivation, comparative physiological studies, and antibiotic resistance tests. ISS isolates were additionally investigated via genome analysis and co-incubation experiments with ISS-relevant materials. A schematic overview is given on the last page of this thesis (Appendix 6).

II Materials & Methods

II.1 Sampling

II.1.1 International space station – ESA and NASA modules

In-flight sampling on board the ISS was conducted by astronaut Jack D. Fischer under the in-flight operation name EXTREMOPHILES. Sampling was distributed over three sampling sessions: session A, session B and session C. The sampling locations of the different sessions are given in Table 1. Sessions A and B targeted seven identical sampling locations in ESA´s research module Columbus and the adjacent Node 2 module, also called Harmony, which houses the sleeping units of ESA and NASA astronauts. In addition to the originally planned samples, we received another sample, A-9, which was sampled as possible replacement for sample A-1 on the RGSH of Node2. Sample A-1 was lost on board ISS during the sampling process while the other samples were performed, but was found again within the following day. Sessions A and B were both sampled with dry wipes (TX 3211 Alpha Wipe, ITW Texwipe) having a time interval of 72 days between the sessions. Session C targeted seven different sampling sites in the modules Cupola, Node 1 (Unity) and Node 3 (Tranquility). In addition to the originally planned samples, we received another sample, C-9, which was identified to have been contaminated on board the ISS in an unknown manner by the astronaut. Session C was sampled with the same type of wipes as session A and session B, but the wipes were pre-moistened on ground with 20ml autoclaved PCR-grade water (LiChrosolv, Merck Millipore). Astronaut Jack D. Fischer sampled areas of approximately 1 m² at the designated sampling locations (Table 1). The sampling procedure for session A is shown in Table 2 and sessions B and C were sampled accordingly, adapted to their own sampling locations. The used material and the sampling procedure on board the ISS is described in more detail in the excerpts from the experiment scientific requirements in Appendix 1. The correct sampling procedure was verified via livestream to Graz during session A. The original order of the session A samples was changed in order to facilitate the livestream and the changed order is given in Table 1. Packaging, pre-processing and logistics of the sample material regarding upload and download from the ISS were managed by the Biotechnology Space Support Center (BIOTESC) of the Lucerne University of Applied Sciences and Arts, Switzerland, in accordance with the prerequisites set by the ESR (Appendix 1). The sampling material was uploaded via a Cygnus transporter during Orbital ATK flight CRS-7 on April 18, 2017, and downloaded via SpaceX Dragon capsules at the end of June 2017 (SpX11, Session A+C) and mid-September 2017 (SpX 12, Session B). After download, Session A and C arrived in the laboratory within a week, while the transport of Session B took two months. An overview regarding the sampling and transport schedule is given in Figure 1.

Table 1: Sampling locations of session A, B, and C (reproduced and updated from Appendix 1)

Dry Wipe Nr.	Sampling Surface	ISS module	Host
A-1	Return Grid Sensor Housing (RGSH)	Columbus	ESA
A-2	SSC Laptop		
A-3	Hand grips		
A-4	Light covers		
A-5	Ambient air		
A-6	Sleeping unit	NODE2	NASA
A-7	Panels (outside surface) close to PFE+PBA		
A-8	Audio terminal unit (ATU)		
A-9	Return Grid Sensor Housing (RGSH)		

Dry Wipe Nr.	Sampling Surface	ISS module	Host
B-1	Ambient air	Columbus	ESA
B-2	Light covers		
B-3	SSC Laptop		
B-4	Hand grips		
B-5	Return Grid Sensor Housing (RGSH)		
B-6	Sleeping unit	NODE2	NASA
B-7	Panels (outside surface) close to PFE+PBA		
B-8	Audio terminal unit (ATU)		

Dry Wipe Nr.	Sampling Surface	ISS module	Host
C-1	Ambient air	Cupola	NASA
C-2	Surface facing a window (higher radiation)		
C-3	Advanced Resistive Exercise Device (ARED)	NODE3	
C-4	Treadmill		
C-5	Waste and hygiene compartment surfaces (WHC)		
C-6	Cover inner side	NODE1	
C-7	Dining table		
C-9	Wipe “contaminated” on board ISS	na	

Table 2: Sampling protocol for session A; Sessions B and C were sampled accordingly (reproduced from Appendix 1).

Step	Activity
1	Put on sterile glove.
2	Using gloved hand, remove wipe A-1 from bag, wave wipe through the air (approx. 20seconds). Put wipe back into its bag and close properly.
3	Change glove.
4	Using gloved hand, take sample using Wipe A-2 according to Table 1. Put wipe back into its bag and close properly.
5-16	Repeat steps 3 and 4 for every new sampling surface according to Table 1.
17	Store dry wipes at ambient temperature and wet wipes at 4°C.

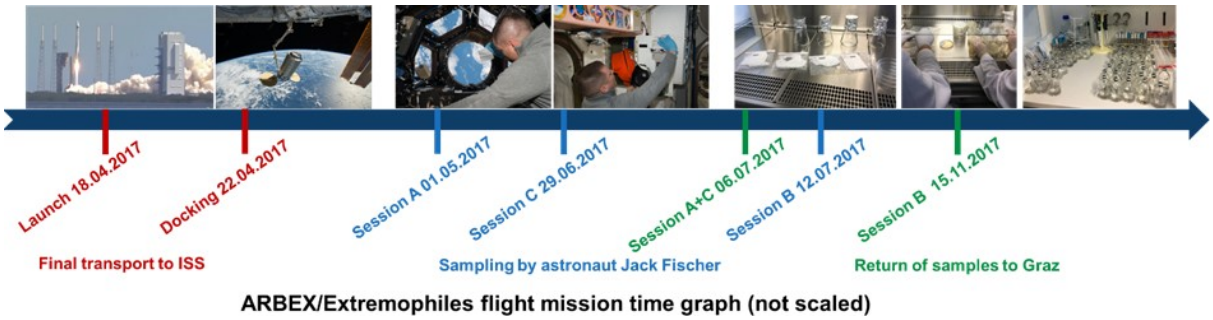

Figure 1: ARBEX/Extremophiles flight mission time graph (not scaled). Images from launch, docking and sampling were provided by NASA and ESA.

II.1.2 International Space Station – Russian modules

The initial plan to take samples in the Russian modules of the ISS with the same method as in the ESA and NASA modules failed because Roscosmos denied sampling in the Russian part of the ISS for the Extremophiles flight project. However, in collaboration with the workgroup of T. Alekhova of the Lomonosov State University in Moscow, Russia, we received old vacuum cleaner bag and dust filter samples, as well as cotton swabs sampled on the Russian service modules of the ISS in September 2017.

Cotton swabs were sampled on surfaces within the Russian service modules in September 2017 and labelled "3 BCS behind the panel 421", "4 SRVK behind the panel 434", "5 BCS behind the panel 461l", "6 Block Electron VM panel 429", "9 SLE - 1 panel 204", "11 Fan socket VV PRK (air duct of the transition chamber)", "12 hermetic case in the control panel under the panel 138", "13 Hermetic case or BCS behind panel 437", "15 behind the panel 428", "18 Hermetic panel behind the panel 135", "19 CM behind the panel F 139". These samples were used for cultivation only, because previous tests before establishing the sampling methods for the Extremophiles flight project showed that microbial DNA yield of cotton swab samples is low and that cotton DNA may overshadow microbial DNA (data not shown).

Vacuum cleaner bag and dust filter samples were retrieved during ISS expedition 9 in October 2004 and during ISS-expedition 16 in April 2008. We received multiple samples of five different sampling locations which were analysed in two sets: Sample 1: "Dust filter-1" (2004), dust filter of ventilation system (internal abbreviation: RISS1); Sample 2: "Dust filter-2" (2004), dust filter of ventilation system (internal abbreviation: RISS4); Sample 3: "Dust collector" (2004), from vacuum cleaner (internal abbreviation: RISS5); Sample 4: "Dust filter" (2008), dust filter of ventilation system (internal abbreviation: RISS3); Sample 5: "Dust collector" (2008), from vacuum cleaner (internal abbreviation: RISS2). During the entire time after retrieval, the vacuum cleaner bags and dust filters were stored sealed (never opened since sampling on the ISS), under dry conditions at ambient temperature (Mora, Perras, *et al.*, 2016).

II.1.3 Clean rooms

Cargo spacecraft are always prepared and loaded in industrial clean rooms on Earth before being sent towards the International Space Station. Several extremotolerant and extremophilic bacteria are known to survive the harsh conditions in spacecraft associated clean rooms (Moissl-Eichinger *et al.*, 2013). To compare if the ISS microbiome composition resembles the composition of clean rooms on ground, and also to evaluate different sampling methods for the Extremophiles flight project, ESA´s clean room S5C at the Centre Spatial Guyanais near Kourou in French Guiana was sampled in 2014. During that time, the S5C clean room housed the cargo spacecraft ATV5 *"Georges Lemaître"* for cargo loading. On March 19, 2014, after the final cleaning procedure of the spacecraft, Stéphanie Raffestin (ESA) sampled five locations within the clean room and spacecraft. The samples arrived three weeks later in our laboratory where they were immediately processed. The sampling locations in the clean room were: K1: "Floor clean room_1"; K2: "REPA platform and fences (access to ICC Pressurized Module)_2"; K3: "TAM (platform access to spacecraft)_3"; K4: "Electrical cupboards inside clean room_4"; K5: "Mechanics cupboards and floor area_5". The sampling locations within the ATV 5 spacecraft were: K6: "Fwd, Hatch/Russian docking system_6"; K7: "Fwd Cone-Primary structure_7", K8: "FWD Rack Inner Panel_8", K9: "AFT Rack Inner Panel_9"; K10: "AFT cone-Primary structure_10". Images of these sampling locations can be viewed in Appendix 2. The clean room was sampled with four different sampling devices: Nylon flocked swabs (FLOQSwabs™, Copan diagnostics, USA) moistened with PCR-grade water (LiChrosolv, Merck Millipore), dry cotton swabs (provided by T. Alekhova), dry wipes (TX 3211 Alpha Wipe , ITW Texwipe), and the same wipes moistened with 20ml PCR-grade water (LiChrosolv, Merck Millipore). The wipe samples in the clean room were taken in duplicates. The sampling locations within the ATV5 spacecraft were sampled once with wet wipes, as access and sampling possibilities in the spacecraft after final cleaning before launch were limited.

Furthermore, the workgroup of Dr. K. Venkateswaran from NASA´s Jet Propulsion Laboratory at the California Institute of Technology (JPL), USA, provided 91 unidentified isolates which were sampled in a North American clean room from the inside and outside surfaces of a Commercial Resupply Vehicle (CRV) destined to ISS. This sampling was done with biological sampling kits (BiSKiT, QuickSilver Analytics, USA) according to the manufacturer´s instructions and isolates were cultivated on commercial R2A, PDA and Blood Agar.

II.2 Extraction

The obtained sample material was either available as wipes (23 cm x 23 cm), swabs or fabric pieces cut out of a vacuum cleaner bag or dust filter (3 cm x 3 cm). These samples were submerged in DNA-free 0.9% NaCl solution (w/v) which contained heat-treated NaCl (24 hours, 250°C), and autoclaved PCR-grade water (LiChrosolv, Merck Millipore). Wipes were submerged in 80 ml of the 0.9% NaCl solution and vacuum cleaner bag / dust filter samples and swabs were submerged in 15 ml of the 0.9% NaCl solution. The sampling material in solution was then vortexed for 10s, manually shaken for 15s, ultra-sonicated at 40 kHz for 2 min, and finally vortexed for 10s to detach microorganisms and particles from the sampling material. The sampling material was aseptically removed from the extraction liquid, and a part of the extraction liquid and all solid sampling material pieces were used for cultivation. If not mentioned otherwise, the remaining extraction liquid was concentrated to final volumes of 200 µl to 500 µl per sample using Amicon® Ultra 15 mL Centrifugal Filters with a cutoff of 50 kDa (Merck, Germany) according to the manufacturer´s instructions. DNA of the concentrated extraction liquid was isolated using a modified XS-Buffer method as described in Moissl-Eichinger 2011 (Moissl-Eichinger, 2011) and used for sequencing based analyses. DNA concentrations were measured via Qubit Fluorometer 2.0 (Invitrogen, USA) according to manufacturer's instructions.

As the extraction fluid of the vacuum cleaner bag and dust filter samples contained a lot of dust particles which would have clogged the Amicon® Ultra 15 mL Centrifugal Filters, these samples were centrifuged at 16.000g to concentrate dust particles and microorganisms. For the first analysis set, the pellets were then re-suspended in 1.5 ml per sample which were divided into three aliquots of 0.5 ml each. One aliquot was directly frozen at −80°C for further DNA extraction and sequencing analyses and one aliquot was treated with an end concentration of 50 µM PMA before freezing. The third aliquot of each sample was mixed with 0.5 ml of pre-warmed 30°C LB medium and incubated at 30°C for 1.5h prior to direct DNA extraction, aiming to increase biomass and possibly trigger germination of expected spores (Mora, Perras, *et al.*, 2016). The second set was divided into two aliquots of which one was directly frozen and one was treated with PMA before freezing.

The concentrated extraction liquid of samples subjected to PMA treatment (S5C clean room wipe samples and first batch of vacuum cleaner bag and dust filter samples) was divided into even aliquots, of which one aliquot was directly frozen at -80°C for further processing and the second was treated with an end concentration of 50 µM propidium monoazide (PMA), according to manufacturer's instructions, to block free DNA of dead cells from downstream applications (Vaishampayan *et al.*, 2013) and then frozen at -80°C.

II.3 Cultivation

Nineteen different media were used for the cultivation assay, targeting a broad diversity of extremotophilic or extremotolerant bacteria and archaea. An overview of the used media is given in Table 3 and detailed recipes of the media are available in Appendix 3. Extraction liquid was used to inoculate each of the media given in Table 3 in duplicates of 500 µl and 250 µl. Wipes were cut into pieces of 40 mm x 40 mm with sterile disposable scalpels, whereas swab heads and fabric pieces of a vacuum cleaner bag or dust filter were aseptically removed in one piece from the extraction solution and placed on each of the solid media. R2A pH5, R2A pH7, RAVAN and ROGOSA were supplemented with a final concentration of 50 µg/ml nystatin to suppress fungal growth. Media targeting archaea (AHM, AAM, MS, MS+organics, MCB-3, N.ex medium) were supplemented with 50 µg/ml streptomycin and 100 µg/ml ampicillin to suppress bacterial growth. Pure cultures were obtained via repeated dilution series in liquid medium and purification streaks on solid media.

Table 3: Overview of used media.

Medium	Target	T [°C]	Phase	Gas phase	Source
R2A pH 4	Acidophiles	32	Solid	Aerobic (ambient)	VWR Chemicals BDH Prolabo®
R2A pH 5	Acidotolerants	32	Solid	Aerobic (ambient)	
R2A pH 7	Mesophiles	32	Solid	Aerobic (ambient)	
	Psychrophiles	4	Solid	Aerobic (ambient)	
	Anaerobes	32	Solid	$N_2H_2CO_2$(80:10:10)	
R2A pH 9	Alkalitolerants	32	Solid	Aerobic (ambient)	
R2A pH 10	Alkaliphiles	32	Solid	Aerobic (ambient)	
RAVAN	Oligotrophs	32	Solid	Aerobic (ambient)	(Watve *et al.*, 2000) (1:100 modified)
Halo	Halophiles	40	Solid	Aerobic (ambient)	DSMZ medium 97
Rogosa	Lactobacilli	32	Solid	Aerobic (ambient)	Merck
TSA	Mesophiles*	32	Solid	Aerobic (ambient)	Sigma Aldrich
YPD	Yeasts & Fungi	32	Solid	Aerobic (ambient)	Sigma Aldrich
PDA	Yeasts & Fungi	25	Solid	Aerobic (ambient)	Oxoid
AHM	Homoacetogen	32	Liquid	H_2CO_2 (20:80)	(Stieglmeier *et al.*, 2009)
AAM	Autotrophs	32	Liquid	N_2CO_2 (80:20)	
ASM	Sulfate reducers	32	Liquid	H_2CO_2(20:80)	
MS	Methanogens	40	Liquid	H_2CO_2(20:80)	(Balch *et al.*, 1979)
MS+organics	Methanogens	40	Liquid	H_2CO_2(20:80)	-
N.ex medium	Thaumarchaea	32	Liquid	Aerobic (ambient)	(Sauder *et al.*, 2017)
MCB-3	Methanogens	40	Liquid	H_2CO_2(20:80)	DSMZ medium322
R2A pH 7 liquid	Thermophiles	65	Liquid	Aerobic (ambient)	VWR Chemicals BDH Prolabo®

II.4 Identification of isolates

Partial 16S rRNA genes of bacterial isolates were amplified using the primers 9bF (5´-GRGTTTGATCCTGGCTCAG-3´) and 1406uR (5´- ACGGGCGGTGTGTRCAA-3´), applying the following cycling conditions: initial denaturation at 95°C for 2min, followed by 10 cycles of denaturing at 96°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 60s, followed by another 25 cycles of denaturing at 94°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 60s and a final elongation step at 72°C for 10 min. The template was either a small fraction of a picked colony in a colony-PCR assay or 5-20ng of DNA purified from culture via the peqGOLD Bacterial DNA Kit (Peqlab, Germany). The 16S rRNA gene amplicons were sequenced using Sanger technology (Eurofins, Germany), and the obtained sequences were classified using the EzBioCloud identification service (Internet reference 1; Yoon *et al.*, 2017).

The ITS sequence of fungal isolates was amplified using the primers ITS1F (5´-CTTGGTCATTTAGAGGAAGTAA-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) using following cycling conditions: initial denaturation at 95°C for 10min, followed by 35 cycles of denaturing at 94°C for 60s, annealing at 51°C for 60s, elongation at 72°C for 60s, and a final elongation step at 72°C for 8min. The amplicons were sequenced using Sanger- technology (Eurofins, Germany) and the obtained sequences were classified using the curated databases UNITE version 7.2 (Kõljalg *et al.*, 2014) and BOLD version 4 (Ratnasingham and Hebert, 2007). Fungal isolates of session A, B and C were not sequenced, but identified according to their phenotypical characteristics at the Lomonosov State University in Moscow, Russia.

Biosafety risk groups of all bacterial and fungal isolates were determined according to the "GESTIS-Stoffdatenbank" (Internet reference 2).

II.5 Antimicrobial resistance tests

Antimicrobial susceptibility tests were performed in duplicates for 37 isolates obtained from the ISS or ESA´s clean room S5C which were selected for testing based on their phylogeny and on differences in phenotypical appearance. Additionally, we also tested the type strains of *Bradyrhizobium erythrophlei* (LMG 28425*), Bradyrhizobium viridifuturi* (LMG 28866) and a *Staphylococcus arlettae* isolate from the Russian service modules Zvesda provided by Rob Van Houdt, Belgian Nuclear Research Centre, Belgium. Antimicrobial susceptibility testing for 17 selected, clinically relevant antibiotics (Table 4) was performed as described in Mora, Perras *et al.* 2016, using Etest® reagent strips (Biomérieux, Germany) according to the manufacturer's instructions. In brief, overnight cultures (2–3-day cultures for slower-growing bacteria) were suspended in 0.9% saline. 100 µl of this suspension was plated on standardized Müller-Hinton agar (Becton Dickinson) for antimicrobial susceptibility testing. If not

mentioned otherwise, Etest® reagent strips were placed on the plates followed by aerobic incubation for 24h at 34°C. Since there were no species-specific breakpoints available, minimal inhibitory concentrations were interpreted according to EUCAST guideline Table version 8.1 for "PK/PD (Non-species related) breakpoints" and for "*Staphylococcus spp.*" (Leclercq *et al.*, 2013).

Table 4: Antibiotics used for antimicrobial susceptibility tests (table reproduced from Mora, Perras *et al*. 2016)

Antibiotic	Type	Mechanism of action	Target group	Concentrations applied [µg/ml]
Amoxicillin/ clavulanic acid	β-lactam antibiotic (penicillin) and β-lactamase inhibitor	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.016-256
Ampicillin	β-lactam antibiotic penicillin	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.016-256
Cefotaxime	β-lactam antibiotic; Cephalosporin	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.016-256
Ceftriaxone	β-lactam antibiotic; Cephalosporin	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.016-256
Ciprofloxacin	Fluoroquinolone	Inhibits bacterial DNA gyrase; bactericidal	Gram+ and Gram- bacteria	0.002-32
Clarithromycin	Macrolide	Inhibits protein synthesis; bacteriostatic	Gram+ and Gram- bacteria	0.016-256
Clindamycin	Lincosamide	inhibits protein synthesis; bacteriostatic	Gram+ bacteria	0.016-256
Colistin	Polypeptide antibiotic; polymyxin	Attacks cell membrane; bactericidal	Gram- bacteria	0.016-256
Doxycycline	Polyketide antibiotic; tetracycline	inhibits protein synthesis; bacteriostatic;	Gram+ and Gram- bacteria	0.016-256
Gentamicin	Aminoglycoside	inhibits protein synthesis; bactericidal	Gram- and some Gram+ bacteria	0.016-256
Levofloxacin	Fluoroquinolone	Inhibits bacterial DNA gyrase; bactericidal	Gram+ and Gram- bacteria	0.002-32
Linezolid	Oxazolidinone	inhibits protein synthesis; bacteriostatic	Gram+ bacteria	0.016-256
Meropenem	β-lactam antibiotic carbapenem	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.002-32
Moxifloxacin	Fluoroquinolone	Inhibits bacterial DNA gyrase; bactericidal;	Gram+ and Gram- bacteria	0.002-32
Penicillin G	β-lactam antibiotic penicillin	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ bacteria	0.016-256
Trimethoprim/ sulfamethoxazole	Dihydrofolate reductase inhibtor and sulfonamide	Inhibits tetrahydrofolate synthesis; bactericidal	Gram+ and Gram- bacteria	0.002-32
Vancomycin	Glycopeptide antibiotic	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ bacteria	0.016-256

II.6 Physiological tests

Before cultivation, 10 x 0.5 ml aliquots of the extraction liquid were sent to the DLR (Deutsches Zentrum für Luft und Raumfahrt) in Cologne to select for radiation resistant isolates. The aliquots were either irradiated by UV-C (254 nm) with an intensity of 50 J/m², 75 J/m², 100 J/m², and 200 J/m², or by X-Rays with an intensity of 125 Gy, 250 Gy, 500 Gy, 750 Gy, or 1000 Gy, and afterwards cultivated in the working group of C.S. Cockell at the University of Edinburgh, UK.

We applied a standardised heat shock to 2.5 ml aliquots of the extraction fluid before cultivation and additionally later on 29 selected strains after growing them in the laboratory for at least five generations. The heat-shock was performed according to ESA standards as also described in Mora, Perras et al. 2016. This test is usually applied for bioburden testing of spacecraft, in order to identify hardy microorganisms

that are potentially able to survive a spaceflight to other solar bodies. In brief, single colonies of 1-2 days old cultures were suspended in two test tubes containing 2.5 ml sterile phosphate buffered saline (PBS). One tube was kept at room temperature during the procedure as control. The other tube was placed in an 80°C water bath and heat shocked for 15 min. Samples were immediately cooled down on ice for 5 min after incubation time. The temperature of 80°C within the test tubes during these 15 minutes was verified by adding a pilot tube containing 2.5 ml PBS in the water bath, and checking temperature thereof. Afterwards 0.5 ml of the heat shocked suspension and 0.5 ml of the room temperature suspension were plated on R2A pH 7 and incubated at 30°C for three days (72h).

For the assessment of the upper and lower boundaries regarding temperature and pH resistance, 29 strains were selected based on their phylogeny and on differences in phenotypical appearance, plated on R2A pH7 agar and incubated overnight at 32°C. Then the cultures were streaked out on a fresh agar plate and the incubation temperature or the pH value of the medium were stepwise decreased and increased by 2°C or pH 0.5. This was repeated until no further growth was observed.

To test if some of the isolates interact with, and possibly damage, materials used on board the ISS, we incubated them together with pieces of NOMEX® fabric, provided by the Biotechnology Space Support Center (BIOTESC) of the Lucerne University of Applied Sciences and Arts, Switzerland, and plates of the aluminum copper magnesium alloy EN AW 2219, provided by Thales Alenia Space (TAS), Italy. Both materials are used on the international space station. NOMEX® is a flexible, flameproof fabric used for most storage bags on board the ISS. The NOMEX® fabric was cut into pieces of 20 mm x 30 mm and autoclaved before incubation. The aluminum alloy EN AW 2219 was cut into small plates of 20 mm x 30 mm x 3 mm by Josef Baumann in Falkenberg, Germany, and evenly polished with a grit size of P240 and partly eloxated by Heuberger Eloxal, Austria. The autoclaved metal platelets, non-eloxated and eloxated, as well as NOMEX® fabric pieces were then incubated together with selected bacteria isolated from the International Space Station: *Cupriavidus metallidurans* (aerobic), *Bacillus paralicheniformis* (aerobic/anaerobic), and *Cutibacterium avidum* (anaerobic). Incubations were performed in triplets over a period of 3 months in liquid R2A medium in Hungate tubes at pH7 and 32°C. 50% of the medium was exchanged every 2 weeks to ensure the survival of the bacteria. After incubation, metal plates an NOMEX® fabric pieces were investigated via electron microscopy.

II.7 Electron microscopy

Metal plates and NOMEX® fabric pieces from the coincubation experiment with selected bacteria were aseptically removed from their respective Hungate tube, carefully rinsed with 1xPBS buffer and then fixated overnight at 4°C in 100 mM sodium cacodylate buffer containing 2,5% glutaraldehyde. Scanning electron microscopy of the samples was performed by A. Klingl at the Biocenter of the Ludwig-Maximilians-Universität Munich, Germany.

II.8 Next generation sequencing

II.8.1 16S rRNA gene amplicon sequencing

To investigate the detectable molecular diversity via 16S rRNA gene sequencing, we used a "universal" and an Archaea-targeting approach. For the universal approach, partial 16S rRNA gene sequences were amplified using Illumina-tagged primers F515 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3′) and R806 (5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAA3`) (Caporaso *et al.*, 2012). Archaeal-specific amplicons were obtained via a nested-PCR approach (Probst *et al.*, 2013): First, a ~550 bp-long 16S rRNA gene amplicon was produced by primers Arch344F (5′-ACGGGGYGCAGCAGGCGCGA-3′) and Arch915R (5′- GTGCTCCCCC GCCAATTCCT-3′) (Stahl and Amann, 1991; Raskin *et al.*, 1994), and in two individual follow-up PCRs, the amplicons for Illumina sequencing were generated by the tagged primer pairs S-D-Arch-0349-a-S-17 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGYGCASCAGKCGMGAAW-3′) and S-D-Arch-0519-a-A-16 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTACCGCGGCKGCTG-3) or S-D-Ar-0519-aS-15_F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGCMGCCGCGGTAA-3′) and S-D-Ba-0785-aA-21_R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) (Klindworth *et al.*, 2012), using the purified product of the first PCR as template. In this thesis, these primer pairs will be referred to as Ar349fwd+Ar519rev and Ar519fwd+Ar785rev. The cycling conditions were the same as described in Mora, Perras *et al.* 2016. Library preparation and sequencing were carried out at the Core Facility for Molecular Biology, Center for Medical Research, Medical University of Graz, Austria. In brief, DNA concentrations were normalized using a SequalPrep™ normalization plate (Invitrogen), and each sample was indexed with a unique barcode sequence (8 cycles index PCR). After pooling of the indexed samples, a gel cut was carried out to purify the products of the index PCR. Sequencing was performed using the Illumina MiSeq device and MS-102-3003 MiSeq® Reagent Kit v3-600cycles (2x251 cycles).

II.8.2 Genome and metagenome sequencing

The genomic DNA of six isolates obtained from vacuum cleaner bag and dust filter samples from the Russian part of the ISS was sequenced. These isolates were *Bacillus pumilus* strain pH7_R2F_2_A, *Bacillus safensis* strain pH9_R2_5_I_C, *Bradyrhizobium viridifuturi* strain pH5_R2_1_I_B, *Cupriavidus metallidurans* strain pH5_R2_1_II_A, *Methylobacterium tardum* strain pH5_R2_1_I_A, and *Paenibacillus campinasensis* strain pH9_R2IIA. Additionally, the remaining purified DNA from the sample "Dust filter-1" (2004) was sequenced using a metagenomic approach. DNA from cultures was isolated from overnight cultures using the peqGOLD bacterial DNA mini kit (Peqlab, Germany). The DNA of sample "Dust filter-1" (2004) was first whole genome amplified via multiple-displacement amplification before shot gun sequencing using the REPLI-g Single Cell Kit (Qiagen, Germany) according to manufacturer´s instructions, because there was not enough DNA available to meet the minimum requirements for sequencing. Double stranded DNA was quantified via Qubit Fluorometer 2.0 (Invitrogen, USA) according to manufacturer's instructions. Library preparation and sequencing was carried out at the Core Facility for Molecular Biology, Center for Medical Research, Medical University of Graz, Austria on an Illumina MiSeq system. Shot gun libraries for Illumina MiSeq sequencing were prepared with the NEBNext® Ultra II DNA Library Prep Kit for Illumina® in combination with the Index Primers Set 1 (NEB, Frankfurt, Germany) according to manufacturer's instructions, and as described in (Thannesberger *et al.*, 2017). Briefly, 500 ng of dsDNA were randomly fragmented by ultrasonication in a microTUBE on a M220 Focused-ultrasonicator™ (Covaris, USA) in a total volume of 130 µl 1xTE for 80 seconds with 200 cycles per burst (140 peak incident power, 10% duty factor). After sharing, 200 ng of shared DNA were used for the end repair and adapter ligation reactions in the NEBNext® Ultra II DNA Library Prep Kit for Illumina® according to manufacturer's instructions. Size selection and purification were performed according to the instructions for 300 to 400bp insert size. Subsequent PCR amplification was performed with 4 cycles and libraries were eluted after successful amplification and purification in 33µl 1xTE buffer pH 8.0. For quality control libraries were analyzed with a DNA High Sensitivity Kit on a 2100 Bioanalyzer system (Agilent Technologies, USA) and again quantified on a Quantus™ Fluorometer (Promega, Germany). An equimolar pool was sequenced on an Illumina MiSeq desktop sequencer (Illumina, CA, USA). Libraries were diluted to 8pM and run with 5% PhiX and v3 600 cycles chemistry according to manufacturer's instructions.

II.9 Sequence data evaluation

16S rRNA amplicons (Illumina)

Demultiplexed, paired reads of 16S rRNA gene sequencing approaches were processed in R (version 3.2.3) using the R package DADA2 as described previously (Callahan *et al.*, 2016; Mora, Perras, *et al.*,

2016). In brief, sequences were quality checked, filtered, and trimmed to a consistent length of ~250 bp (forward reads) and ~210 bp (reverse reads) and filtered with a maximum of two expected errors per read. Passed sequences were dereplicated and subjected to the DADA2 algorithm. Afterwards reads were merged and chimeras removed. Resulting RSVs (Ribosomal sequence variants) were classified via the SILVA database v123, and RSVs assigned to eukaryotes, mitochondria and chloroplasts were removed. Visualization was carried out using the online software suite "Calypso" (Zakrzewski *et al.*, 2017), Microsoft Excel, and GIMP v2.8 (GNU Image Manipulation Program version 2.8). For bar plots data was normalized by total sum normalization (TSS) and for diversity analyses by TSS combined with square root transformation.

16S rRNA amplicons (Sanger)

For phylogenetic tree construction based on long 16SrRNA gene fragments obtained from cultures, the forward and reverse sequences were merged via the HVDR Online Fragment Merger tool (Bell and Kramvis, 2013). Merged partial 16SrRNA gene sequences were aligned using the SINA Aligner v1.2.11 (Pruesse *et al.* 2012) and phylogenetic trees were calculated via Fast Tree (Price *et al.*, 2010), and visualized by the Interactive Tree of Life online tool iTOL v3 (Letunic and Bork, 2016).

Genomes and metagenomes

Genomic and metagenomic reads were quality checked with FastQC (Internet reference 3) and then filtered with Trimmomatic (removed all adapter sequences, SLIDINGWINDOW 4:20, MINLEN 50) (Bolger, *et al.*, 2014). Genomes were assembled with SPADES in careful mode (Bankevich *et al.*, 2012), and checked for completeness via CheckM (Parks *et al.*, 2015). The assemblies were annotated and compared to closely related reference strains via the microbial genome annotation & analysis platform MicroScope (Internet reference 4) (Vallenet *et al.*, 2006, 2009, 2017). Metagenomic datasets were assembled with megahit (meta-sensitive presets) (Li *et al.*, 2016), binned via maxbin (Wu *et al.*, 2014) and checked for completeness via CheckM (Parks et al., 2015) and Amphora2 (Wu and Scott, 2012). Additionally, the filtered reads of sample "Dust filter-1" (2004) were mapped on the reference genome of *Nitrososphaera viennensis* (Tourna *et al.*, 2011) using bowtie2 (Langmead and Salzberg, 2012).

II.10 Negative controls

Negative controls were performed throughout the whole study. Each sampling approach had also a blank sample which was used as negative control for the complete process and additional negative controls were added for each cultivation assay, each DNA isolation and in each PCR. All cultivation negative controls showed no growth and RSVs which appeared in according sequenced PCR negative controls were removed.

III Results

III.1 Cultivation

III.1.1 Overview of all cultivated isolates

A wide variety of media (see II.3) was inoculated with extraction fluid and pieces of sampling hardware of recent samples from the ISS (sessions A, B, C, and Russian swab samples), 10-12 years old samples from the ISS (Russian dust samples from vacuum cleaner bags and dust filters) and samples from ESA´s clean room S5C in Kourou, French Guiana. Additionally, we received unidentified bacterial cultures from the inside and outside surfaces of a Commercial Resupply Vehicle (CRV) destined to ISS which were isolated by the workgroup of K. Venkateswaran, Jet Propulsion Laboratory, California Institute of Technology, USA. In total more than 500 isolates were cultivated and identified and attributed to 55 bacterial and 26 fungal genera. Table 5 gives an overview of the amount of isolates and distinct genera retrieved from each sampling event. Of all isolates, 473 were bacteria, which belong to 145 distinct species according to their partial 16S rRNA gene sequence. If a species was isolated multiple times, one representative sequence was kept for the clean room samples, the recent ISS samples (Session A, B, C, and Russian Swabs), and the past ISS samples (Russian dust from vacuum cleaner and dust filter samples). Multiple identical isolates from one of these three sources were deemed redundant. This resulted in 170 non-redundant sequences which are displayed in a phylogenetic tree in Figure 2. Most of the non-redundant isolates belonged to the phylum Firmicutes (76 isolates; 44.7%), followed by the phyla Proteobacteria (48 isolates; 28.2%) and Actinobacteria (39 isolates; 23%) whereas the phyla Bacteroidetes (6 isolates; 3.5%) and Deinococci (1 isolate; 0.6%) made up less than 5% of the non-redundant isolates. The recent ISS samples contributed 82 of the non-redundant isolates, ISS dust and vacuum cleaner samples contributed 18 non-redundant isolates and the clean rooms contributed 33 (CRV) and 37 (ESA) non-redundant isolates respectively. Figure 2 also gives an overview regarding the extremotolerant/extremophilic properties which were tested in the course of this study and which of the isolates are human associated, spore formers, or classified to be biosafety risk group S2. Of the 170 distinct isolates, 50 were observed to be acidotolerant and able to grow on pH 5 or lower, 43 were observed to be alkalitolerant and able to grow on pH 9 or higher and 27 of these were acido- as well as alkalitolerant. The temperature tests showed that 22 of the isolates were cryotolerant and could grow below 10°C, 11 were thermotolerant and could grow above 50°C and 8 could grow at both conditions. The two isolates belonging to the genus *Thermaerobacter* were found to be real thermophiles which could not grow below 50°C. *Sphingomonas aerolata* and *Microbacterium lemovicicum* were the only

Table 5: Overview of Isolates per sampling event: BI = Number of total bacterial isolates; **BG** = Number of different bacterial genera; **FI =** Number of total fungal isolates; **BG** = Number of different fungal genera; **nd** = not done.

Sampling	BI	BG	FI	FG
Clean room (ESA)	66	21	14	10
Clean room (CRV)	47	18	33	16
ISS Russian dust	87	8	1	1
ISS Session A	91	11	16	5
ISS Session B	72	21	8	1
ISS Session C	94	16	6	1
ISS Russian Swabs	16	5	nd	nd

two isolates which were actually isolated only by the 4°C assay, but these two strains grew quicker at higher temperatures. Furthermore, 11 isolates could also be isolated using the low-nutrient RAVAN medium, 12 were (facultative) anaerobes, 16 were resistant at least to the minimal dose of X-rays, 10 were resistant at least to the minimal dose of UV-C irradiation, and 2 were isolated via the medium for halophiles. Of the latter, *Salinibacillus aidingensis* was a real halophile while *Brevibacterium sediminis* was also able to grow well on R2A. As not all isolates were tested regarding the extremotolerant/extremophilic properties because of time reasons, the properties listed in Figure 2 are not exhaustive for all non-redundant isolates. For example, most of the 57 spore formers can be expected to survive a heat shock and at least the lower UV-C and X-ray irradiation doses which were tested during this study. None of the isolates were unexpectedly dangerous to human health, but 20 of the 170 bacterial isolates belonged to the safety risk group S2 and 38 are known to be human associated. *Bacillus anthracis* is usually classified as biosafety risk group S3 but cannot be distinguished from *B. cereus* or *B. thuringiensis* strains based on 16S rRNA gene sequences alone and a recent study on *B. anthracis/cereus/thuringiensis* strains isolated from other ISS samples has shown that none of these carry anthrax toxin-encoding plasmids (Venkateswaran *et al.*, 2017). For this reason, it is assumed that the isolates classified as *B. anthracis* based on their 16S rRNA gene sequences by the EzBioCloud identification service which were obtained from our samples were wrongly classified as *B. anthracis* and can be classified S2 as done in Figure 2.

