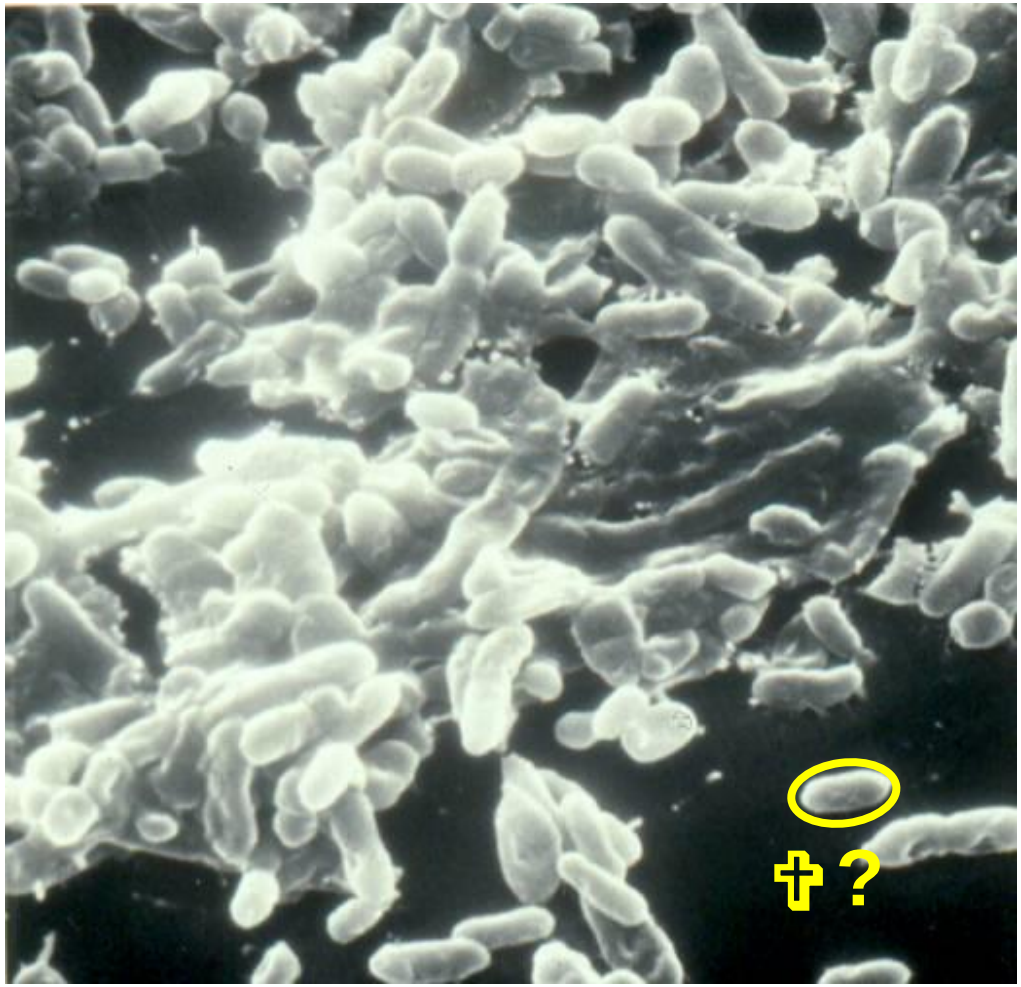
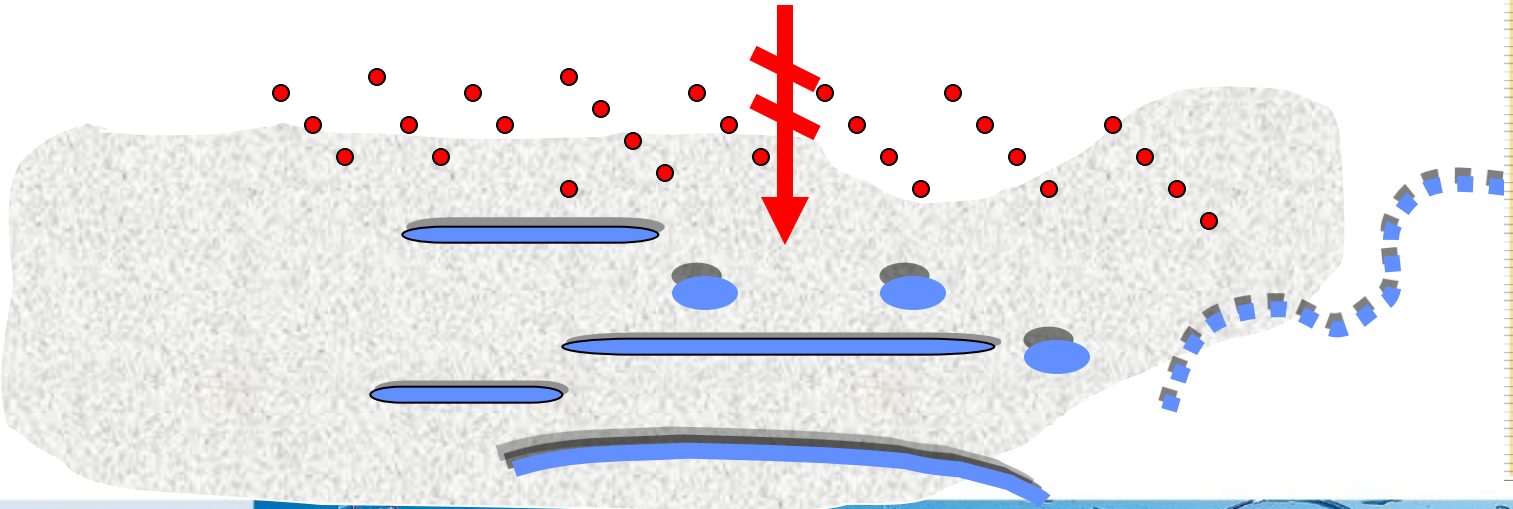
The eternal life of bacteria

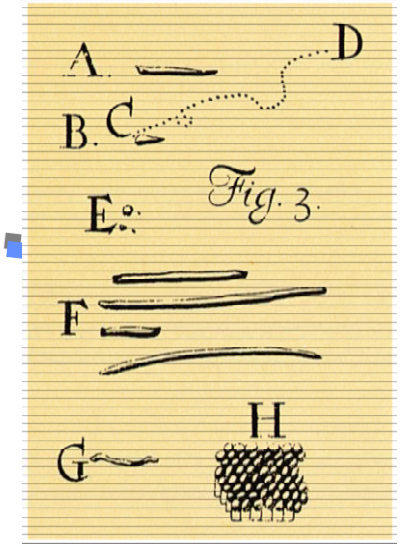


Hans-Curt Flemming, Biofilm Centre, University of Duisburg-Essen

Are they immortal?

1684 discovered by van Leeuwenhoek
"From whence I conclude, that the Vinegar with which I washt my teeth, kill'd only those Animals which were on the outside of the scruf, but did not pass thro the whole substance of it."

