

IS THE OCCURRENCE OF MICROORGANISM LIQUID NITROGEN STORAGE TANKS A CHALLENGE FOR CRYOBANKING?



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ECCO XL 2022 Braunschweig September 27 – 29



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Evaluate the potential risk of microbial cross-contaminations during cryostorage

1) Systematic screen to assess organisms in LN storage tanks

- GDK (Association of German Cryobanks)
“Hygiene-Project”

Bajerski *et al.*, 2020 , Appl Microbiol Biotech.



2) Literature Review & short survey among different culture collections

Bajerski, Nagel & Overmann, 2021, Appl Microbiol Biotech.



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Relevance of contaminations in storage containers

- Anecdotal reports of bacterial isolates & sample contamination (*Bielanski et al.*, *Fountain et al.*)
 - Organisms may persist in liquid nitrogen (LN) storage tanks
- Bone marrow samples stored in LN contaminated with hepatitis B virus after direct contact with tank detritus
(*Tedder et al. 1995*)
 - Transmission route not clear, but awareness raised

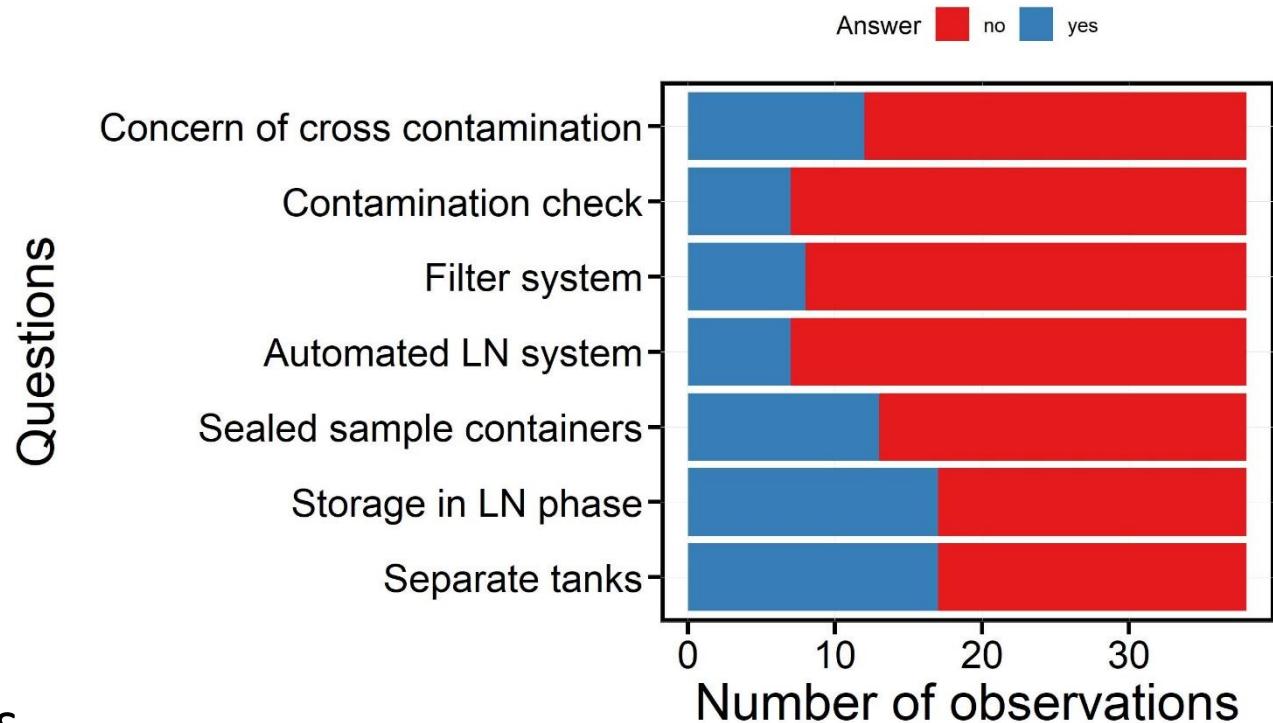


Awareness for microbial contaminations of storage containers

- Survey among cryo-collections and biobanks January 20th and April 6th 2021
- 39 participants of 34 biobanks from 10 countries of 5 continents
- 7 yes/no questions on cryostorage and biobanking



Awareness for microbial contaminations of storage containers



Measures

- Sealed devices
- Separate tanks
- Storage in Gaseous LN phase
- LN-free systems (– 150 °C freezers)

Bajerski, Nagel & Overmann, 2021, Appl Microbiol Biotech.