The remaining 78 isolates are fungal isolates and comprise 42 non-redundant isolates. These 42 non-redundant fungal isolates and their origin and biosafety risk group are listed in Table 6. Only *Exophiala xenobiotica, Aspergillus niger,* and *Phialemoniopsis dimorphosporum* belong to biosafety risk group S2, and *Rhizopus stolonifera, Rhodotorula mucilaginosa, Penicillium brevicompactum, Penicillium chrysogenum, and Penicillium crustosum* are known to sometimes cause allergenic reactions according to GESTIS. *Phoma multirostrata, Penicilium expansum*, and *Microsphaeropsis arundinis* are not harmful to humans but pathogenic for plants. *Alternaria alternata* was the only isolate obtained from a sample within the ATV cargo transporter.

The fungal isolates obtained from this study are currently further investigated regarding their physiologic properties and their ability to degrade or corrode metal by the workgroup of T. Alekhova, Lomonosov State University, Moscow, Russia.

Micrococcus yunnanensis is the only species of all 186 distinct bacterial and fungal species which appeared in all sampling events. None of the cultivation assays successfully enriched Archaea.

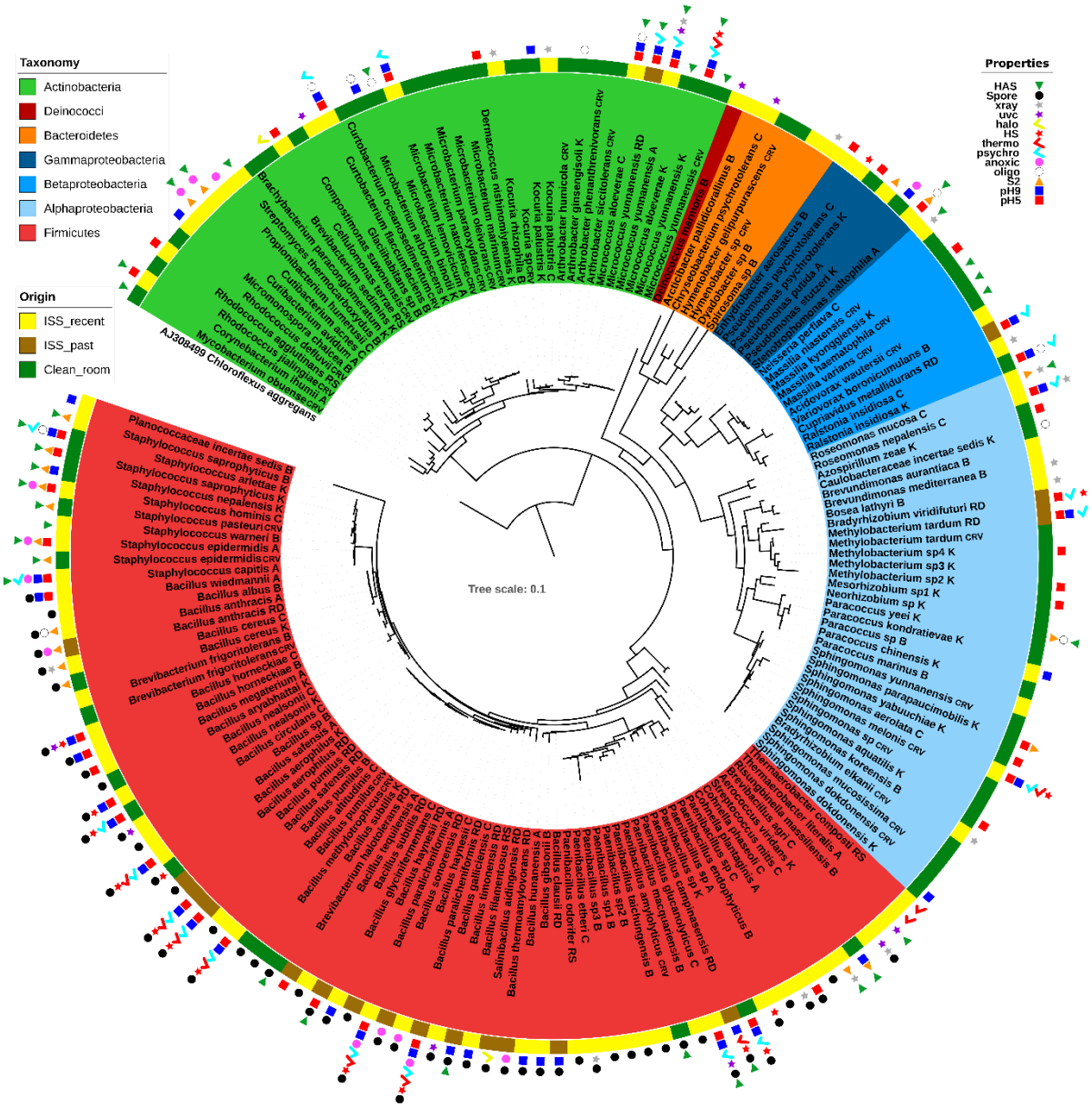

Figure 2: Phylogenetic tree of the 170 non-redundant isolates obtained during this study. The color of the species name indicates the higher taxonomic classification of the isolate and the ring colored in yellow brown and green around the isolates indicates their origin: **ISS_recent**: Session A, B, C, and Russian swab samples; **ISS_past:** Russian dust samples from vacuum cleaner bags and dust filters; **Clean_room:** ESA´s S5C clean room and CRV clean room; The outer layer of the circle indicates properties of interest for each isolate: **HAS:** Human associated microorganism: **Spore:** Sporeformer; **xray:** Isolate was able to withstand at least the minimum dose of applied X-ray irradiation before cultivation; **uvc:** Isolate was able to withstand at least the minimum dose of applied UV-C irradiation before cultivation; **halo:** Isolate was isolated from medium for halophilic microorganisms; **HS:** Isolate survived a heat shock, either prior to cultivation or after being grown in the laboratory for at least 5 generations; **thermo:** Isolate was able to grow at a temperature >50°C; **psychro:** Isolate was able to grow at a temperature <10°C; **anoxic:** Isolate was isolated via oxygen free medium; **oligo:** Isolate was isolated via RAV medium for oligotrophic microorganisms; **S2:** isolate belongs to the biosafety risk group S2; **ph9**: isolate was able to grow on medium pH9 or higher; **pH5:** isolate was able to grow on medium pH5 or lower; The tree is rooted on the type strain 16S rRNA gene sequence AJ308499 of *Chloroflexus aggregans* which was not isolated during this study.

Table 6: List of fungal isolates in alphabetical order. Higher taxonomy is given in divisions and classes. When no biosafety group is given (-), the isolate was not present in GESTIS.

Higher taxonomy	Isolate	Origin	Biosafety risk group
Ascomycota	*Ascomycete incertae sedis*	CRV	-
Chaetothyriomycetes	*Exophiala xenobiotica*	CRV	S2
Dothideomycetes	*Alternaria alternata*	K (ATV)	S1
	Alternaria botrytis	RD	S1
	Alternaria sp.	CRV	-
	Aureobasidium pullulans	K, CRV	S1
	Cladosporium aphidis	CRV	-
	Cladosporium sp.	CRV	-
	Curvularia eragrostidis	K	-
	Curvularia lunata	K	S1
	Epicoccum sorghinum	K	-
	Microsphaeropsis arundinis	K	S1
	Periconia sp.	CRV	-
	Phoma multirostrata	K	-
Eurotiomycetes	*Aspergillus flavipes*	K	S1
	Aspergillus niger	K	S2
	Aspergillus sydowii	A	S1
	Aspergillus unguis	A, K	S1
	Penicillium aurantiogriseum	A, C	S1
	Penicillium brevicompactum	C	S1 (allergenic)
	Penicillium chermesinum	K	-
	Penicillium chrysogenum	A, B, C	S1 (allergenic)
	Penicillium crustosum	A	S1 (allergenic)
	Penicillium expansum	A, C	S1
	Penicillium sp.	K	-
	Phitomyces sp.	CRV	-
	Talaromyces sp.	CRV	-
Sordariomycetes	*Chaetomium globosum*	A	S1
	Colletotrichum sp.	CRV	-
	Pestalotiopsis sp.	K	-
	Phialemoniopsis dimorphosporum	CRV	S2
	Trichoderma reesei	K	S1
Basidiomycota	-	-	-
Agaricomycetes	*Phlebia acerina*	CRV	S1
Microbotryomycetes	*Rhodotorula mucilaginosa*	A, CRV	S1 (allergenic, opportunistic pathogen)
Tremellomycetes	*Cryptococcus aureus*	CRV	-
	Cryptococcus rajasthanensis	CRV	-
	Dioszegia sp.	CRV	-
	Hannaella sp.	CRV	-
	Naganishia sp.	CRV	S1
	Papiliotrema pseudoalba	CRV	S1
Zygomycota	-	-	-
Zygomycetes	*Rhizopus stolonifera*	A	S1 (allergenic)

III.1.2 Cultivable diversity on board the International Space Station

The broad cultivation approach resulted in a high diversity of microbial isolates from the ISS, and to the authors knowledge 22 of the bacterial genera obtained during this study have not been isolated from ISS samples before, although most of them have been detected by molecular methods (Venkateswaran *et al.*, 2014; Checinska *et al.*, 2015; Lang *et al.*, 2017). The new bacterial genera isolated are: *Arcticibacter, Bosea, Brevundimonas, Chryseobacterium, Cohnella, Curtobacterium, Cutibacterium, Deinococcus, Dyadobacter, Enhydrobacter, Glaciihabitans, Micromonospora, Neisseria, Paracoccus, Planococcus, Propionibacterium, Risungbinella, Roseomonas, Spirosoma, Stenotrophomonas, Thermaerobacter,* and *Variovorax.* Of the fungal isolates, only *Aspergillus unguis* was not found on the ISS before. However, it is interesting to note that this species was also identified in the samples of the S5C clean room in Kourou. Figure 3 shows the local distribution of all bacterial isolates sampled in 2017 within the ISS on genus level, as well as how many different species of the respective genera were found. There is only a comparably low diversity of isolates from the Russian modules in 2017, which is most likely due to the different sampling technique used here, as the wipes always covered an area of approx. $1m^2$ which is not feasible with swabs. Columbus and Node 2 show the highest diversity which correlates with the fact that these modules were the only modules which were sampled twice (Session A and B).

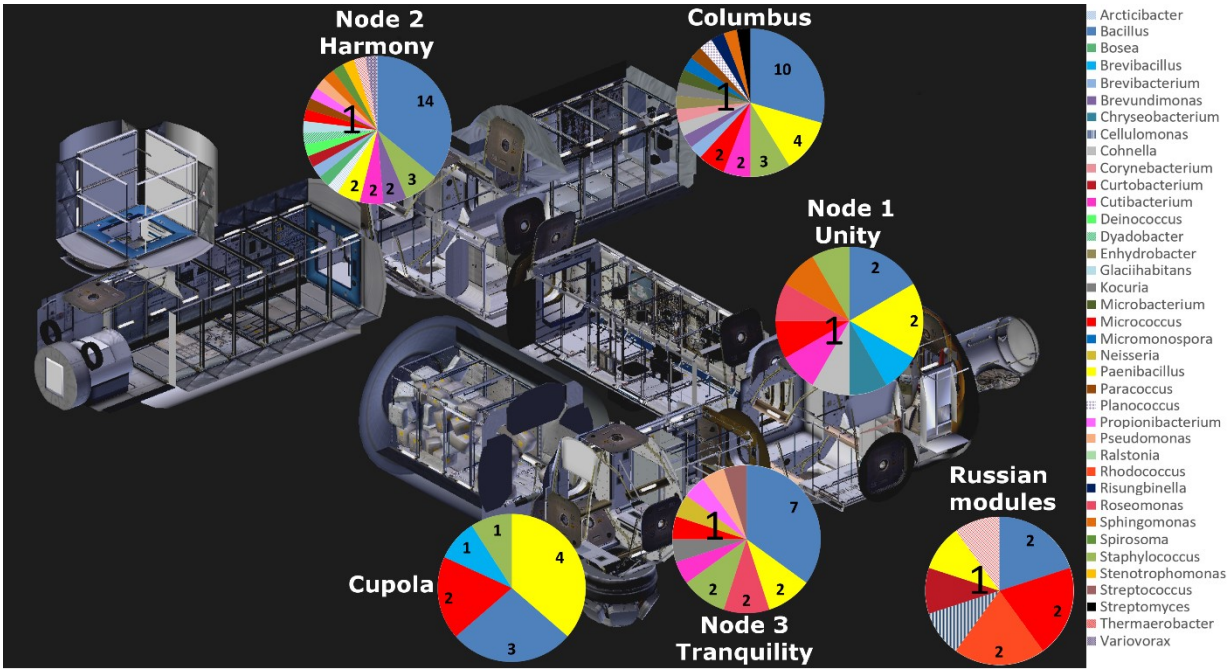

Figure 3: 3D-Model of NASA, ESA and JAXA modules of the ISS (background source: NASA; Internet reference 5); Circle diagrams show the diversity of obtained Isolates in 2017 (Session A, B, C and Russian swabs) on genus level for each sampled module. Modules of the Russian part of the ISS which are not shown on the 3D-model are summarized in an additional circle diagram.

In the recent sampling sessions in 2017 as well as in vacuum cleaner and dustfilter samples from 2004 and 2008, isolates of the spore forming genus *Bacillus* were the most frequently isolated. The older vacuum cleaner and dust filter samples shared four *Bacillus* species with the recent samples: *Bacillus anthracis/cereus* (A,C), *Bacillus haynesii* (B,C), *Bacillus paralicheniformis* (A,C), and *Bacillus safensis* (A,B, Russian Swabs).

Sessions A, B, and C amounted to 74 distinct bacterial species and 10 distinct fungal species, but only the bacteria *Bacillus hunanensis*, *Micrococcus yunnanensis* and *Staphylococcus epidermidis* and the fungus *Penicillium chrysogenum* appeared constantly in all three ISS sampling sessions. Besides these three, session A and B shared two bacteria: *Bacillus megaterium* and *Bacillus safensis. S*ession A and C shared two fungal species, *Penicillium aurantiogriseum* and *Penicillium expansum*, and six bacterial species: *Bacillus anthracis/cereus, Bacillus paralicheniformis*, *Cutibacterium acnes, Propionibacterium humerusii, Staphylococcus capitis,* and *Staphylococcus hominis.* Session B and C shared six bacterial species: *Bacillus altitudinis, Bacillus haynesii, Bacillus horneckiae, Bacillus nealsonii, Micrococcus aloeverae,* and *Paenibacillus taichungensis.* The Russian swab samples shared three species with all wipe samples: *Bacillus safensis* (A,B) *Micrococcus aloeverae*(B,C), and *Curtobacterium flaccumfaciens* (B). Based on the observation that most isolates were not retrieved during all sampling sessions, there seems to be a constant fluctuation in the ISS microbiota.

III.1.3 Cultivable diversity within clean rooms

The S5C clean room from ESA in Kourou, French Guiana, housing an Automated Transfer Vehicle (ATV) and a clean room in North America housing a Commercial Resupply Vehicle (CRV) were sampled to compare the obtained isolates with isolates obtained from the ISS. The hypothesis behind was that microorganisms may be transferred from clean rooms to the ISS via cargo spacecraft. Isolates of the CRV samples were cultivated at the Jet Propulsion Laboratory, California, USA, and sent after cultivation to Graz. Only 80 of the 91 CRV isolates sent could be cultivated and identified in Graz, the remaining 11 could not be re-cultured. The samples of both clean rooms combined yielded 68 distinct bacterial and 33 distinct fungal species, but there was almost no species overlap between the two clean rooms apart from the ubiquitous *M. yunnanensis, Sphingomonas dokdonensis,* and the fungus *Aureobasidium pullulans.* This is most likely due to the different applied sampling and cultivation methods and the spatial separation of these two clean rooms. The CRV samples shared four bacterial species besides *M. yunnanensis* and one fungal species with ISS samples: *Bacillus pumilus* (B),Russian dust)*, Brevibacterium frigoritolerans* (B)*, Methylobacterium tardum* (Russian dust)*, Staphylococcus epidermidis* (A,B,C), and *Rhodotorula mucilaginosa* (A). The ESA clean room shared seven bacterial and one fungal species with the ISS samples: *Bacillus aerophilus* (Russian dust), *Bacillus anthracis/cereus/thuringiensis* (A, C, Russian dust)*, Bacillus nealsonii* (B, C)*, Bacillus subtilis* (Russian dust)*, Kocuria palustris* (C)*, Micrococcus aloeverae* (B, C, Russian swabs) *Staphylococcus*

saprophyticus (B), and *Aspergillus unguis* (A). As *Aspergillus unguis* was detected for the first time on the ISS after this clean room sampling was performed, a transfer from the ESA clean room towards the ISS cannot be excluded. However, it is unlikely that this happened via ATV 5, because the fungus *Alternaria alternata* was the only isolate which was obtained from inside the ATV cargo spacecraft, otherwise all samples from inside the spacecraft were negative. Since the spacecraft was sampled after the final cleaning procedure, this is not unexpected and verifies the efficacy of the final ESA cleaning protocol. Based on these results there seems to be only a minor transfer of living microorganism from clean rooms to the ISS, if at all.

III.1.4 Radiation experiments

To estimate the resistance potential of ISS isolates against different kinds of radiation, extraction liquid aliquots of Session B and C were either irradiated by UV-C radiation (254 nm) or by X-Rays with different intensities before cultivation. It has to be clarified that UV-C radiation is not expected to be an issue within the ISS, but isolates able to withstand high UV-radiation would be expected to have also otherwise higher resistances, as they should have a sophisticated DNA damage repair system. The isolates obtained by this approach are listed in Table 7 and Table 8. In total we obtained 14 distinct isolates in the UV-C radiation assay and 16 distinct isolates in the X-ray radiation assay. The maximum applied UV-C radiation of 200 J/m² was survived by 4 isolates: *Brevibacillus agri, Curtobacterium flaccumfaciens, Deinococcus marmoris,* and *Paenibacillus taichungensis.* The maximum applied X-ray radiation of 1000 Gy was survived by 5 isolates: *Kocuria palustris, Micrococcus yunnanensis, Roseomonas nepalensis, Roseomonas mucosa,* and *Paenibacillus* species. It is noteworthy that most of the maximum resistant strains (1 x UV-C and 2 x X-ray) stem from sample C1, the ambient air within the Cupola. While the complete ISS is of course protected against solar radiation, the Cupola is one of the modules with the highest background radiation (personal communication with Frits de Jong, ESA). Otherwise the resistant isolates were quite evenly distributed over all sampling sites.

During the sampling period (01.05.2017 – 15.07.2017) the absorbed dose measured within the Columbus module was in average 328 µGy/day, which is converted to an effective dose equivalent of 713 µSv/day (see Appendix 4), resulting in an annual dose of 120 Gy or 260 mSv/a. This is higher than previously reported measurements from July 2009 - June 2016 which were in the range from 208 µGy/day to 320 µGy/day (Berger et al. 2017). In comparison, the annual dose on Earth ranges from 1-13 mSv/a and averages on 2.4 mSv/a (UNSCEAR, 2008).

Table 7: Isolates obtained after irradiating sample aliquots with UV-C_{254nm}

Bacterial species	UV-C_{254nm}	Origin
Risungbinella massiliensis	50 J/m²	B4 - Hand grips
Bacillus galliciensis	75 J/m²	C9 - contaminated
Micrococcus yunnanensis	75 J/m²	B6 - Sleeping unit
Paenibacillus etheri	75 J/m²	C2 – Cupola surface
Bacillus circulans	100 J/m²	C4 - Treadmill
Bacillus sp.	100 J/m²	C7 – Dining table
Chryseobacterium psychrotolerans	100 J/m²	C6 – Cover panel Node1
Glaciihabitans sp.	100 J/m²	B7 – Cover panel Node2
Paenibacillus glucanolyticus	100 J/m²	C7 – Dining table
Roseomonas mucosa	100 J/m²	C3 - ARED
Brevibacillus agri	75 J/m² 200 J/m²	C6 – Cover panel Node1 C9 - contaminated
Curtobacterium flaccumfaciens	200 J/m²	B7 – Cover panel Node2
Deinococcus marmoris	100 J/m² 200 J/m²	B7 – Cover panel Node2 B7 – Cover panel Node2
Paenibacillus taichungensis	100 J/m² 200 J/m²	C1 – Cupola air C1 – Cupola air

Table 8: Isolates obtained after irradiating sample aliquots with X-rays

Bacterial species	X-Ray intensity	Origin
Bacillus anthracis/cereus/thuringiensis	125 Gy	C1 – Cupola air
Bosea lathyri	125 Gy	B7 – Cover panel Node2
Brevundimonas mediterranea	125 Gy	B7 – Cover panel Node2
Neisseria perflava	125 Gy	C4 - Treadmill
Sphingomonas koreensis	125 Gy	B7 – Cover panel Node2
Spirosoma sp.	125 Gy	B7 – Cover panel Node2
Streptococcus mitis	125 Gy	C4 - Treadmill
Kocuria rhizophila	250 Gy	B4 - Hand grips
Paenibacillus etheri	250 Gy	C6 – Cover panel Node1
Ralstonia insidiosa	250 Gy	C9 - contaminated
Brevibacillus agri	500 Gy	C2 – Cupola surface
Kocuria palustris	250 Gy 1000 Gy	C4 - Treadmill C9 - contaminated
Micrococcus yunnanensis	250 Gy 1000 Gy	B1 – Columbus air C1 – Cupola air
Roseomonas nepalensis	500 Gy 1000 Gy	C6 – Cover panel Node1 C5 - WHC
Roseomonas mucosa	750 Gy 1000 Gy	C3 - ARED C4 - Treadmill
Paenibacillus sp.	1000 Gy	C1 – Cupola air

III.1.5 Antimicrobial susceptibility testing

To discover if the bacterial ISS isolates exhibit unusual antimicrobial resistances, antimicrobial susceptibility testing for 17 clinically relevant antibiotics was performed on 37 selected isolates and three available reference strains and evaluated according to the EUCAST PK/PD (Non-species related) and *Staphylococcus* ssp. breakpoints when applicable. The results are given in Table 9 part 1 and 2. All tested isolates revealed resistance against at least one antibiotic compound above the non-species specific EUCAST threshold except *Bacillus nealsonii* R9_B8_IIIB. The three *Bradyrhizobium* strains and *Roseomonas nepalensis* C63 were resistant against all antibiotics for which PK/PD breakpoints are defined and often even completely unaffected by the tested antibiotics and growing even at the maximum tested concentrations. The only exception here is *Bradyrhizobium erythrophlei* LMG 28425, which was sensitive against Moxifloxacin. The ISS isolate *Bradyrhizobium viridifuturi* strain pH5_R2_1_I_B was tested twice, once together with other ISS isolates from Russian dust and vacuum cleaner samples and once again almost 2 years later together with Isolates from session A, B, and C and the two reference *Bradyrhizobium* strains. While *Bradyrhizobium viridifuturi* strain pH5_R2_1_I_B retained its general resistance profile, some of the observed resistances were not as strong as during the first tests (see Table 9).

The cephalosporins Ceftriaxone and Cefotaxime were the least effective of the tested antibiotics. Of the tested strains 11 (27.5%) were sensitive, 5 (12.5%) were intermediate, and 24 (60%) were resistant against Ceftriaxone, and 5 (12.5%) were sensitive, 6 (15%) were intermediate, and 29 (72.5%) were resistant against Cefotaxime. The three most effective antibiotics against the tested strains were the carbapenem Meropenem, the fluorchinolone Levofloxacin, and the oxazolidinone Linezolid. Of the tested strains 31 (77.5%) were sensitive, 1 (2.5%) were intermediate, and 8 (20%) were resistant against Meropenem, 30 (75%) were sensitive, 4 (10%) were intermediate, and 6 (15%) were resistant against Levofloxacin, and 29 (72.5%) were sensitive, 3 (7.5%) were intermediate, and 8 (20%) were resistant against Linezolid.

It has to be stressed that a guaranteed evaluation for a strain to be resistant, intermediate, or sensitive regarding a certain antibiotic is in general only possible when defined breakpoints for the tested species exist. In most cases, such defined breakpoints exist only for common pathogens. Nevertheless, it is possible to evaluate the antimicrobial susceptibility of species for which defined breakpoints do not exist by using PK/PD (non-species related) breakpoints (The European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2016). When one of the tested organisms grew at the highest tested concentration of a specific antibiotic, it was evaluated to be resistant against this antibiotic, also in abstinence of a defined PK/PD breakpoint. For Colistin, Clarithromycin, Clindamycin, Doxycycline, Gentamicin, Vancomycin, and Trimethoprim/Sulfamethoxazole exist no defined PK/PD breakpoints and for Colistin as well as Trimethoprim/Sulfamethoxazole there also exist no defined *Staphylococcus* spp. Breakpoints. These are marked “most likely resistant” in Table 9 according to the rationale for

“most likely resistant“ rating mentioned later. However, these are assumptions of the author based on breakpoints given for other species in the EUCAST table as a guiding value and will have to be verified in future when the EUCAST table is updated regarding these antibiotics. This interpretation approach is in compliance with the EUCAST guidelines, but as these assumptions are not based on real evidence they have to be taken with caution.

The used PK/PD breakpoints in µg/ml, as given in the EUCAST breakpoint tables v8.1, were in alphabetical order:

Amoxicillin/clavulanic acid: S<2 R>8; Ampicillin: Cefotaxime: S<1 R>2; Ceftriaxone: S<1 R>2; Ciprofloxacin: S<0,25 R>5; Levofloxacin: S<0,5 R>1; Linezolid: S<2 R>4; Meropenem: S<2 R>8; Moxifloxacin: S<0,25 R>0,25; Penicillin G: S<0,25 R>2.

The defined *Staphylococcus* spp. breakpoints used for the tested *Staphylococcus* strains differ from the PK/PD breakpoints regarding the following antibiotics:

Ciprofloxacin: S<1 R>1; Clarithromycin S<1 R>2, Clindamycin S<0,25 R>0,5, Doxycycline: S<1 R>2, Gentamicin: S<1 R>1, Levofloxacin: S<1 R>1; Linezolid: S<4 R>4; Penicillin G: S<0,125 R>0,125; Trimethoprim/sulfamethoxazole: S<4 R>4; Vancomycin: S<4 R>4.

The tested *Bradyrhizobium* spp., *Methylobacterium* sp., *Ralstonia* sp., and *Roseomonas* sp. were not able to grow fast enough for a valid minimum inhibitory concentration (MIC) definition after over-night incubation. For this reason, they were incubated up to 48 hours which does not comply with the EUCAST guidelines. To exclude that the high resistance values measured for *Bradyrhizobium* spp., *Methylobacterium* sp., and *Roseomonas* spp. were based on the longer incubation time, a random assortment of the other tested isolates - which had been already evaluated after over-night incubation - was also incubated for 48 hours and none of these showed an increase of their measurable MIC after 48 hours incubation time compared to their respective first evaluation.

Rationale for "most likely resistant" rating

Clarithromycin, as most macrolides, acts against a wide range of Gram-positive but only a narrow range of Gram-negative organisms via inhibiting their protein synthesis (Table 4). Defined EUCAST breakpoints for individual pathogens and pathogenic groups vary. The defined breakpoint for *Staphylococcus* sp. of R>2 µg/ml is used as guiding value for Gram-positive isolates, and the breakpoint for *Haemophilus influenzae* of R> 32 µg/ml, one of the few Gram-negatives with a defined breakpoint for clarithromycin, is used for Gram-negative isolates.

Clindamycin inhibits the protein synthesis of Gram-positive and Gram-negative bacteria (Table 4). Individual EUCAST breakpoints of clindamycin vary slightly but have a resistance MIC breakpoint of R>0.5 µg/ml in common for aerobic microorganisms (e.g. for *Staphylococcus* sp.) which is used as guiding value here, as all tested isolates grew aerobic.

Colistin (or polymyxin B) targets the outer membrane of Gram-negative bacteria, which renders it ineffective against Gram-positive bacteria (Table 4). EUCAST defines a sharp breakpoint against *Acinetobacter* sp. and other susceptible bacteria at a MIC of R>2 µg/ml. Not surprisingly most Gram-positive isolates are way above this value.

Doxycycline belongs to the tetracyclines and inhibits protein synthesis of a wide range of Gram-positive and Gram-negative bacteria (Table 4). Where individually defined, the EUCAST MIC breakpoints are generally the same as for *Staphylococcus* sp., namely R>2 µg/ml which is used as guiding value here.

Gentamicin is active against aerobic Gram-positive and Gram-negative bacteria (Table 4) of which it inhibits the protein biosynthesis. For gentamicin are almost no individual EUCAST breakpoints defined, here we will use R>1 µg/ml as defined for *S. aureus* as guiding value for the Gram-positive isolates and R>4 µg/ml as defined for *Acinetobacter* sp. as guiding value for Gram-negative isolates.

Trimethoprim and sulfamethoxazole disturb the folate metabolism of Gram-positive and Gram-negative cells (Table 4). Individual EUCAST breakpoints vary but are in most cases R>4 µg/ml (e.g. for *Staphylococcus* sp. and *Enterobacteriaceae*) and sometimes lower (e.g. R>2 µg/ml for *Streptococcus* sp.). The highest measured MIC of the ISS Isolates in this study for the trimethoprim/sulfamethoxazole combination besides complete resistance was *P. campinasensis* with 0.750 µg/ml. It should be safe to say that all isolates tested in this study which did not grow at the maximum concentration of 32 µg/ml were not resistant to trimethoprim/sulfamethoxazole.

Vancomycin is a glycopeptide antibiotic which inhibits bacterial cell wall synthesis and is therefore mostly active against Gram-positive bacteria (Table 4). The defined EUCAST breakpoint for *Staphylococcus* sp. is R>2 µg/ml. Using 2 µg/ml as a guiding value, most Gram-positive isolates and interestingly also several Gram-negative isolates can be rated not resistant against vancomycin.

Table 9 part 1: Minimal inhibitory concentrations for the tested isolates and evaluation according to PK/PD (non-species related) breakpoints and *Staphylococcus* spp. breakpoints of EUCAST. Green: sensitive; **Yellow:** intermediate; **Red:** resistant; **Orange:** most likely resistant; **Bold:** grew at the tested maximum concentration of the respective antibiotic; concentrations given in µg/ml. Colistin, clarithromycin, clindamycin, doxycycline, gentamicin, and vancomycin have no defined PK/PD breakpoints and colistin also has no defined *Staphylococcus* spp. breakpoints

	Max. Concentration: 256 µg/ml											Max. Concentration: 32 µg/ml					
Antibiotics / Isolates	Amoxicillin/ clavulanic acid	Ceftriaxone	Linezolid	Penicillin G	Ampicillin	Colistin	Clarithromycin	Clindamycin	Doxycycline	Gentamicin	Vancomycin	Cefotaxime	Ciprofloxacin	Levofloxacin	Meropenem	Moxifloxacin	Trimethoprim/ sulfamethoxazole
Bacillus aerophilus pH7_R2F_2_C	0.25	16	1.5	0.064	0.25	12	0.064	2	0.094	0.064	1	**32**	0.094	0.125	0.094	0.016	0.004
Bacillus altitudinis R10_C4_IIIB	0.25	12	1	0.023	0.047	16	0.047	1	0.19	0.094	0.25	**32**	0.125	0.125	0.125	0.064	0.003
Bacillus clausii pH9_R2_5_IIB	0.19	12	1	24	3	3	**256**	**256**	0.25	0.064	0.75	**32**	0.75	2	1	0.5	0.047
Bacillus horneckiae C78	0.75	48	0.5	1	6	3	4	2	0.5	0.25	0.5	**32**	0.125	0.19	0.75	0.094	**32**
Bacillus nealsonii pH7_CW1_HS0.2A	0.125	2	1	0.032	0.032	4	0.125	0.038	0.032	0.125	0.094	4	0.19	0.25	0.016	0.19	0.012
Bacillus nealsonii R5_C4_III_A	1.5	**256**	0.38	1	0.5	2	0.064	0.125	0.016	0.023	0.094	**32**	0.19	0.25	0.38	0.125	0.008
Bacillus nealsonii R9_B8_IIIB	0.016	2	2	0.016	0.016	0.5	3	0.25	0.064	0.125	0.125	0.25	0.125	0.064	0.02	0.032	0.008
Bacillus paralicheniformis pH5_R2_5_I_B	7	**256**	1	**256**	**256**	64	0.38	12	0.25	0.38	3	**32**	0.125	0.19	0.19	0.032	0.19
Bacillus paralicheniformis pH7_anox_R2	3	64	2	0.75	3	24	0.032	4	0.38	0.064	2	**32**	0.125	0.125	0.38	0.025	0.006
Bacillus paralicheniformis r2a5R0,2	16	24	1.5	24	24	32	0.064	4	0.125	0.19	1	24	0.094	0.094	19	16	0.032
Bacillus paralicheniformis r2a5R0,5	12	12	1	6	8	32	**256**	8	0.25	0.75	8	**32**	0.047	0.064	19	0.012	0.032
Bacillus paralicheniformis R9_A2_III_B	0.75	8	1	8	1.5	32	**256**	24	1.5	0.5	0.125	**32**	0.032	0.023	0.125	0.032	0.016
Bacillus pumilus pH7_R2F_2_A	3	32	2	0.38	2	48	0.125	3	0.19	0.125	1.5	**32**	0.19	0.25	0.25	0.64	0.004
Bacillus pumilus pH9_R2_5_I_B	2	4	0.75	**256**	0.15	32	0.064	24	0.064	0.5	2	16	0.032	0.094	0.094	0.008	0.032
Bacillus safensis pH9_R2_5_I_C	3	24	2	0.25	2	24	0.5	4	0.125	0.094	0.75	**32**	0.094	0.125	0.25	0.023	0.094
Bacillus safensis R10_A9_IIIB	0.5	0.25	0.38	0.047	0.125	8	0.047	1.5	0.125	0.064	0.25	**32**	0.064	0.064	0.094	0.047	0.002
Bacillus subtilis pH5_R2_2_I_A	3	0.38	1	0.75	3	32	0.064	0.75	0.094	0.094	1	8	0.064	0.125	0.19	0.047	0.19
Bacillus subtilis pH5_R2_2_II_A	0.5	12	0.75	0.5	16	64	0.064	0.5	0.19	0.75	2	16	0.064	0.094	0.094	0.023	0.19
Bacillus thermoamylovorans ms2R0,5	0.19	8	1	4	2	64	**256**	1	0.75	0.125	2	**32**	0.19	0.38	0.38	0.032	0.094
Bacillus timonensis pH9_R2IID	2	48	1	1	2	16	0.032	2	0.19	0.064	1.5	**32**	0.125	0.094	0.38	0.023	0.094
Bradyrhizobium erythrophlei LMG 28425	12	128	**256**	8	64	**256**	64	**256**	16	**256**	**256**	**32**	**32**	**32**	**32**	0.19	**32**
Bradyrhizobium viridifuturi LMG 28866	16	**256**	**256**	**256**	**256**	**256**	32	**256**	**256**	16	**256**	**32**	**32**	**12**	**32**	16	**32**
Bradyrhizobium viridifuturi pH5_R2_1_I_B	**256**	**256**	**256**	**256**	**256**	**256**	**256**	**256**	**256**	96	**256**	**32**	**32**	**32**	**32**	**32**	**32**
	32	**256**	**256**	**256**	**256**	**256**	128	192	48	24	**256**	**32**	16	8	**32**	4	**32**

Table 9 part 2: Minimal inhibitory concentrations for the tested isolates and evaluation according to PK/PD (non-species related) breakpoints and *Staphylococcus* spp. breakpoints of EUCAST. Green: sensitive; **Yellow:** intermediate; **Red:** resistant; **Orange:** most likely resistant; **Bold:** grew at the tested maximum concentration of the respective antibiotic; concentrations given in µg/ml. Colistin, clarithromycin, clindamycin, doxycycline, gentamicin, and vancomycin have no defined PK/PD breakpoints and colistin also has no defined *Staphylococcus* spp. breakpoints

Antibiotics / Isolates	Max. Concentration: 256 µg/ml											Max. Concentration: 32 µg/ml					
	Amoxicillin/ clavulanic acid	Ceftriaxone	Linezolid	Penicillin G	Ampicillin	Colistin	Clarithromycin	Clindamycin	Doxycycline	Gentamicin	Vancomycin	Cefotaxime	Ciprofloxacin	Levofloxacin	Meropenem	Moxifloxacin	Trimethoprim/ sulfamethoxazole
Brevibacterium halotolerans pH5_R2_2_I_B	2	1.5	1	8	2	32	0.094	0.5	0.047	0.094	**256**	2	0.047	0.094	0.064	0.016	0.125
Cupriavidus metallidurans pH5_R2_1_II_A	1.5	0.5	**256**	3	1.5	24	2	**256**	0.064	32	0.75	1	0.016	0.047	0.125	0.023	0.064
Methylobacterium tardum pH5_R2_1_I_A	2	1.5	**256**	**256**	3	**256**	2	**256**	1.5	8	**256**	12	6	3	**32**	0.25	**32**
Microbacterium lemovicicum R7K_A1_IA	0.064	0.25	0.25	0.047	0.023	32	0.016	0.016	0.032	0.125	0.19	**32**	**32**	**32**	0.5	24	0.16
Micrococcus yunnanensis pH9_CD3_II_A	0.5	0.25	0.38	0.094	0.047	1.5	0.125	0.094	0.19	0.75	0.38	0.25	0.5	1	0.064	0.38	0.023
Micrococcus yunnanensis pH9_R2_3_II_A	0.75	0.19	1	0.094	0.5	2	4	0.25	0.25	0.38	0.19	0.75	0.75	1.5	0.47	0.38	0.5
Micrococcus yunnanensis R10_A6_III_A	0.75	0.75	0.5	0.5	0.25	16	0.064	0.5	0.125	1	0.25	1	0.75	0.75	0.25	0.38	0.064
Paenibacillus campinasensis pH9_R2IIA	**256**	12	6	24	**256**	48	**256**	**256**	0.125	0.75	4	12	0.125	0.094	3	0.047	0.75
Paenibacillus glucanolyticus R7_C3_IIIWC	**256**	0.38	1.5	12	12	8	0.125	12	32	0.38	1	1	0.125	0.25	0.75	0.094	0.023
Paenibacillus pasadenensis pH7_NFS1_IV_C	4	0.25	0.047	0.38	1.5	2	0.125	0.032	0.047	1	0.75	1	1	0.38	0.5	0.125	0.032
Ralstonia insidiosa RAV_CW4_II_A	**256**	0.38	**256**	**256**	**256**	12	8	**256**	2	24	**256**	2	0.094	0.38	16	0.19	0.032
Roseomonas nepalensis C63	**256**	**256**	**256**	**256**	32	7	**256**	**256**	**256**	6	**256**	**32**	**32**	**32**	**32**	**32**	**32**
Roseomonas nepalensis C55	0.016	0.094	0.38	0.125	0.5	**256**	0.023	1	0.064	0.5	**256**	0.125	0.003	0.006	0.25	0.008	**32**
Sphingomonas aerolata R7K_C6_IIA	0.19	16	0.19	0.023	0.032	24	0.047	1	0.032	0.125	0.032	**32**	0.094	0.047	0.064	0.016	0.002
Staphylococcus arlettae RAV_CD2_II_A	6	192	1	1	2	4	8	0.125	0.25	0.016	1	**32**	1	0.38	0.38	0.19	0.064
Staphylococcus arlettae Zvesda	6	12	2	0.75	2	3	128	2	0.5	0.016	1.5	8	1	1	0.25	0.38	0.25
Staphylococcus capitis R7A_A8_IB	0.38	1	0.38	0.032	0.032	12	0.125	0.064	0.19	0.047	1	0.5	0.125	0.25	0.032	0.094	0.047

III.1.6 Temperature and pH tests

For 26 of the 40 isolates selected for the antibiotic susceptibility testing and the three reference strains, also the upper and lower boundaries of their temperature and pH growth range were tested, to check if these isolates have a broader growth spectrum than their reference strains or the literature values of the respective type strains (Table 10).