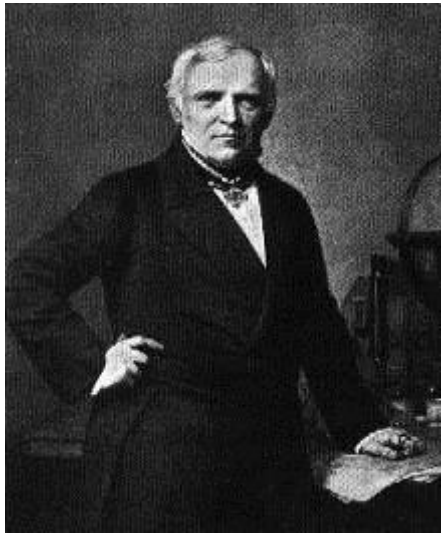




The eternal life of microorganisms

Lazzaro Spallanzani, Professor for physics and natural philosophy in Bologna, Rom, St. Petersburg (1776):

„Animalcula [microorganisms] **can be killed by complete desiccation, elevated temperature, electrical shock, treatment with various deadly chemicals and resurrect afterwards!“**



Christian Gottfried Ehrenberg, Professor for micropaleontology und mycology in Berlin (1838):

„This is not resurrection but an extreme deceleration of life processes“

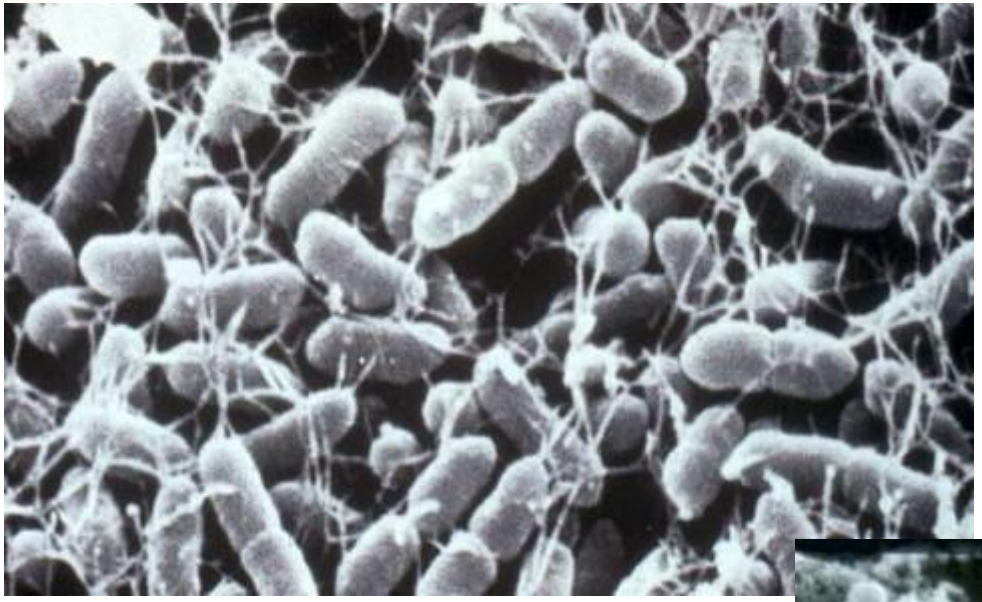
„Anabiosis“ (Wilhelm Preyer, 1872): Phenomon of resuscitation of microorganisms

„Cryptobiosis“ = hidden life (Keilin (1959)

Tod = not viable state – but: how determined?

So: How dead is dead?

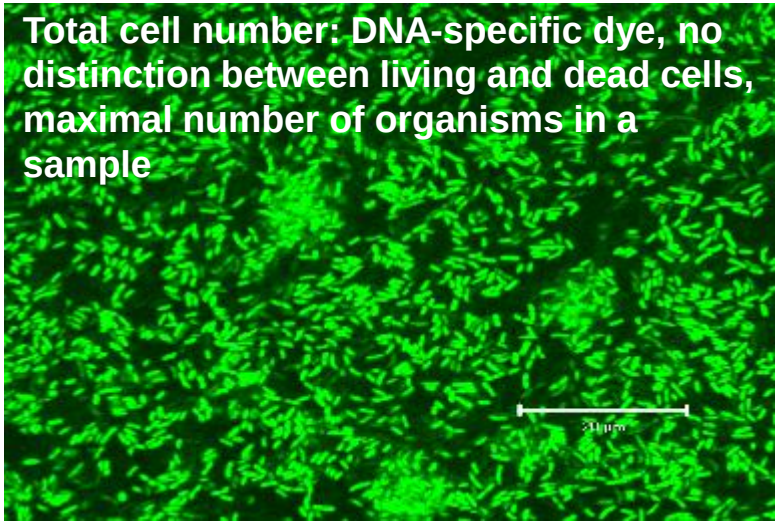
Safest life sign: Colony formation



Colony (cfu)

Only those which are fully alive

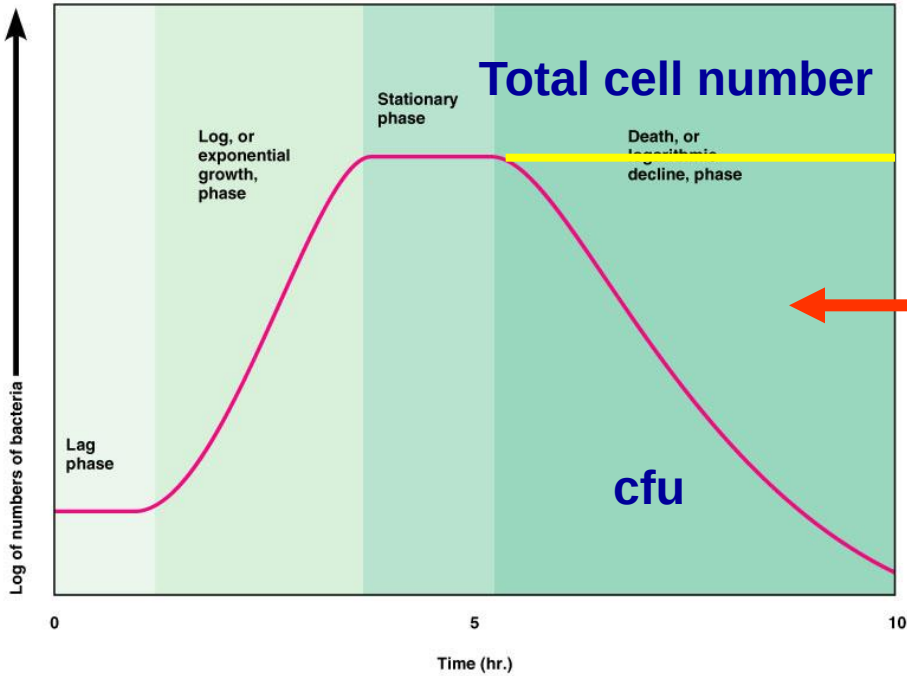
Between the colonies



G. Tuschewitzki

Considerable proportion of microorganisms: Viable but not culturable (VBNC) – not dead!