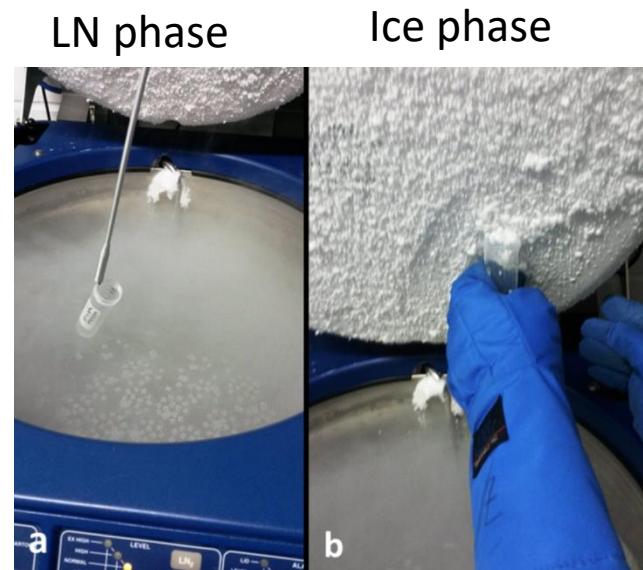
Assess organisms in LN storage tanks

Systematic screen

- 10 biobank facilities
- Bacteria (incl. Mycoplasma), fungi, human cells, chloroplasts (plants)
- Different phases of LN storage tanks

Approach

- State-of-the-art microscopy (morphology, cell counts)
- Group-specific marker gene amplification
- High-throughput sequencing of bacterial ribosomal genes



Sampling

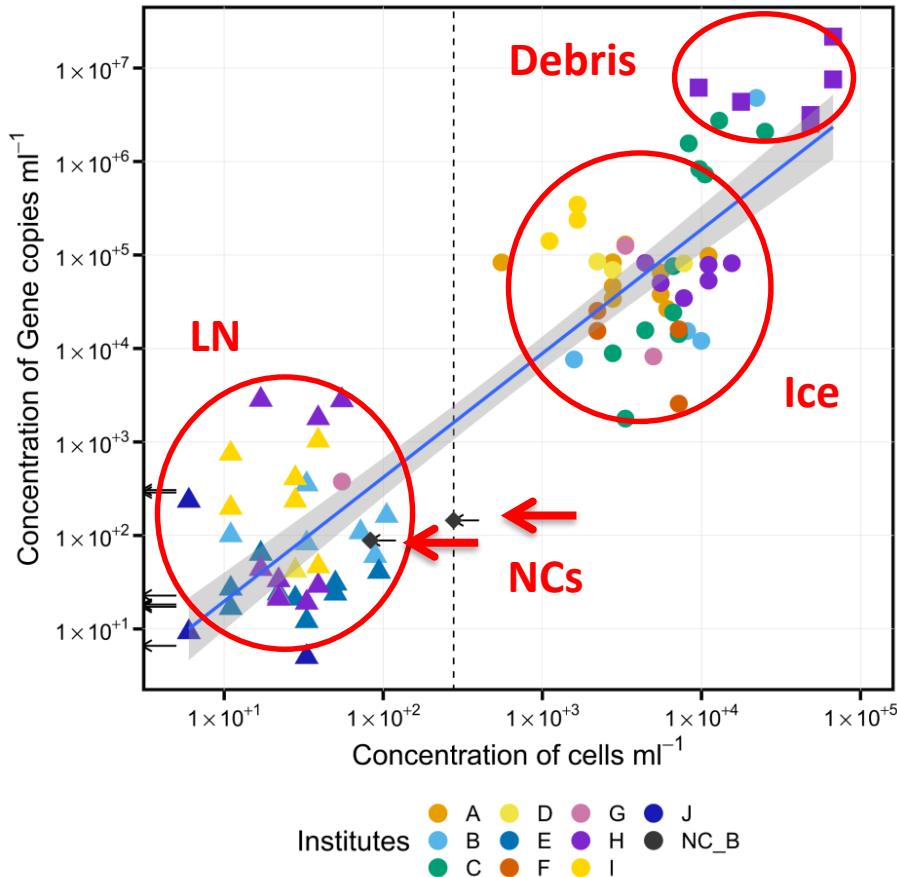
Bajerski *et al.*, 2020 , Appl Microbiol Biotech.