The tested isolate species and their respective type strain publications are given as follows: *Bacillus paralicheniformis* (Dunlap *et al.*, 2015), *Bacillus altitudinis* (Shivaji *et al.*, 2006), *Bacillus horneckiae* (Vaishampayan *et al.*, 2010), *Bacillus nealsonii* (Venkateswaran *et al.*, 2003), *Bacillus safensis* (Satomi *et al.* 2006), *Bradyrhizobium erythrophlei* (Yao *et al.*, 2015), *Bradyrhizobium viridifuturi* (Helene *et al.*, 2015), *Cupriavidus metallidurans* (Goris *et al.*, 2001), *Methylobacterium tardum* (Kato *et al.*, 2008), *Microbacterium lemovicicum* (Mondani *et al.*, 2013), *Micrococcus yunnanensis* (Zhao *et al.*, 2009), *Paenibacillus campinasensis* (Yoon *et al.*, 1998), *Paenibacillus glucanolyticus* (Alexander and Priest, 1989), *Paenibacillus pasadenensis* (Osman *et al.* 2006), *Ralstonia insidiosa* (Coenye *et al.*, 2003), *Roseomonas nepalensis* (Chaudhary and Kim, 2017), *Sphingomonas aerolata* (Busse *et al.*, 2003), *Staphylococcus arlettae* (Schleifer *et al.* 1984), and *Staphylococcus capitis* (Schleifer and Kloos, 1975).

The growth temperature span of the tested strains ranged from 18° (14-32°C, *Roseomonas* C63) to 52° (4-56°C, *Bacillus pumilus* pH7_R2F_2_A) and the pH span ranged from 4 (*Bradyrhizobium erythroplei* LMG28425, pH 3-7) to 10 pH values (*Bacillus altitudinis* R10_C4_IIIB, pH 2-12). Neither the total ranges nor the minimal or maximal values for pH or temperature differed significantly between ISS isolates and ground control isolates (Mann-Whitney U Tests). Based on these data, the null hypothesis that strains obtained from the ISS are hardier than strains from Earth regarding the upper and lower boundaries of their temperature and/or pH growth ranges has to be rejected.

Further comparison with literature values was hampered, because for some type strains no reference values for the lower and upper boundaries are defined, for these the (optimum) temperatures and pH values as used in the type strain description are given in Table 10. The growth spectra for pH and temperature of our strains do in all cases differ from those reported in literature. However, most of the species descriptions are not exhaustive regarding the boundaries of the growth ranges (e.g. no pH tests were performed at all as in Busse *et al.*, 2003, or temperature range was tested only partially as in Vaishampayan *et al.*, 2010) which tremendously decreases the information value gained by comparing the results obtained in these tests to literature values. In case of *Bradyrhizobium erythrophlei* LMG 28425 the same strain was tested as in the original species description but the observed temperature growth range was narrower (6°C -43°C vs 4°C-60°C) and pH growth range was slightly more acidic as described in Yao et al. 2015, which might be because a different medium was used. Furthermore, even different isolates of the same species which were all isolated on the ISS showed individual temperature and pH growth range boundaries (see for example the *B. nealsonii* strains R5_C4_III_A + R9_B8_IIIB, and *R. nepalensis* strains C63 + C55 in Table 10).

Table 10: Temperature and pH growth ranges and heat shock (HS) results of selected isolates. Literature values of temperature and pH growth ranges of the respective type strains are given in brackets;

Taxonomy	Strain	Growth range [°C]	Growth range [pH]	HS survival
Bacillus paralicheniformis	pH5_R2_5_I_B	8-57 (15-60°C)	4-12 (6-11)	Yes
Bacillus paralicheniformis	R9_A2_III_B	8-57 (15-60°C)	5-12 (6-11)	Yes
Bacillus altitudinis	R10_C4_IIIB	6-51 (8-45)	2-12 (5-8)	Yes
Bacillus horneckiae	C78	14- 45 (4-32)	5-12 (7-10.8)	Yes
Bacillus nealsonii	R5_C4_III_A	12-45 (25-60)	4-12 (6-10)	Yes
Bacillus nealsonii	pH7_CW1_HS0.2A	8-43 (25-60)	5-12 (6-10)	Yes
Bacillus nealsonii	R9_B8_IIIB	20-43 (25-60)	6-12 (6-10)	Yes
Bacillus pumilus	pH7_R2F_2_A	4-56 (30)	5-12 (6)	Yes
Bacillus safensis	pH9_R2_5_I_C	6-53 (10-50)	3-12 (5.6)	Yes
Bacillus safensis	R10_A9_IIIB	6-51 (10-50)	5-12 (5.6)	Yes
Bradyrhizobium erythrophlei	LMG 28425	6-43 (4-60)	3-7 (5-8)	No
Bradyrhizobium viridifuturi	LMG 28866	15-40 (28)	2-8 (4.5-8)	No
Bradyrhizobium viridifuturi	pH5_R2_1_I_B	7-37 (28)	5-8 (4.5-8)	Yes
Cupriavidus metallidurans	pH5_R2_1_II_A	6-40 (20-37)	4-12 (7.3)	No
Methylobacterium tardum	pH5_R2_1_I_A	4-43 (15-35)	3-9 (7.2)	No
Microbacterium lemovicicum	R7K_A1_IA	4-32 (15-37)	5-12 (7)	No
Micrococcus yunnanensis	R10_A6_III_A	8-45 (4-45)	3-11 (6-8)	No
Micrococcus yunnanensis	pH9_R2_3_II_A	4-45 (4-45)	3-12 (6-8)	No
Micrococcus yunnanensis	pH9_CD3_II_A	6-56 (4-45)	3-12 (6-8)	Yes
Paenibacillus campinasensis	pH9_R2IIA	12-54 (10-45)	6-11 (7.5-10.5)	Yes
Paenibacillus glucanolyticus	R7_C3_IIIWC	7-45 (17-45)	6-10 (5.7)	No
Paenibacillus pasadenensis	pH7_NFS1_IV_C	8-40 (25-32)	5-12 (7.2)	Yes
Ralstonia insidiosa	RAV_CW4_II_A	4-40 (28-37)	5-12 (7.3)	Yes
Roseomonas nepalensis	C63	14- 32 (10-37)	4-9 (6-10)	No
Roseomonas nepalensis	C55	6-37 (10-37)	5-9 (6-10)	No
Sphingomonas aerolata	R7K_C6_IIA	4-51 (4-28)	5-12 (7)	Yes
Staphylococcus arlettae	RAV_CD2_II_A	7-43 (25-42)	3-12 (7.4)	No
Staphylococcus arlettae	Zvesda	10-56 (25-42)	5-12 (7.4)	Yes
Staphylococcus capitis	R7A_A8_IB	9-45 (30-45)	3-12 (4.2-6.8)	No

Additionally, these 29 bacterial strains were also subjugated to a heat shock according to ESA standards. Generally, it would be expected that spore formers survive the heat shock and non-spore formers do not. This was true for most cases with some exceptions: The spore former *Paenibacillus glucanolyticus* did not survive the heat shock treatment and the non-spore forming strains *Bradyrhizobium viridifuturi* pH5_R2_1_I_B, *Micrococcus yunnanensis* pH9_CD3_II_A, *Ralstonia insidiosa* RAV_CW4_II_A, *Sphingomonas aerolata* R7K_C6_IIA, and *Staphylococcus arlettae* Zvesda survived the heat shock. Interestingly the ability to survive the heat shock seems to be strain specific as other tested strains of the same (non sporeforming) species did not survive the heat shock. It was not tested if the spore forming isolates actually formed spores before the heat shock, therefore it is most likely that *Paenibacillus glucanolyticus* did not survive the heat shock because it did not form spores within the 48h incubation period before the heat shock.

III.1.7 Co-incubation with ISS material

To investigate if the bacteria isolated from ISS can interact with materials present on the ISS, *Cupriavidus metallidurans* strain pH5_R2_1_II_A, *Bacillus paralicheniformis* strain R2A_5R_0.5, and *Cutibacterium avidum* strain R7A_A1_IIIA were incubated together with untreated aluminum alloy platelets, eloxated aluminum alloy platelets, and pieces of NOMEX® fabric. Unfortunately, *C. avidum* did not grow in the assays containing NOMEX® fabric and *B. paralicheniformis* did not grow in the anaerobic assay. As the used NOMEX® fabric is not known to have antimicrobial properties per se and this *B. paralicheniformis* strain was originally isolated via the anaerobic cultivation assay, this unexpected lack of growth cannot be explained.

After incubation, the co-incubated materials were analysed via scanning electron microscopy. The pictures presented here in this thesis are all taken of only one sample of each available triplicate but a more detailed investigation is planned to follow. Figure 4 A-D shows scanning electron micrographs of NOMEX® fabric co-incubated with *B. paralicheniformis*. There were visible flocks of *B. paralicheniformis* biofilm sporadically attached to the NOMEX® fabric. In contrast, *C. metallidurans* did not form a biofilm, but single cells were attached all over the NOMEX® fabric as shown in Figure 4 E-F. Nevertheless, the co-incubated NOMEX® fabric fibers were still as intact as those of the respective negative control. The negative control, which consisted of NOMEX® fabric incubated in sterile medium for six weeks was for the most part clean, as shown in Figure 4 I, but there were also particles found within the tissue (Figure 4 J). However, as seen in the magnifications of these particles in Figure 4 K-L, these particles were too small and irregular to be living microbial cells. They might represent remnants of a bacterial biofilm which was alive prior to autoclaving the material, as the piece of NOMEX® fabric at our disposal was not handled sterile before it was autoclaved for this experiment.

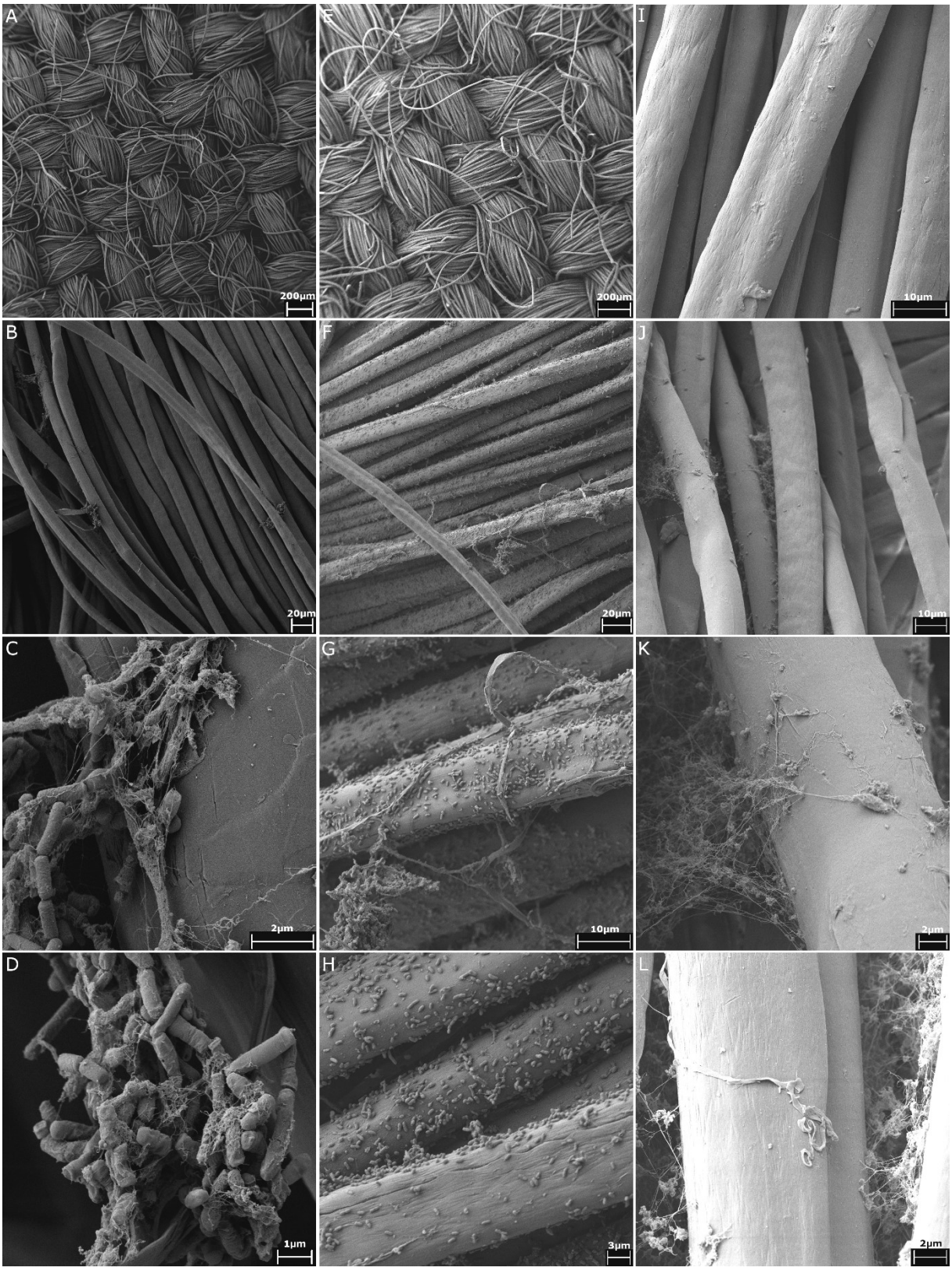

Figure 4: Scanning electron micrographs of NOMEX® fabric which was co-incubated for six weeks with bacteria isolated from the ISS: A-D: Co-incubation with *Bacillus paralicheniformis*; **E-H:** Co-incubation with *Cupriavidus metallidurans*; **I-L:** Negative control of NOMEX® fabric kept in sterile medium for six weeks.

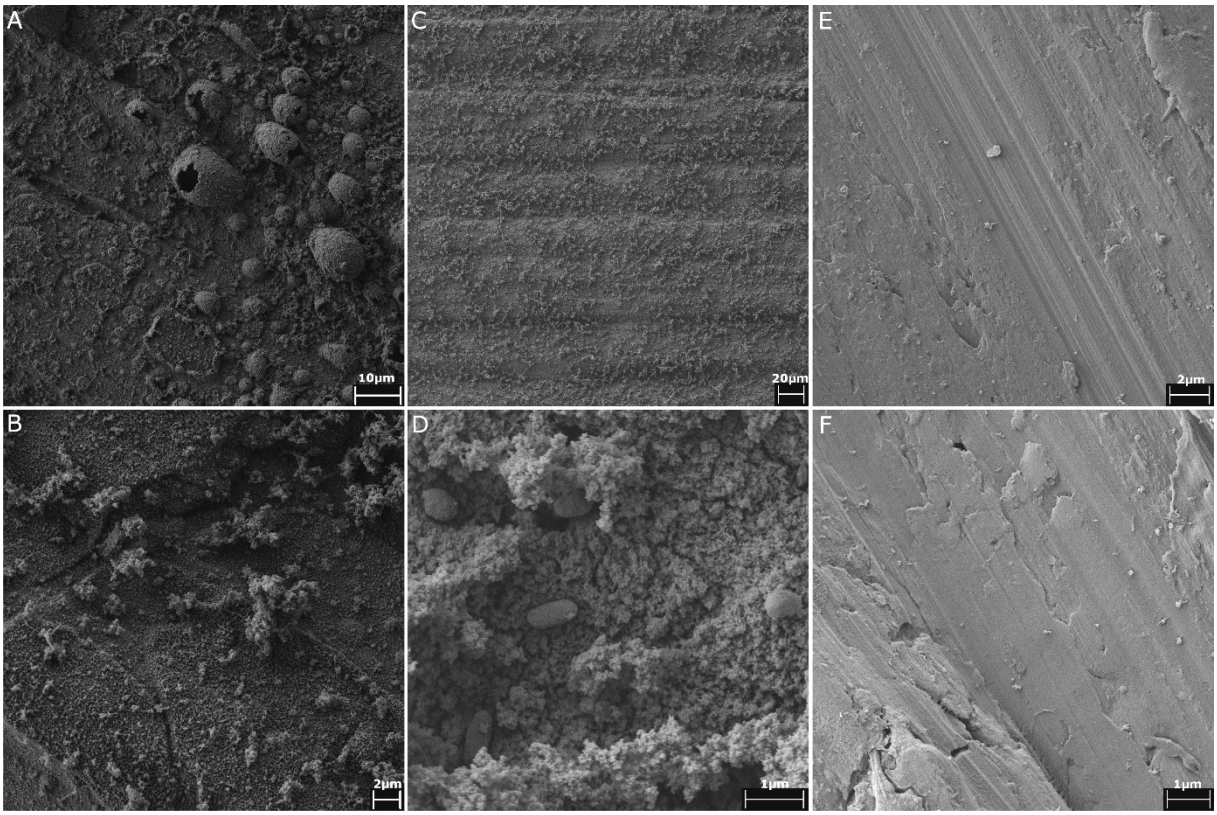

Figure 5: Scanning electron micrographs of polished and untreated aluminum copper magnesium alloy EN AW 2219 which was co-incubated for six weeks with bacteria isolated from the ISS: A-B: Co-incubation with *Bacillus paralicheniformis*; **C-D:** Co-incubation with *Cupriavidus metallidurans*; **E-F:** Negative control kept in sterile medium.

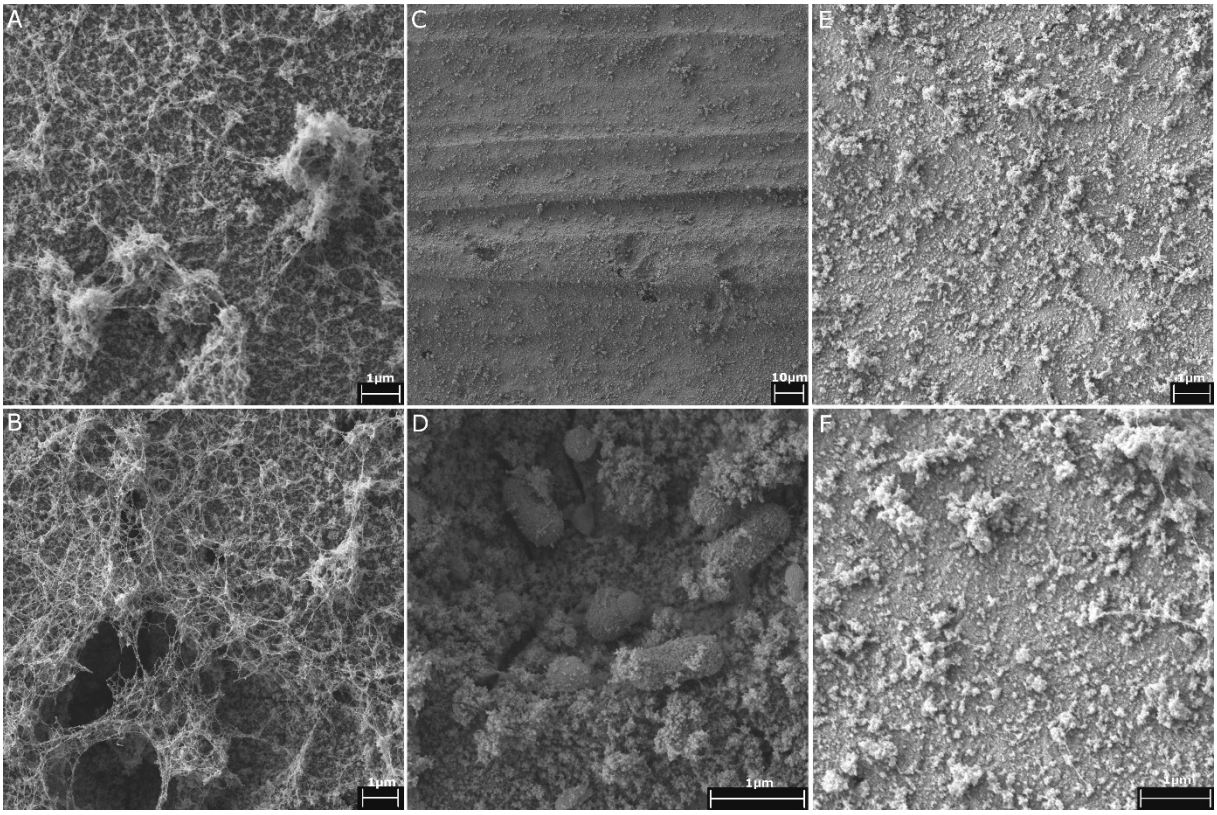

Figure 6: Scanning electron micrographs of polished and eloxated aluminum copper magnesium alloy EN AW 2219 which was co-incubated for six weeks with bacteria isolated from the ISS: A-B: Co-incubation with *Bacillus paralicheniformis*; **C-D:** Co-incubation with *Cupriavidus metallidurans*; **E-F:** Negative control kept in sterile medium.

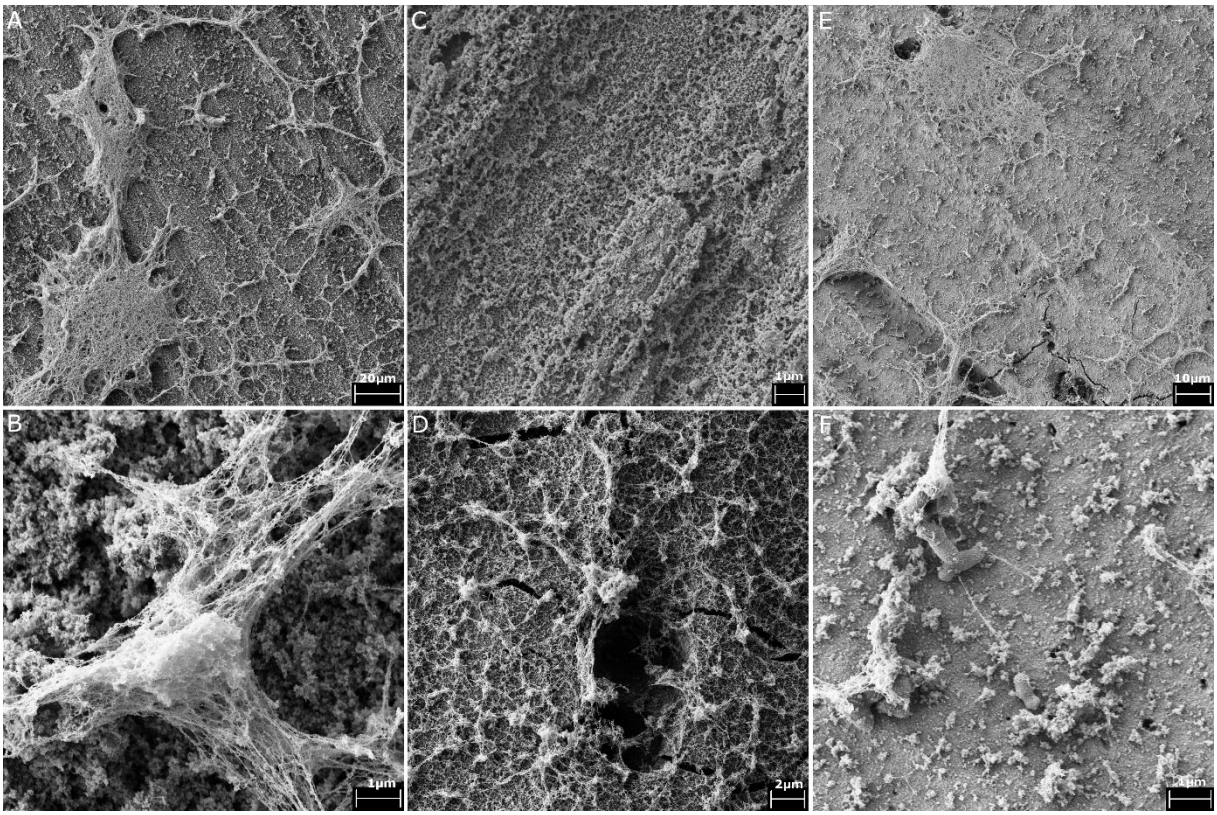

Figure 7: Scanning electron micrographs of polished untreated and of eloxated aluminum copper magnesium alloy EN AW 2219 which was anaerobically co-incubated for six weeks with bacteria isolated from the ISS: A-B: Untreated alloy co-incubated with *Cutibacterium avidum***; C:** Negative control of untreated alloy kept in sterile medium for six weeks; **D;** Negative control of eloxated alloy kept in sterile medium for six weeks; **E-F:** Eloxated alloy co-incubated with *Cutibacterium avidum***.**

B. paralicheniformis did neither adhere to the untreated nor to the eloxated aluminum alloy, as seen in Figure 5 A-B and Figure 6 A-B respectively. However, the untreated aluminum alloy which was co-incubated with *B. paralicheniformis* showed sporadic signs of corrosion (Figure 5 A) compared to the untreated negative control incubated in sterile medium (Figure 5 E-F) and at least on the eloxated aluminum alloy surface, which was incubated together with *B. paralicheniformis* (Figure 6 A-B), there seems to be an additional surface coating next to the ubiquitous debris, which might be remnants of a washed away biofilm (extracellular matrix or attached flagella or pili). This uncertainty will most likely be clarified in near future after investigating the other two samples of the triplicate. All eloxated aluminum surfaces had attached debris regardless if the incubation in sterile medium was performed with or without oxygen (see Figure 6 E-F and Figure 7D respectively). Single cells of *C. metallidurans* also attached to the surfaces of untreated and eloxated aluminum alloys, and their co-incubated alluminium alloys had a unique background surface pattern, which was distinct from their respective negative controls, as shown in Figure 5D and 6D. The anaerobic *C. avidum* formed a biofilm attached to the surface of both, untreated and eloxated, aluminum alloys (see Figure 6 A,B,E,F).

III.2 Microbial diversity detected by 16S rRNA amplicon sequencing

To analyse the ISS indoor microbiome in a more comprehensive manner, including the uncultivable diversity, DNA of all samples from session A, session B, and session C, as well as of the vacuum cleaner bag and dust filter samples was extracted. The hypervariable region V4 of the 16S rRNA gene was amplified using a universal primer pair, targeting bacterial and archaeal 16S rRNA genes in the same reaction, and using an additional Archaea-targeting approach, aiming to capture the archaeal diversity in more detail. The amplicons were sequenced on an Illumina MiSeq device (see II.8). The same methodology was used for incubated and PMA treated vacuum cleaner bag and dust filter samples. During the early stage of this thesis only samples which showed a visible band on an electrophoresis gel after the 16S rRNA gene amplification were sequenced. For this reason, only the wet wipe samples K1, K2 and K3 of the clean room in Kourou were included in the sequencing analysis whereas the rest of the clean room samples and all PMA treated samples of the clean room were interpreted negative. This procedure changed while the vacuum cleaner bag and dust filter samples were analysed of which we received two sets which were analysed independently from each other. In the first approach only the non PMA-treated samples RISS1, RISS2 and RISS3 and the incubated samples were sequenced, because these samples showed a positive signal on the electrophoresis gel. In the second approach no incubation experiment was conducted, but all untreated and PMA-treated vacuum cleaner bag and dust filter samples were sequenced independently of their electrophoresis gel signal and produced evaluable results. This approach was also used for the samples from session A, B, and C with the exception that the extraction fluid was not split and no PMA-treatment was done to maximise the expected low DNA yield. In total, 16S rRNA gene amplicons of 46 samples were sequenced.

III.2.1 Microbial diversity detected by the universal approach

After processing the sequence reads of the universal approach with DADA2, all sequences assigned to mitochondria, chloroplasts, or RSV´s present in the respective negative controls were removed from the dataset. Alltogether, 684,189 high quality sequences distributed over the 46 samples were obtained and the read counts per sample varied from 649 (A8) to 60864 (RISS2, incubated dust collector) as shown in Figure 8. In general, the clean room samples and the vacuum cleaner bag and dust filter samples yielded more sequence reads than the ISS indoor wipe samples.

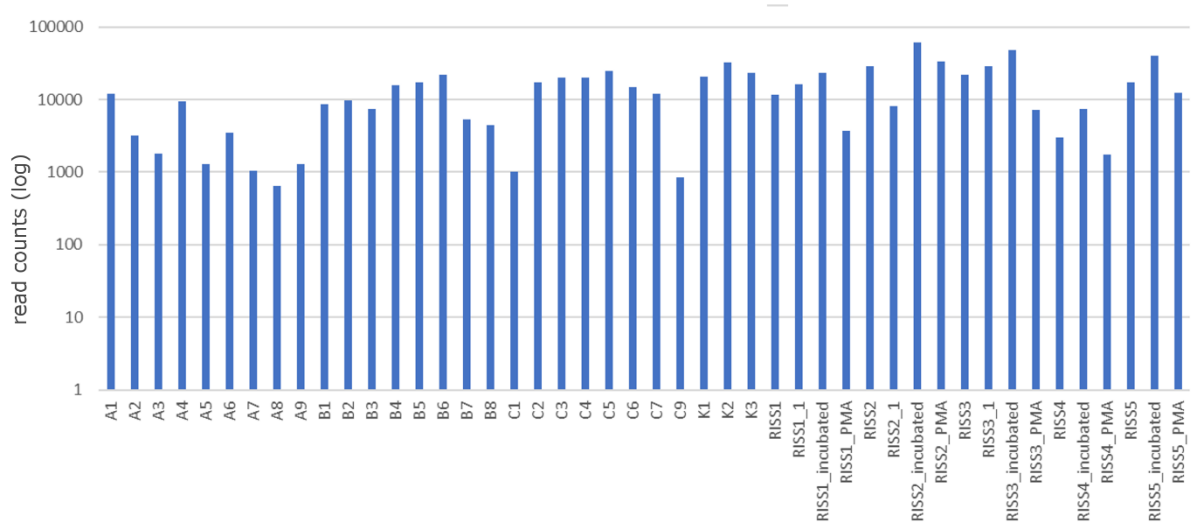

Figure 8: Filtered read counts for all sequenced samples.

The sequence reads were taxonomically assigned according to the SILVA database v123 and an overview of the relative abundances of the most abundant RSVs on kingdom, phylum, and genus level is given in Figure 9. Across all samples, 2,611 distinct RSVs were detected, of which 1,148 RSVs (367 genera) are attributed to sessions A, B, and C, 1,830 RSVs (529 genera) are attributed to the vacuum cleaner and dust samples, and 64 RSVs (34 genera) are attributed to the clean room samples. As expected, Bacteria dominated all samples on kingdom level, but the universal approach was able to detect Archaea in 13 samples, most of which were detected in the first sequencing of the dustfilter1_2004 (39% of 16,033 reads). The detected Archaea will be covered in more detail in chapter III 2.4. The clean room samples were dominated mainly by the Proteobacteria *Sphingomonas* and *Methylobacterium* whereas the ISS samples were dominated by various genera of the phyla Actinobacteria, Firmicutes, and Bacteroidetes.

The clean room samples differed strongly from the ISS samples: The observed diversity in the clean room samples was significantly lower than in the ISS wipe samples (ANOVA; p=0.012; Shannon diversity index; Figure 11) and also the multivariate analyses in Figure 12 show that the clean room samples cluster apart from the ISS samples.

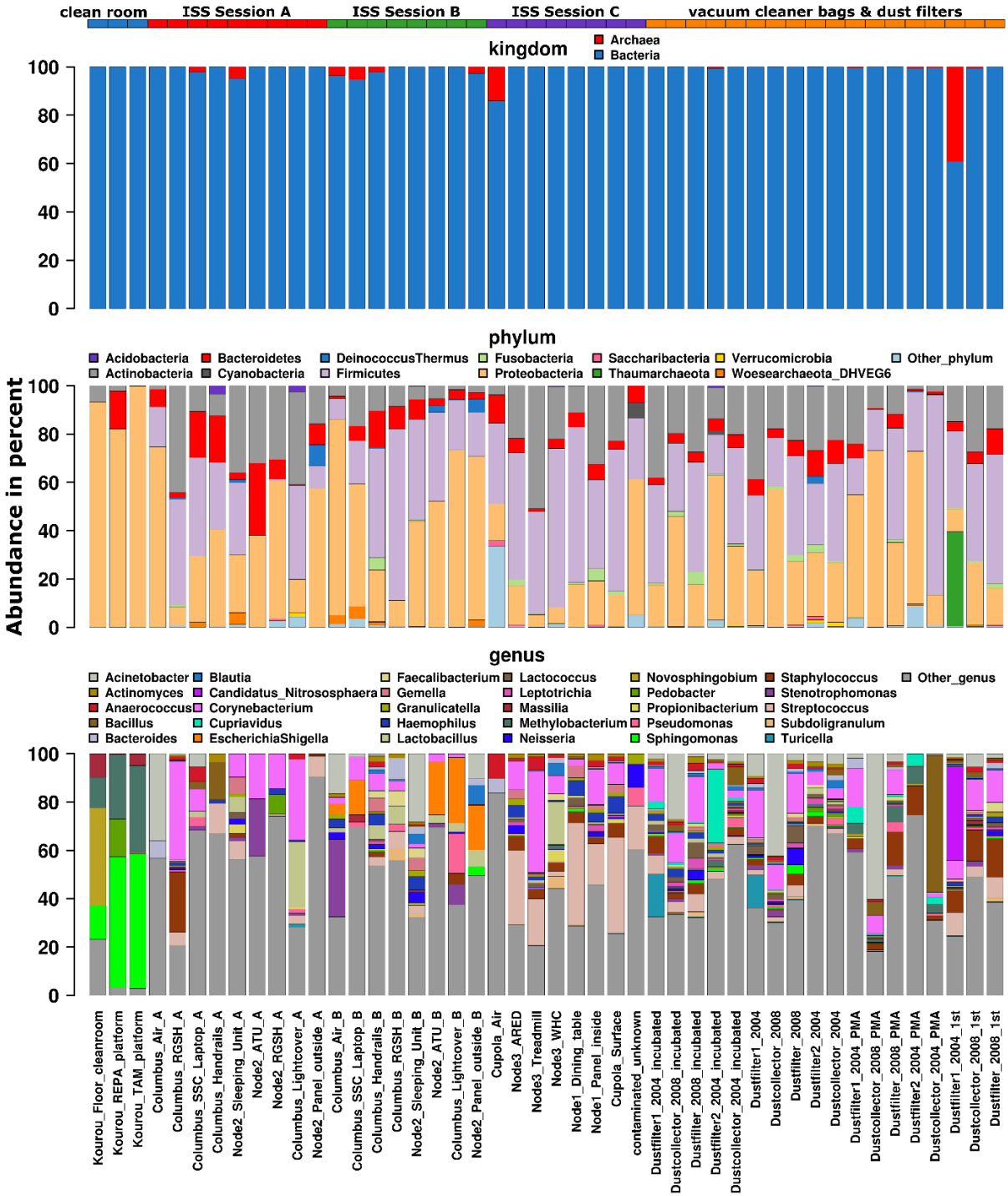

Figure 9: Barcharts showing the relative taxonomical abundance of the universal approach for each sample on kingdom, phylum, and genus level. Only the top 12 phyla and the Top 30 genera across all samples are shown.

Session A, B, and C were sampled within a timeframe of two and a half months and by the same method, with the exception that wipes used in Session C were premoistened. The samples were grouped in three different ways: 1) according to the individual sampling events, to evaluate if the ISS microbiota changed over this relatively short timeframe, 2) the localization of the sampled areas, to evaluate if the ISS microbiota differ in different modules, and 3) the functional type of the sampled areas, to evaluate if different interaction patterns of the astronauts have a measurable effect on the microbiota. The different types of samples are defined as follows: "Air" includes all ambient air samples and return grid sensor housing (RGSH) samples, where the air within a module is sucked in for recycling. "Exercise" samples were taken from the treadmill and the Advanced Resistive Exercise Device (ARED), where active shedding of particles while training was expected. "Intimate" includes the samples taken from the sleeping unit and the Waste & Hygiene Compartment (WHC). "Frequent interaction" are all other samples with an expected high, although not intense, interaction with humans, namely audio terminal units (ATU), laptops, handrails, and the dining table. "Low interaction" are surfaces which are rather seldomly touched by the crew, i.e. lightcovers, wall panels on the inside and outside, and the sampled cupola surface. The diversity was significantly different regarding the sampling events (ANOVA, p=0.0031) but neither regarding to the type (ANOVA, p=0.16) nor localization (ANOVA, p=0.19) of the sampled areas (Figure 11).