The Great Plate Count Anomaly



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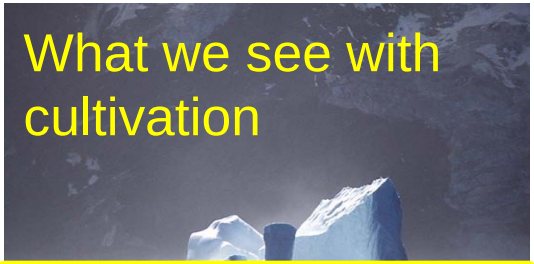
Cells which are not culturable but not necessarily dead

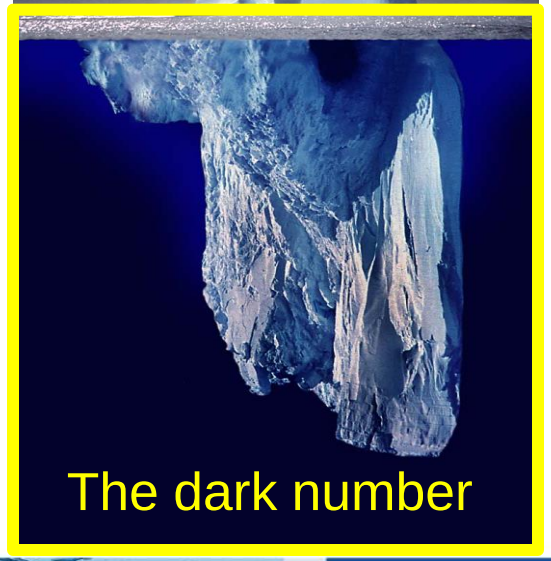
- Bacteria in VBNC state do not grow on usual microbial media on which they usually would grow and develop colonies
- However, they are alive and can pick up metabolic activity
- Cells in VBNC state show extremely low metabolism but are reculturable after resuscitation
- **VBNC state occurs particularly frequently in biofilms!**

Definitions: „Viable but not culturable“ (VBNC)

The current definition of VBNC is operational:

- “A bacterial cell in the VBNC state may be defined as one which fails to grow at the routine bacteriological cultivation conditions under which it would normally grow, but which is in fact alive and has still metabolic activity (after Oliver, 1999, 2005, 2010, modified)
- This can be understood as a stress response.
- In that sense, it applies for cells, called, e.g.:
  - Injured cells
  - Dormant cells, sleepers
  - Inactive, passive cells
  - Cryptic growth
  - Moribund cells
- There are many reasons not to grow, but:
  VBNC-cells can recover, resuscitate,
  regain culturability & become infectious again





Cultivation methods: current gold standard

- ⇨ Assessment of sterilization
- ⇨ Assessment of disinfection efficacy
- ⇨ Assessment of drinking water quality
- ⇨ Assessment of food and beverage safety
- ⇨ Assessment of pharmaceutical safety
- ⇨ Assessment of catheter, implant etc. safety

➢ **Until now, risk assessment is based *only* on cultivation methods**

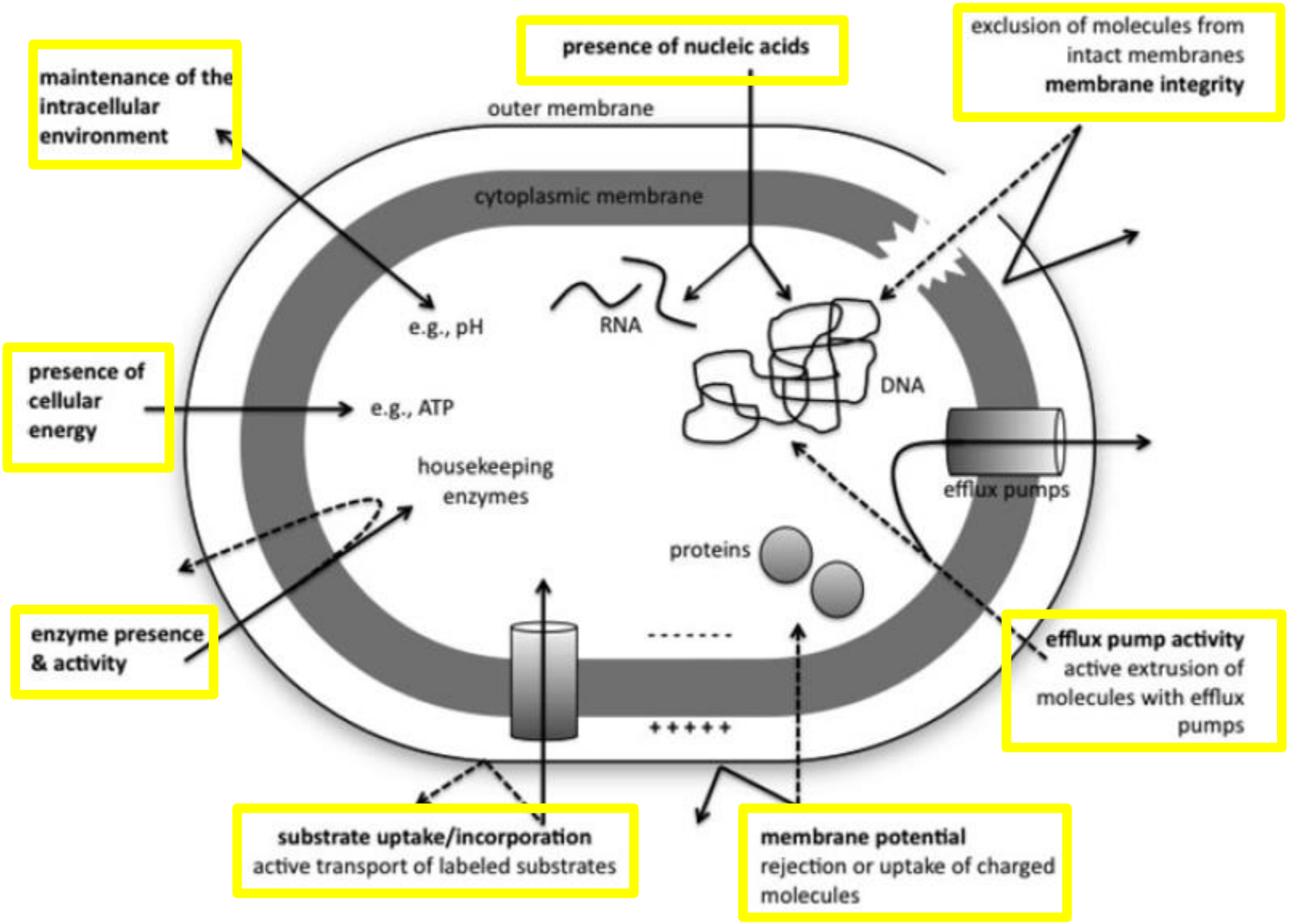
This is a considerable health risk:

- ⇨Underestimating presence of pathogens
- ⇨Overestimating efficacy of disinfection

Questions about the VBNC state:

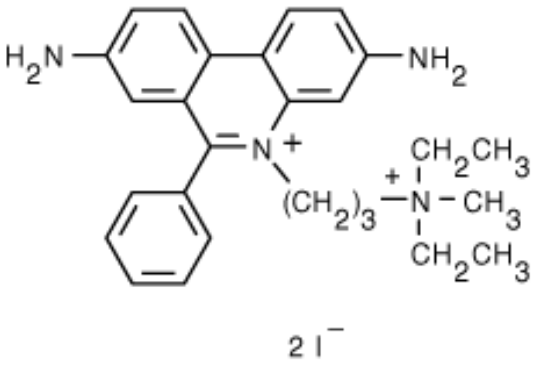
- **Why do bacteria not grow in media?**
  - ⇨ Oxidative stress (e.g., oxidizing disinfectants)
  - ⇨ Metal stress
  - ⇨ Irradiation stress
  - ⇨ Pressure
  - ⇨ pH value, ionic strength
  - ⇨ Starvation
  - ⇨ Population stress, grazers
  - ⇨ Unsuitable temperature
- **Which level of remaining integrity and activity is detected by which method?**
- **What are the stress responses on a cellular and genetic level?**
  - ⇨ Repair mechanisms activated, return after success
  - ⇨ Inactivity, „waiting“, return after more favourable conditions
- **Are the cells cytotoxic and infectious in the VBNC state?**
- **If not – how can we keep pathogens in the VBNC state as long as possible?**