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Bacterial load in LN storage devices is low (ice) or not detectable (LN samples)

Correlation of gene copy numbers and cell counts



- Separation of ice and LN
- LN samples in the range of the negative controls
- Debris samples with highest gene copy numbers and cell counts
- 10^2 and 10^5 colony forming units per ml melted Dewar sediment (Morris 2005)

Bajerski *et al.*, 2020 , Appl Microbiol Biotech.



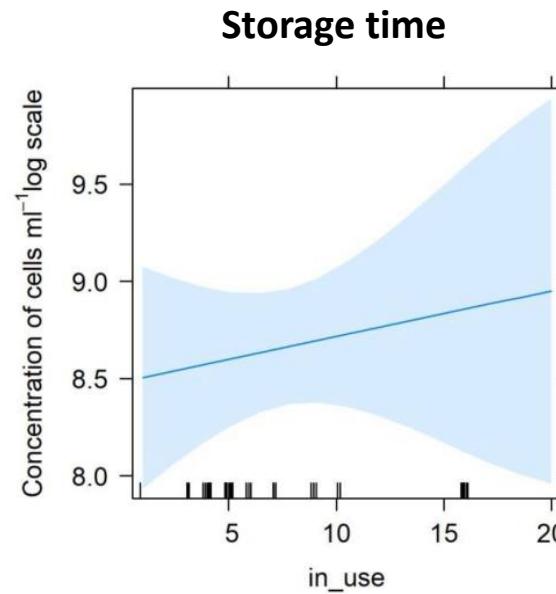
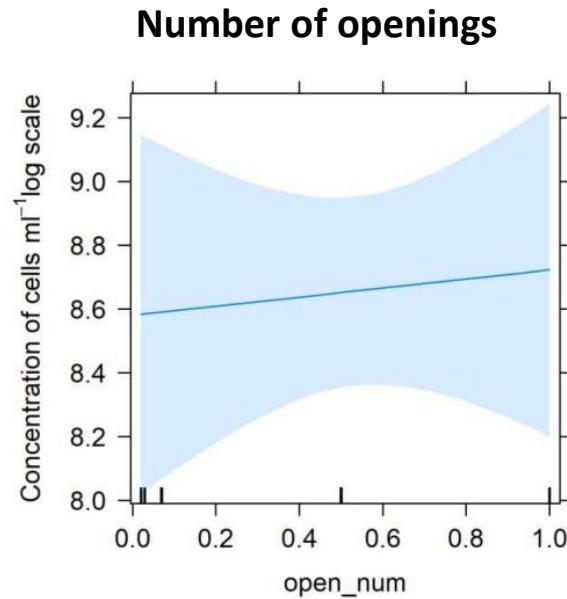
Gemeinschaft
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Kryobanken
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Cells and gene copies increased with storage time and number of openings