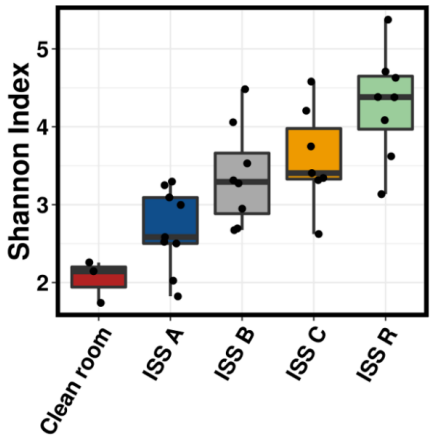

Figure 10: Shannon diversity indices of all sampling events (ISS R = vacuum cleaner & dust filter samples). The difference is significant (ANOVA, p<0.00001, read count rarefied to 649).

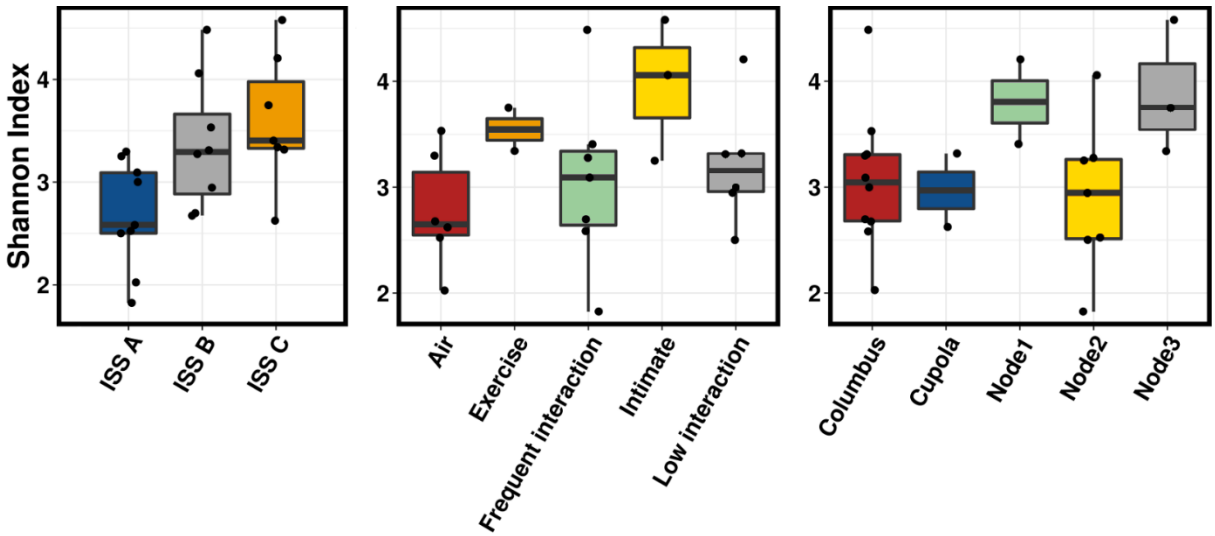

Figure 11: Shannon diversity indices of Session A, B, and C grouped according to the individual sampling events (left), the type of the sampled areas (middle) and localization of the sampled areas within the ISS (right). The difference is significant regarding to the sampling events (ANOVA, p=0.0031) but not regarding to the type (ANOVA, p=0.16) or localization (ANOVA, p=0.19) of the sampled areas. Read count was rarefied to 649.

The PCoA and NMDS plots in Fig. 12 also show that the ISS samples are distinct regarding the given sampling events but not regarding the originating modules or the type of the sampled surfaces. This can be expected in such a small closed environment when assuming that the ISS microbiota change over time.

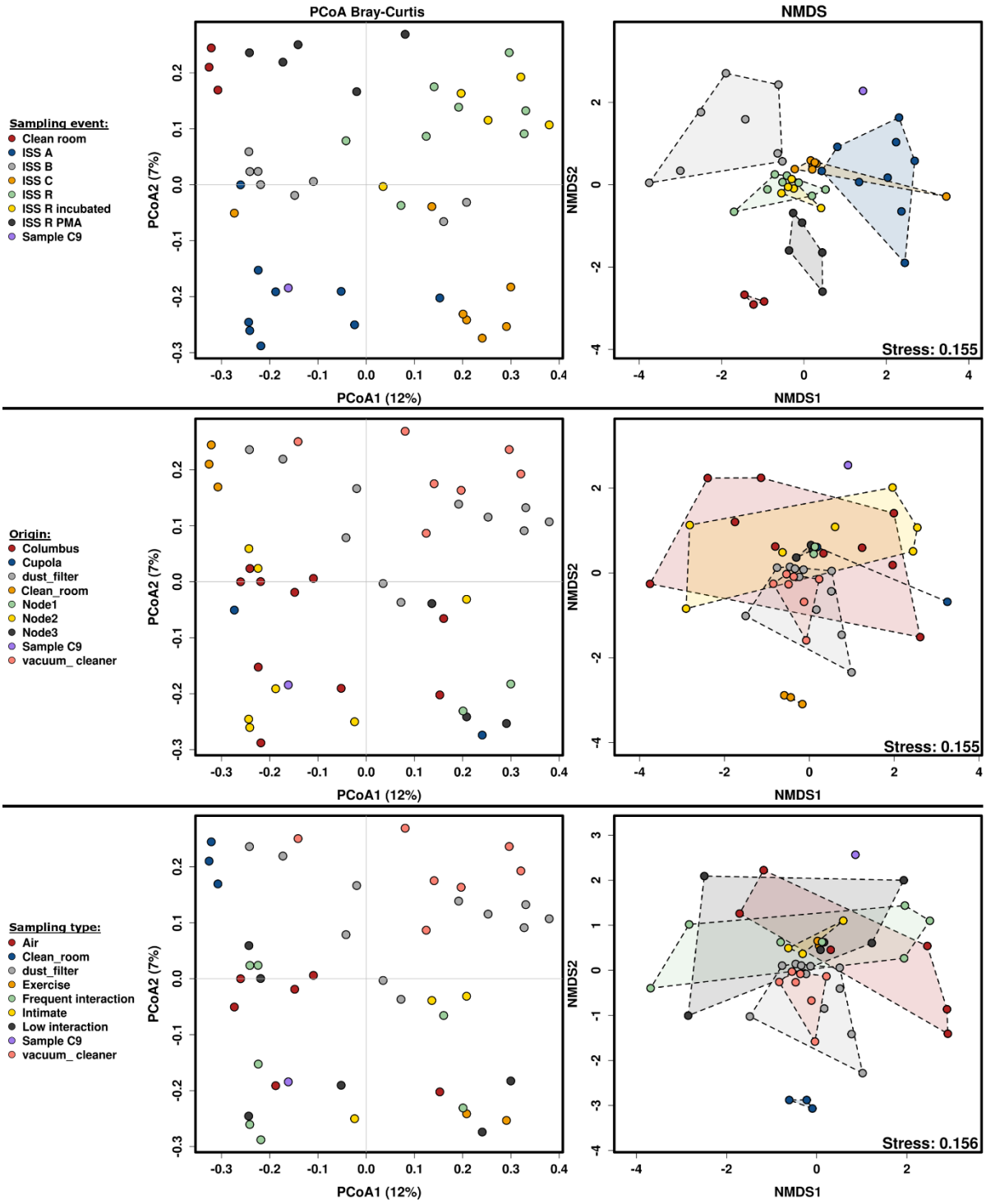

Figure 12: PCoA plots (Bray-Curtis index) of all sequenced isolates, colored according to their respective sampling event (top), the origin of the samples within the ISS (middle), and the different type of their respective sampling location (bottom). Only the different sampling events show a distinct dissimilarity pattern.

III.2.2 Comparison over time (Session A and B)

Session A and B were taken at the same locations by the same astronaut and with exactly the same method, to specifically address if and how the ISS microbiome changes over time. The timespan between these two sampling events was two months and eleven days, which was the longest logistically feasible timespan without crew exchange. The samples had to be taken without crew exchange, as the arrival of new astronauts and their individual microbiomes would cause a shift in the ISS microbiome by itself. During these two and a half months, two resupply cargoes arrived at the ISS, the SpaceX CRS-11 on the 5th of June 2017 (resupply from USA), and the Progress 67 on the 16th of June 2017 (resupply from Russia). For direct comparison, sample A9 was not considered, as it does not have an equivalent in the session B samples. Indeed, the overall microbial diversity detected in session A was significantly different from the diversity detected in session B (Shannon index; ANOVA p=0.041) and also in the principal coordinate analysis the samples of session A can be clearly distinguished from the samples of session B along the PCoA1 axis (Figure 13).

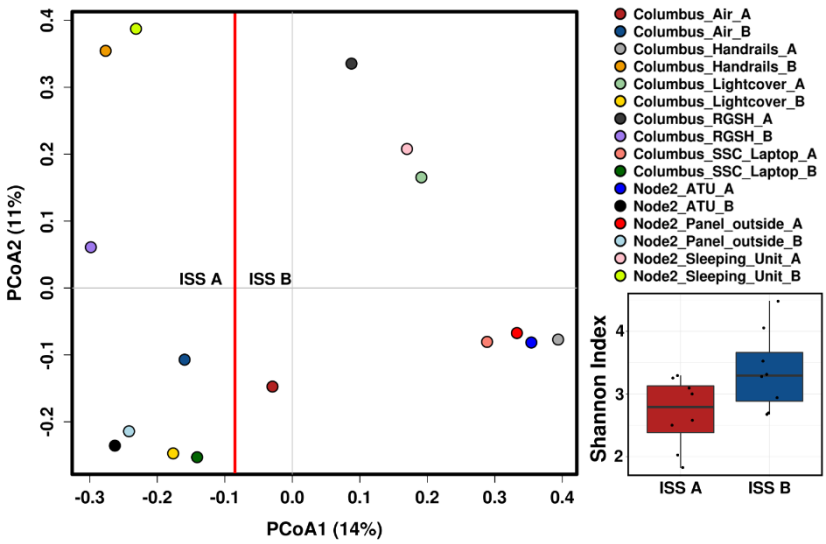

Figure 13: PCoA plot (Bray-Curtis index; RSV level) of all individual samples of session A and B. All samples on the left side of the red line belong to session A, all samples on the right side of the red line belong to session B. Dotplot on the bottom right shows the significantly different diversity of session A and B (ANOVA p=0.041, samples rarefied to 649 reads)

A LEfSe (Linear discriminant analysis effect size) evaluation on genus level indicates that this difference is mainly due to the genera *Escherichia/Shigella, Pseudobutyrivibrio, Ruminococcus_2, Lachnoclostridium*, and *Tepidicella* which are significantly more abundant in the session B samples, and the genus *Cloacibacterium* which is significantly more abundant in session A samples (all log 10 LDA Scores >4). *Escherichia/Shigella*, *Pseudobutyrivibrio, Ruminococcus_2*, and *Lachnoclostridium* are regularly found in human digestive tract samples (Segata *et al.*, 2012) whereas the only known *Tepidicella* species has been found in a hot spring runoff (França *et al.*, 2006) and *Cloacibacterium* species are mostly wastewater associated (Allen *et al.*, 2006; Chun *et al.*, 2017). When comparing only session A and B and omitting C, the samples were even less significantly different regarding their location (ANOVA p=0.8) or type (ANOVA p=0.62). Still, LEfSe shows that the genus *Pseudomonas*

was found more often in the Columbus module than in Node 2. And in the sleeping units (intimate type samples) the human associated genera *Gemella, Rothia* and *Lactococcus* were more abundant than in the other session A and B samples (log 10 LDA Scores >4). When comparing all corresponding samples from session A and B individually, the observed Shannon diversity index is in almost all cases higher in the respective sample of session B as also reflected in the overall diversity. The exception is the sample pair taken from the Columbus SSC laptops, which carries a higher microbial diversity during session A (Shannon index; see Figure 14). Figure 14 also shows all unique genera per sample (corresponding unique genera = cug) present during session A and absent in the corresponding session B samples, which were taken two and a half months later, and vice versa. Samples taken from the SSC laptop (A: 17 cug; B: 11 cug), RGSH (A: 75 cug; B: 19 cug), and lightcover (A: 20 cug; B: 19 cug) contained more cug in session A, whereas samples taken from air (A: 8 cug; B: 21 cug), handrails (A: 9 cug; B: 71 cug), ATU (A: 3 cug; B: 17 cug), Node2 panel(A: 8 cug; B: 16 cug) and sleeping unit contained more cug in session B. A higher number of cug naturally also correlates with a higher richness (not shown).
In accordance with aforementioned LEfSe evaluation, the genera *Escherichia/Shigella, Pseudobutyrivibrio, Ruminococcus_2, Lachnoclostridium*, and *Tepidicella* were unique to at least four session B samples and the genus *Cloacibacterium* to six session A samples.

According to a hierarchical cluster analysis based on Pearson´s correlation, some signatures of microbial phyla positively correlated with certain sampling locations (see Figure 15). For example, Verrucomicrobia, Planctomycetales, and Spirochaetae correlated with the lightcovers in Columbus module, and Tenericutes and Deferribacteres correlated with Columbus RGSH. Even archaeal phyla could be correlated to specific surfaces: Thaumarchaea were correlated with the Node 2 sleeping unit and Euryarchaeota were correlated with Columbus handrails. The outside panel and the ATU of Node 2 also are more similar to each other than to the Columbus samples, but the Node 2 sleeping unit was different from other Node 2 samples. However, this was not found to be significant.

Two logistical factors most likely also influenced the difference of the samples: 1) Session A was sampled on a Monday evening, about 36 hours after the last cleaning procedure on board the ISS, and Session B was sampled on a Wednesday, about three days after the last cleaning procedure, which means the ISS indoor microbiota had more time to regenerate from the last surface cleaning in the session B samples. 2) Session A samples were delivered to the laboratory within one month and five days after the actual sampling and session B samples were delivered to the laboratory within four months and three days after the actual sampling. This bigger time difference might enable certain microbes to proliferate or die and degenerate in session B samples compared to session A samples, although we expect this to be only a minor issue since the samples of session A and B were taken with dry wipes and kept dry during transport which should have kept the microbes within the samples in a physiologically inactive state.

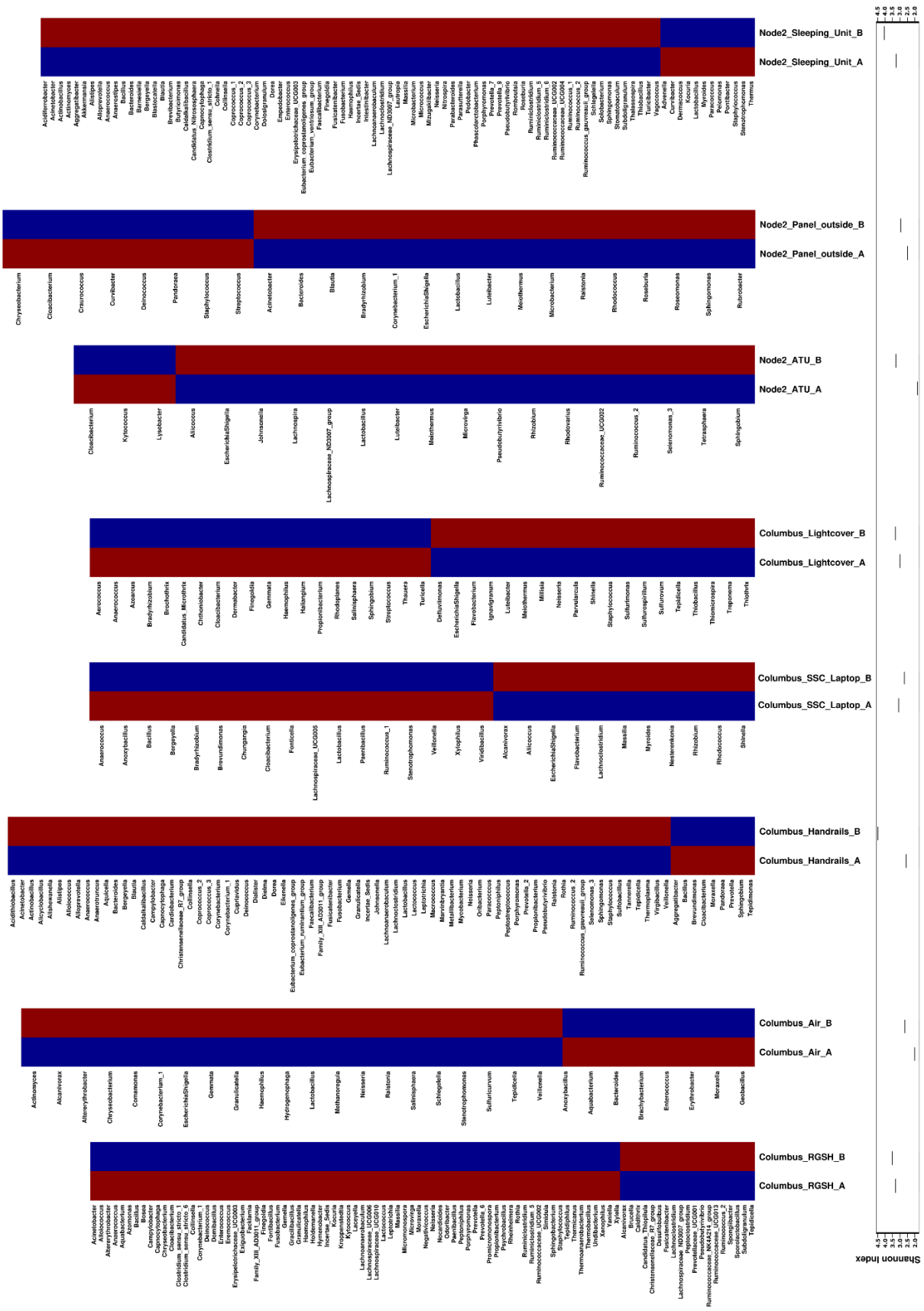

Figure 14: Present (red) and absent (blue) genera, when comparing the individual samples of sessions A and B which were taken at the same location and their individual Shannon index (samples rarefied to 649). This image is recommended to be viewed in landscape.

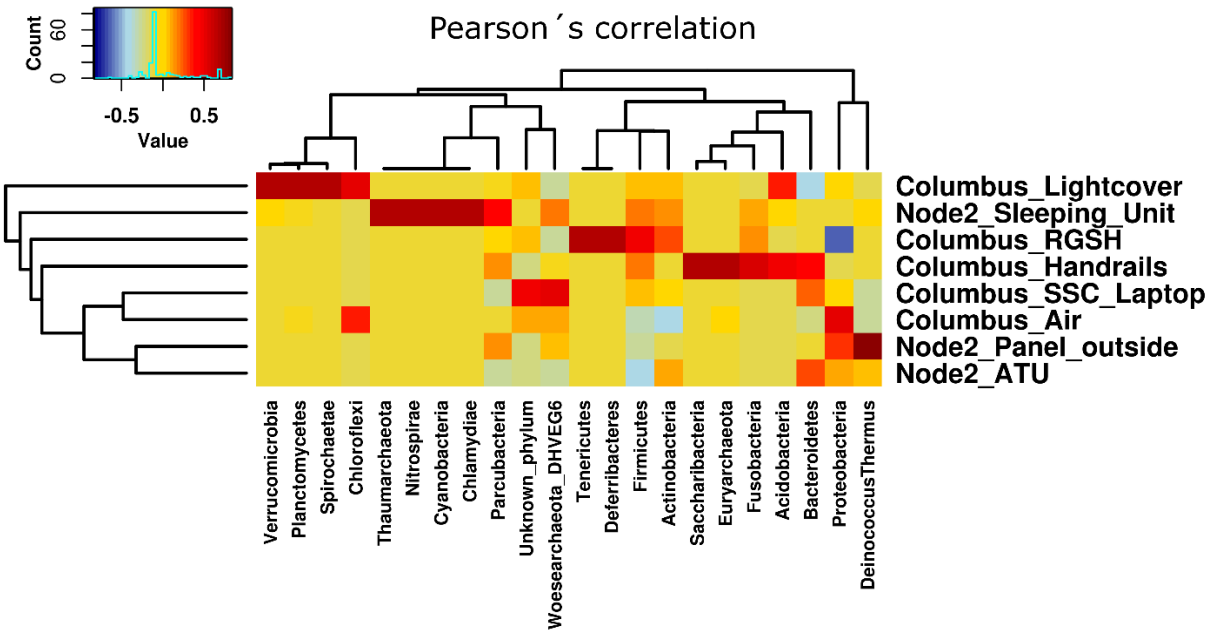

Figure 15: Heatmap with dendrograms of Pearson's correlation analysis between normalized square root transformed abundance of detected phyla and the individual samples of sessions A and B. Positive correlations are represented by the intensity of shades of red and negative correlations are represented by the intensity of shades of blue (reproduced from Mora *et al.*, 2019).

III.2.3 Microbial diversity of the vacuum cleaner bag and dust filter samples

Vacuum cleaner bag and dust filter samples collected from the Russian part of the ISS in 2004 and 2008 were analysed in two batches. The first batch included the incubation experiment, where dormant bacteria were induced to germinate before DNA extraction to increase the yield, but only three of the untreated samples and no PMA-samples were sequenced. For the second batch no incubation experiment was performed, but all untreated samples and PMA-treated samples were sequenced.

The incubated samples were not significantly different from the untreated samples in diversity (Shannon index, ANOVA p=0.38 regarding only the three correspondent samples from the same batch and p=0.85 regarding all untreated samples, also no significant diversity detected by a Wilcoxon rank test for dependent samples) but a LEfSe analysis revealed that the genera *Cupriavidus, Mesorhizobium, Peredibacter, Pantoea, Slackia, Turicibacter, Enterococcus, Collinsella*, and *Solirubrobacter* were significantly stronger represented in the incubated samples (log 10 LDA scores > 3). The incubation experiment did not succeed in increasing the relative abundance of sequences attributed to spore forming genera.

Independent of the incubation treatment, the dust samples of the two analysis sets were most similar to their origin (ANOVA, p=0.0046) and according to the LEfSe analysis each sample had a variety of individual genera as shown in Figure 16. PMA treatment significantly lowered the detected diversity

compared to the correspondent untreated samples (Shannon index; ANOVA p=0.0072*)* but also significantly improved the detection of intact representatives of the genera *Staphylococcus, Methylobacterium* and *Cupriavidus* according to a LEfSe evaluation (see Figure 17). This is in accordance with the cultivation results, as isolates of these three genera were also retrieved. Several genera were also significantly stronger represented in the untreated samples compared to the PMA treated samples which was expected as PMA blocks free DNA for downstream reactions such as PCR. Most likely the representatives of these genera did not exist in a viable state within the vacuum cleaner bag and dust filter samples.

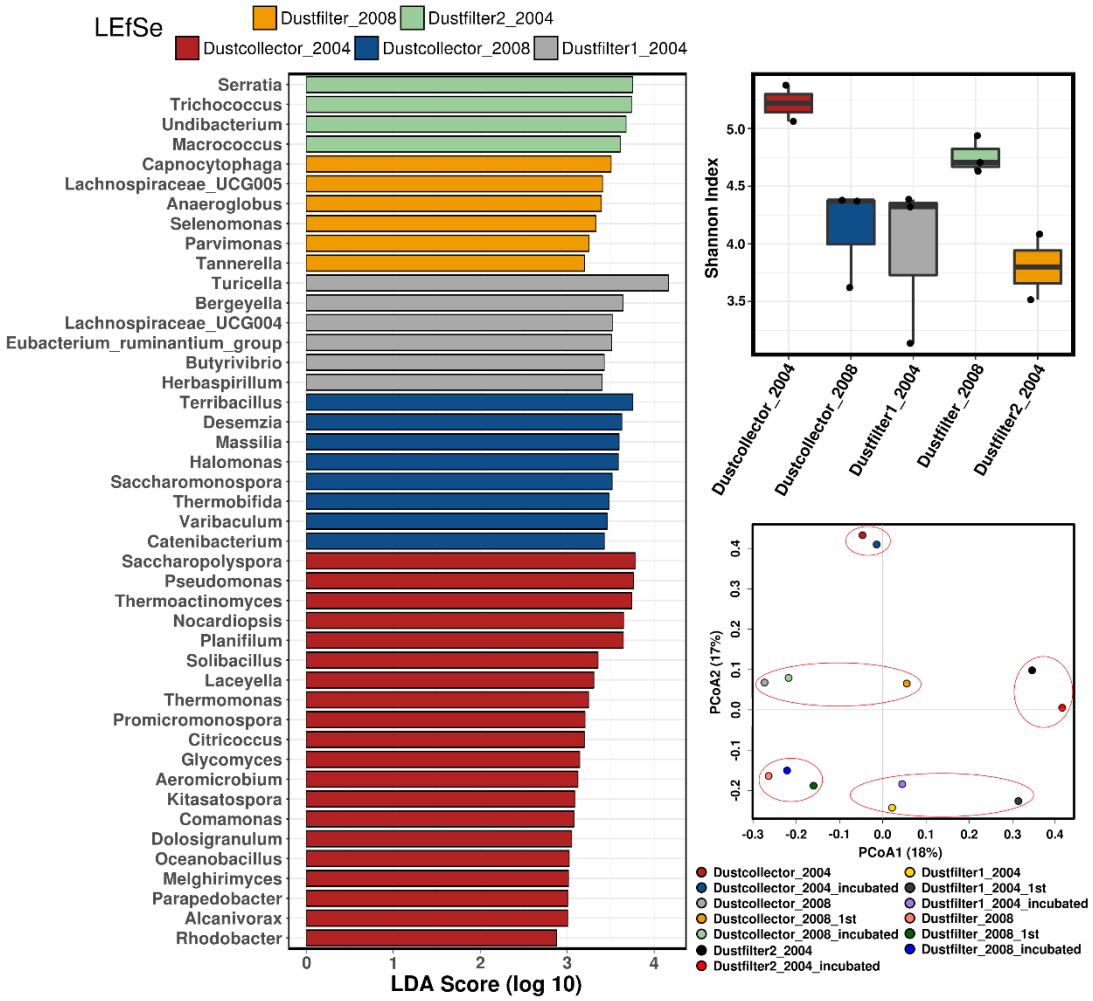

Figure 16: Comparison of incubated vacuum cleaner and dustfilter samples with untreated samples. On the left are the genera which were found to have a significantly different representation by LEfSe. The comparison of their Shannon indices on the right top shows that the detected diversity was significantly dependent on the origin of the samples (ANOVA, p=0.0046). The PCoA plot (Bray-Curtis index) on the bottom right also shows that the samples of the same original vacuum cleaner bag/dust filter tend to cluster together. Red circles highlight samples of the same origin.

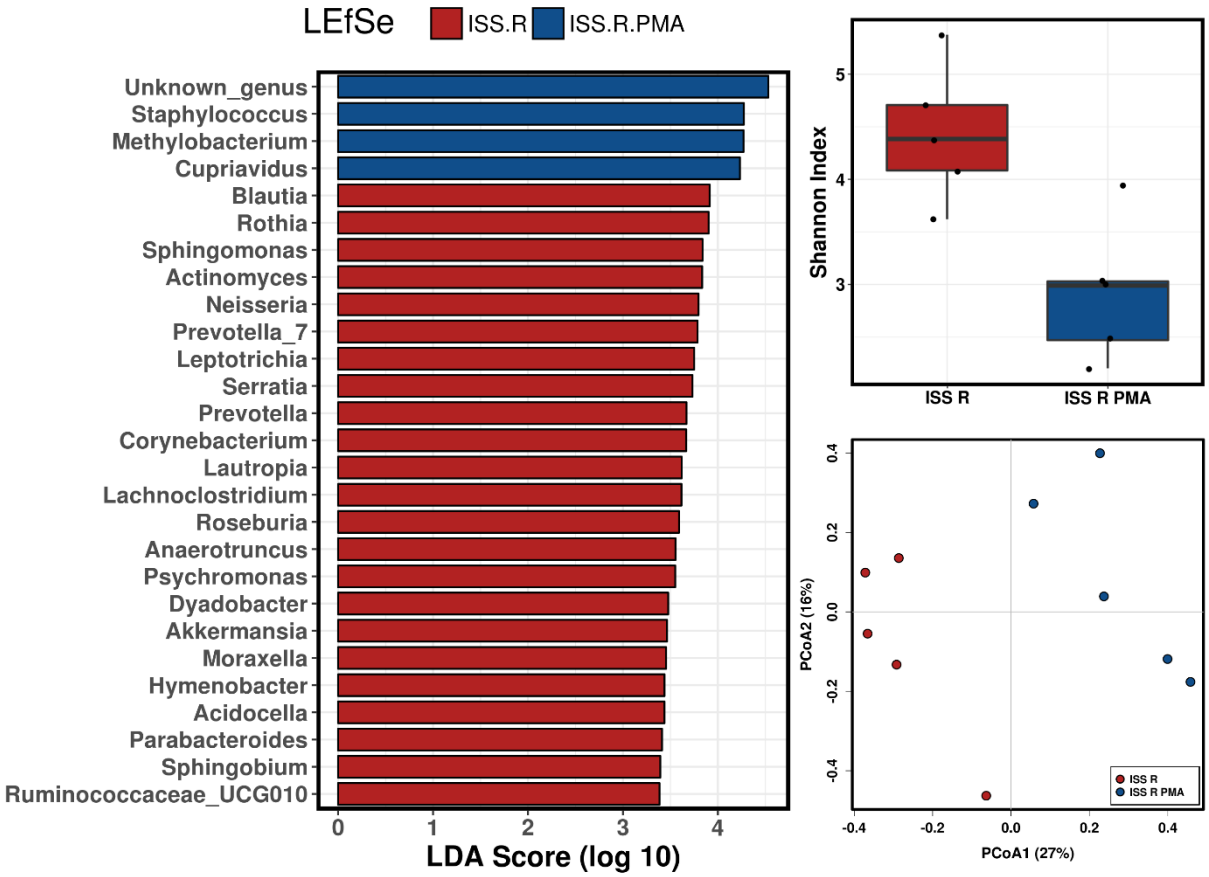

Figure 17: Comparison of the PMA treated vacuum cleaner and dustfilter samples with their correspondent untreated samples from the second analysed set. On the left are the genera which were found to have a significantly different representation by LEfSe. The comparison of their Shannon indices on the right top shows that the detected diversity was significantly lowered by the PMA treatment (ANOVA, p=0.0072). The PCoA plot (Bray-Curtis index) on the bottom right shows segregation of the samples into two clusters according to the treatment.

III.2.4 Microbial diversity detected by the Archaea-targeting approach

One of the main goals of the ARBEX project was, as the name "Archaea and Bacterial Extremophiles on board the ISS" implies, to prove the existence of Archaea on board the ISS. For this purpose, the extracted DNA of ISS samples was analysed with the universal primer pair F515+R806, targeting Bacteria as well as Archaea and additionally with the Archaea-targeting primer pairs Ar349fwd+Ar519rev and Ar519fwd+Ar785rev in a nested approach to amplify the 16S rRNA gene of rare Archaea which were expected to be overshadowed by Bacteria in the universal approach. The Russian dust filter and vacuum cleaner samples were only analysed with the Archaea-targeting primer pair Ar349fwd+Ar519rev in the 1st sample set and the session A, B, and C samples were analyzed with both Archaea-targeting primer pairs. However, primer pair Ar349fwd+Ar519rev did not detect Archaea in the session A, B, and C samples. Overall, 19 of 30 ISS samples were Archaea positive by at least one of these approaches (see Table 11).

As mentioned before, the universal approach detected Archaea in 13 samples: All five Russian dust filter and vacuum cleaner samples, the "SSC Laptop Columbus" and "Sleeping unit Node2" samples from

session A and B, C1 - “Ambient air Cupola”, B1 - “Ambient air Columbus”, B4 - “Hand grips Columbus”, and B7 - “Panels Node2” (see Figure 9 and Table 11).

The Archaea-targeting approach detected archaeal signatures in 12 samples: All Russian dust filter and vacuum cleaner samples with the exception of RISS4 – “Dust filter-2 2004” as well as in both “Audio Terminal unit Columbus” samples from session A and B, A2 - “SSC Laptop Columbus”, B4 - “Hand grips Columbus”, C2 - “Surface Cupola”, C5 - “WHC surfaces Node3”, C7 - “Dining table Node1”, C9 - “contaminated wipe” (see Table 11 and Figure 18). When Archaea were detected by the Archaea-targeting approach, the samples were generally dominated by single taxa, which rendered statistical analyses or visualizing relative abundances impractical. Instead, the phylogenetic relations of the detected RSV´s across all samples are given in Figure 11 together with their read count values as a tentative proxy for the individual RSV abundances.

Table 11: All archaeal signatures detected via 16S rRNA gene amplification in the ISS samples. For archaeal signatures detected via the universal approach, their relative abundance value in comparison with the detected bacterial signatures is given in percent. For the Archaea-targeting approach, only positive (+) or negative (-) detection is shown.

Sample		**Universal approach**	**Archaea-targeting approach**
RISS1 - Dust filter-1 2004	1st	39.1 %	+
	2nd	<0.1%	
	PMA	0.3%	
	incubated	<0.1%	
RISS2 - Dust collector 2008	1st	0.7%	+
	PMA	<0.1%	
RISS3 - Dust filter 2008	1st	<0.1%	+
	2nd	<0.1%	
	incubated	<0.1%	
RISS4 - Dust filter-2 2004	PMA	0.7%	-
	incubated	0.6%	
RISS5 - Dust collector 2004	2nd	<0.1%	+
	PMA	0.4%	
	incubated	<0.1%	
A2 - SSC Laptop Columbus		2.1%	+
A6 - Sleeping unit Node2		4.9%	-
A8 - Audio terminal unit Node2		-	+
B1 - Ambient air Columbus		3.6%	-
B3 - SSC Laptop Columbus		5.2%	-
B4 - Hand grips Columbus		2.1%	+
B6 - Sleeping unit Node2		<0.1%	-
B7 - Panels Node2		2.7%	-
B8 - Audio terminal unit Node2		-	+
C1 - Ambient air Cupola		14.1%	-
C2 - Surface Cupola		-	+
C5 - WHC surfaces Node3		-	+
C7 - Dining table Node1		-	+
C9 - contaminated wipe		-	+

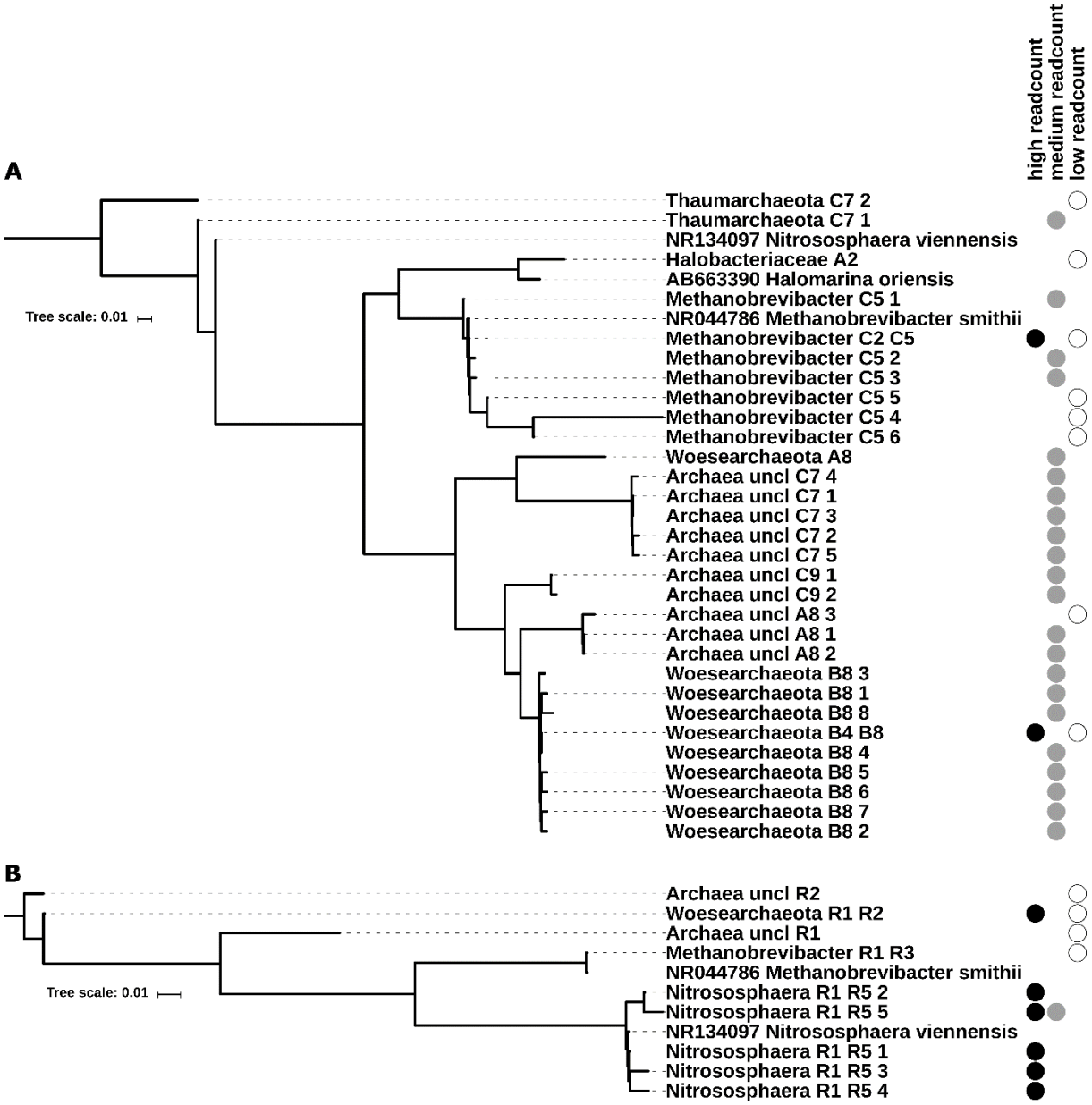

Figure 18: Maximum likelihood trees of the archaeal sequences detected by the Archaea-targeting 16S rRNA amplicon assays. A: Archaeal sequences detected by Ar519fwd+Ar785rev (reproduced from Mora *et al.*, 2019); **B:** Archaeal sequences detected by Ar349fwd+Ar519rev**. high read count:** >10k reads**; medium read count:** 100-10k reads; **low read counts:** <100 reads**.** AB663390 *Halomarina oriensis*, NR044786 *Methanobrevibacter smithii* and NR134097 *Nitrososphaera viennenis* are the sequences of type strains of the closest cultured neighbours and were not detected in this study.