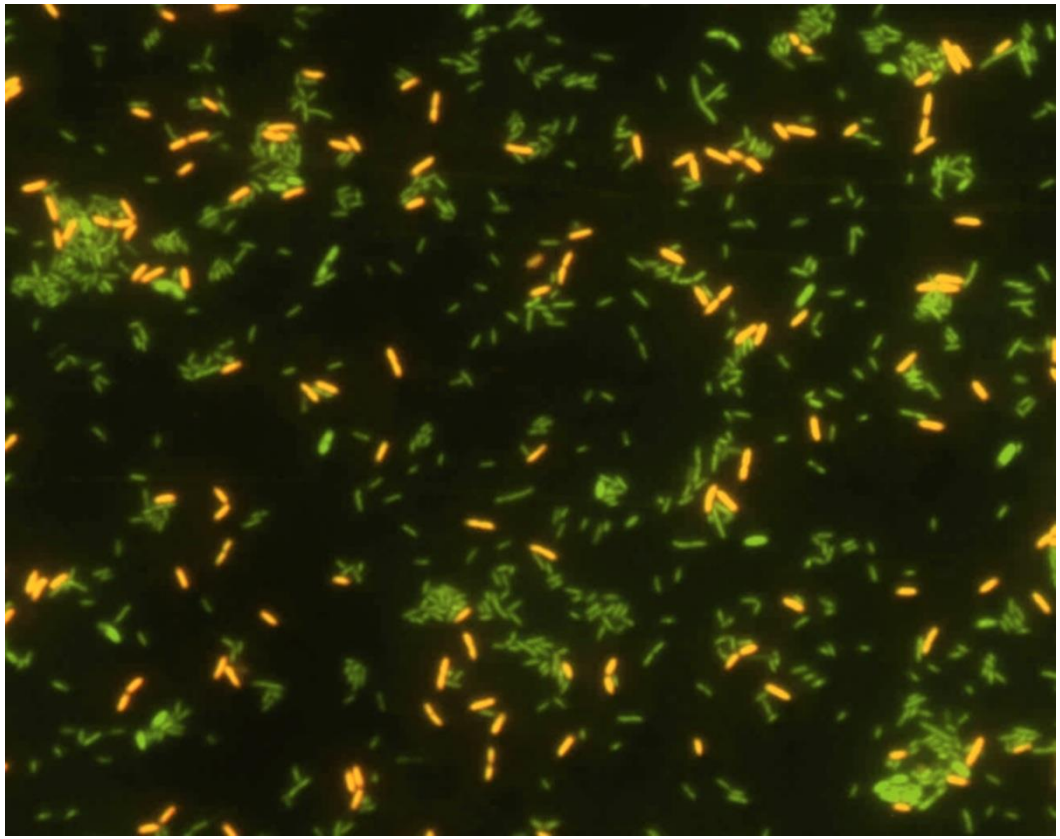
Life signs: Evaluating various cellular processes in order to assess viability status



Hammes et al., Adv. Biochem. Engin/Biotechnol. 124, 123-150 (2011)

Determination of viability: Membrane integrity





Caution:

Red is not necessarily „dead“

➢PI sensitivity varies with organisms and depends on PI concentration

Example: *P. aeruginosa*

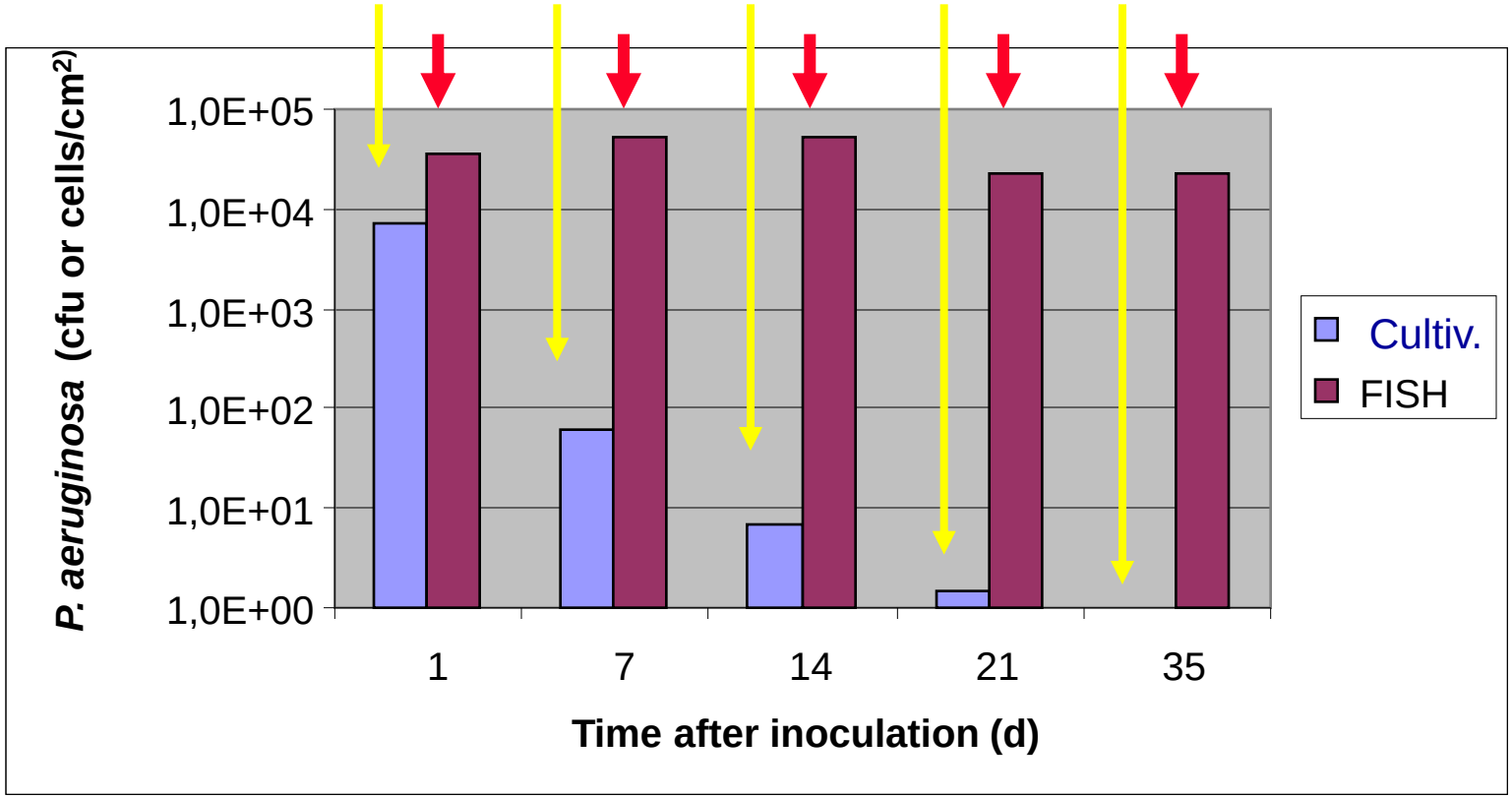
Observation: at log phase, 85 % of total cell number culturable, 80 % „dead“

Consequence: propidium iodide diluted 1:200

Example: pathogens in biofilms

Establishment of *P. aeruginosa* AdS, isolate from contamination case

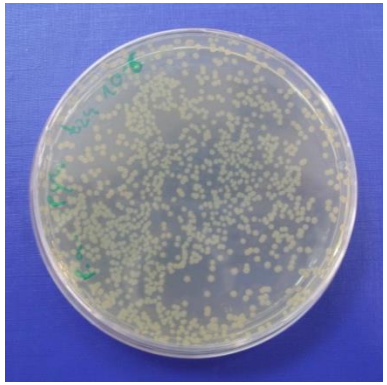
- In biofilm (EPDM, W270) cultivated on CN-agar
- FISH (Fluorescence-*in-situ*-hybridization), gene probe Psae16S-182.