Effect plots of a generalized linear model with Gaussian distribution



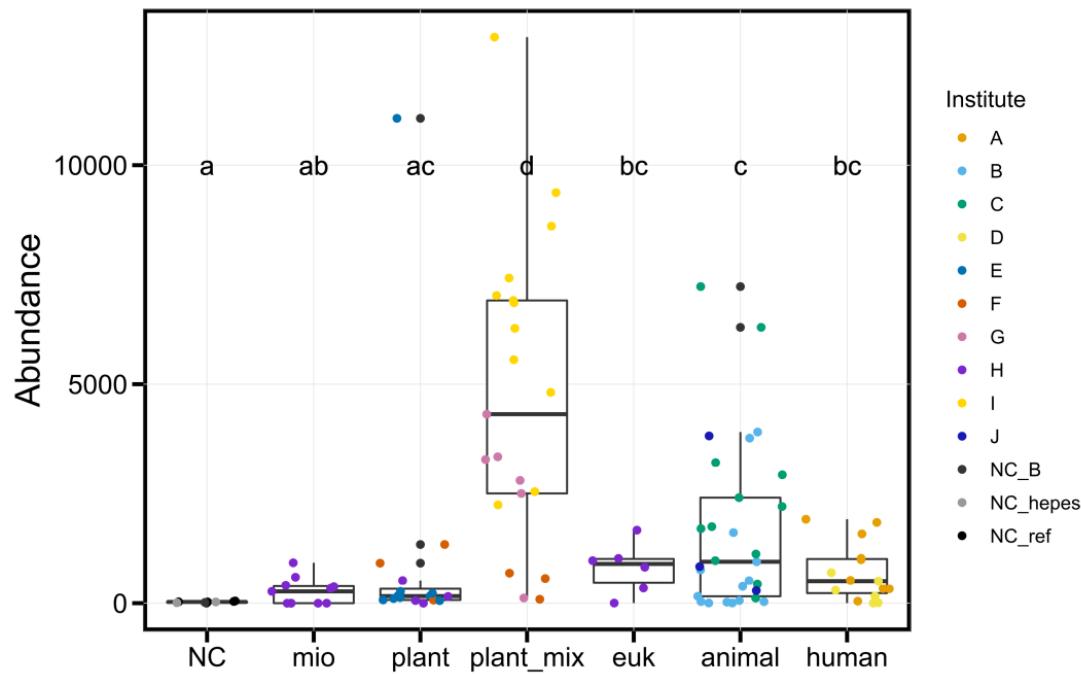
Bajerski *et al.*, 2020 , Appl Microbiol Biotech.



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Occurrence of sequences related to stored material

Abundance of sequences affiliated with the phylum
Cyanobacteria/chloroplasts in relation to the stored material



- Compact letter display: significance of differences
- NC, negative control; mio, microorganisms; plant_mix, plant and other eukaryotes; euk, eukaryotes (animal, human)



Occurrence of sequences related to stored material

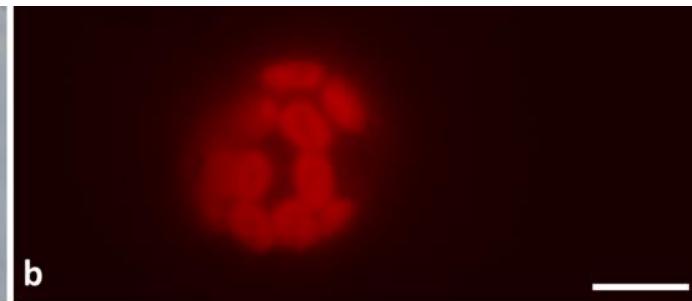
Morphology of detected green algae and diatoms

Cells of *Chlorella* spec

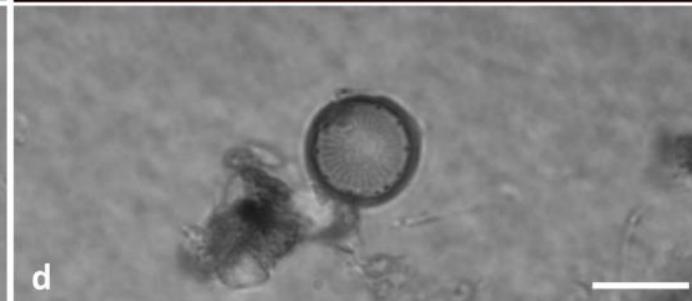
a) bright field color



b) autofluorescence



c) pennate



d) centric

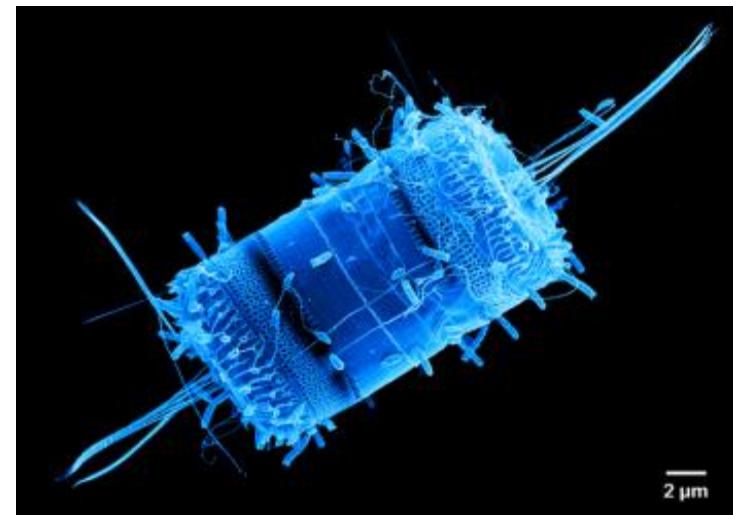
Diatoms



Biobank material and their possible contaminants: Microorganisms (Culture Collections)