While these results show, that Archaea are definitely present on board the ISS, Archaea detection across all samples and methods was inconsistent to a certain extent as specified in detail in the following passages and summarized in Table 11.

The sleeping unit in Node2 was Archaea positive for session A and B considering the universal approach, but sample A6 had 168/3,455 reads classified as Woesearchaeota whereas sample B6 had 13/21,959 reads of an unclassified archaeon. No Archaea could be detected in both samples via the Archaea-targeting approach.

The SSC Laptop in Columbus module was also Archaea positive for session A and B considering the universal approach, and in both samples the obtained RSVs were classified as Woesearchaeota (A2:

68/3,201 reads; B3: 386/7,445 reads), but the Archaea-targeting approach did not detect Archaea in B3 and solely 24 reads classified as euryarchaeotic *Halobacteriaceae* in A2.

The Audio terminal unit in Node2 was Archaea negative for session A and B considering the universal approach but Archaea positive for both sessions considering the Archaea-targeting approach. Sample A8 yielded 1,144 archaeal reads of which 219 were classified as Woesearchaeota and the rest were unclassifiable Archaea. Sample B8 was the sample which yielded the most reads via the Archaea-targeting approach (106885) and all of these were classified as Woesearchaeota.

The Hand grips in Columbus module were Archaea positive only for session B but via the universal as well as the Archaea-targeting approach. The universal approach detected Woesearchaeota (201/15,621 reads) and euryarchaeotic Thermoplasmaceae (131/15,621 reads) whereas the Archaea-targeting approach detected only Woesearchaeota (30 reads).

The ambient Air in Columbus module (B1) and in Cupola, and the panels in Node2 (B7) were Archaea positive via the universal approach for session B only and Archaea negative via the Archaea-targeting approach. In sample B1 310/8630 reads were classified as Woesearchaeota and 2/8630 reads were classified as euryarchaeotic *Methanobrevibacter*, in sample C1 141/1002 reads were classified as euryarchaeotic *Methanobrevibacter*, and in sample B7 140/5,241 reads were classified as Woesearchaeota.

The Surface in Cupola (C2), the waste and hygiene compartment surfaces in Node3 (C5), the dining table in Node1 (C7) and the wipe contaminated on board ISS (C9) were Archaea positive only via the Archaea-targeting approach. The detected reads In C2 (71 reads) and C5 (30,775 reads) were classified as *Methanobrevibacter*, in C7 unclassified Archaea (7,389 reads) and Thaumarchaeota (6,942 reads) were detected, and in C9 all 3,168 reads were unclassifiable Archaea.

Only the 1st set of the Russian dust filter and vacuum cleaner samples was analysed with the Archaea-targeting approach and was positive for RISS1, RISS2, RISS3, and RISS5, while RISS4 and all PMA treated versions of these samples were Archaea negative. Sample RISS1 yielded 71,203 archaeal reads of which 71,143 reads were classified as *Nitrososphaera* (Thaumarchaeota) 42 reads were classified as Woesearchaeota, 14 reads were classified as *Methanobrevibacter*, and 4 reads were unclassifiable Archaea. Sample RISS2 yielded 29,548 reads classified as Woesearchaeota as well as 52 reads of unclassifiable Archaea, RISS3 yielded 82 reads classified as *Methanobrevibacter*, and RISS5 yielded 102,782 reads classified as *Nitrososphaera* (Thaumarchaeota). This is in accordance with the data obtained via the universal approach of the 1st sample set, where RISS1 yielded 6,272/16,033 reads classified as *Nitrososphaera* (Thaumarchaeota), RISS2 yielded 58/8,181 reads of which 35 reads were classified as Woesearchaeota and 23 reads were classified as *Nitrososphaera*, and RISS3 yielded 13/28,296 reads classified as genus *Methanobrevibacter*. However, there is a huge divergence in comparison with the data obtained via the universal approach of the 2nd sample set regarding archaeal detection: RISS1 yielded only 5/11,640 sequences classified as *Nitrososphaera*, RISS5 only 7/17,278 reads classified as *Methanobrevibacter*, and RISS2 yielded no archaeal reads at all. RISS4, which was

Archaea negative again, and RISS3 with 6/21,963 reads classified as *Methanobrevibacter* were similar to the 1st sample set. Surprisingly, the universal approach detected Archaea also in the PMA treated samples of the 2nd sample set. In detail these were RISS1-PMA (13/3,736 reads classified as *Nitrososphaera*), RISS2-PMA (5/33,616 reads classified as *Methanobrevibacter*), RISS5-PMA (49/12,382 archaeal reads of which 36 reads were classified as *Halococcus* and 13 reads were classified as *Methanobrevibacter*), and even in RISS4-PMA which was Archaea-negative in the other approaches (12/1,753 reads classified as Woesearchaeota).

The incubated dust filter and vacuum cleaner samples were of little interest regarding archaeal detection, as this approach was meant to ease DNA extraction from bacterial spores. For this reason the incubated samples were also not analysed with the Archaea-targeting approach. For sake of completeness it shall be mentioned that the universal approach detected Archaea in all incubated samples except RISS2. In RISS1 6/23,050 reads of unclassifiable Archaea were detected and all other detected archaeal reads were classified as *Methanobrevibacter* (RISS3: 24/47,442 reads; RISS4: 45/7,361 reads; RISS5: 22/39,524 reads).

III.3 Metagenomic approach for Archaea detection

The purified DNA of sample RISS1 Dust filter-1 2004 in the 1st sample set was chosen for a shotgun sequencing metagenomic approach because of its unexpectedly high proportion of archaeal sequences obtained by the 16S rRNA gene sequencing approach. Because the initial amount of DNA available did not meet the minimum requirements for sequencing (<0.2 ng), it was whole genome amplified via multiple-displacement amplification before shot gun sequencing. Sequencing yielded 1,394,991 raw paired end reads of a length of 35-301 basepairs. After removing all adapter sequences, which represented about 40% of the total reads, and quality filtering the dataset consisted of 705,679 high quality paired end sequences of a length of at least 50 bp. The assembly resulted in 31,753 contigs with a total length of 11,846,018 bp. The average length per contig constituted 373 bp (min: 200 bp; max: 48,514; N50: 359 bp). Binning of this data did not yield an evaluable result (1,3% complete bacterial genome according to CheckM). However, analysing the contigs with Amphora2 showed that the assembled data set contained 25 thaumarchaeal marker genes. The three longest contigs containing a thaumarchaeal marker gene (960 bp, 507bp, and 557bp) were subjected to a standard nucleotide BLAST (Internet reference 6; Altschul *et al.*, 1990) and surprisingly shared 100% sequence identity with the genome of the *Nitrososphaera viennensis* type strain. Because of this high sequence identity, all filtered high quality reads were matched against the type genome of *N. viennensis* and all aligned forward and reverse reads (1.62% of all filtered reads) were merged. This resulted in 11,256 archaeal sequences with an average length of 200 bp (min: 50 bp; max 497 bp). Checking this “artificial bin” with Amphora and CheckM revealed 53 thaumarchaeal marker genes according to Amphora and 46 archaeal marker genes according to CheckM with the estimation of 29.75% complete archaeal genome. Unfortunately, these

sequences could not be further assembled to an incomplete archaeal genome, but as all these sequences were identical with the *N. viennensis* genome it can be assumed that the Thaumarchaeota we detected on board the ISS are *N. viennensis* or a close relative thereof.

III.4 Genome analysis of ISS isolates

Six isolates obtained from the Russian dust filter and vacuum cleaner samples were selected for genome sequencing to check if these ISS microorganisms have specific genomic characteristics which distinguish from Earth based isolates. These isolates were *Bacillus pumilus* strain pH7_R2F_2_A, *Bacillus safensis* strain pH9_R2_5_I_C, *Bradyrhizobium viridifuturi* strain pH5_R2_1_I_B, *Cupriavidus metallidurans* strain pH5_R2_1_II_A, *Methylobacterium tardum* strain pH5_R2_1_I_A, and *Paenibacillus campinasensis* strain pH9_R2IIA. Their assemblies were compared to publicly available genomes on the MicroScope – Microbial genome annotation & Analysis platform (Internet reference 4).

All the antibiotic resistances inferred from the detected antibiotic resistance genes (ARG) of these six isolates are summarized in Figure 19. Both *Bacillus* isolates had the same ARG-profile and *C. metallidurans* and *B. viridifuturi* had the highest numbers of ARG´s. Most of their ARG´s were annotated to multidrug exporter genes which increased the number of inferred antibiotics resistances even further. Not all of the antibiotics listed in Figure 19 were also tested by the antibiotic susceptibility test, but those which were tested conformed largely with the results detected in the genomes with the exception of some discrepancies which are discussed in chapter IV.7.

The strain-specific-genomes of the ISS Isolates comprised from 315 (*B. safensis* strain pH9_R2_5_I_C) to 4219 (*P. campinasensis* strain pH9_R2IIA) annotated genes. An overview of the pangenomic comparison with public available reference genomes is given in Figure 20. The strain-specific-genomes have been thoroughly examined with regard to genes which would indicate a higher resistance potential of the ISS isolates. However, almost no genes were found which would render the ISS Isolates more resistant to ISS relevant stressors than their compared ground control strains. No virulence has been reported for the six sequenced species up to date and also in this study no indications have been found in this regard.

The individual assessments of the genomes were published in Mora et al. 2019 as follows:

“The genome of *Bacillus pumilus* strain pH7_R2F_2_A was retrieved 99.59% complete, with a %GC of 41.6. The overall genome length was 3.7 Mbp. *Bacillus pumilus* SAFR-032 (3.7 Mbp, 41.3 %GC; ENA study ID: PRJNA20391), whose genome was analysed for comparative reasons as well, possessed the same antibiotics resistance capacity. The ISS strain possessed all necessary genes for flagellum assembly and CAS-TypeIIIB (with cmr5_TypeIIIB missing); the latter was not found in *Bacillus*

pumilus SAFR-032. Looking at the metabolic profiles, the ISS isolate of *Bacillus pumilus* (comparison to SAFR-032 and ATCC 7061 (3.8 Mbp, 41.7 %GC; ENA study ID: PRJNA29785) possessed the genomic capacity to perform choline and methionine degradation, but no other peculiarities were identified.

The genome of *Bacillus safensis* strain pH9_R2_5_I_C was found to be 99.59% complete, with a %GC of 41.5. The overall genome length was 3.7 Mbp. It possessed all necessary genes for flagellum assembly and CAS-TypeIIIB, as did next neighbour *Bacillus safensis* FO-36b. Looking at the metabolic profiles, the ISS isolate of *Bacillus safensis* (comparison to CFA06 (3.7 Mbp, 41.5 %GC; ENA Study ID: PRJNA246604) and FO-36b (3.7 Mbp, 41.6 %GC; ENA Study ID: PRJNA270528) did not show certain peculiarities.

The genome of *Bradyrhizobium viridifuturi* strain pH5_R2_1_I_B was found to be 99.96% complete, with a %GC of 64.3. The overall genome length was 7.9 Mbp. The genome carried several copies of the efflux pump membrane transporter BepG as well as other multidrug efflux transporters and β-lactamase genes, which largely explained the overall stable antibiotic resistance observed in our experiments. The observed resistances against linezolid and vancomycin could not be directly inferred from the genomic data. These features were also found in *Bradyrhizobium viridifuturi* SEMIA 690 (8.8 Mbp, 64.0 %GC; ENA Study ID: PRJNA290320), the next phylogenetic neighbour. Overall, the genetic features of our ISS isolate were widely similar to known *Bradyrhizobium* species"(Mora *et al.*, 2019). The strain-specific-genome of *B. viridifuturi* strain pH5_R2_1_I_B contained several genes coding for copper and silver ion resistance, cold and heat shock proteins, and a modular protein for DNA protection during starvation. The latter might partly explain how this strain was able to survive for 10-12 years in dry dust samples without being able to form spores and the variety of heat shock proteins explains how this isolate was able to survive the heat shock test applied in this study. Heat shock proteins have also been reported for *B. japonicum,* another species of this genus which was not investigated in this study (Münchbach *et al.* 1999).

"The genome of *Cupriavidus metallidurans* strain pH5_R2_1_II_A was found to be 99.94% complete, with a GC content of 63.7 %. The overall genome length was 6.9 Mbp. This strain carries three bepE efflux pump membrane transporters, and also a multidrug efflux system protein (acrB). However, the bepE efflux pumps were not detected in the genome of its closest relative *C. metallidurans* CH34. The genome showed full potential for type IV pili and flagella formation and numerous secretion systems, but this was not a unique feature for the ISS strain. With respect to the metabolic profile, *C. metallidurans* strain pH5_R2_1_II_A showed a number of different features when being compared to the next relatives (*C. basilensis* OR16, ENA Study ID: PRJNA79047; *C. metallidurans* CH34, ENA Study ID: PRJNA250; *C. necator* N-1, ENA Study ID: PRJNA67893; *C. taiwanensis* LMG19424, ENA Study ID: PRJNA15733), which included the predicted capacity for 5,6-dimethylbenzimidazole biosynthesis and butanediol degradation/synthesis.

The genome of *Methylobacterium tardum* strain pH5_R2_1_I_A was found to be 100% complete with a GC content of 69.2%, and a total genome length of 6.5 Mbp. Also *M. tardum* pH5_R2_1_I_A carried the efflux pump membrane transporter BepE and the genetic capacity for flagellum formation and several secretion systems. The strain showed a number of differential features when we compared the genomic potential with other members of the genus (*M. extorquens* DSM13060, ENA Study ID: PRJNA62143*; M. mesophilicum* SR1.6/6*,* ENA Study ID: PRJNA175757; *M. nodulans* ORS2060*,* ENA Study ID: PRJNA20477; *M. radiotolerans* JCM 2831, ENA Study ID: PRJNA18817; 5,6-dimethylbenzimidazole biosynthesis, base-degraded thiamine salvage, cytidylyl molybdenum cofactor biosynthesis, L-dopachrome biosynthesis); however, it shall be noted, that another genome of the species was not available at the time of analysis.

The genome of *Paenibacillus campinasensis* strain pH9_R2IIA could be retrieved with a 99.84% completeness. It showed a GC content of 52.26%, and a genome length of 5.4 Mbp. The genome revealed a potential for lincosamide (clindamycin), macrolide (clarithromycin), fluorquinolone (moxifloxacin, levofloxacin, ciprofloxacin), and glycopeptide (vancomycin) resistance which could all be verified by the antimicrobial susceptibility tests with the exception of the vancomycin resistance (no PK/PD breakpoint in the EUCAST Table). Nevertheless, the observed MIC for vancomycin was 4 µg/ml, which was [one of] the highest observed MIC for vancomycin besides the seven isolates which were completely resistant [see Table 9]. The genome did not show any β-lactam resistances but in spite of this, *Paenibacillus campinasensis* strain pH9_R2IIA was resistant against all β-lactam antibiotics with the exception of meropenem in the antimicrobial susceptibility tests. The strain showed the potential for flagella and pilus formation, and the presence of CAS type III. At the time of the analysis there was no other genome of this species publically available, but the metabolic potential was not found to be strikingly different from other genome-sequenced members of the *Paenibacillus* genus"(Mora *et al.*, 2019) (*Paenibacillus polymyxa* ATCC15970, ENA Study ID: PRJNA277390; *Paenibacillus dendritiformis* C454, ENA Study ID: PRJNA80855; *Paenibacillus elgii* B69, ENA Study ID: PRJNA65565; *Paenibacillus terrae* HPL-003, ENA Study ID: PRJNA67295). Furthermore, *Paenibacillus campinasensis* strain pH9_R2IIA carried genes for heat shock proteins (Hsp33 and Hsp20), catalases, and a variety of general stress proteins and DNA-damage and repair proteins in its strain-specific-genome which may be of assistance in coping with the extreme conditions on board the ISS. However, similar genes were also found in the strain-specific-genomes of the other *Paenibacillus* species with the exception of catalase genes which may provide a certain resistance against hydrogen peroxide-based cleaning agents.

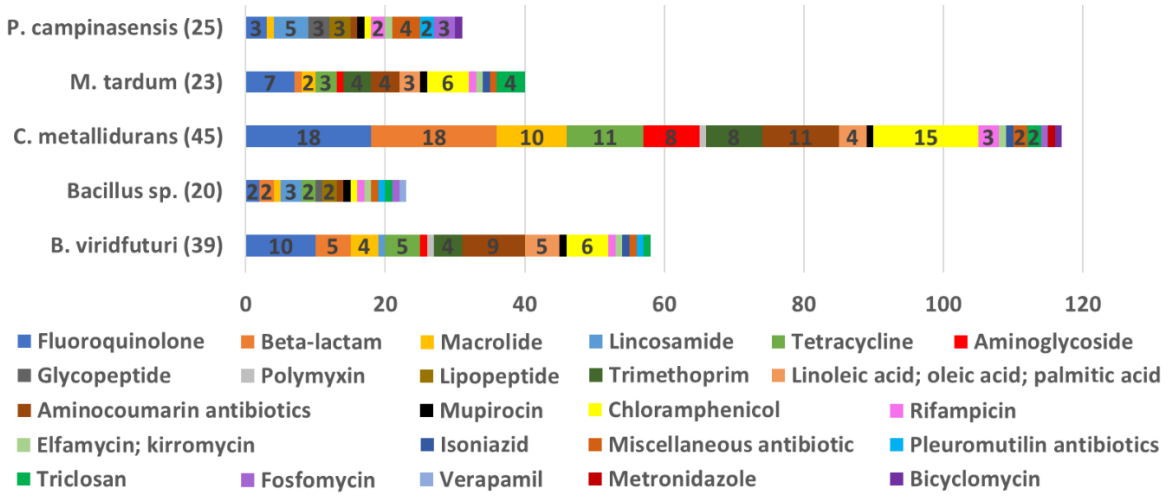

Figure 19: Summary of the antibiotic resistance genes (ARG) detected in the sequenced genomes. The number behind a species name is the total number of detected ARG in the respective genome and the colored columns show the groups of antibiotics against which the detected ARG are known to provide resistance. Numbers within a colored column indicate how many of the detected ARG provide resistance against this type of antibiotic; no number equals to 1. As some ARG, especially the multidrug efflux pumps which were found in a high number in *C. metallidurans* and *B. viridfuturi* may be able to provide resistance against multiple kinds of antibiotics, the sum of the numbers within the colored columns may exceed the total number of detected antibiotic resistance genes per organism. The number of ARG as well as of inferred antiobiotic resistances was the same for the sequenced *Bacillus* genomes, which is why they are summarized as one *Bacillus sp.* in this graph (reproduced from Mora *et al.*, 2019).

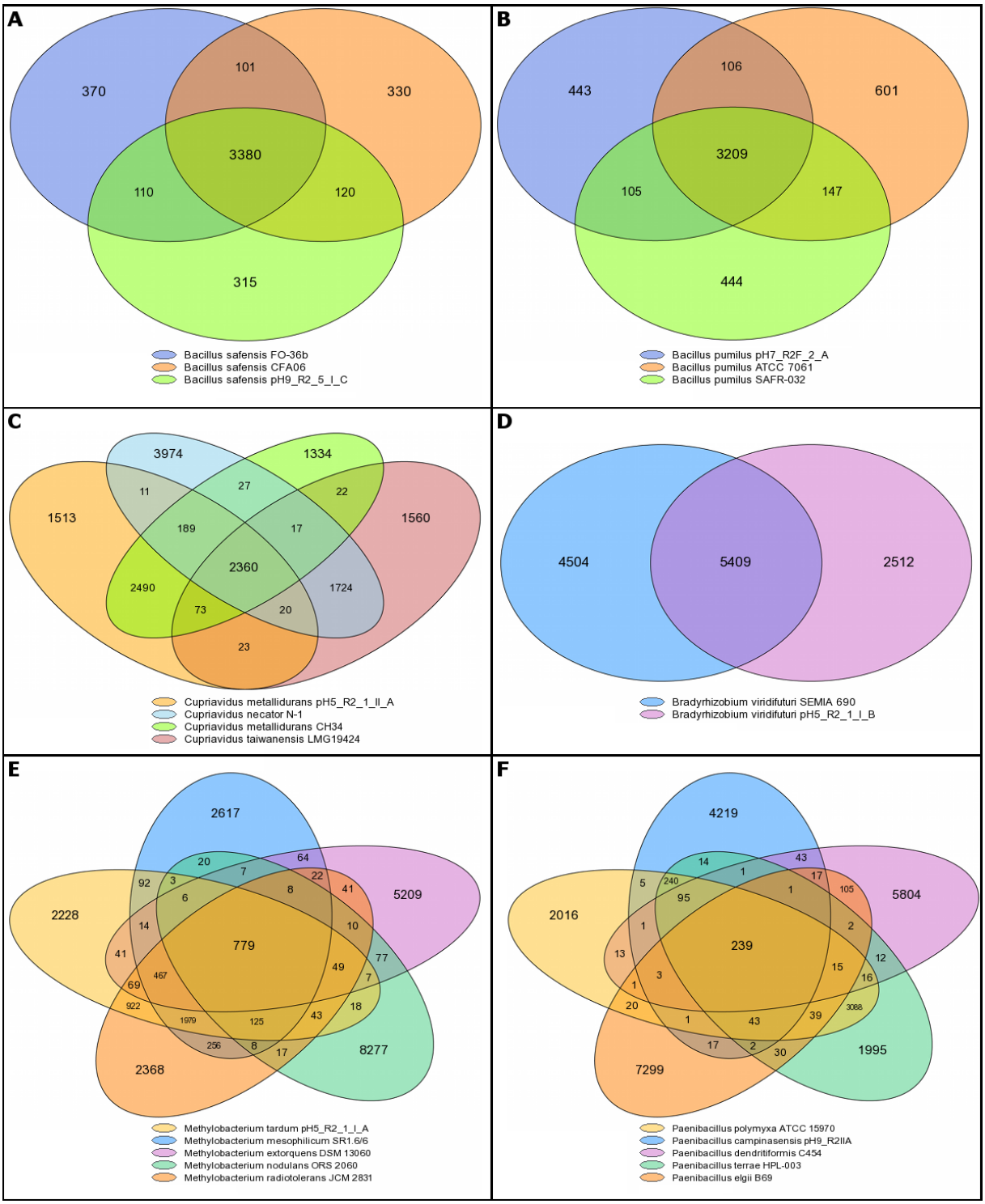

Figure 20: Pangenomic overview of the sequenced genomes and public available genomes of close neighbours: A: *Bacillus safensis* strain pH9_R2_5_I_C **B:** *Bacillus pumilus* strain pH7_R2F_2_A **C:** *Cupriavidus metallidurans* strain pH5_R2_1_II_A **D:** *Bradyrhizobium viridifuturi* strain pH5_R2_1_I_B **E:** *Methylobacterium tardum* strain pH5_R2_1_I_A **F:** *Paenibacillus campinasensis* strain pH9_R2IIA.

IV Discussion

The International Space Station (ISS) is a unique and extreme-situated habitat for the human crew and co-inhabiting microorganisms. This thesis is based on the ARBEX project (ARchaea and Bacterial Extremophiles on board the ISS) which included: 1) The sampling of an ESA clean room in Kourou as ground control and method validation 2) the analysis of 10-12 years old dust samples from the Russian part of the ISS, and 3) an ISS flight project named "EXTREMOPHILES". During this thesis the complete ISS flight project was conducted, from adjusting the original experiment design from 2008, planning implementation of the project on board the ISS together with ESA and BIOTESC, to the actual sampling sessions on board ISS by astronaut Jack D. Fischer during the increments 51 and 52 (2017), and the analysis of the samples at the Medical University of Graz. It was possible to assess the molecular detectable as well as the cultivable microbial diversity on board the ISS and to investigate it with regard to spatial and temporal parameters. Special focus hereby was set on the evaluation of the ISS microbial community with regard to its potential of withstanding extreme parameters and with regard to the diversity and abundance of Archaea on board the ISS. Furthermore, the antibiotics-resistance potential of chosen isolates was evaluated by antibiotic susceptibility testing and genome sequencing, and the microbial behavior of ISS isolates towards ISS-relevant materials was investigated with regard to potential biofilm formation on these materials and potential degradation or corrosion of these materials.

IV.1 General Diversity of the ISS microbiome

The ISS indoor microbiome is known to be strongly influenced by the human microbiome which has already been shown in several studies before (Venkateswaran *et al.*, 2014; Yamaguchi *et al.*, 2014; Checinska *et al.*, 2015; Mora, Mahnert, *et al.*, 2016; Mora, Perras, *et al.*, 2016; Lang *et al.*, 2017). The data obtained in this study are in that regard in accordance with earlier 16S rRNA amplicon studies on the ISS indoor microbiome. The universal 16S rRNA amplicon approach showed a high presence of human associated bacteria throughout all ISS samples. These included, but are not limited to: *Acinetobacter, Corynebacterium, Escherichia/Shigella, Faecalibacterium, Propionibacterium, Pseudomonas, Staphylococcus, Stenotrophomonas,* and *Streptococcus.* All of these genera are often found on human skin, in oral and nasal caves, or the gut (Grice *et al.*, 2008; Grice and Segre, 2011; Huttenhower *et al.*, 2012; Segata *et al.*, 2012). The genus *Propionibacterium* has recently been reclassified into several other genera (Scholz and Kilian, 2016), including *Cutibacterium* of which we also obtained isolates during this study. Due to this taxonomic reclassification, it can be expected that the relative abundance of detected signatures classified as *Propionibacterium* in future indoor or human microbiome studies will be observed to seemingly decrease in proportion when reference databases get updated in this regard.

The data obtained in this study further support the finding from Lang *et al.* 2017 that the genus *Staphylococcus* does not utterly dominate the ISS microbiome as described in Venkateswaran *et al.* 2014. As this genus contains several opportunistic pathogens, such as *Staphylococcus aureus* (Tong *et al.*, 2015), the lower proportion of *Staphylococcus* sequences may be advantageous with regard to potential health risks of the ISS crew. Whether these contradicting data between different studies are based on methodical differences or maybe due to a better microbial control management on board the ISS after the findings of Venkateswaran *et al.* 2014 is not known. The high presence of human associated bacteria confirms the indoor airborne microbial community of the ISS to be to a major extent derived directly from the ISS crew, as also discussed in other studies (Hospodsky *et al.*, 2012; Checinska *et al.*, 2015). Because Lang et al. 2017 also reported that the ISS microbiome composition is very similar to mechanically ventilated hospital environments, the results obtained in this study were compared to a recent hospital microbiome study (Lax *et al.*, 2017). Indeed, all top 20 genera described in the hospital study were also detected in the ISS microbiome and most of them even in high relative abundance. Only the genus *Enhydrobacter* was not detected by our molecular approach, but an *Enhydrobacter aerosaccus* isolate was obtained from the Columbus RGSH sample in session B. The human associated genera *Actinomyces, Aerococcus, Bacteroides, Blautia, Enterobacter, Gemella, Granulicatella, Haemophilus, Propionibacterium (Cutibacterium), Stenotrophomonas,* and *Veillonella,* were relatively higher abundant in the ISS or even specific for the ISS as they were not in the top 20 hospital list (Mora *et al.*, 2019).

A different picture was obtained from the cultivable diversity of the ISS microbial community. Although some human associated isolates were obtained, most isolates represented hardy (spore-forming) environmental organisms (Figure 2). All human associated isolates were human skin associated microorganisms, whereas typical gut associated microorganisms could not be retrieved by cultivation. However, as most of the cultivation approaches used in this study were designed to target rather extremotolerant environmental microorganisms (with the exception of the methanogen approach) this was also not unexpected. Most gut associated microorganisms would need very rich medium and a slightly higher incubation temperature of 37°C instead of 32°C. This discrepance between the cultivation- and molecular-based microbial community analysis emphasizes again the non-comparability of these two methods (Mora *et al.*, 2019). Nevertheless, the dominance of Firmicutes which was detected by the molecular approach is also reflected in the retrieved isolates to some extent: The genera *Bacillus, Paenibacillus, Micrococcus, and Staphylococcus* were ubiquitously retrieved. However, the high abundance of these genera (especially of *Bacillus* sp.) is not reflected on the genus level of the molecular data (see Figure 3 & Figure 9). This can be explained by the fact that it can be more difficult to isolate DNA from bacterial spores because these tend to resist the cell lysis part of DNA extraction methods, as also discussed in another ISS study on the ISS microbiome, where no *Bacillus* sequences were retrieved at all (Venkateswaran *et al.*, 2014). For this reason, in this work it was attempted to trigger the germination of spores by incubation in warm medium before DNA

extraction with an aliquot of the Russian dust-filter and vacuum cleaner samples, but also this approach did not yield a higher relative abundance of *Bacillus* sequences.

Generally, molecular analysis was able to detect a much wider diversity than the cultivation approaches, which was completely expected, as only about 1% of all microorganism are estimated to be cultivable from a certain habitat (Amann *et al.* 1995; Stewart, 2012) and the 16S rRNA gene analysis also covers uncultivable organisms. However, with the broad cultivation approach conducted in this study it was also possible to cultivate organisms which were overlooked by the molecular analysis. On the ISS, the bacterial genera *Curtobacterium, Glaciihabitans, Enhydrobacter, Risungbinella, Spirosoma, Thermaerobacter,* and *Variovorax* of which we obtained isolates in this study were not detected at all by the molecular approach. This was also the case for the ESA clean room isolates of the genera *Aerococcus, Arthrobacter, Azospirillum* and *Caulobacter*. Other ISS isolates were detected in the complete sample set but not in the specific respective sample which yielded the isolate. Amongst others, for example the genera *Bosea* and *Brevundimonas* were isolated from session B samples but detected only in session C samples by molecular methods. *Cohnella* isolates were obtained from session A and C but this genus was detected only in session B by molecular methods and there exist similar cases also for the clean room and Russian dust samples. These examples show that neither a molecular approach nor a cultivation approach can cover the complete microbial (i.e. bacterial) diversity of a specific environment. However, these different methods, while not being really comparable to each other, complement each other very well and give a more comprehensive picture of the microbial diversity in a given sample or environment when combined than any of these methods could give on their own. Whether a combination of molecular detection and cultivation actually elucidates the complete microbial diversity of a given environment is another question and very dependent on the actual sampling technique, the primers which are used for molecular detection, and the amount and combination of different media which are used for cultivation. Nevertheless, a polyphasic approach as done in this work definitely has more informative value as studies which are based solely on cultivation (e.g. Novikova *et al.*, 2006) or molecular detection (e.g. Checinska *et al.*, 2015; Ichijo *et al.*, 2016) and is hereby recommended for comparable future studies.

IV.2 Temporal and spatial differences of the ISS microbiome

The ISS microbiome was not stable with regard to composition and diversity. This was especially shown by the cultivation approach since only four of 84 isolated species were found in all samples of sessions A, B, and C (*Micrococcus yunnanensis, Bacillus hunanensis, Penicillium chrysogenum*, and *Staphylococcus epidermidis*). The comparison of session A and B regarding the molecular data (Figure 13) shows that the microbial composition of the ISS microbiome changes already significantly within the timespan of one increment. Only 57 of the 367 genera detected by the molecular approach in session

A, B, and C were constantly present in all samples. However, 56 of these genera, with the exception of *Finegoldia*, were already present in the 529 genera detected in the 10-12 years Russian dust and vacuum cleaner samples. This suggests that 10%-15% of the detectable genera comprise a well-established ISS core microbiome which is stable also over a decade while the rest of the ISS microbiome is subject to a constant change and fails to properly settle down in this extreme environment. The ten most abundant genera of this core microbiome were again all human associated: *Acinetobacter, Anaerococcus, Bacteroides, Corynebacterium, Gemella, Haemophilus, Lactobacillus, Staphylococcus, Stenotrophomonas,* and *Streptococcus*.

In spatial terms, the microbial composition does not differ much throughout the ISS, as there was no statistically significant difference observable between the samples of different modules. Most likely this is caused by the astronauts who are constantly moving between different modules and thereby evenly distribute the ISS microbiota. Since particulates within the ISS keep floating in Air for a long time because of microgravity, they are easily distributed throughout the ISS. However, specific spatial microbial patterns could be identified for some of the individual sampling locations of session A and B, as shown in Figure 15. In particular the association of Thaumarchaea with the intimate sleeping unit is pointed out here, since this supports the theory that the Thaumarchaea on board are human derived, as also discussed below (IV.5). In general, these spatial microbial patterns reflect the observations of Ruiz-Calderon et al. on terrestrial indoor environments that indoor surfaces reflect space use and also show an increased content of human associated microbial signatures with increasing urbanization (or less environmental influence) (Ruiz-Calderon *et al.*, 2016). With the exception of the signals detected in the sleeping unit, which might differ because it is a place for individual retreat, also the Node 2 samples seem to be more similar to each other than to the Columbus samples according to Figure 15, but this was found to be not statistically significant. Furthermore, aforementioned correlations of Figure 15 were also largely in agreement with the cultivation results. For example, *Deinococcus* sp. was (only) isolated from Node2_Panel_Outside, the location with strongest correlation do the Deinococcus-Thermus phylum.

The Archaea-targeting 16S rRNA amplicon assay was, however, not in accordance with these spatial microbial patterns detected by the universal approach. It did not detect signatures of Euryarchaeota, but of Woesearchaeota on the Columbus handrails and did not detect signatures of Thaumarchaea in the sleeping unit or of Woesarchaeota on the Columbus SSC laptop. But the Archaea-targeting assay detected another spatial peculiarity: The second most abundant archaeal sequences of all EXTREMOPHILES flight project samples were obtained from the waste and hygiene compartment (WHC). These archaeal signatures were solely comprised of several gut associated *Methanobrevibacter* RSV´s (Figure 18). The WHC also contains NASA´s space toilet and this finding is in compliance with personal experience reports of several astronauts, who report that, due to toilet malfunctions, feces are sometimes distributed in the immediate vicinity of the toilet (Internet reference 7 + 8). Such mishaps

naturally lead to increased signatures of gut associated microorganisms in nearest surroundings. These big differences between the universal and archaeal molecular approach reflect the difficulties of detecting Archaea in general, and are due to the fact that different primer sets tend to detect different Archaea as also described elsewhere (Pausan *et al.*, 2018).

The cultivation assays were not suited to infer a spatial distribution pattern (Figure 3) which is mainly due to the fact that more samples from a certain module lead to a broader isolate diversity and the Columbus module and Node 2 were sampled more often than the other modules. However, it has to be noted that the cultivable diversity in Node 1 was unexpectedly very low. Node 1 includes the dining area and food waste bin, which implies a bigger nutrient availability for growing microbes. The reason for the low cultivable diversity from Node 1 might be because the chosen sample surfaces were not optimal to reflect the Node 1 diversity: One of the two sampled areas in Node 1 is a "low interaction" surface behind a wall panel and the second, theoretical "high interaction" surface, is the dining table which might be cleaned more often than other surfaces within the ISS.

IV. 3 Diversity of extremophilic and extremotolerant microorganisms on board the ISS

The ISS indoor environment is characterized by low nutrient availability for microorganisms, regular cleaning with antimicrobial agents and uniquely extreme but stable conditions such as higher radiation impact than on Earth, microgravity and a constant vibration which in combination are expected to exert a constant selection pressure on the microbial population on board (Taylor, 2015; Mora, Mahnert, *et al.*, 2016). Due to this selection pressure, one of the main hypotheses of the ARBEX project was, that the indoor environment of the ISS is expected to carry various extremophilic or extremotolerant microbes, and that also the mesophilic bacteria found on board the ISS would be more extremotolerant than their Earth-based representatives. Several extremotolerant and some extremophilic bacteria were discovered among the isolates. However, they only constitute a minor part of the ISS microbiota according to the molecular analyses, since the microbial diversity detected using molecular techniques was dominated by human associated bacteria. It could not be verified that bacterial strains isolated from the ISS have a higher extremotolerance than Earth-based strains of the same species.

Most of the obtained isolates had a variety of extremotolerant properties and were able to grow at at pH of 5 or lower and/or at pH of 9 or higher as well as at a relatively wide temperature range (see Figure 2 and Table 10). With the exception of *Bradyrhizobium viridifuturi* pH5_R2_1_I_B, all tested ISS isolates were able to grow at pH 9 or higher, which might be due to a selection pressure caused by alkaline cleaning reagents used on board the ISS. More than half of all obtained isolates belonged to the spore forming genera *Bacillus, Brevibacillus, Paenibacillus* and *Cohnella* which are known to be resistant against a variety of extreme influences, including desiccation, extreme temperatures, UV and γ-

radiation, high pressure, and toxic chemicals (Setlow, 2014). However, also non spore forming isolates were able to survive heat shocks and the maximum applied intensities of UV-C and X-ray irradiation (see Table 7 and Table 10). All isolates from the Russian dust filter and vacuum cleaner samples can be considered desiccation tolerant as they survived for more than 10 years in dry dust (Mora, Perras, *et al.*, 2016). Additionally, three real extremophiles - two thermophilic isolates (*Thermaerobacter litoralis, T. composti*) and one halophilic isolate (*Salinibacillus aidingensis*) - were acquired from the ISS samples. These findings were unexpected, as the ISS indoor environment is neither very hot nor saline at the sampled locations. *S. aidingensis* has originally been isolated from a saline lake (Ren and Zhou, 2005) and it has also been detected in human stool samples (Endesfelder *et al.*, 2016) although the authors did suspect an erroneous taxonomic classification. Therefore *S. aidingensis* might also be of human origin on board the ISS in spite of not being a typical human associated bacterium. *T. litoralis* was originally isolated from a coastal hydrothermal field (Tanaka *et al.*, 2006) and *T. composti* from compost which can achieve temperatures of over 80°C by microbial-self-heating during fermentation (Yabe *et al.*, 2009). Based on this information, a possible explanation would be that this *T. litoralis* isolate obtained from the ISS might have a similar physiology than the *T. composti* isolate (although they do have only 98.0% 16S rRNA gene sequence identity) and that both originate from a space bioconversion device for organic matter. However, for now this remains only speculation. While it is known that NASA is interested in developing a space bioconvertor for more than 10 years (Internet reference 9), no publicly available information was found, if such a device has actually been installed already on the ISS. All waste is supposed to be sealed in individual bags and then loaded into cargo transporters which either bring the waste back to ground or burn on reentry into the Earth´s atmosphere.