- **Persistence of *P. aeruginosa* in drinking water biofilm on EPDM in viable but not culturable (VBNC) state**

J. Wingender, M. Moritz, A. Dannehl, Biofilm Centre

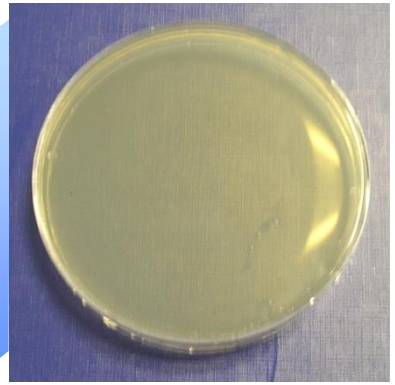
Example: *P. aeruginosa* loses culturability in copper-containing drinking water from plumbing systems.



Drinking water

containing Cu^{2+} ions

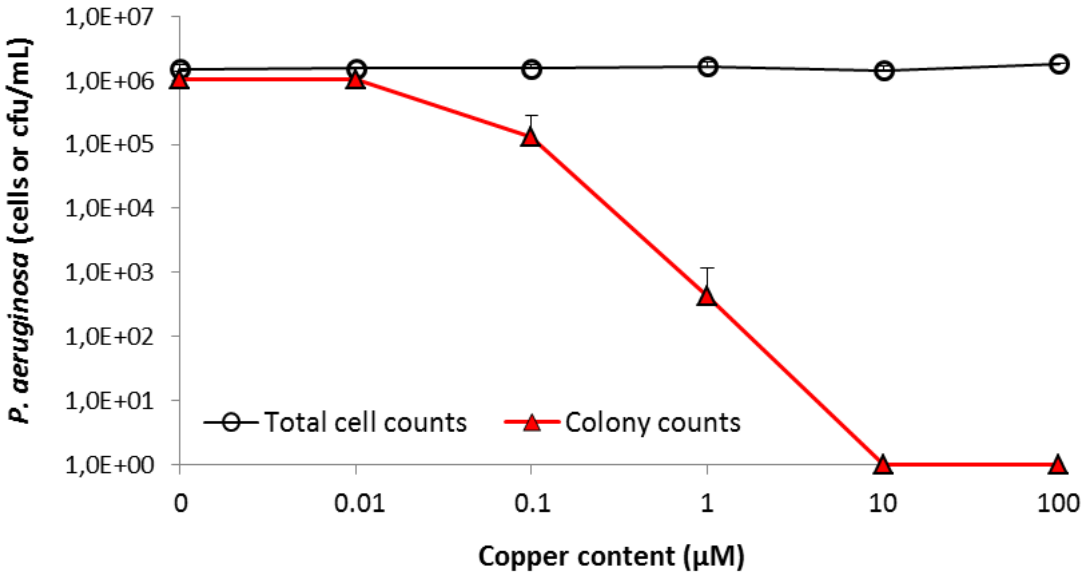
60 µg L^{-1}



- Are copper ions in drinking water the cause of the loss of culturability of *P. aeruginosa*?
- **Are the cells after copper exposure really dead?**
- Can copper-stressed bacteria return to their culturable state upon resuscitation?
- Are copper-stressed and resuscitated *P. aeruginosa* cytotoxic to eukaryotic (mammalian) cells?

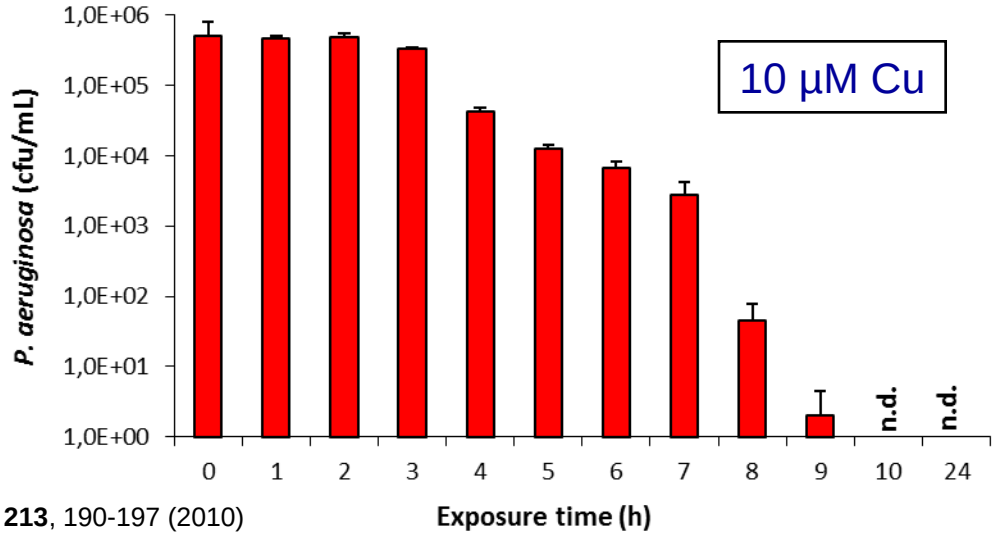
Moritz et al., Int. J. Hyg. Environ. Health 213, 190-197 (2010)
Dwidjosiswojo et al., Int. J. Hyg. Environ. Health 214, 485-492, (2011)