- Microorganisms survive severe conditions, grow at subzero temperatures down to $-15\text{ }^{\circ}\text{C}$ (Bajerski et al. 2017; Georlette et al. 2004, Mykytczuk et al. 2013)
 - not expected to actively metabolise in LN storage tanks
 - Survive without cryoprotectant (Georlette et al. 2004; Shimkets 2013)
 - *Citrobacter freundii* and *Staphylococcus aureus* survived the direct exposure to LN (Ramin et al. 2014)
- Carbon-rich media & supplemented sugars can act as cryoprotectants (Vekeman et al. 2013)
- Microorganisms can occur as mixture of organisms

Scanning electron micrograph of the non-axenic diatom *Thalassiosira rotula*. (Pradella, S., Päuker, O., Rohde, M., unpublished)



Biobank material and their possible contaminants:

Plants

- Healthy plant tissues commonly colonised by bacteria or fungi which do not damage the host or elicit defence responses (Wilson 1995)
 - Stressful conditions during cryopreservation triggers proliferation and outbreaks of endophytes
 - Colonisation of these microbes emerges around the shoot tips during rewarming and compromises the ability of explants to regrow to a fully developed plant (Köpnick et al. 2018; Senula et al. 2018; Senula and Keller 2011)
- Plants diseases, e.g. viruses
(Knierim et al. 2017)



- Plant-associated *Methylobacterium populi* detected in LN tanks, might originate from plant material stored in non-hermetically sealed containers (Bajerski et al. 2020)

Biobank material and their possible contaminants:

Human biomaterials, Cell lines

- Material and the sampling process usually not sterile (Bielanski and Vajta 2009; Ramin et al. 2014; Vitrenko et al. 2017)
- Microorganisms colonise all parts of the body as commensals or opportunistic pathogens
 - number of bacteria in the human body 10^{13} with more than 1000 different species (Dekaboruah et al. 2020, Sender et al. 2016)
- Cell lines: Mycoplasma as known contaminant during cell culturing
 - renders the biological resource useless due to the production of artefacts, such as altered cell metabolism, protein, RNA, or DNA levels (Drexler and Uphof 2002)