The presence of these extremotolerant and extremophilic isolates indicates that the ISS environment supports the selection of microorganisms best-adapted to the partially extreme physical and chemical environmental conditions. However, it was not possible to confirm the null hypothesis that strains obtained from the ISS are more extremotolereant/extremophilic than closely related strains from Earth regarding the upper and lower boundaries of their temperature and pH growth ranges. The boundaries for pH and temperature determined in this study were in most cases broader than reported for the respective reference strains (if reported), but in most species descriptions of these boundaries are not extensively defined (see Table 10). Consequently, drawing conclusions about the effect of extreme conditions on board ISS by comparing with literature values is not reliable. The *Bradyrhizobium* and *Staphylococcus* reference strains included in our tests did not differ considerably from their ISS counterparts regarding pH and temperature growth ranges. Moreover, different ISS strains of the same species showed also varying growth range profiles (e.g. *B. nealsonii*) which leads to the conclusion that a study comparing several ISS and Earth strains of the same species would be needed to properly assess this question. However, all tested strains were able to grow in a broad temperature range which should also cover unexpected temperature fluctuation on board the ISS, which is generally kept at 22°C-24°C

(Coil et al., 2016) (T. Alekhova personal communication), or a spacecraft in general. With the exception of *Bradyrhizobium viridifuturi* pH5_R2_1_I_B, all tested ISS isolates were able to grow at pH 9 or higher, which is in most case higher than reported for the respective reference strains (if reported). This might be due to a selection pressure caused by alkaline cleaning reagents used on board the ISS.
The complete genomes of isolates *Bacillus pumilus* strain pH7_R2F_2_A, *Bacillus safensis* strain pH9_R2_5_I_C, *Bradyrhizobium viridifuturi* strain pH5_R2_1_I_B, *Cupriavidus metallidurans* strain pH5_R2_1_II_A, *Methylobacterium tardum* strain pH5_R2_1_I_A, and *Paenibacillus campinasensis* strain pH9_R2IIA were shot gun sequenced to check if these ISS microorganisms have specific genomic characteristics which differ from terrestrial isolates. However, even the genomic inventory of whole genome sequenced ISS isolates did not show any indications that the ISS isolates differ from closely related Earth isolates when compared to their respective publicly available genomes. This is in accordance with a recently published pangenome study stating that genomes of ISS strains do not differ much from genomes of Earth based built environment strains of the same species (Blaustein *et al.*, 2019). Nevertheless, it is likely that gene expression patterns of microorganisms change in response to the external stressors on board the ISS (Taylor, 2015), but an investigation thereof is methodically challenging as RNA is very unstable und would ideally needed to be purified and sequenced during actual space flight.

Taking all these results together, it can be concluded that ISS microorganisms are not more extremotolerant or resistant than their ground relatives, neither with respect to growth behavior, nor antibiotics resistances, nor their genomic inventory. They might temporarily adapt their gene expression profile to better cope with the ISS environment during actual space flight - which still needs to be proven - but if these changes happen, they are quickly reversed when returning to Earth.

A recent study reported the detection of 16S rRNA genes of bacterial genus *Delftia* on the outside of the ISS (Grebennikova *et al.*, 2018). This raises the question if these signatures might have originated from the ISS indoor microbiome. However, no *Delftia* isolate was obtained within this study and while one *Delftia* RSV was detected in all samples, it was also present in all negative controls. For this reason, the data presented here can neither rule out nor verify the presence of *Delftia* within the ISS. It has to be stressed that Grebennikova et al. 2018 also performed negative controls which were reportedly free of *Delftia* signatures.

IV.4 Novel ISS isolates

Describing a new bacterial species is no trivial task and not possible by using solely the 16S rRNA gene sequence information. Nevertheless, the 16S rRNA gene sequence information is an excellent indicator to estimate if an isolate might potentially be a new species. It is still debated which sequence similarity cutoff should be used to have the highest probability of identifying a novel species without obtaining

too many false positive results, especially as these cutoffs vary between different genera (Rodriguez-R *et al.*, 2018). The classical cutoff based on comparisons with DNA-DNA hybridization experiments is 97% (Stackebrandt and Goebel, 1994) whereas a more recent comparison based on average nucleotide identity over the complete 16S rRNA gene sequence suggests a higher cutoff of 98.65% (Kim *et al.*, 2014). As the 16S rRNA sequences obtained in this study are not full-length sequences, a cutoff of 98.5% is used here as also discussed in Rodriguez-R et al., 2018. Most of the obtained isolates could be assigned to already described microorganisms. However, 13 of the unique isolates obtained in this study qualify to comprise possible novel, hitherto undescribed bacterial species as their 16S rRNA gene sequence similarity to their respective closest described neighbor was below 98.5%. These isolates are: Several *Paenibacillus* sp. (*Paenibacillus durus* 95.26%; *Paenibacillus tarimensis* 95.54%; *Paenibacillus tritici* 96.41%), *Dyadobacter* sp. (*Dyadobacter sediminis* 95.56%), *Spirosoma* sp. (*Spirosoma lacussanchae* 95.7%), *Glaciihabitans* sp. (*Glaciihabitans tibetensis* 97.7%), and *Paracoccus* sp. (Paracoccus chinensis 98.23%) from ISS samples, and *Caulobacter* sp. (*Caulobacter mirabilis* 97.2%), several *Mesorhizobium* sp. (*Mesorhizobium huakuii* 97.6%; *Mesorhizobium loti* 98%), and several *Methylobacterium* sp. (*Methylobacterium platani* 96.61%; *Methylobacterium dankookense* 98.14%) from clean room samples. Additionally, the isolate with the largest similarity gap, ISS isolate R9_B3_IA *Planococcaceae* incertae sedis (*Planococcus halocryophilus* 87.36%) might even comprise a new genus. It has to be kept in mind that these are only assessments based on their 16S rRNA genes and that there are still future experiments necessary to verify these assumptions.

IV.5 Archaea on board the ISS

Life on Earth can be divided into three domains: Eukarya, Bacteria and Archaea. Archaea resemble Bacteria in shape, size and organization, but since their discovery in the 1970s (Woese and Fox, 1977) it became obvious that their genetic makeup, biochemistry and structure is very different. Archaea possess unique cell walls, cell surface appendages, (co-) enzymes and metabolic pathways (Woese *et al.* 1990) . A wide range of extremophilic Archaea (like hyperthermophiles and methanogens) have been cultivated successfully and studied extensively (Stetter, 1999). Archaea were for long erroneously considered as extremophiles per se, requiring extreme environments for growth and thriving exclusively in very special habitats. Consequently, they were considered as residues from early Earth conditions. Later, analyses using molecular methods have shown that Archaea can be found in every ecological niche explored so far: e.g. in lakes, rivers, soil, fresh water springs, rice fields, the sea, and on glaciers (Bintrim *et al.*, 1997; Großkopf *et al.* 1998; Pernthaler *et al.*, 1998; Battin *et al.*, 2001; Zarsky *et al.*, 2013). Archaea have also been found in human intestines, mouth cave, nose, lung, and on skin, but our knowledge about these human associated Archaea remains limited, and their role is currently unclear (Horz and Conrads, 2011; Hulcr *et al.*, 2012; Probst *et al.* 2013; Koskinen *et al.*, 2017; Pausan *et al.*,

2018). Furthermore, Archaea have also been found in spacecraft assembly clean rooms (Moissl *et al.* 2008; Moissl-Eichinger, 2011), where they are most likely of human origin.

When the ARBEX project was originally devised in year 2009, nothing was known about the presence of Archaea on board the ISS, and to demonstrate that Archaea can also exist and persist in this spacecraft environment was one of the main goals of the project. Although it is now known that Archaea are also human associated and could theoretically also reach the ISS via cargo transports which are loaded in spacecraft assembly clean rooms, Archaea have still not been considered as a significant contributor to the microbiome that inhabits the ISS. Nevertheless, recent studies did report the detection of archaeal signatures on board the ISS but did not further discuss the meaning of their existence in this environment (Lang *et al.*, 2017; Singh *et al.*, 2018).

In this study, archaeal signatures were detected in 19 of the 30 ISS samples by the combination of universal and Archaea-targeting 16S rRNA amplicon approaches (see Table 11). The relative abundance of detected archaea was relatively low compared to the bacterial signatures, but that was expected as Archaea, although known to be a part of the human microbiome and surrounding environments appear in most cases in a lower relative abundance than Bacteria, roughly by a factor of ten (Bang and Schmitz, 2018; Moissl-Eichinger *et al.*, 2018). According to the data presented here, archaeal signatures were found to be good markers for most surfaces with regular human contact (see Figure 15). The most abundant archaeal signatures on surfaces were Thaumarchaea, Woesearchaea and euryarchaeal *Methanobrevibacter*, which all have previously been attributed to the human microbiome (Probst *et al.* 2013; Koskinen *et al.*, 2017; Pausan *et al.*, 2018). Therefore, the Archaea detected on board the ISS are likely shed by the human occupants. However, as the human associated Thaumarchaea and Woesarchaea have not yet been successfully cultivated or sequenced, the knowledge about their ecology and growth requirements remains elusive. *Methanobrevibacter* species are strictly anaerobic and do not survive for longer than 6h in an aerobic milieu (Pausan *et al.* unpublished). This explains why our archaeal cultivation assays were not successful. Obtaining an archaeal isolate from the ISS might however be possible by anaerobic *in situ* cultivation.

Because of the expected low total biomass, it was decided not to split the extraction fluid obtained from session A, B, and C samples for additional PMA-analysis. Nevertheless, the PMA analysis of the second batch of Russian dust filter and vacuum cleaner samples showed that even in 10-12 years old ISS samples there were still intact (assumingly alive) archaeal cells (see Table 11 and Figure 9), although the PMA analysis of the Archaea-targeting approach of these samples was negative.

The highest proportion of archaeal signatures was detected in sample RISS1 “Dust filter-1 2004” (39.1%) which was therefore also subjected to metagenomic analysis to study the functional potential and phylogenetic position of these archaea in more detail. The fact that so many metagenomic reads of sample RISS1 “Dust filter-1 2004” were exact matches to the *Nitrososphaera viennensis* genome, and most of the 16S rRNA gene sequences obtained from Russian dust and vacuum cleaner samples could be attributed to the genus *Nitrososphaera* (Figure 18 B), validates the conclusion that the most likely

human associated Thaumarchaea on board the ISS are actually *N. viennensis* or close relatives thereof. Unfortunately, due to the low biomass of the original sample, the metagenomic dataset was very small with only 1,394,991 paired raw reads and therefore not sufficient to bin genomes. Analysis of the metagenomic dataset was additionally impeded as whole genome amplification (WGA) via multiple displacement amplification (MDA) is known to be prone to introducing coverage shifts and possible chimeric sequences although it is still the most reliable WGA method (Pinard *et al.*, 2006). This is most likely also the reason, why the archaeal sequences could not be binned in the first approach. The functional potential was not further analysed, as all sequences obtained in the end belong to *N. viennensis* reference genome.

N. viennensis was originally isolated from garden soil (Tourna *et al.*, 2011). The presence of soil microorganisms on board the ISS is scarce in comparison with human associated microorganisms. Considering this difference in prevalence and the fact that the presence of Thaumarchaea was correlated with the sleeping unit in the ISS, it is highly probable that the Thaumarchaea detected were also human associated, and therefore possibly related to the Thaumarchaea previously detected on human skin (Probst *et al.* 2013).

IV.6 Clean rooms as possible contamination source

Clean rooms are particulate controlled facilities which ensure quality and safety of many production processes as reviewed in detail in Mora, Mahnert et al 2016 (Mora, Mahnert, *et al.*, 2016). Of importance regarding the ISS microbiome is, that all crew resupply vehicles which transport cargo to the ISS are loaded in spacecraft assembly clean rooms, which are known to select for hardy microbes (Moissl-Eichinger *et al.*, 2013; Vaishampayan *et al.*, 2013). Some of these harsh clean room conditions, e.g. mechanical ventilation combined with air particle filtering, low nutrient availability and strong cleaning agents, resemble the conditions on board the ISS. Therefore, it can be expected that clean room derived microorganisms are able to settle down in the ISS indoor environment if they are transported to the ISS together with crew resupply cargo. For this reason, an ESA clean room (S5C clean room in Kourou, French Guiana) was sampled in the same way as the ISS samples of session A, B, and C, and additionally K. Venkateswaran from NASA´s Jet Propulsion Laboratory at the California Institute of Technology (JPL), USA, provided us with isolates from a North American clean room housing a crew resupply vehicle (CRV). These samples and isolates were used as ground control references to assess if the ISS microbiome is influenced by clean room microbiota.

The microbial composition of the sampled ESA clean room harboring ISS cargo was found to be different from the ISS microbiome. The microbial diversity detected in clean rooms was significantly lower than observed in ISS samples and clustered separately in multivariate analyses indicating to be different from the ISS samples (Figure 12). The amount of detected genera in the ESA clean room

samples was an order of magnitude smaller than in the ISS samples. One reason for this might be of course the fact that far less clean room samples were sequenced in this study than ISS samples, but it has to be kept in mind that operating clean rooms are also stricter biocontrolled than the ISS (Mora, Mahnert, *et al.*, 2016). The clean room microbiome was specifically characterized by a predominant abundance of signatures of α- Proteobacteria such as *Novosphingobium*, *Sphingomonas* and *Methylobacterium* whereas most ISS samples were dominated by signatures of Firmicutes and Actinobacteria (Figure 9). This observation is in concordance with a previously published comparison of dust from NASA modules of the ISS and a NASA clean room (Checinska *et al.*, 2015) although some of the detected genera in the clean rooms vary which might be because of the big spatial separation of the NASA clean room in Pasadena, California, USA, and the ESA clean room in Kourou, French Guyana, South America. A similar difference can be observed when comparing the cultivable diversities of the ESA clean room and the CRV clean room, as most obtained isolates were distinct for each of the clean rooms. Previous publications have also shown that spacecraft assembly clean rooms are influenced to a certain extent by their local surroundings and that for this reason e.g. ESA and NASA clean rooms harbour distinct microbiota (Stieglmeier *et al.*, 2012; Moissl-Eichinger *et al.*, 2013). However, the difference observed here is additionally influenced by the fact that the media used to obtain the CRV isolates were also less diverse than the media used for the ESA clean room isolates. Unfortunately, there is no available molecular data available for the CRV clean room which could be compared with the ESA clean room or the ISS data.

Based on the molecular 16S rRNA gene comparison, it seems that the cargo deliveries from terrestrial clean rooms to the ISS are most likely not a (relevant) ISS indoor microbiome source.

However, a different picture was obtained, when looking at the cultivable diversity retrieved from all ISS samples and both clean rooms: When comparing all non-redundant ISS isolates with all non-redundant clean room isolates, an overlap of two fungal isolates, namely *Aspergillus unguis* and *Rhodotorula mucilaginosa*, and 15 bacterial isolates, namely *Bacillus aerophilus, Bacillus cereus, Bacillus nealsonii, Bacillus pumilus, Bacillus subtilis Brevibacterium frigoritolerans, Kocuria palustris, Methylobacterium tardum, Micrococcus aloeverae, Micrococcus yunnanensis, Paenibacillus sp., Pseudomonas psychrotolerans, Ralstonia insidiosa, Staphylococcus epidermidis* and *Staphylococcus saprophyticus* was found. Furthermore, the bacterial species *Bacillus horneckiae, Bacillus safensis*, and *Paenibacillus pasadenensis* obtained from the ISS in this study are classical, frequently detected spacecraft assembly clean room contaminants which have originally been described in this setting (Osman *et al.* 2006; Satomi *et al.* 2006; Vaishampayan *et al.*, 2010). The human associated *Staphylococcus* and *Micrococcus* species were more likely introduced by humans into both environments than transported via cargo from clean rooms to the ISS. Nevertheless, this comparison shows that it is very likely that clean room associated microorganisms have been transported onto the ISS and established themselves as a part of the ISS microbial community. However, also based on the

total cultivable diversity they comprise only a minor fraction of the ISS microbial community (Mora *et al.*, 2019). It has to be mentioned that all samples obtained from the inside of the ATV cargo transporter in the ESA clean room were exceptionally clean. No bacterial isolate was obtained by cultivation and no detectable DNA was obtained from these samples. The only obtained isolate, indicating that a microbial transport from clean rooms to the ISS might be possible, was a fungal *Alternaria alternata* isolate.

IV.7 Antibiotic resistance potential and health risks

Resistance against antibiotics can be seen as an extremotolerant trait with impact on human health. As discussed previously the ISS microbiome composition resembles microbiome compositions in medical care environments, which are known to harbour increasing proportions of multiresistant bacteria (Dennesen *et al.* 1998; Hu *et al.*, 2015). It is known that antibiotics producing microorganisms are able to produce more antibiotics during spaceflight stress (Taylor, 2015) which may in turn promote selection for antibiotics resistant ecological competitors in a confined environment such as the ISS. Indeed, recent publications came to the alarming conclusion that the prevalence of antibiotics resistance genes on board the ISS is rising (Singh *et al.*, 2018; Urbaniak *et al.*, 2018). Furthermore, it has been shown for rifampicin resistance in *Staphylococcus*, that already a short term stay in space can trigger the development of antimicrobial resistance (Fajardo-Cavazos and Nicholson, 2016). To address this prominent problem within this study, 37 isolates obtained during this study and three reference strains were selected and tested against 17 clinically relevant antibiotics with regard to their antimicrobial susceptibility (see Table 9). The isolates chosen for the antimicrobial susceptibility tests included the six isolates which genomes were sequenced and these six sequenced genomes were also specifically analysed with regard to their antibiotic resistances to evaluate if predicted resistances conform with actually observed resistances and compared to public genomes to evaluate if the ISS strains are more resistant than terrestrial strains.

Based on the antimicrobial susceptibility tests (see Table 9), antibiotic resistance/susceptibility was in some cases strain-specific: for example, the high clarythromycin resistance of the *Staphylococcus arlettae* Zvesda isolate, the complete clindamycin resistance of *Bacillus clausii* strain pH9_R2_5_IIB, or the cefotaxime sensitivity of *Bacillus nealsonii* strain R9_B8_IIIB. The majority of the observed resistances however, was species or even genus specific and also independent from their isolation source (ISS or ground control reference). For example, all *Bradyrhizobium* strains were resistant against almost all tested antibiotics. The ISS strains were not significantly more resistant (number of antibiotics or concentration) than their terrestrial counterparts and there was also no noteworthy difference between ISS strains from 10-12 years old dust and recent isolates from session A, B, and C.

Most of the isolates were resistant against cephalosporins ceftriaxone and cefotaxime which should in theory be efficient against Gram-positive as well as Gram-negative bacteria by inhibiting murein cross-linking and thereby cell wall synthesis (Table 4). Half of the tested isolates belonged to the genus *Bacillus*. *Bacillus* species, in particular *B. anthracis* due to its clinical importance, have been previously reported to be rather resistant against cefotaxime and ceftriaxone (Doğanay and Aydin, 1991; Mohammed *et al.*, 2002; Turnbull *et al.*, 2004) which explains the relatively high amount of resistant *Bacillus* isolates against these antibiotics in this study. However, four of the *Bacillus* isolates tested here were not resistant against ceftriaxone (*B. safensis* R10_A9_IIIB, *B. subtilis* pH5_R2_2_I_A, and *B. nealsonii* strains pH7_CW1_HS0.2A and R9_B8_IIIB) and one was not resistant against cefotaxime (*B. nealsonii* R9_B8_IIIB) which shows that these resistances are not inherent to all *Bacillus* strains.

In particular the ISS isolates *Bradyrhizobium viridifuturi* pH5_R2_1_I_B and *Roseomonas nepalensis* C63 showed a vast resistance against numerous antibiotics. Both of these isolates are not pathogenic and therefore their high resistance profile poses no direct threat to the ISS crew. However, a non-pathogenic multiresistant strain could be problematic if it acts as a reservoir for resistance genes, e.g. on a long-term spaceflight or in other closed environments on Earth. Signatures of the *Bradyrhizobium* genus are also found in other restricted environments such as in clean rooms (Vaishampayan *et al.*, 2013) or hospitals (Oberauner *et al.*, 2013; Soto-Giron *et al.*, 2016) where they are generally not considered to pose a problem. However, in a theoretical worst-case scenario, antibiotic resistances could be transferred to (opportunistic) pathogens by horizontal gene transfer, which would enhance for example the risk of multiresistant hospital acquired infections. A transfer of antibiotic resistance genes to (opportunistic) pathogens could also pose a problem in long-term space flights considering the impaired immune systems of humans in space.

The most interesting antibiotics with regard to the ISS indoor microbiome were amoxicillin (without clavulanic acid), ceftriaxone, ciprofloxacin, clindamycin, doxycycline, levofloxacin, sulfamethoxazole/trimethoprim, and moxifloxacin, which are also ingredients of the ISS medical inventory and can thus be used for treatment of bacterial infections aboard (Mora, Perras, *et al.*, 2016).. In this study, the environmental isolates from ISS were susceptible to amoxicillin (with clavulanic acid), ciprofloxacin, doxycycline, levofloxacin, sulfamethoxazole/ trimethoprim, and moxifloxacin. We further confirmed that ceftriaxone is not very effective against *Bacillus* sp., but it was effective against most of the other isolates. There exist no non-species-specific breakpoints for clindamycin in the EUCAST Table but when the measured clindamycin MICs are compared to breakpoints defined for other species (see rationale in III.1.4), 27 of 40 isolates (67.5%) could also be rated potentially resistant against clindamycin.

The resistances observed in the antibiotic susceptibility tests indicate that the antibiotics already present in the ISS medical inventory should provide a sufficient arsenal to battle infections, assuming that a similar resistance profile is shared by potential pathogens. However, all tested isolates were non-pathogenic and these results should therefore not be used for clinical risk assessment.

The antibiotic resistance genes (ARG) detected in the genomes of six isolates conformed for the most part with the results from the antimicrobial susceptibility tests. The broad resistance profile of *Bradyrhizobium* species can be explained by a vast amount of multiple drug exporter systems in their genomes. However, there were also some discrepancies: Both *Bacillus* strains possessed genes for the transcription-repair coupling factor mfd and the efflux transporter blt which should provide resistance against fluorquinolones. Still, *Bacillus pumilus* strain pH7_R2F_2_A was only resistant against moxifloxacin and not against ciprofloxacin or levofloxain, while *Bacillus safensis* strain pH9_R2_5_I_C was sensitive against all tested fluorquinolones. *C. metallidurans* was unharmed by the lincosamide clindamycin and the oxazolidinone linezolid and grew at the maximal tested concentrations of these antibiotics, but these resistances could not be inferred from the ARG´s. Furthermore, *C. metallidurans* was sensitive to all fluorquinolones in spite of possessing several efflux transporter genes from which a resistance against fluorquinolones can be inferred. It was also sensitive to all beta-lactam antibiotics besides penicillin G in spite of possessing genes for beta-lactamase AmpC which is a specialized cephalosporinase and infers resistance against the tested antibiotics cefotaxime and ceftriaxone. However, it is known that these two cephalosporins, while being sensitive to AmpC, are only weak inducers for actual AmpC expression (Jacoby, 2009).
Overall, the data presented here show that the molecular detection of antibiotic resistance genes, while being a good approximation of the resistance potential of an organism or microbial community, does on the one hand overestimate the antibiotic resistance potential because some resistance factors might not be expressed at all, and on the other hand it does not necessarily cover all antibiotic-resistances which a microorganism actually has. This is another example that molecular investigations should be coupled with traditional cultivation methods to get an impression of the full picture of a given microbiome (Mora *et al.*, 2019). As already mentioned in the chapter IV.3, the recent pangenome-based observation (Blaustein *et al.*, 2019), that ISS microorganisms are not necessarily more resistant than their ground-based relatives was also confirmed for antibiotic resistances in this study.

With the cultivation approaches, in total 11 ISS isolates belonging to biosafety risk group S2 were obtained during this study. “However, nine of these are generally human associated bacteria which have only opportunistic pathogenic potential. Especially in the light of a weakened human immune system in space conditions, the presence and abundance of such opportunistic pathogens has of course to be carefully monitored, but as these do thrive in and on the human body and are shed into the environment by the crew itself, such opportunistic pathogens will always exist in the vicinity of humans and their presence per se is not alarming. The two non-human associated S2 rated isolates were *Pseudomonas putida* isolated from the RGSH in Node2 and isolates of the *Bacillus cereus/anthracis/thuringensis* clade isolated from the RGSHs in Node2 and Columbus, from the hand grips in Columbus, and from the sleeping unit in Node2. *P. putida* is an environmental opportunistic pathogen with a low virulence that may cause infections via open wounds or catheters (Fernández *et al.*, 2015) and the presence of *B. cereus/anthracis/thuringensis* within the ISS is already known for several years and a recent study

showed that ISS isolates of this clade generally lack toxin-producing plasmids (Venkateswaran *et al.*, 2017)." (Mora *et al.*, 2019). None of the fungal isolates obtained from ISS samples were classified S2 in this study, but some of the *Penicillium* isolates, *Rhodotorula mucilaginosa*, and *Rhizopus stolonifera* are known to be able to induce allergic reactions according to GESTIS (see Table 6). This might be of importance because allergic reactions of astronauts on board the ISS are reported roughly once per year (Crucian *et al.*, 2016). However, the isolates with pathogenic potential found in this study, do not give reason for elevated concern and the ISS can still be considered a microbially safe working and living environment (Mora *et al.*, 2019).

IV.8 Microbial surface interaction with ISS materials

Some microorganisms are known pose a risk to the material integrity of a spacecraft. Especially biofilm formation may pose a threat for spacecraft materials with the potential to induce instrument malfunctioning and therefore compromise mission success. So-called technophilic microorganisms, are able to corrode alloys and polymers used in spacecraft assembly, either by direct interaction with the surfaces or passively by producing corrosive metabolites. This has mainly been shown for fungi, but also for bacterial *Bacillus* isolates from the ISS (Alekhova *et al.*, 2005, 2010, 2015). Technophilic microorganisms caused major problems on the former Russian space station MIR (Novikova *et al.*, 2001; Novikova, 2004) and the same species have already also been found on the ISS, although in a very low abundance (Alekhova *et al.*, 2008).

With the aim to investigate if the ISS strains isolated in this study also have technophilic potential, three isolates were incubated together with untreated and eloxated metal platelets consisting of aluminum alloy EN AW 2219 which was also used for the construction of the Cupola (Thales Alenia Space, personal communication), and with pieces of NOMEX® fabric which is widely used on board the ISS (mainly for storage purposes; BIOTESC, personal communication). The chosen isolates for this experiment were classified as *Bacillus paralicheniformis, Cupriavidus metallidurans,* and *Cutibacterium avidum*. *B. paralicheniformis* was selected because *Bacillus* species are known to possibly have technophilic potential (Alekhova *et al.*, 2015), because it was almost ubiquitously isolated (Session A, C, and Russian dust samples), and because *B. paralicheniformis* was observed to form a widespread biofilm on agar during cultivation approaches. *C. metallidurans* was selected because it is known to resist to - and interact with - different kinds of metals (Janssen *et al.*, 2010). *C. avidum* was selected because it is a typical human skin associated bacterium which is known to produce acidic metabolites under anaerobic conditions (Corvec, 2018) which could indirectly cause corrosion.

This experiment showed that that the aerobic *C. metallidurans* and the anaerobic *C. avidum* are able to attach to untreated and eloxated aluminum alloy surfaces, while the aerobically incubated *B. paralicheniformis* most likely cannot, and that *C. metallidurans* and *B. paralicheniformis* can attach to

NOMEX® fabric under aerobic conditions (see Figures 4-7). The ability of *C. metallidurans* to attach to surfaces was also encoded in the genome of *C. metallidurans* strains pH5_R2_1_II_A and CH34.

NOMEX® fabric was not visibly damaged by interaction with bacteria in this test. Nevertheless, the observed fact that microorganisms can easily adhere to this material might be problematic, especially if the NOMEX® fabric gets wet, for example due to the high humidity aboard ISS or leaking pipes as very recently reported (Internet reference 10). Wet NOMEX® fabric may provide the necessary conditions for microbial growth. With regard to the isolates found on the ISS in this study, the fabric could theoretically be a reservoir for allergenic microorganisms, which could also explain the reported allergic reactions of astronauts (Venkateswaran *et al.*, 2014; Crucian *et al.*, 2016).

The untreated aluminum alloy showed an altered surface structure after being incubated together with *B. paralicheniformis* and *C. metallidurans* for 6 weeks compared to the negative control. The used experiment setup cannot reliably explain if this is caused by direct interaction with the microorganisms or by metabolites of the microorganisms. While both options are possible regarding *C. metallidurans,* which is adhered to the surface, it seems not to be caused by direct interaction in the case of *B. paralicheniformis*, because this organism did not adhere to the surface. Surfaces on board the ISS consist of course not of untreated material, but microorganisms could get access to untreated aluminium alloys after a surface is mechanically damaged.

A similarly altered surface was, however, also observed on the eloxated aluminum alloy coincubated with *C. metallidurans*. A trustworthy comparison with the negative control is hindered in this case because the negative controls were covered in debris. Still, based on the retrieved electron microscopy images it can be tentatively stated that *C. metallidurans* damages eloxated and untreated aluminum copper magnesium alloy EN AW 2219 which is used among other aluminum alloys on board the ISS and other spacecraft. This might pose a problem for long-term material integrity. If this result can be replicated, *C. metallidurans* should be considered when designing future disinfectants intended to be used on spacecraft.

C. avidum formed biofilms on untreated and eloxated aluminium alloy surfaces. There was no visible structural difference of the material surfaces compared to the negative controls, but if a biofilm would damage the surface, this would be expected to happen mainly below the biofilm, an area which was not visible in our experimental setup. However, it is generally not likely for an anaerobic microorganism to form a biofilm in an aerobic indoor environment such as the ISS, but it might be possible for *C. avidum* to co-inhabit a multispecies biofilm. Such multispecies biofilms are known to have oxic and anoxic zones and much higher biocorrosive potential than biofilms of pure cultures (Beech and Sunner, 2004).

Considering the high cultivable diversity obtained from ISS indoor environment in this study, the material interaction experiment should be replicated with larger number of distinct isolates to discover the overall technophilic potential of ISS microbiota. Unfortunately, it was not possible to test more than three isolates in the frame of this thesis, because the amount of available testing material and the electron

microscopy time were restricted. This experiment was performed in liquid medium and not under ISS indoor atmospheric condition, which might overexaggerate the severity of the results.

IV.9 Conclusion & Outlook

Within this study it was possible to confirm the first hypothesis of the ARBEX project that Archaea can be detected on board the International Space Station and are an integral part of the ISS microbiome. The second hypothesis of the ARBEX project, that microorganisms adapt to the extreme ISS conditions and that isolates from the ISS are more resistant than respective ground control strains, was not confirmed. The ISS indoor environment was found to select for resistant microorganisms which can cope with the harsh ISS conditions, but ISS strains were not more resistant than respective terrestrial strains. Furthermore, it was found that clean room derived microorganisms do most likely influence the ISS microbiome but only to a minor extent and that isolates found on board the ISS are able to adhere to - and maybe damage - ISS relevant materials, which may pose a problem with regard to long-term material integrity and possibly crew health, especially if these materials get wet. The most relevant finding with regard to the health of ISS crew members was that, with respect to the microbial community on board, the ISS can still be considered a safe workplace.

The composition of the microbiome was strongly fluctuating, most likely because it was still adapting to the last crew change which happened before the first sampling and due to the arrival of new cargo during the sampling period. On a longer spaceflight towards other celestial bodies, with a constant crew composition and no additional cargo deliveries, such fluctuations over time are expected to be rather low. However, depending on the size of crew and spacecraft, spatial differences within such a spacecraft might be more pronounced than observed in this dataset. Despite these temporal fluctuations, a core microbiome comprising 56 genera was identified in this work which was stable for longer than a decade on board the ISS. These genera can also be expected to establish themselves in future spacecraft and should be investigated in more detail. A meta-analysis across all publicly available datasets published by projects of ESA, NASA, JAXA and Roscosmos could show if the core microbiome composition detected in this study is indeed present during all performed ISS sampling events and might additionally be able to clear some of the contradictory results regarding these studies. Another option can be by targeted cultivation approaches followed by genomic and physiological analyses to obtain a better understanding how these established microbes may positively or negatively interact with crew and spacecraft material. If some of them are found to be beneficial for crew health or to counteract the risk for harmful biofilm formation on spacecraft material, it could be advantageous to abolish the concept of a sterile spacecraft at launch and to spread such beneficial microbes within a spacecraft prior to launch in a controlled manner. This would enhance the chance for such beneficial microbes to establish themselves in the indoor environment and at the same time decrease the possibility for harmful microbes

to settle down inside the spacecraft later. Such a “beneficial kick-off microbiome” concept could also be applied in inhabited confined indoor environments on Earth or even in newly built hospitals.

It is getting more and more clear nowadays that Archaea are a part of the human microbiome and therefore constant companions of humans. However, the role of most Archaea in the human microbiome as well as their interplay with humans, other microbes and the surrounding environment is still elusive. It was possible to prove in this study that Archaea even follow humans into space habitats but it was not possible to enrich them with the cultivation approaches presented here. The next step to elucidate the role of Archaea within the ISS microbiome would be to try and cultivate for example *Methanobrevibacter* sp. with in situ cultivation approaches directly on board the ISS to prove that they are physiologically capable to survive and live in a space indoor environment.

Most of the extremotolerant and extremophilic bacteria identified in this study are not expected to harm crew or equipment. However, such microorganisms are of high interest for planetary protection. When spacecraft are sent to other celestial bodies it is important not to contaminate them upon arrival, as this could destroy possibly existing ecosystems, compromise life detection missions, or have other unforeseen consequences. (Nicholson *et al.* 2009). Extremotolerant and extremophilic microorganisms have a higher risk of establishing themselves on other celestial bodies (Cockell *et al.*, 2017). For this reason, some of the isolates obtained in this study will be investigated with regard to their abilities to withstand outer space conditions and analogue Mars conditions in future studies.

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Screenshots of Internet references are given in Appendix 5.

Internet references 5 and 7-10 do not meet the criteria of peer-reviewed scientific content.

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VI Appendices

Appendix 1: Excerpts from the Experiment Scientific Requirements of the EXTREMOPHILES flight project.

Reference: ESA-HSO-ESR-ARBEX
Issue 3, Rev. 2
Date: 2017

3 SCIENTIFIC REQUIREMENTS ON THE HARDWARE

All items listed below are commercially available off-the shelf and can be provided by the Extremophiles science team if needed with a lead time of approximately 1 month.

3.1 Sterilized wipes

The ARBEX samples shall be collected on board using sterilized wipes.

Manufacturer:	ITW Texwipe, Kernersville (US)
Type:	TX 3211 Alpha Wipe
Size per wipe:	23 cm x 23 cm (unfolded)
Minimum quantity:	20 dry wipes 10 wet wipes, pre-moistened with 20 ml PCR-grade H_2O

There is no stowage limitation for the wipes. However, the wet wipes should not dry out, which means that they have to remain properly packed all the time.

Fig. 2: Folded Alpha Wipe in Metal Closure bag

3.2 Metal Closure bags

Before and after sampling, each wipe shall individually be contained in a transparent Metal Closure bag.

Manufacturer:	GML-alfaplast GmbH, München (DE)
Type:	Art. Nr. 10-01682 Drahtverschlußbeutel 650ml o.Schriftfeld *which translates into:* Cat. No. 10-01682 Metal Closure Bags 650ml w/o labelling field

Size per bag:	140 mm x 229 mm x 10 mm (with folded wipe inside)
Mass per bag:	max. 15 gram (dry wipe inside) max. 35 gram (wet wipe inside)
Minimum quantity:	20 dry wipes + 10 wet wipes = 30 Metal Closure Bags
Minimum total mass:	(20 x 15) + (10 x 35) = 650 gram

3.3 Sterile, DNA-free, disposable gloves

The crew that collects the samples shall wear sterile, DNA-free gloves to prevent the wipes to be contaminated by microbes from the human skin.

Manufacturer:	ABF Diagnostics GmbH, Kranzberg (DE)
Type:	DNA-free nitrile gloves
Glove size:	Small (S), medium (M) or large (L), dependent on the size of the astronaut's hands.
Size per bag:	180 mm x 130 mm x 10 mm (one glove in rectangular bag)
Mass per glove:	15 gram
Minimum quantity:	12 + 12 + 12 = 36 (single) gloves
Minimum total mass:	36 x 15 gram = 540 gram

Fig. 3: One pair of DNA-free gloves
(http://shop.voigtlaendertechnik.de)

4 SCIENTIFIC REQUIREMENTS ON OPERATIONS

4.1 Pre-flight tests and crew training

The proposed sampling technique has been tried out successfully at the EAC on 1 June 2015. It shall be reviewed for gravity independency. In case the microgravity environment on board is deemed to interfere with the handling of the wipes and gloves, the existing technique would require an adaptation. Irrespective of the necessity to tailor the sampling technique to the microgravity environment, the crew member(s) assigned to collect the Extremophiles samples will have to practise the sampling procedure. A training session is advised to take place.