Cu inactivates *P. aeruginosa* time and concentration depending



Concentration-dependent loss of culturability

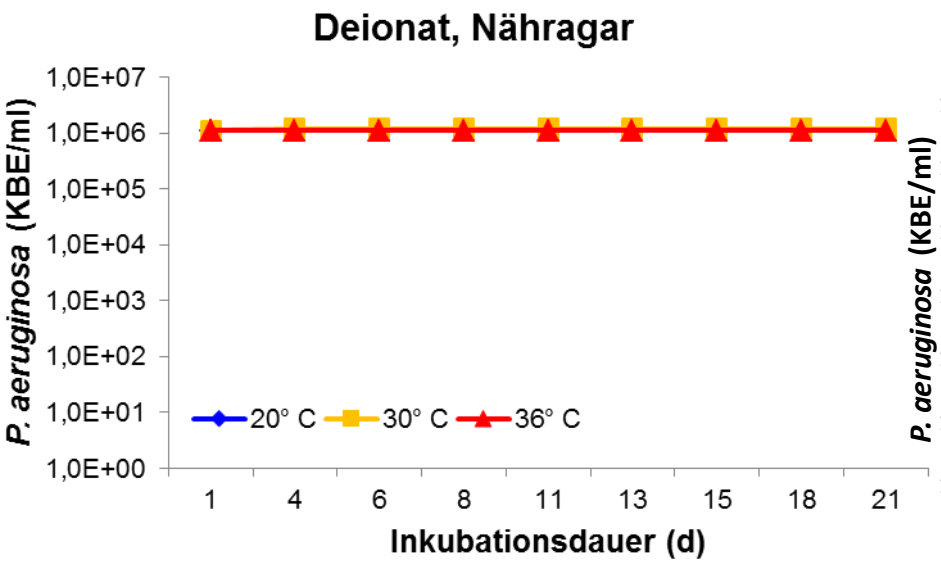
Time-dependent loss of culturability

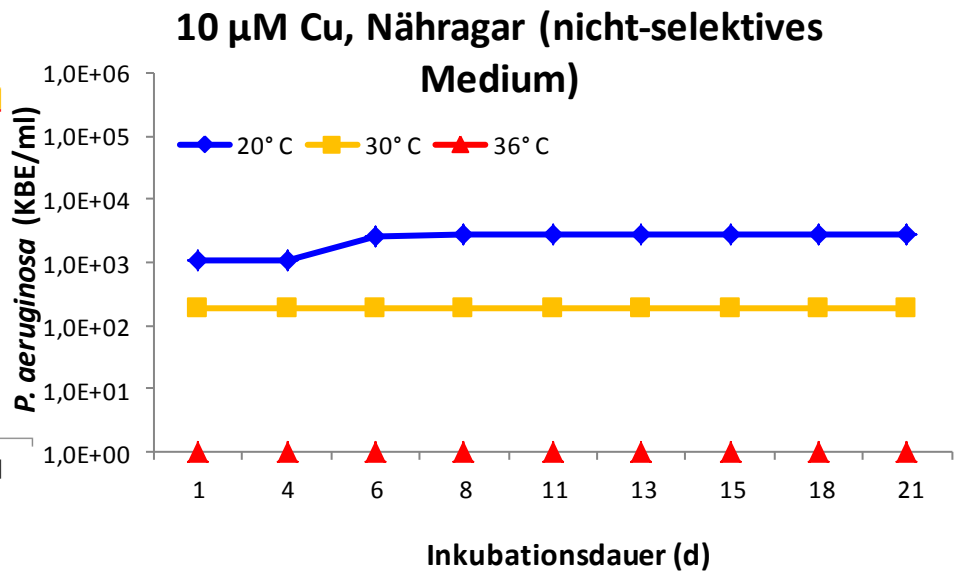


Moritz et al., Int. J. Hyg. Environ. Health 213, 190-197 (2010)
Dwidjosiswojo et al., Int. J. Hyg. Environ. Health 214, 485-492, (2011)

Recovery after longer incubation times?

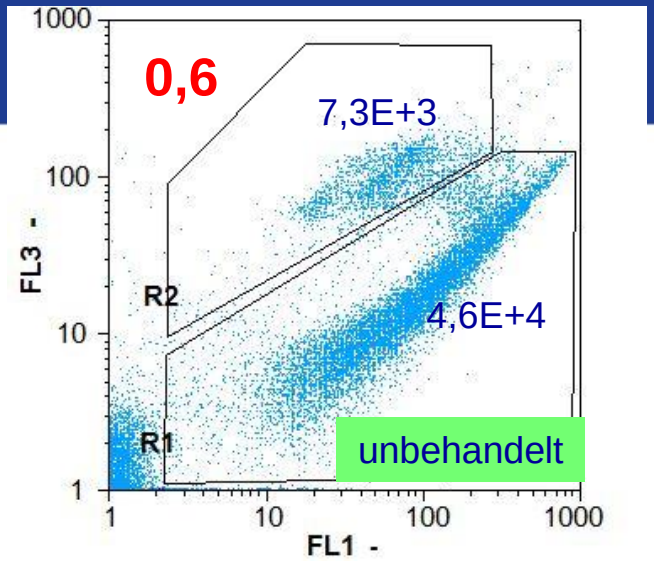
Control

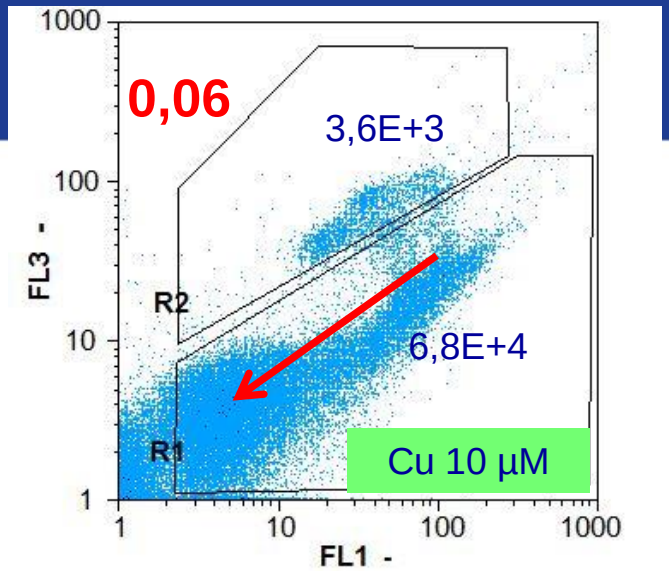




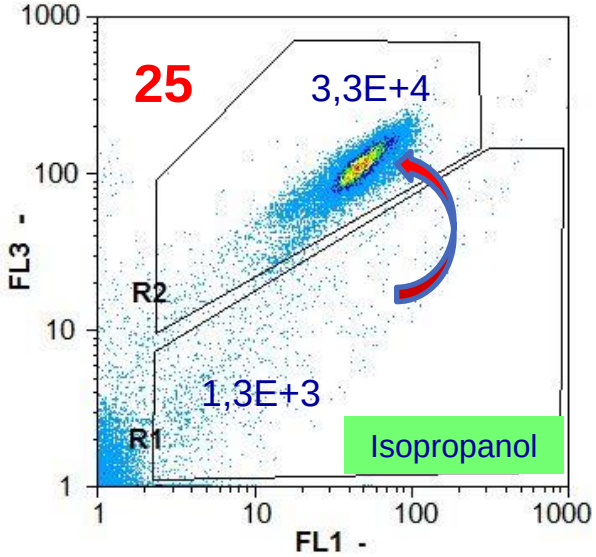
- No increase of cfu of copper-stressed cells on non-selective agar after extended incubation (3 weeks) at **36 °C**.
- BUT: at lower incubation temperatures (**20 °C** und **30 °C**), slightly higher level of cfu on non-selective medium observed

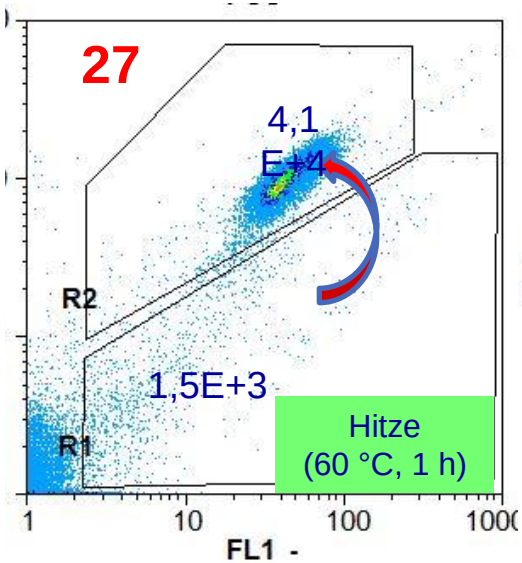
Relative intensity or fluoreszence (Propidiumiodid)