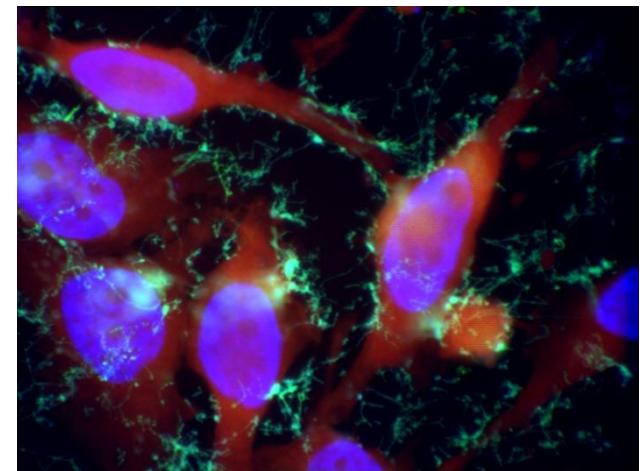
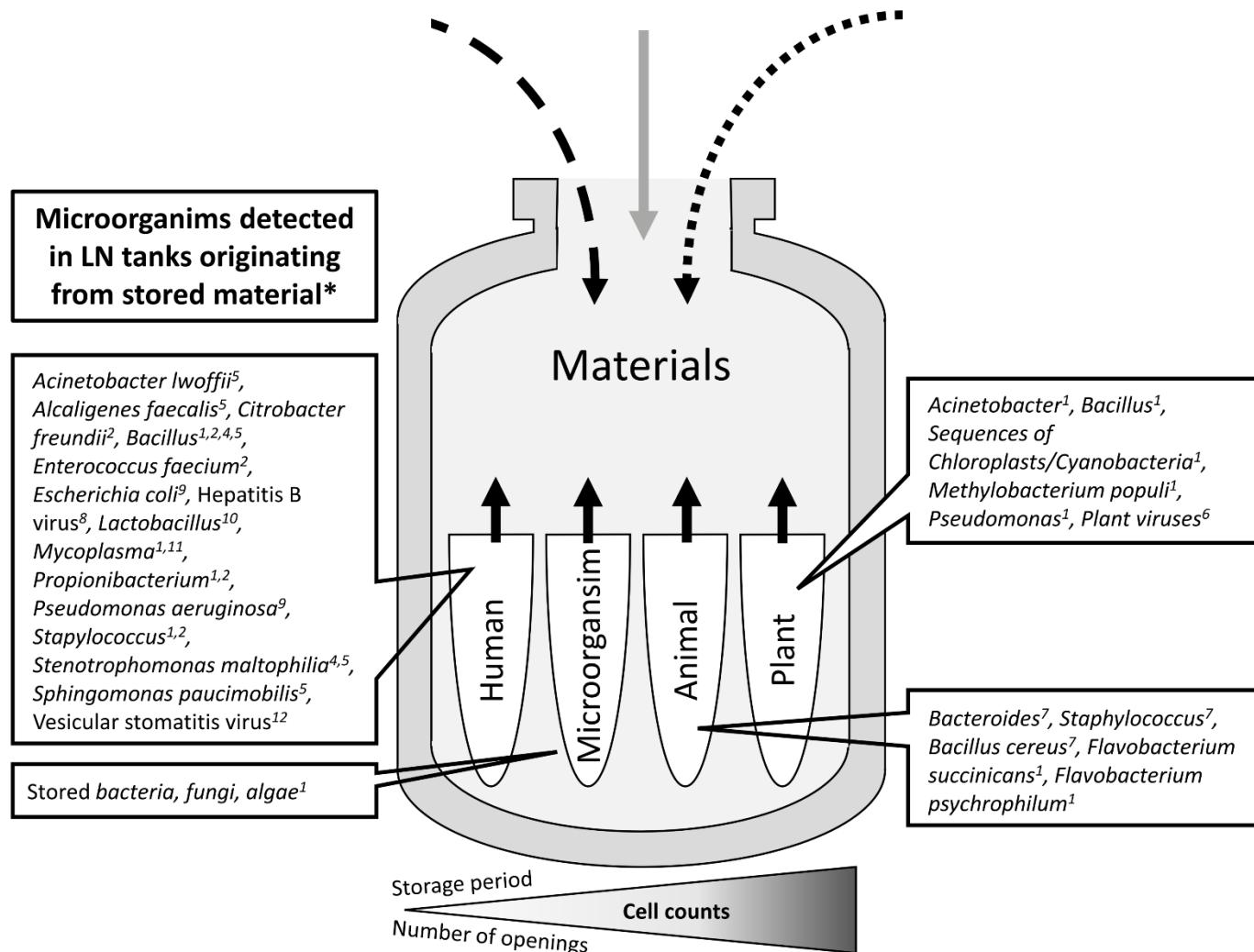