4.2 Transport and storage before upload

Before flight the Extremophiles cargo contains no biological materials. No restrictions exist concerning the environmental conditions.

4.3 Upload and on-board storage before sampling

Before sampling starts the Extremophiles cargo contains no biological materials. No restrictions exist concerning the environmental conditions.

4.4 Conduction of the experiment on board

4.4.1. Sampling Sessions

The wiping is distributed over three sampling sessions:

Session A: The first sampling session shall preferably happen soon after upload. Only dry wipes are involved.

Session B: Session B is a replication of Session A, at a later point in time. Only dry wipes are involved. By comparing the results from A and B, the development of microbial life over time can be studied.

Session C: Session C is different. The objective is to acquire as much as possible biomass. Only wet wipes are involved and the sampling surfaces are different from A and B. The results from C will provide detailed information about the identity and diversity of microbial life on the ISS.

Timing:

- The time gap between Session A and Session B shall be minimally 2 months and maximally 9 months.
  The longest possible duration is preferred but can be overruled by other, prioritized requirements. See next page.
- As the wet samples are time critical, the time period between Session C and the hand-over of the samples to the science team shall be kept within a maximum of 16 days. See Table 8.
- Session C shall only be started when Session A is over. This to benefit from the video footage acquired during Session A, see Section 4.8.1. Apart from this restriction, the timing of Session C is independent of Sessions A and B.
- None of the samples shall be taken from a surface that was recently sanitized. Sanitizing (= disinfection) is part of the standard on-board cleaning procedures and occurs at a frequency of approximately once per week. The interval between sanitizing and sampling shall be minimally 3 days.

Other specific requirements:

- Between Session A and Session B there should preferably be no crew change, because a different composition of the crew will disturb the microbiome.
- To optimize the consistency in the sampling process, it is recommended to have the same crew member involved in Session A, Session B (B is considered as a replica of A, see above) and Session C.
- In case sampling during Session A and Session B is not conducted by the same crew member, the video requirements need to be extended. See Section 4.8.1.

Prioritization of the above requirements:

1. No crew exchange between Session A and Session B;
2. Session A, Session B and Session C performed by the same crew member;
3. Duration between Session A and Session B as long as possible.

4.5 Storage of wipes after sampling

- After sampling each wipe shall be returned into its bag. The bag shall be closed properly.
- After sampling, the dry wipes (Sessions A and B) and the wet wipes (Session C) shall be stored under different conditions. The dry wipes can be maintained at ambient whereas the wet wipes shall be stored in a refrigerator to minimize metabolic activity in the moist wipe.
- After sampling it is recommended to have none of the wipes X-rayed during security checks at the airport because X-rays can have an influence on the collected microbes.

*Table 7: Storage of **dry wipes** after sampling*

	temperature			duration (counting from sampling)
	optimal	min	max	
storage on board	20–25°C	2°C	30°C	max. one year
download to Earth				
transport to Extremophiles lab				

*Table 8: Storage of **wet wipes** after sampling*

	temperature			duration (counting from sampling)
	optimal	min	max	
storage on board	4°C	2°C	10°C	max. 16 days (if permanently cooled)
download to Earth	4°C	2°C	ambient*	
transport to Extremophiles lab	4°C	2°C	10°C	

* An increase to ambient can be tolerated for maximally 24 hours.

Reference: ESA-HSO-ESR-ARBEX
Issue 3, Rev. 2
Date: 2017

Appendix 1: Timeline Sampling Session A (= B)

as recorded during simulation test performed on 1 June 2015 at EAC, Cologne

Activity	Time (as recorded)
Unstow materials	15 min
Perform positive control sampling (in COL): Remove a dry wipe from bag (with ungloved hands), wipe hands thoroughly, put wipe back into bag and close properly.	10 min
Open one bag containing a dry wipe for field blank sampling. Touch bag only at the outside, do not remove wipe yet. Put on sterile glove (one hand). Dispose of glove packaging with ungloved hand.	
Perform field blank sampling (in COL): Remove dry wipe from pre-opened bag with gloved hand, wave wipe through the air for approx. 20 seconds, put wipe back into its bag and close properly, dispose of glove.	
Perform sampling in COL Open four metal closure bags containing fresh dry wipes, touch bags only on the outside, do not remove wipes yet.	20 min
Put on sterile glove (one hand). Dispose of the glove packaging with ungloved hand.	
Sample four locations in COL with wipes from pre-opened bags, one wipe for each location; sampling surface approx. 1 square meter per location. Touch wipes only with gloved hand; put each wipe back into its bag; keep the glove sterile (touch only the wipes, and only touch on the side which has no contact with the sampling surface). Locations: - Light covers - SSC Laptop - Hand grips - Return Grid Sensor Housing (RGSH)	
After sampling, properly close all metal closure bags containing used wipes and put them into the "finished samples bag". Dispose of glove.	
Move from COL to NODE2	10 min
Perform sampling in NODE2 Open three metal closure bags containing fresh dry wipes, touch bags only on the outside, do not remove wipes yet.	20 min
Put on sterile glove (one hand). Dispose of the glove packaging with ungloved hand.	
Sample three locations in NODE2 with wipes from the pre-opened bags; one wipe for each location; sampling surface approx. 1 square meter per location. Touch wipes only with gloved hand; put each wipe back into its bag and close properly; keep the glove sterile (touch only the wipes, and only touch on the side which has no contact with the sampling surface). Locations: - Sleeping Unit - Panels (outside surface) close to PFE+PBA - Audio terminal unit (ATU)	
After sampling, properly close all metal closure bags containing used wipes and put them into the "finished samples bag".	
Store materials appropriately.	15 min
Total time	1 h 30 min

37

Reference: ESA-HSO-ESR-ARBEX
Issue 3, Rev. 2
Date: 2017

Appendix 2: Estimated Timeline Sampling Session C

Activity	Time (estimated)
Unstow materials, move to Cupola.	15 min
Open one bag containing a wet wipe for field blank sampling, touch bag only on the outside, do not remove wipe yet. Put on sterile glove (one hand). Dispose of the glove packaging with ungloved hand.	
Perform field blank sampling (in Cupola): Remove wet wipe from bag with gloved hand from pre-opened bag, wave wipe through the air for approx. 20 seconds, put wipe back into its bag and close properly; dispose of glove.	
Perform sampling in Cupola Open a metal closure bag containing a fresh wet wipe, touch bag only on the outside, do not remove wipe yet.	
Put on sterile glove (one hand). Dispose of the glove packaging with ungloved hand.	
Sample one or more surfaces facing a window (needs to be exposed to natural light regularly) in Cupola with wipes from pre-opened bags; one wipe for each location; sampling surface approx. 2 square meters. Touch wipe only with gloved hand; put wipe back into its bag; keep the glove sterile (touch only the wipe).	15 min
After sampling, properly close the metal closure bag containing the used wipe and put it into the "finished samples bag". Dispose of glove.	
Move from Cupola to NODE3	10 min
Perform sampling in NODE3 Open three metal closure bags containing fresh wet wipes, touch bags only on the outside, do not remove wipes yet.	
Put on sterile glove (one hand). Dispose of the glove packaging with ungloved hand.	
Sample three locations in NODE3 with wipes from pre-opened bags; one wipe for each location; sampling surface approx. 1 square meter per location. Touch wipes only with gloved hand; put each wipe back into its bag; keep the glove sterile (touch only the wipes and only on the side which has no contact with the surface). Locations: - ARED - Treadmill - Waste and hygiene compartment: surface areas	20 min
After sampling, properly close all metal closure bags containing used wipes and put them into the "finished samples bag". Dispose of glove.	
Move from NODE3 to NODE1	10 min
Perform sampling in NODE1 Open two metal closure bags containing fresh wet wipes, touch bags only on the outside, do not remove wipes yet.	
Put on sterile glove (one hand). Dispose of the glove packaging with ungloved hand.	
Sample two locations in NODE1 with wipes from pre-opened bags; one wipe for each location; sampling surface approx. 1 square meter per location. Touch wipes only with gloved hand; put each wipe back into its bag; keep the glove sterile (touch only the wipes and only on the side which has no contact with the surface).	20 min

Locations: - Cover inner side (e.g. of PBA) - Dining table	
After sampling, close all metal closure bags containing used wipes properly and put them into the "finished samples bag". Dispose of glove.	
Store materials appropriately.	15 min
Total time	1 h 55 min

Appendix 2: Memo of the ARBEX-ATV5 S5C clean room sampling in Kourou, French Guiana.

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MEMO

Date	02/04/2014	**Ref**	TEC-MMG/2014/110
From	Stephanie Raffestin	**Visa**	
To	Christine Moissl-Eichinger	**Copy**	D. Siruguet, M. Cislaghi, K. MacDonell, R. Lindner, C. Lasseur, M. Zell, J. Hatton

Subject: ARBEX sampling_ATV5 launch campaign

1 INTRODUCTION

ARBEX experiment is part of the ELIPS research pool which is a candidate for implementation as an ESA-ROSCOMOS joint experiment. The primary objective of this experiment is to sample various locations of the interior of ISS to assess the population of Archea and Extremophiles, a class of micro-organisms which have not been studied in detail before onboard the ISS. The proposed implementation would initially via sampling sharing from the currently ongoing Russian experiment on the RS-ISS, led by a team at Moscow State University.

One objective the science team would like to address are the changes in the Archea/Extremophile microbial population occurring on a vehicle launched to ISS. Hence ATV-5 is potentially an excellent opportunity to implement this part of the study, with preflight & inflight sampling.

It was agreed that ESA TEC-MMG will take samples for ARBEX experiment in the frame of their support to ATV5 launch campaign in Kourou.

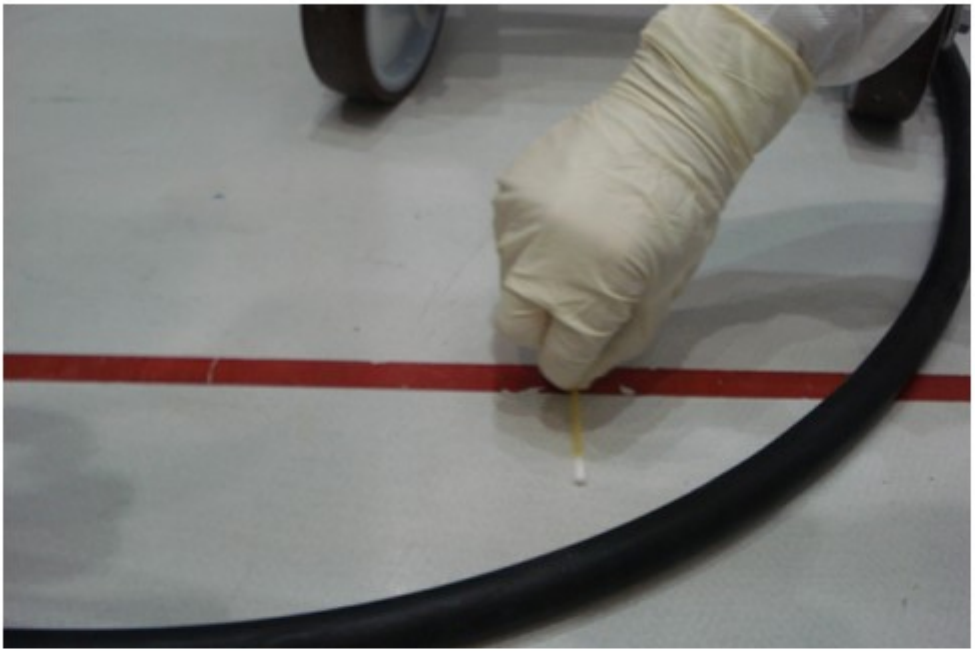
This document presents the ARBEX sampling performed during nominal activities of ATV5 preparation in S5c building.

2 MONITORING OF S5C CLEAN ROOM

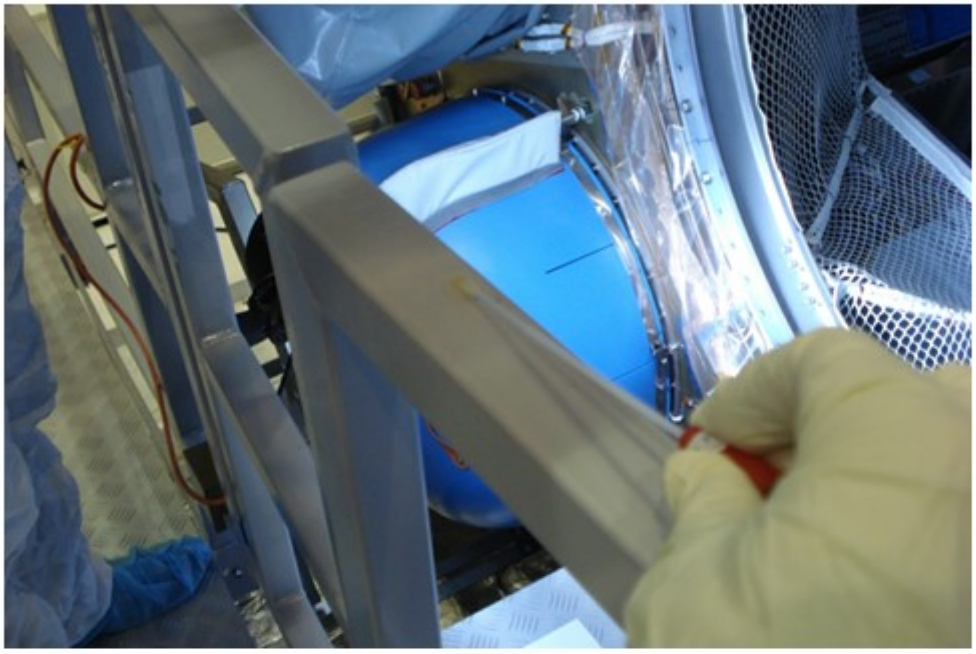
Samples were taken in 5 locations inside the S5c clean room class 100 000 on 19th March 2014. After sampling, samples were stored at 4C until shipment on the 24th March 2014. Per sampling location 6 different types of samples were performed:

-Swab Copan moistened with sterile water,
-Russian swab (dried),
-Wet Wipes for cultivation,
-Wet wipes for molecular analysis,
-Dried wipe for cultivation,
-Dried wipe for molecular analysis.

2.1 Floor clean room_1



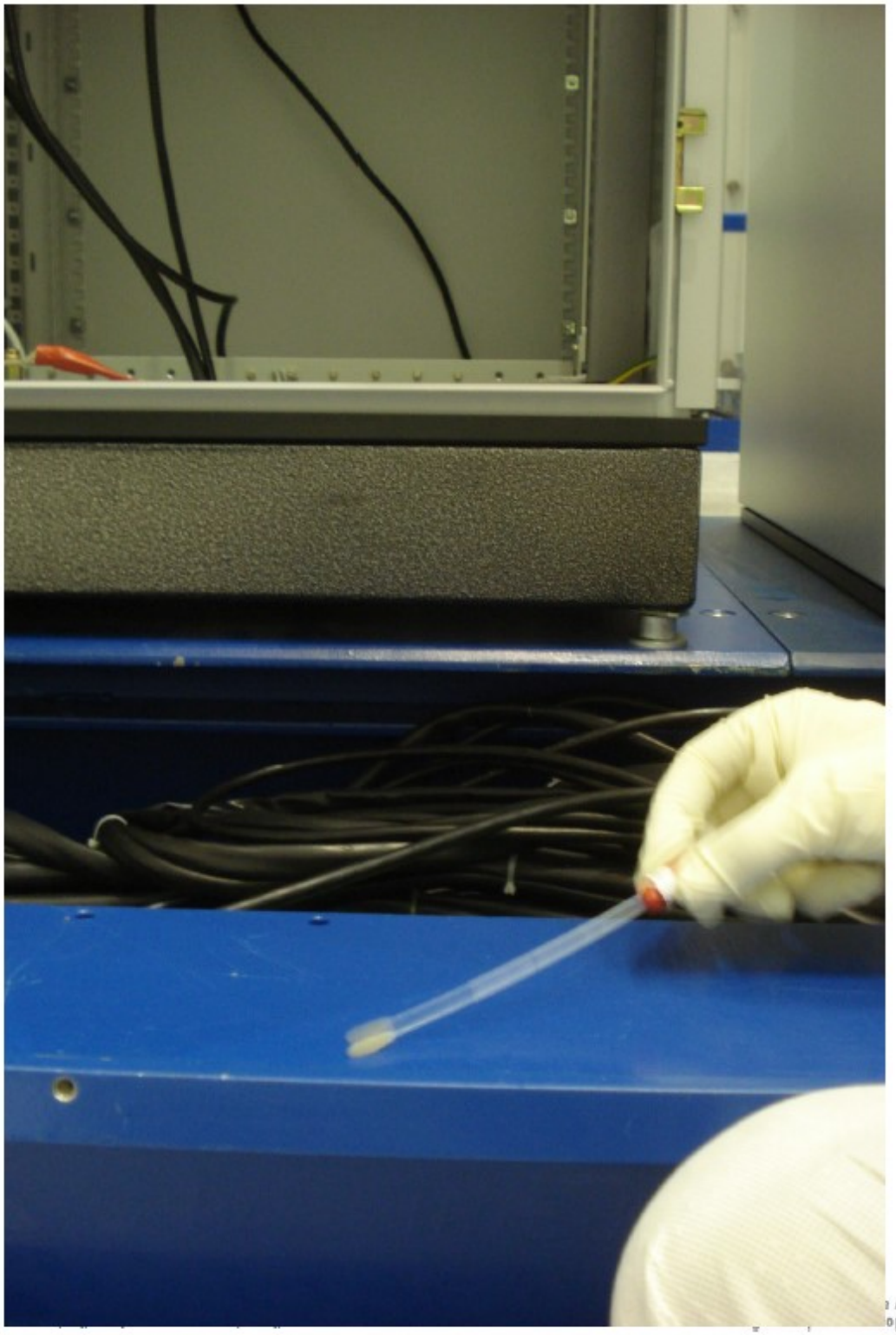
2.2 REPA platform and fences (access to ICC Pressurized Module)_2



2.3 TAM (platform access to spacecraft)_3


ASTRIUM
Dutch Space
REMOVE BEFORE FLIGHT
atv5
esa
Georges Lemaître

2.4 Electrical cupboards inside clean room_4



2.5 ''Mechanics'' cupboards and floor area_5

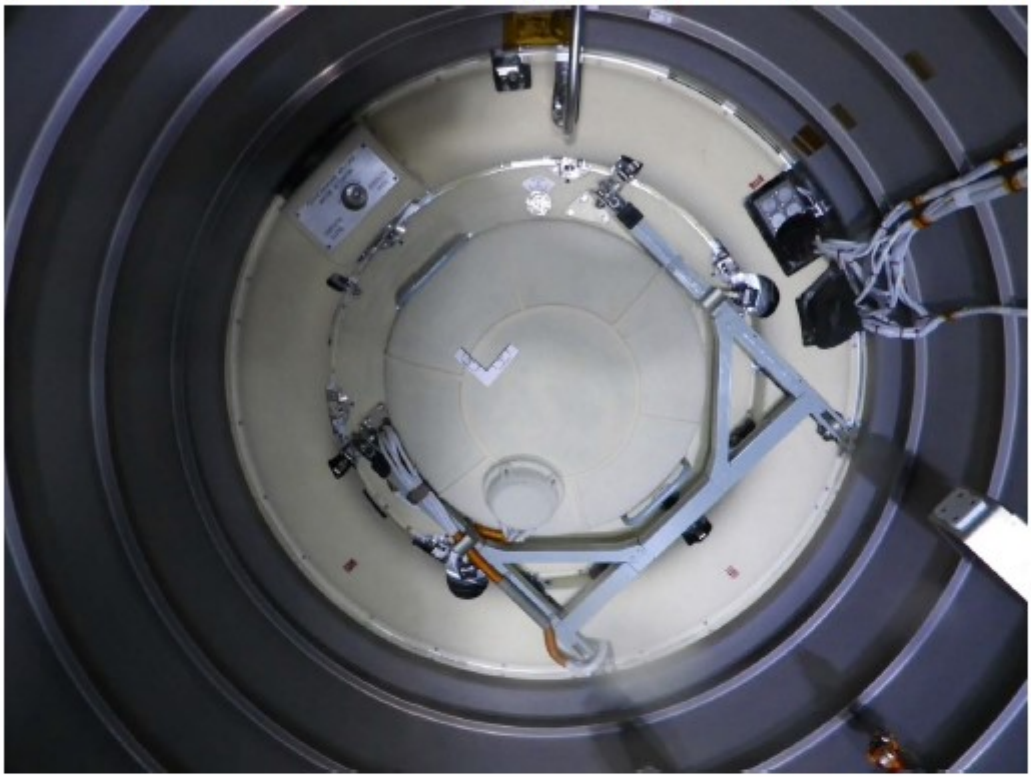
3 MONITORING OF ATV5 ICC PRESURRIZED MODULE

Samples were taken in 5 locations inside the ICC Pressurized Module after disinfection before cargo loading on the 21th March 2014. After sampling, samples were stored at 4C until shipment on the 24th March 2014.

Per sampling location, 2 different types of samples were performed:

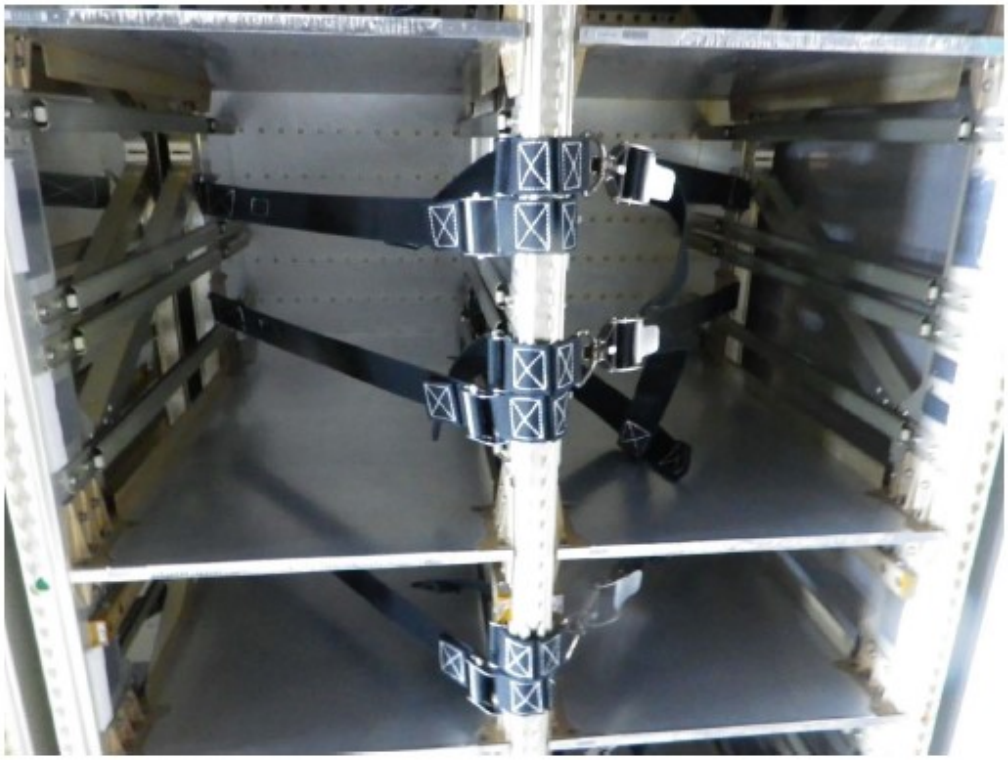
-Wet wipes for cultivation,
-Wet wipes for molecular analysis.

3.1 Fwd, Hatch/Russian docking system, on the yellow cover_6

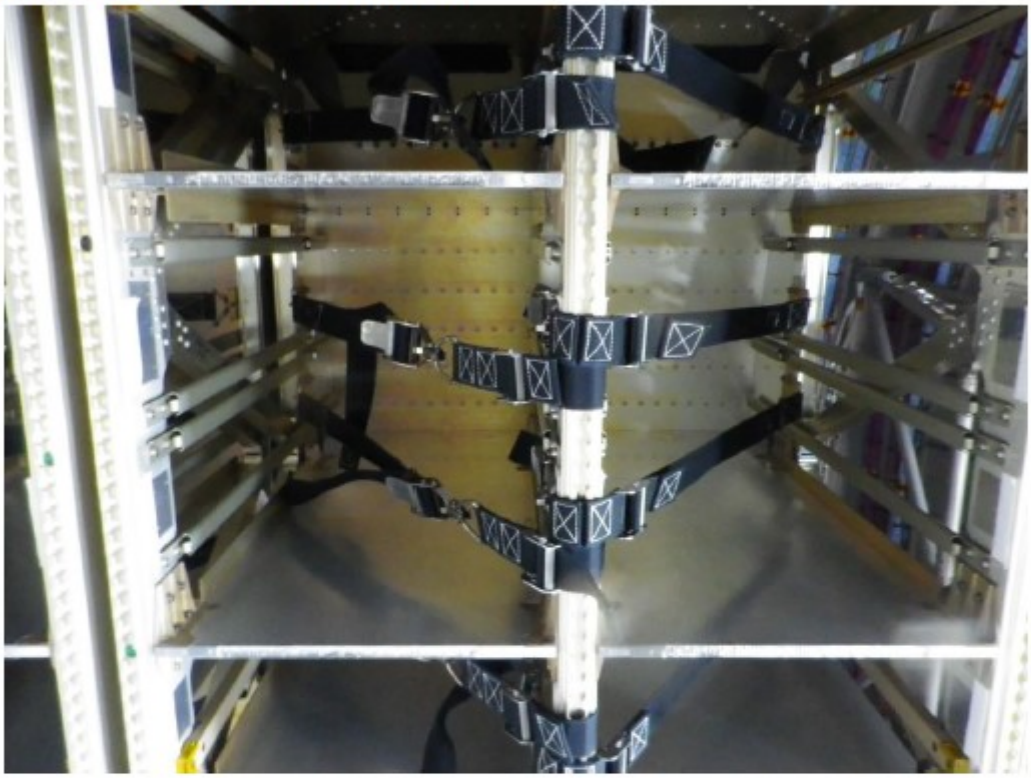


3.2 Fwd Cone-Primary structure_7

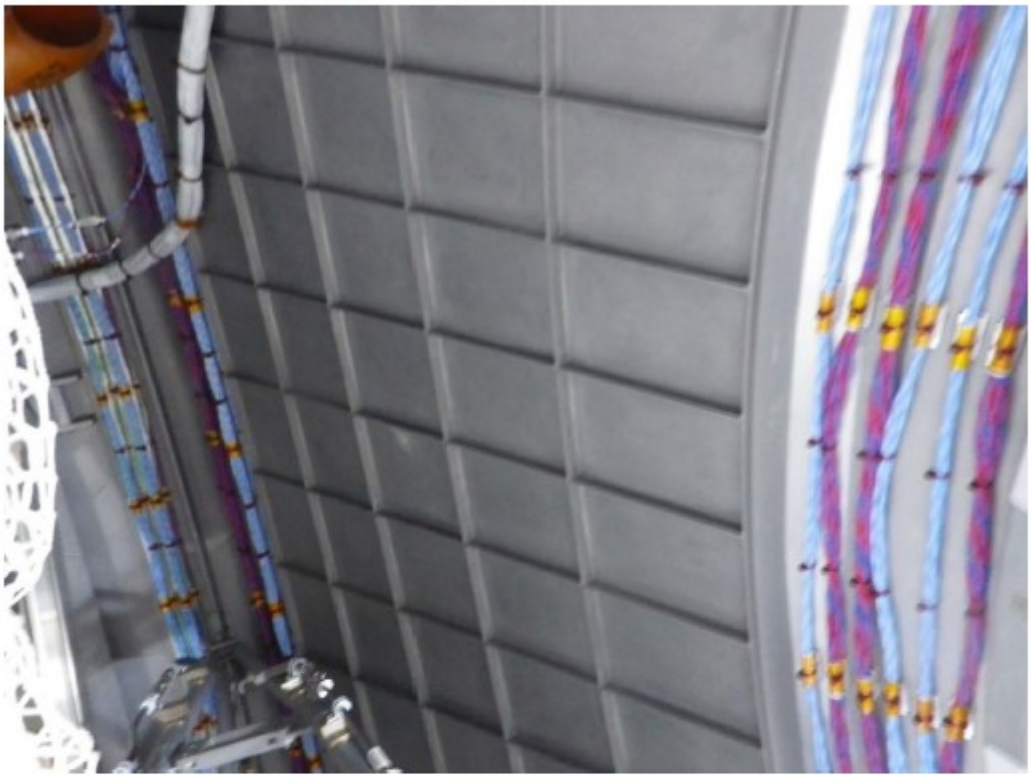
3.3 FWD Rack Inner Panel_8



3.4 AFT Rack Inner Panel_9



3.5 AFT cone-Primary structure_10



Appendix 3: Detailed list of all used media and ingredients.

Overview media + target group

R2A, pH5 + pH4, incubation at 30-32°C : acidophilic/acidotolerant

R2A, pH9, pH10, incubation at 30-32°C: alkaliphilic/alkalitolerant

R2A, pH7, incubation at 4°C : psychrophilic/psychrotolerant

R2A,pH7, anaerobic: (facultative) anaerobes

R2A,pH7, Heatshocked ESA-standard: extreme thermotolerant

R2A liquid, pH7, incubation at 65°C: thermophilic/thermotolerant

RAVAN, pH7, incubation at 30-32°C: oligotrophic

TSA, Radiation experiments

PDA, incubation at 25°C: Yeasts & Fungi

AAM, ASM, AHM, incubation at 30-32°C: Autotrophs. Sulfate reducers, Homoacetogens

Halo DSM97(less YE), incubation at 40°C: halophilic

MS, MS+organics (pH7)/AB, DSMZ_322 MRB-3, incubation at 40°C: Methanogens

N.exaquare medium: Thaumarchaeota 30-32C

ROGOSA-Agar, incubation at 30-32°C: Lactobacilli

YPD, incubation at 30-32°C: Yeasts & Fungi

R2A liquid, pH7, incubation at 30-32°C: Enrichments for NGS-boost-experiment

Media list

Aerobic, solid (+Nystatin)

R2A, pH7

BDH Prolabo, prepare according to manufacturers instructions

R2A, pH5

BDH Prolabo, prepare according to manufacturers instructions , adjust to pH5 with 1M HCl

R2A, pH4

BDH Prolabo, prepare according to manufacturers instructions , adjust to pH4 with 1M HCl

R2A, pH9

BDH Prolabo, prepare according to manufacturers instructions , adjust to pH9 with Sodiumsesquicarbonate

R2A, pH10

BDH Prolabo, prepare according to manufacturers instructions, adjust to pH9 with 1M NaOH

ROGOSA-Agar

Merck, prepare according to manufacturers instructions

PDA (without Nystatin)

Oxoid, prepare according to manufacturers instructions

TSA

Sigma Aldrich, prepare according to manufacturers instructions

YPD (without Nystatin)

Bacto Yeast Extract	10 g
Bacto Peptone	20 g
Bacto Agar	15 g
Distilled H2O	1000 ml

20% D-Glucose 100 ml/l filter sterilize, add after autoclave, 2% final

Add AB-Mix

RAVAN

Glucose	50 mg
Peptone	50 mg
Na-Acetate-Trihydrat	83 mg
3-Na-Citrate-Dihydrat	57 mg
Yeast extract	50 mg
Na-pyruvate	50 mg
Agarose	10.0 g
H2O, ASTM grade 3	Ad 1000 ml

Adjust to pH 6.5-7 with 1M HCl/NaOH

Aerobic, liquid (+Nystatin)

Halo

Halophile medium DSM97 (1/10 YE+AA)

NaCl	180 g
MgSO4 x 7H2O	20g
Sodium citrate dihydrate HOC(COONa)(CH2COONa)2 • 2H2O	3 g
KCl	2 g
FeSO4 x 7H2O	0.05 g
MnSO4 x H2O	0.20 mg
Casamino Acid	**0.75g**
Yeast Extract	**1g**
Distilled H2O	1000 ml

Adjust pH to 7.4 (HCl/NaOH). Add the agar after dissolving all ingredients in the water and adjustment of pH

Liquid R2A

Ingredients as BDH Prolabo, no Agar, adjust pH to 6.5-7

N.ex medium

Ca. *N. exaquare* G61 was cultivated in a growth medium described previously (Sauder *et al.*, 2017)

Sauder, L. A. et al. (2017) 'Cultivation and characterization of Candidatus Nitrosocosmicus exaquare, an ammonia-oxidizing archaeon from a municipal wastewater treatment system'; I*SME Journal,* 11(5), pp. 1142–1157. doi: 10.1038/ismej.2016.192.

KH2PO4	0.05 g
KCl	0.075 g
MgSO4×7H2O	0.05 g
NaCl	0.58 g
CaCO3	4 g
NH4Cl	0.107g
H2O bidest,	ad 1000,0 ml

After autoclaving, the medium was supplemented with filter-sterilized NH4Cl (0.5–1mM), 1ml selenite-tungstate solution and 1ml trace element solution (Könneke *et al.*, 2005) . The resulting medium pH was ~ 8.5.

Anaerobic, solid (no AB)

Anaerobic R2A

BDH Prolabo, prepare according to manufacturers instructions, bubble with N2 and add Na2S x 7-9 H2O (0,5 g) or Na2S x 2 H2O (0,25 g) before autoclaving; adjust to pH 6,5 to 7 with NaOH or HCl

Pour plates in anaerobic bench

Anaerobic, liquid

AAM (autotrophic allrounder medium)

KH2PO4	0,40 g
CaCl2 x 2 H2O	0,05 g
NaHCO3	1,50 g
Fe3O4	0,35 g
NaNO3	0,50 g
Na2S2O3 x 5 H2O	1,56 g
MgCl2 x 6 H2O	0,15 g
Wolfe's mineral solution 10x	1,00 ml
Wolfe's vitamine solution 10x	1,00 ml
Na2S x 9 H2O	0,50 g
Resazurin, 0,1%	1,0 ml
H2O bidest	ad 1000 ml

adjust to pH7 with 1M HCl

Gas phase: N2/CO2

AHM (autotrophic homoacetogenics medium)

NH4Cl	0,50 g
KH2PO4	0,40 g
MgCl2 x 6 H2O	0,15 g
CaCl2 x 2 H2O	0,05 g
NaHCO3	1,00 g
Wolfe's mineral solution 10x	1,00 ml
Wolfe's vitamine solution 10x	1,00 ml
Resazurin, 0,1%	1,0 ml
Na2S x 9 H2O	0,50 g
Cystein-HCl	0,50 g
H2O bidest	ad 1000 ml

adjust to pH7 with 1M HCl

Gas phase: H2/CO2

ASM (autotrophic sulfate reducer medium)

KH2PO4	0,47 g
NH4Cl	1,00 g
CaCl2 x 2 H2O	0,10 g
NaHCO3	1,50 g
Na2SO4	1,00 g
MgSO4 x 7 H2O	2,00 g
FeSO4 x 7 H2O	4,00 mg
Na2S x 9 H2O	0,50 g
Resazurin, 0,1%	1,0 ml
H2O bidest	ad 1000 ml

adjust to pH7 with 1M HCl.