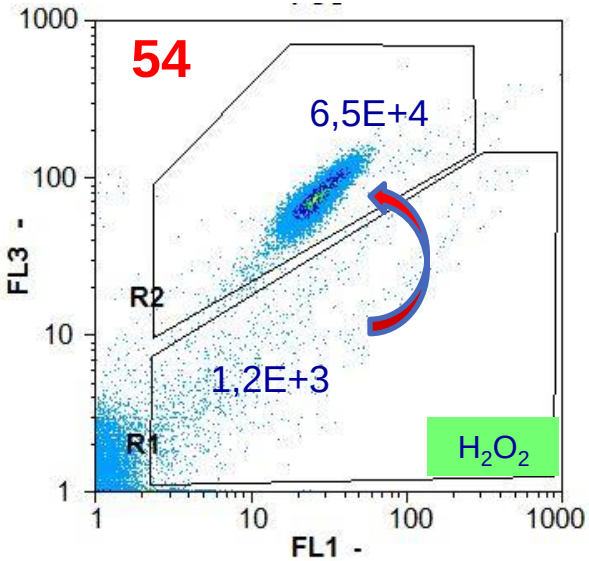




Flow cytometry: distribution pattern R2:R1

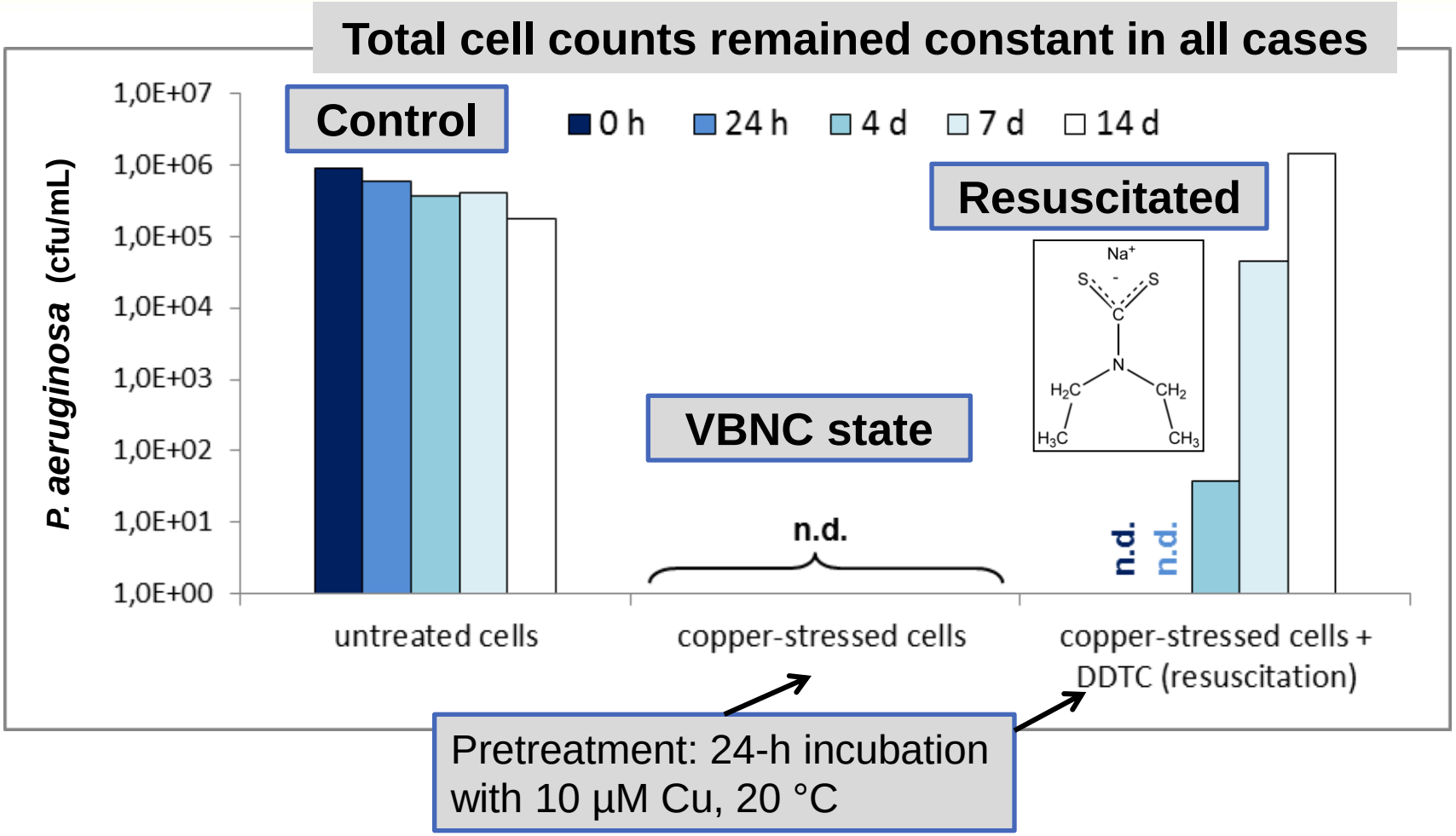






Relative intensity green fluorescence (SYTO9)