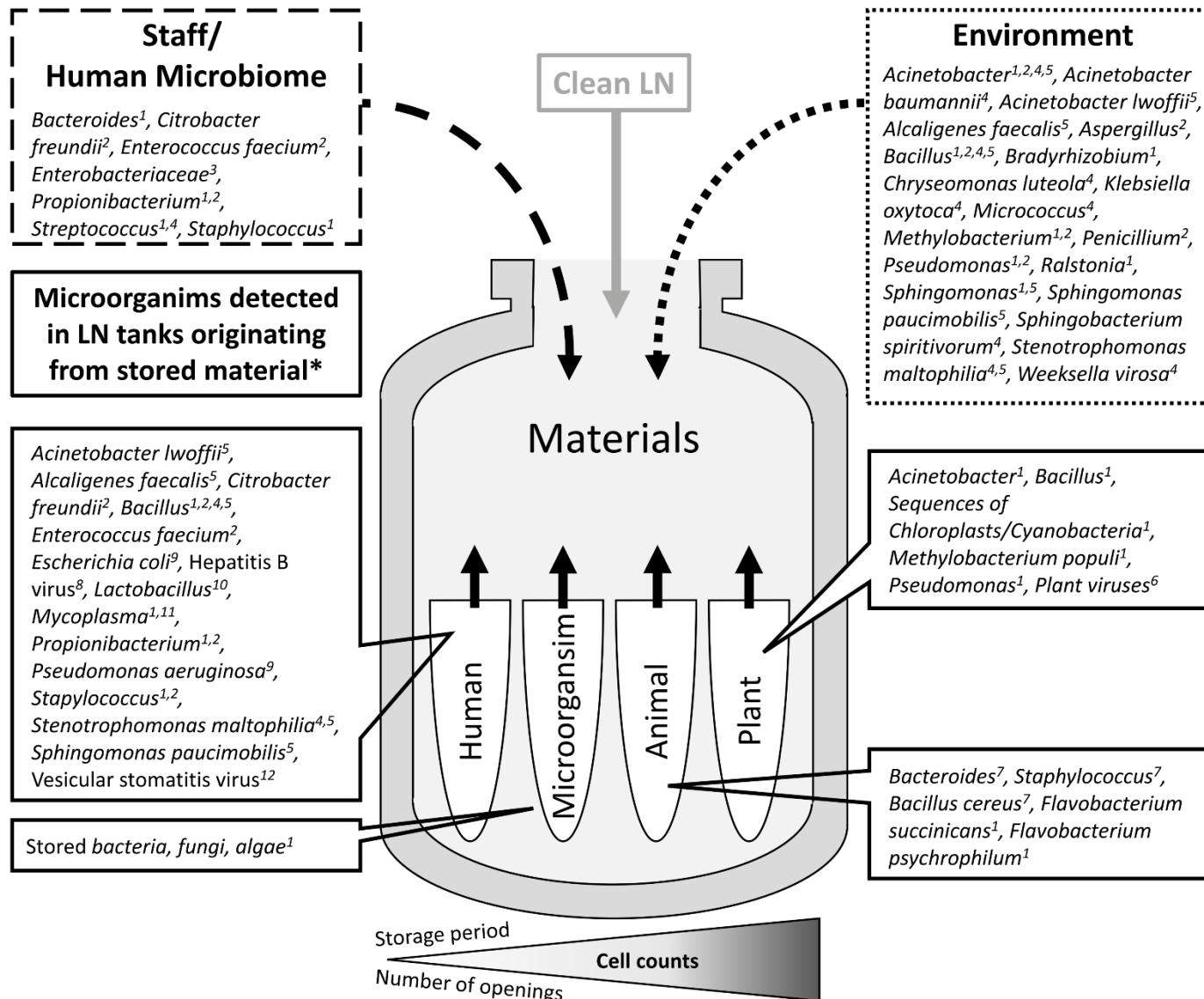
Fig.: Fluorescence image of a mycoplasma-infected cell line (mycoplasma green, cell nuclei blue and cytoplasm red), ©Cord Uphoff

Potential contaminants & routes of contamination



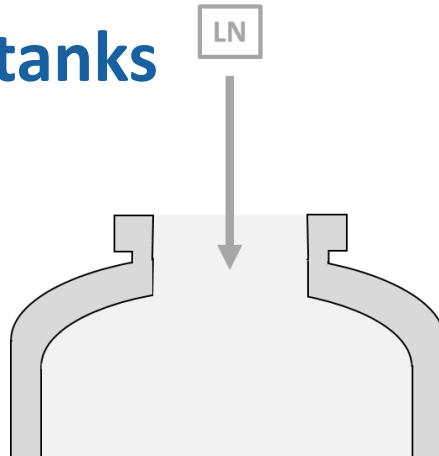
Bajerski, Nagel & Overmann, 2021, Appl Microbiol Biotech.