Gas phase: H2/CO2

MS

(NH4)2 Ni(SO4)2	0,002 g
(NH4)2SO4	0,225 g
CaCl2 x 2 H2O	0,06 g
FeSO4 x 7 H2O	0,002 g
K2HPO4 x 3 H2O	0,3 g
KH2PO4	0,225 g
MgSO4 x 7 H2O	0,10 g
NaCl	0,450 g
NaHCO3	5,00 g
Wolfe's mineral solution 10x	1,0 ml
Wolfe's vitamine solution 10x	1,0 ml
Resazurin, 0,1%	1,0 ml
Na2S x 7-9 H2O	0,5 g
alternativ:	
Na2S x 2 H2O	0,25 g
H2O bidest,	ad 1000,0 ml

MS + organics + Antiobiotics

As MS, add AB-Mix, NaAc(10%) and YE(10%) to a final concentration of 0,1% after autoclaving

DSMZ_322 MRB-3

Rumen fluid, clarified (see medium 1310)	100.00 ml
Trypticase peptone (BD BBL)	2.00 g
Yeast extract	2.00 g
Na-acetate	0.50 g
Na-formate	0.50 g
Trace element solution (see medium 141)	10.00 ml
Na2SeO4 solution (0.1% w/v)	1.90 ml
NiCl2 x 6 H2O solution (0.1% w/v) 0.	70 ml
FeSO4 x 7 H2O solution (0.1% w/v in 0.1 N H2SO4)	3.00 ml
K2HPO4	0.60 g
KH2PO4	2.80 g
(NH4)2SO4	0.30 g
NH4Cl	1.00 g
NaCl	0.60 g
MgSO4 x 7 H2O	0.15 g
CaCl2 x 2 H2O	0.08 g
Na-resazurin solution (0.1% w/v)	0.50 ml
NaHCO3	4.00 g
Methanol	5.00 ml
Vitamin solution (see medium 141)	20.00 ml
L-Cysteine-HCl x H2O	0.50 g
Na2S x 9 H2O	0.50 g
Distilled water	900.00 ml

Dissolve ingredients except bicarbonate, methanol, cysteine and sulfide, then sparge medium with 80% H2 and 20% CO2 gas mixture for 30 – 45 min to make it anoxic. Add and dissolve bicarbonate, adjust pH of medium to 6.8 – 7.0, then dispense medium under 80% H2 and 20% CO2 gas atmosphere into anoxic Hungate-type tubes or serum vials and autoclave. Add methanol, vitamins (sterilized by filtration), cysteine and sulfide from sterile anoxic stock solutions prepared under 100% N2 gas. Prior to use check pH of complete medium and adjust to 6.7 - 6.9, if necessary. After inoculation add sterile 80% H2 and 20% CO2 gas mixture to 1 bar overpressure. Add AB-Mix

Solutions

AB-Mix

Ampicillin 100mg

Streptomycin 50mg

H2O ad 20 ml

→filter 0,2µm pores (Nylon or cellulose membrane)

Nystatin

Nystatin 50mg/ml in DMSO

→ filter 0,2µm pores (Nylon or PTFE membrane)

Add 1:1000 to medium <60°C after autoclaving for final concentration of 50µg/ml

NaAc(10%)

2g ad 20ml H20, bubble with N2

YE(10%)

2g ad 20ml H20, bubble with N2

NH4Cl(0.1M)

0.1 g ad 20 ml H20, aerobic

Wolfe's mineral solution 10x

$MgSO_4 \times 7 H_2O$	30,0 g
$MnSO_4 \times H_2O$	5,0 g
NaCl	10,0 g
$FeSO_4 \times 7 H_2O$	1,0 g
$CoSO_4 \times 7 H_2O$	1,8 g
$CaCl_2 \times 2 H_2O$	1,0 g
$ZnSO_4 \times 7 H_2O$	1,8 g
$CuSO_4 \times 5 H_2O$	0,1 g
$KAl(SO_4)_2 \times 12 H_2O$	0,18 g
H_3BO_3	0,1 g
$Na_2MoO_4 \times 2 H_2O$	0,1 g
$(NH_4)_2Ni(SO_4)_2 \times 6 H_2O$	2,80 g
$Na_2WO_4 \times 2 H_2O$	0,1 g
Na_2SeO_4	0,1 g
H_2O bidest, ad	1000,0 ml

Wolfe's vitamine solution10x

Biotin	20 mg
Folic acid	20 mg
Pyridoxamine dihydrochloride	100 mg
Thiamine hydrochloride	50 mg
Riboflavin	50 mg
Niacin	50 mg
D-Calcium pantothenate	50 mg
Cyanocobalamin	1 mg
4-Aminobenzoic acid	50 mg
DL-alpha-lipoic acid	50 mg
H_2O bidest, ad	1000 ml

Selenite-tungstate solution:

NaOH	0.5 g
Na2SeO3 x 5 H2O	3.0 mg
Na2WO4 x 2 H2O	4.0 mg
Distilled water	1000.0 ml

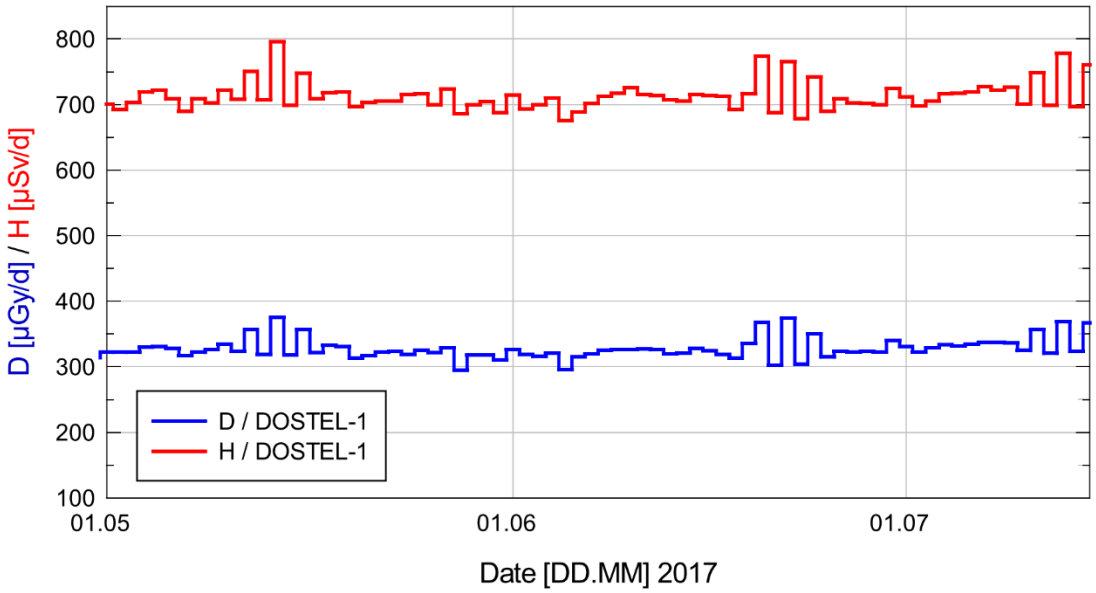
Widdel TES: Trace element solution (Widdel & Bak 1992)

Widdel F., Bak F. (1992). Gram-negative mesophilic sulfate-reducing bacteria, in The Prokaryotes, eds Balows A., Trüper H. G., Dworkin M., Harder W., Schleifer K.-H., editors. (New York, NY: Springer;), 3352–3378 (vol2)

HCl (25%=7.7M)	12.5 ml
FeSO4 x 7H2O	2.1 g
ZnSO4 x 7H2O	0.144 g
MnCl2 x 4H2O	0.1 g
H3BO3	0.03 g
CoCl2 x 6H2O	0.19 g
CuCl2 x 2H2O	0.002 g
NiCl2 x 6H2O	0.024 g
Na2MoO4 x 2H2O	0.036 g
Distilled water	ad 1000 ml

Appendix 4: DOSIS 3D radiation report for the time of the EXTREMOPHILES flight project.

Date	Absorbed dose [mGy]	Dose equivalent [mSv]
01.05.2017 – 15.07.2017	24.91	54.21
	328 µG/day	713 µGy/day



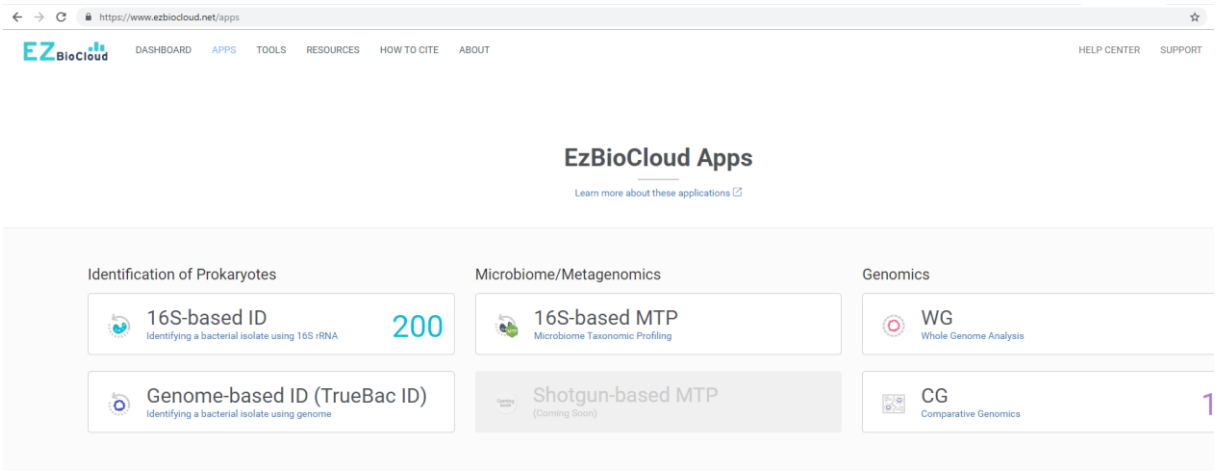
2/12/2018

DOSIS 3D / DLR-ME-SBA
Thomas Berger thomas.berger@dlr.de

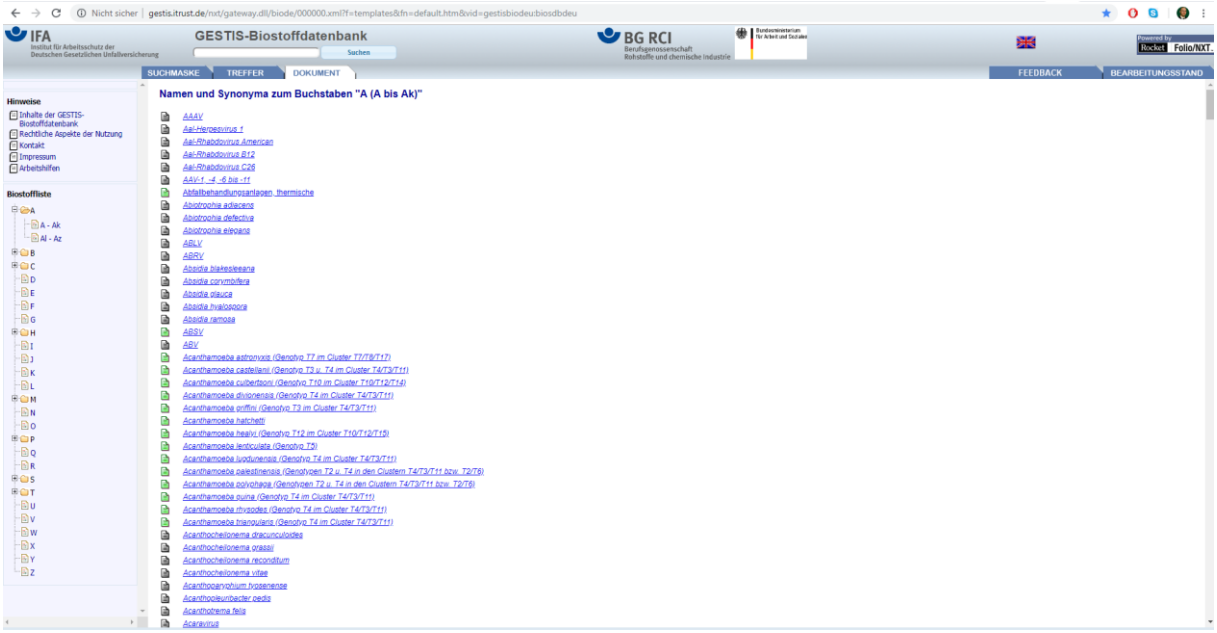
DLR

Appendix 5: Screenshots of referenced websites.

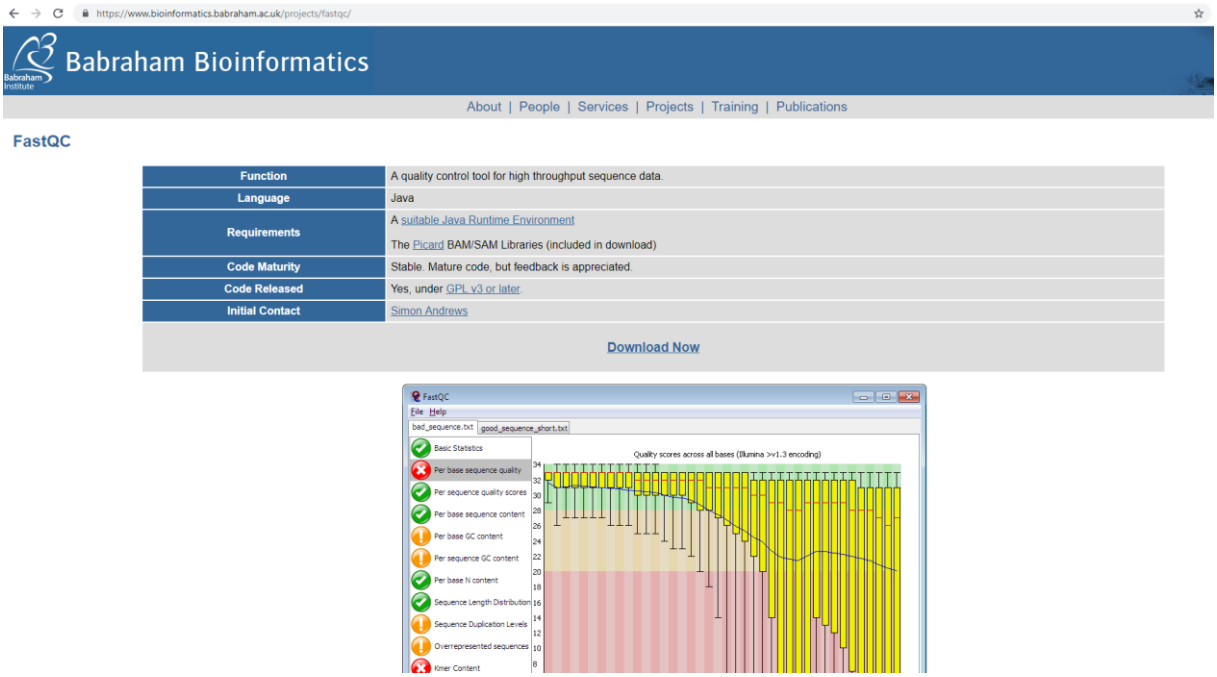
Internet reference 1: last accessed January 2019: http://www.ezbiocloud.net/apps



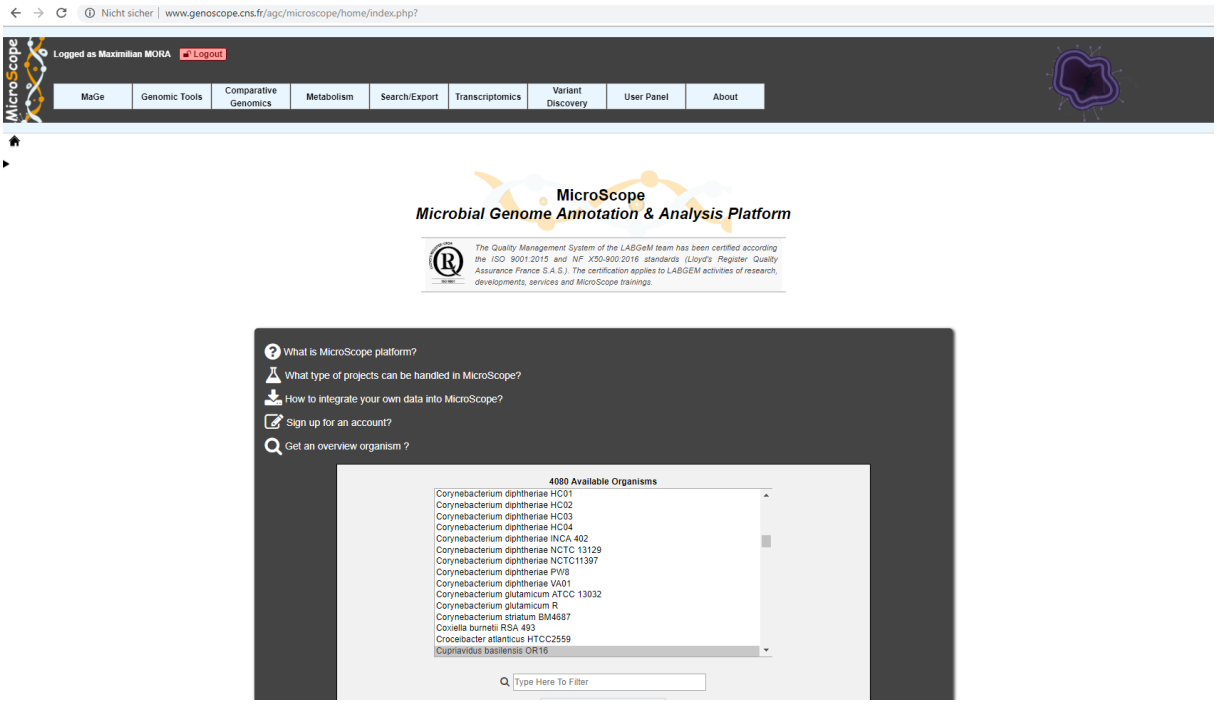
Internet reference 2; last accessed January 2019: http://gestis.itrust.de



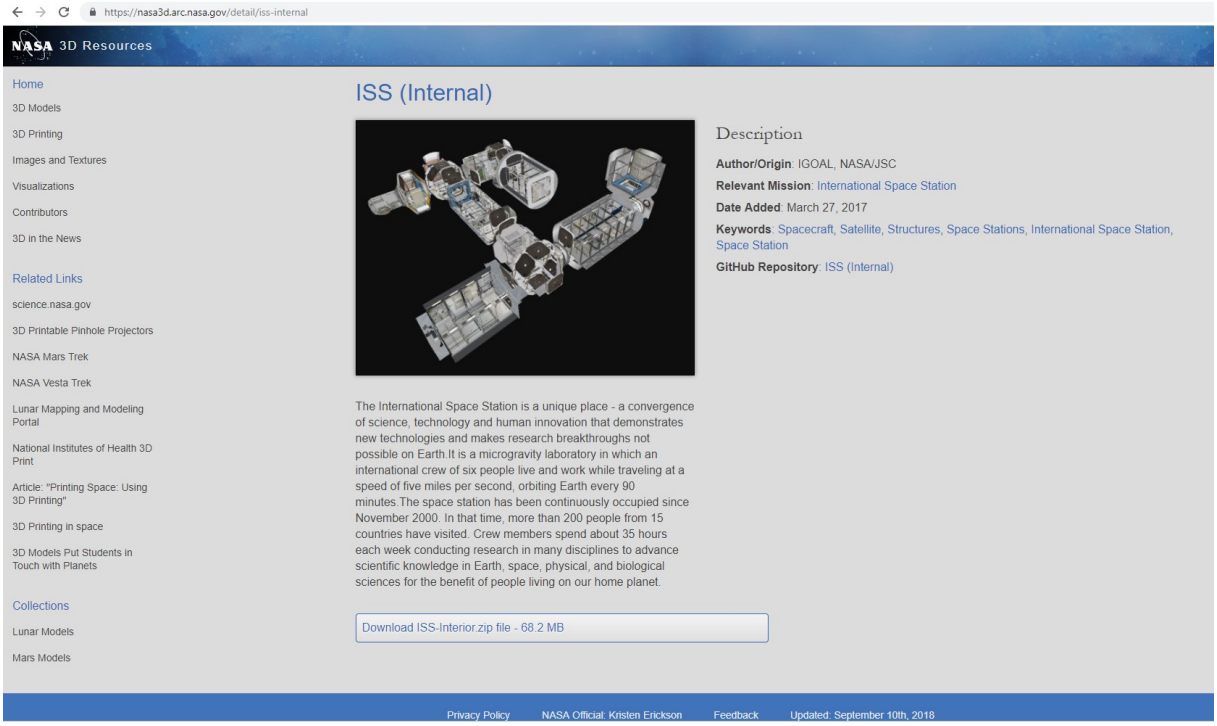
Internet reference 3; last accessed January 2019:
https://www.bioinformatics.babraham.ac.uk/projects/fastqc/



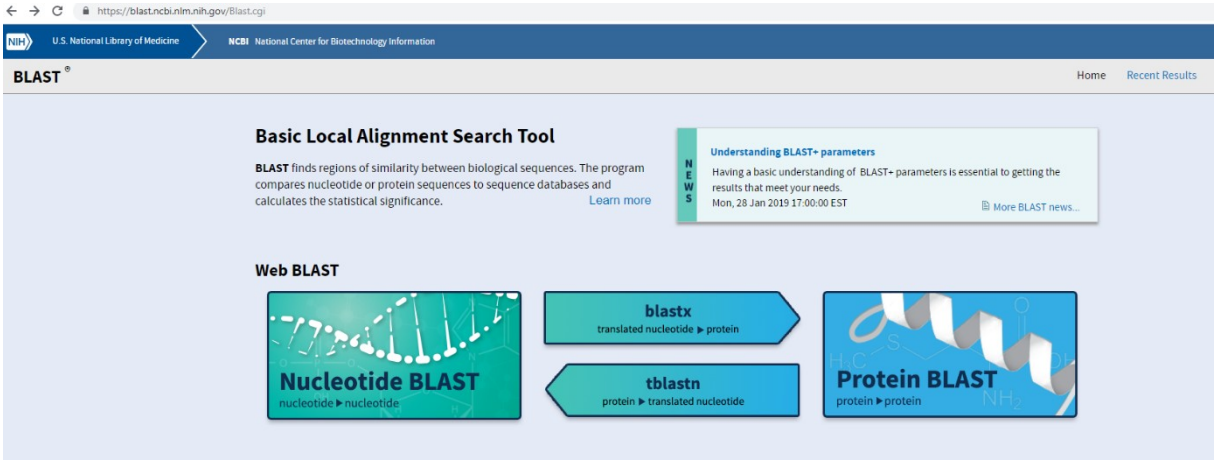
Internet reference 4; last accessed January 2019: http://www.genoscope.cns.fr/agc/microscope



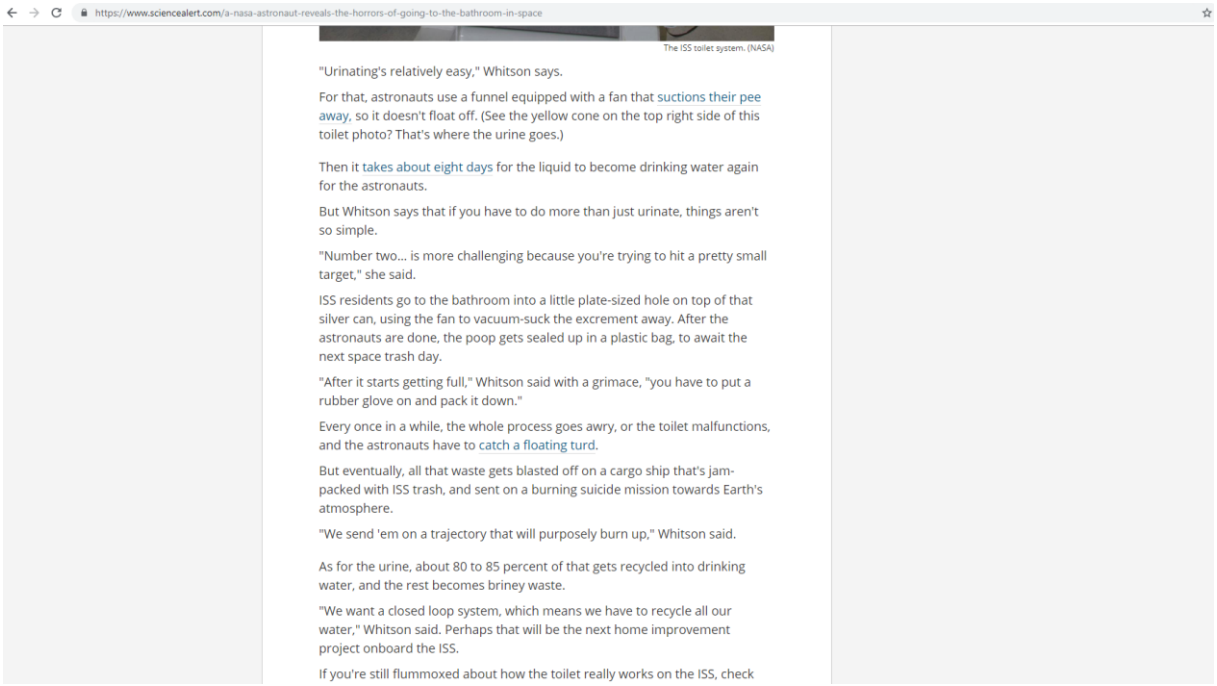
Internet reference 5; last accessed January 2019: https://nasa3d.arc.nasa.gov/detail/iss-internal



Internet reference 6; last accessed January 2019: https://blast.ncbi.nlm.nih.gov/



Internet reference 7; last accessed January 2019: https://www.sciencealert.com/a-nasa-astronaut-reveals-the-horrors-of-going-to-the-bathroom-in-space



https://www.sciencealert.com/a-nasa-astronaut-reveals-the-horrors-of-going-to-the-bathroom-in-space

The ISS toilet system. (NASA)

"Urinating's relatively easy," Whitson says.

For that, astronauts use a funnel equipped with a fan that suctions their pee away, so it doesn't float off. (See the yellow cone on the top right side of this toilet photo? That's where the urine goes.)

Then it takes about eight days for the liquid to become drinking water again for the astronauts.

But Whitson says that if you have to do more than just urinate, things aren't so simple.

"Number two... is more challenging because you're trying to hit a pretty small target," she said.

ISS residents go to the bathroom into a little plate-sized hole on top of that silver can, using the fan to vacuum-suck the excrement away. After the astronauts are done, the poop gets sealed up in a plastic bag, to await the next space trash day.

"After it starts getting full," Whitson said with a grimace, "you have to put a rubber glove on and pack it down."

Every once in a while, the whole process goes awry, or the toilet malfunctions, and the astronauts have to catch a floating turd.

But eventually, all that waste gets blasted off on a cargo ship that's jam-packed with ISS trash, and sent on a burning suicide mission towards Earth's atmosphere.

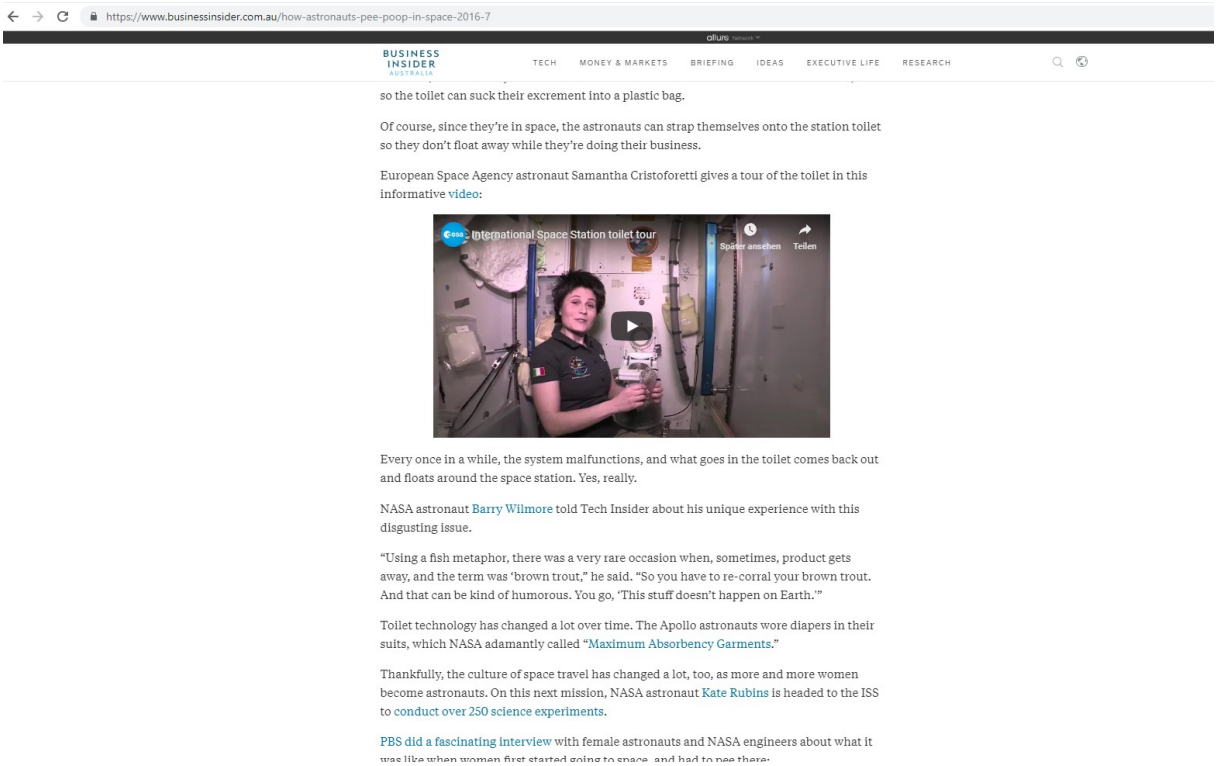
"We send 'em on a trajectory that will purposely burn up," Whitson said.

As for the urine, about 80 to 85 percent of that gets recycled into drinking water, and the rest becomes briney waste.

"We want a closed loop system, which means we have to recycle all our water," Whitson said. Perhaps that will be the next home improvement project onboard the ISS.

If you're still flummoxed about how the toilet really works on the ISS, check

Internet reference 8; accessed January 2019: https://www.businessinsider.com.au/how-astronauts-pee-poop-in-space-2016-7



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so the toilet can suck their excrement into a plastic bag.

Of course, since they're in space, the astronauts can strap themselves onto the station toilet so they don't float away while they're doing their business.

European Space Agency astronaut Samantha Cristoforetti gives a tour of the toilet in this informative video:

Every once in a while, the system malfunctions, and what goes in the toilet comes back out and floats around the space station. Yes, really.

NASA astronaut Barry Wilmore told Tech Insider about his unique experience with this disgusting issue.

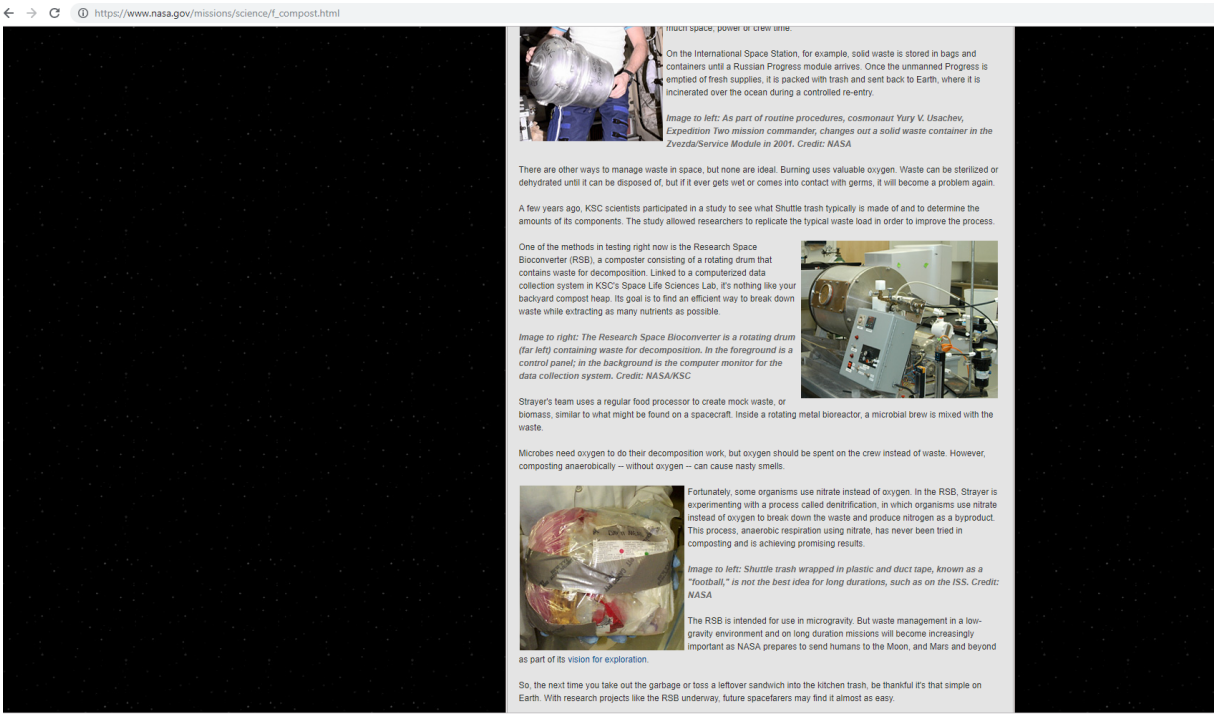
"Using a fish metaphor, there was a very rare occasion when, sometimes, product gets away, and the term was 'brown trout," he said. "So you have to re-corral your brown trout. And that can be kind of humorous. You go, 'This stuff doesn't happen on Earth.'"

Toilet technology has changed a lot over time. The Apollo astronauts wore diapers in their suits, which NASA adamantly called "Maximum Absorbency Garments."

Thankfully, the culture of space travel has changed a lot, too, as more and more women become astronauts. On this next mission, NASA astronaut Kate Rubins is headed to the ISS to conduct over 250 science experiments.

PBS did a fascinating interview with female astronauts and NASA engineers about what it

Internet reference 9; last accessed January 2019:
https://www.nasa.gov/missions/science/f_compost.html



https://www.nasa.gov/missions/science/f_compost.html

much space, power or crew time.

On the International Space Station, for example, solid waste is stored in bags and containers until a Russian Progress module arrives. Once the unmanned Progress is emptied of fresh supplies, it is packed with trash and sent back to Earth, where it is incinerated over the ocean during a controlled re-entry.

Image to left: As part of routine procedures, cosmonaut Yury V. Usachev, Expedition Two mission commander, changes out a solid waste container in the Zvezda/Service Module in 2001. Credit: NASA

There are other ways to manage waste in space, but none are ideal. Burning uses valuable oxygen. Waste can be sterilized or dehydrated until it can be disposed of, but if it ever gets wet or comes into contact with germs, it will become a problem again.

A few years ago, KSC scientists participated in a study to see what Shuttle trash typically is made of and to determine the amounts of its components. The study allowed researchers to replicate the typical waste load in order to improve the process.

One of the methods in testing right now is the Research Space Bioconverter (RSB), a composter consisting of a rotating drum that contains waste for decomposition. Linked to a computerized data collection system in KSC's Space Life Sciences Lab, it's nothing like your backyard compost heap. Its goal is to find an efficient way to break down waste while extracting as many nutrients as possible.

Image to right: The Research Space Bioconverter is a rotating drum (far left) containing waste for decomposition. In the foreground is a control panel; in the background is the computer monitor for the data collection system. Credit: NASA/KSC

Strayer's team uses a regular food processor to create mock waste, or biomass, similar to what might be found on a spacecraft. Inside a rotating metal bioreactor, a microbial brew is mixed with the waste.

Microbes need oxygen to do their decomposition work, but oxygen should be spent on the crew instead of waste. However, composting anaerobically -- without oxygen -- can cause nasty smells.

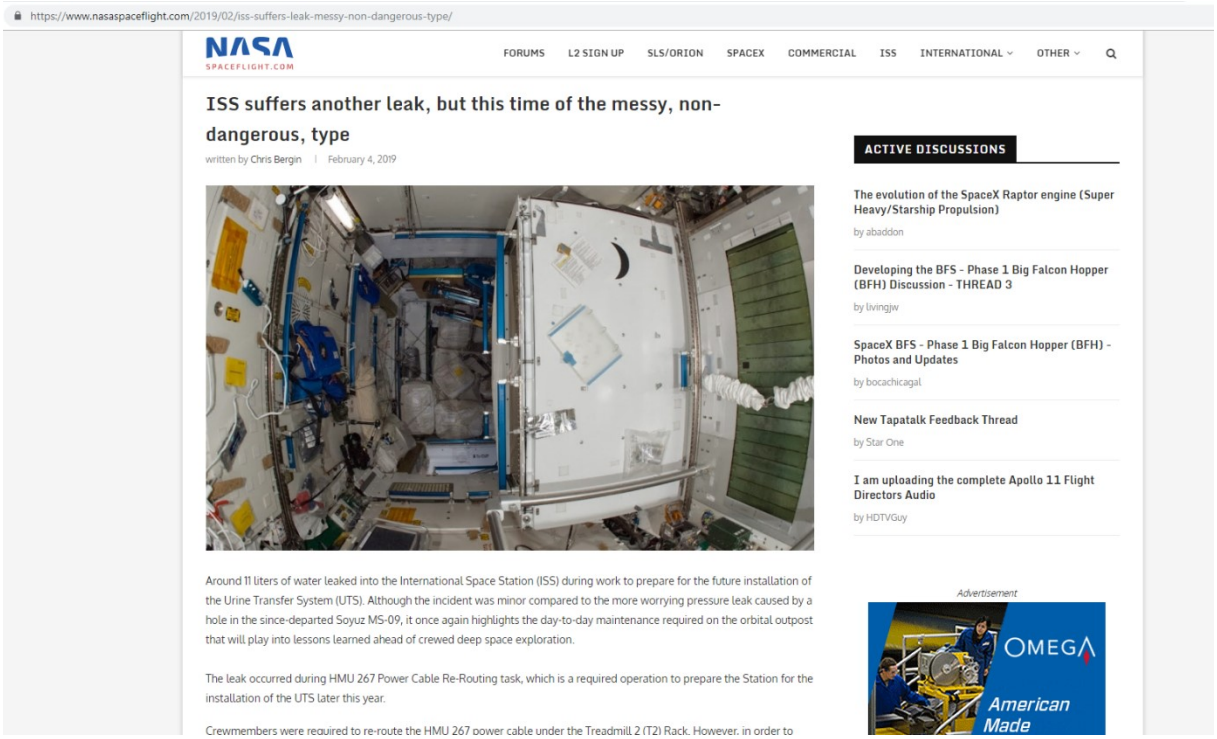
Fortunately, some organisms use nitrate instead of oxygen. In the RSB, Strayer is experimenting with a process called denitrification, in which organisms use nitrate instead of oxygen to break down the waste and produce nitrogen as a byproduct. This process, anaerobic respiration using nitrate, has never been tried in composting and is achieving promising results.

Image to left: Shuttle trash wrapped in plastic and duct tape, known as a "football," is not the best idea for long durations, such as on the ISS. Credit: NASA

The RSB is intended for use in microgravity. But waste management in a low-gravity environment and on long duration missions will become increasingly important as NASA prepares to send humans to the Moon, and Mars and beyond as part of its vision for exploration.

So, the next time you take out the garbage or toss a leftover sandwich into the kitchen trash, be thankful it's that simple on Earth. With research projects like the RSB underway, future spacefarers may find it almost as easy.

Internet reference 10; last accessed February 2019:
https://www.nasaspaceflight.com/2019/02/iss-suffers-leak-messy-non-dangerous-type/



https://www.nasaspaceflight.com/2019/02/iss-suffers-leak-messy-non-dangerous-type/

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ISS suffers another leak, but this time of the messy, non-dangerous, type

written by Chris Bergin | February 4, 2019

Around 11 liters of water leaked into the International Space Station (ISS) during work to prepare for the future installation of the Urine Transfer System (UTS). Although the incident was minor compared to the more worrying pressure leak caused by a hole in the since-departed Soyuz MS-09, it once again highlights the day-to-day maintenance required on the orbital outpost that will play into lessons learned ahead of crewed deep space exploration.

The leak occurred during HMU 267 Power Cable Re-Routing task, which is a required operation to prepare the Station for the installation of the UTS later this year.

Crewmembers were required to re-route the HMU 267 power cable under the Treadmill 2 (T2) Rack. However, in order to

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Appendix 6: Schematic overview of the study