Potential contaminants & routes of contamination



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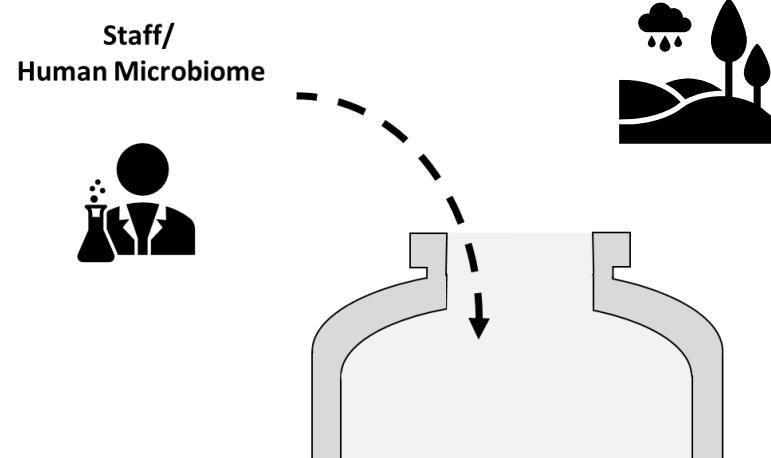
Routes of cross contamination in LN storage tanks



- LN as a source
 - freshly manufactured LN typically considered as free of microbial contaminants (Molina et al. 2016)
 - microbial contamination might occur during transport or transfer ubiquitous microorganisms can be expected to occur in the storage tanks (Bielanski and Vajta 2009)
 - Gaseous and LN as transfer media shared by different samples
 - Contaminated LN can become source for contamination (Bielanski et al. 2000; Fountain et al. 1997)
 - Microbial load of the LN phases is low (ice and sediment in cryotanks) or below the detection limit (LN in cryotanks (Bajerski et al. 2020))

Routes of cross contamination in LN storage tanks

- Storage system
 - Type of storage (in LN or above LN in the vapour phase)
 - Use of open or closed storage devices
- Sampling & tank environment
 - Handling by staff, e.g. skin-colonising organisms (Bajerski et al. 2020; Fountain et al. 1997)
 - In foetal tissues, the typical vaginal microbiota (e.g. *Lactobacillus* sp.) and airborne or hospital contaminants were detected, likely entering the samples during surgery and handling (Vitrenko et al. 2017)
 - Environmental organisms from surrounding atmosphere (Bajerski et al. 2020; Fountain et al. 1997; Molina et al. 2016; Morris 2005)
 - Ice crystals that form underneath the lids and on the rim of the tanks entrap microorganisms (Bajerski et al. 2020; Morris 2005; Schafer et al. 1976)
 - represent a major risk factor for contamination



Summary

Presence of the microorganisms is low and can be explained by their

- (1) high abundance in the environment
- (2) association with operating personnel
- (3) the introduction into LN tanks through the stored biomaterials



Recommendation

- Avoid contaminations with human microbiome
- Install air filtered systems in labs to avoid environmental intakes
- Screen for diseases & microorganisms

Sample collection

- Sterile handling
- Use filtered LN
- Regular cleaning of instruments & automated systems
- Use of hermetically sealed containers and/or secondary packaging
- Avoid glass containers

Sample processing

- Track samples via barcode
- Decontaminate tanks & transfer devices regularly
- Avoid ice formation
- Use the LN vapour phase for storage
- Use quarantine tanks
- Check sample devices after longer storage for leakage

Cryostorage

Withdrawal & distribution

- Sterile handling
- Decontaminate tubes before opening
- If required, use antibiotics to protect patients

Bajerski, Nagel & Overmann, 2021, Appl Microbiol Biotech.



Acknowledgements

Microbial Ecology and Diversity Department

- Johannes Sikorski
- Sixing Huang
- Technical Assistance (Anika Methner, Franziska Klann)
- Microscopy (Petra Henke)



Gemeinschaft

Deutscher
Kryobanken
e.V.

GDK „Hygiene“-Project

Manuela Nagel
(IPK, Gatersleben)

Prof. Jörg Overmann

Literature

- ¹Bajerski, Bürger et al. 2020,
- ² Fountain, Ralston et al. 1997,
- ³Ramin, Bürger et al. 2014,
- ⁴Morris 2005,
- ⁵Molina, Mari 2016,
- ⁶Knierim, Menzel et al. 2017,
- ⁷Pessoa, Rubin et al. 2014,
- ⁸Tedder, Zuckerman et al. 1995,
- ⁹Bielanski, Bergeron et al. 2003,
- ¹⁰Vitrenko, Kostenko et al. 2017,
- ¹¹Drexler and Uphoff 2002,
- ¹²Schafer, Everett et al. 1976.

* Microorganisms detected in LN tanks most probably originating from the stored material itself.



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