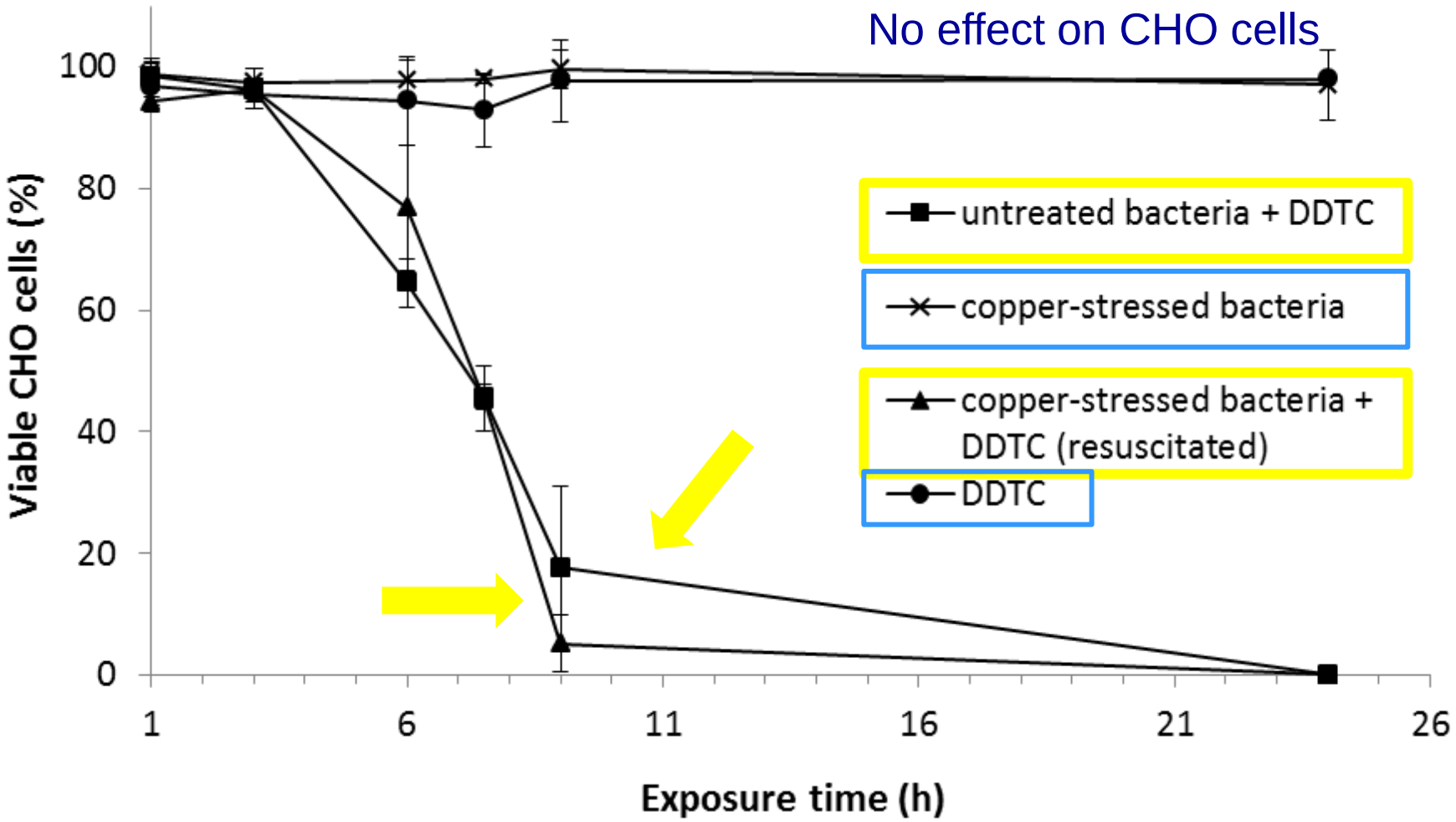
Resuscitation



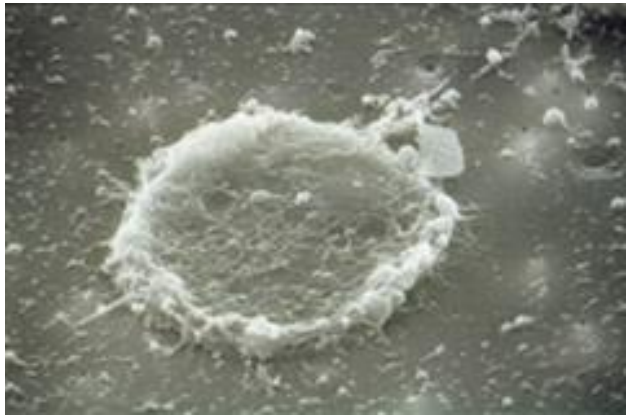
Pretreatment: 24-h incubation with 10 µM Cu, 20 °C

Copper-treated *P. aeruginosa* (VBNC bacteria) can be resuscitated, when copper stress is abolished.

Infectivity after resuscitation (CHO cells)



When are bacteria really dead?



- Loss of cultivability
- Loss of membrane integrity
- Loss of membrane potential
- Loss of enzymatic activity
- Loss of cell division
- Loss of protein production
- Loss of DNA
- Energy production terminated
- Physical destruction

⇨ **Verification of cell death depends only upon the method applied**

Conclusions

- Verification of cell death depends only on method
- Current gold standard is Cultivation: not cultivable=dead
- Applies only for cells in growth metabolism
- Many cells are in maintenance metabolism
- Examples for life signs which can be detected (⇨tool box),
- These cells may resuscitate and pathogens can regain infectivity
- This is the case for adoption of advanced methods in health surveillance
- *Research demand in validation and relevance of data in order to avoid data inflation*
- Further questions:
  - ⇨ Which organisms are infectious in the VBNC state?
  - ⇨ Expression of virulence factors in VBNC state?
  - ⇨ Which conditions support resuscitation?
  - ⇨ Does quorum sensing blocking work in VBNC?
  - ⇨ What happens in the infection process of VBNC cells?

How dead are cultivation methods?

- Current methods of cultivation do not mirror the content of viable organisms
- No „standard method“ for hygienic assessment in sight but a number of methods on lab scale level
- Therefore: Cultivation cannot be replaced but has to be considered with great care
- Employment of advanced methods suitable for cases of long-term contamination
- Crucial questions: Conventions, public health consequences – overdetection, overreaction?
- Biggest problem: *interpretation* of data gained by new methods

Is the danger to be hit by a falling star higher with stronger telescopes?

(Thomas Kistemann, Univ. Bonn)

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Options for resuscitation

- **Abolishment of stress factors**
  - ⇨ More suitable temperature
  - ⇨ Removal of inhibiting metals (e.g., by copper chelator)
  - ⇨ Termination of stress (e.g., UV, radioactive irridiation)
  - ⇨ Neutralization of antibiotics, biocides
  - ⇨ Lowering of osmotic stress

- **Exposure to more favourable conditions**
  - ⇨ Improvement of nutrient situation
  - ⇨ Resiccation
  - ⇨ Presence of „helpers“ (Lewis), „scouts“ (Slava Epstein)

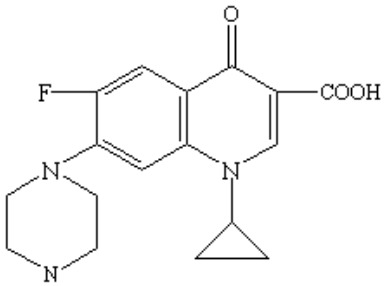
- **Other options**
  - ⇨ Uptake of resistance plasmids
  - ⇨ Repair mechanisms (e.g., UV or membrane damage)
  - ⇨ Uptake by amoebae or other hosts (animals, humans, plants?)

Toolbox for detection of viability of cells

Method	What is detected?	Interpretation
DAPI, Syto 9, PCR	Nucleic acid	All cells, dead or alive
PI+Syto 9, PMA	Membrane integrity	Principially viable
Rhodamin 123	Proton motive force	Energy conservation
Resazurin, CTC	Fluorescent resorufin, fluorescent formazan	Metabolic activity
Oxonol ($DiBAC_4$)	Membrane penetration	Membrane potential
Fluorescein diacetate	Hydrolysis of FDA, fluorescein formation	Intracellular metabolic activity
rtPCR	Reverse transcriptase	Protein expression
Direct viable count	Cell elongation	Growth sign
FISH, DVC-FISH	ribosomal RNA	Protein production
ATP	ATP	Energetical state of cell
Ethidium bromide	Expelling of EB	Efflux pump activity
^{13}C uptake	Cell-bound isotope activity	Assimilation, metabolic activity

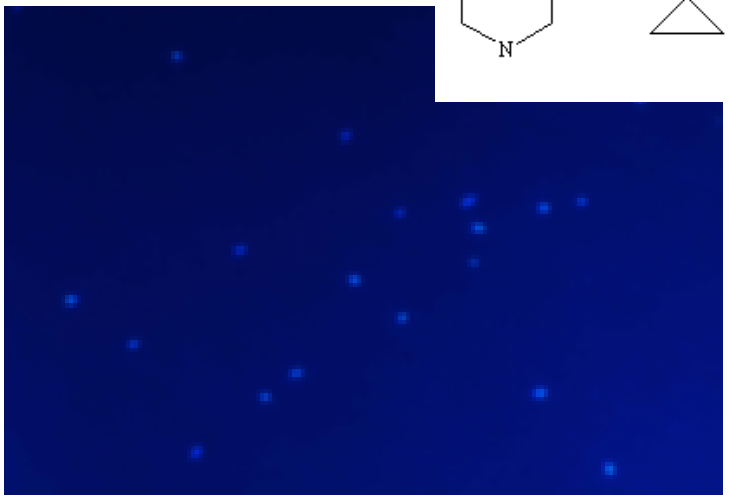
Life sign (2) Cell elongation [DVD]

- Metabolic response to R2A medium to pipimedinic acid
- Gyrase inhibitor, prevents cell division
- Live sign: cell elongation



24 h, 10 mg/mL pipemidic acid, 20 °C

Untreated (absence of copper)

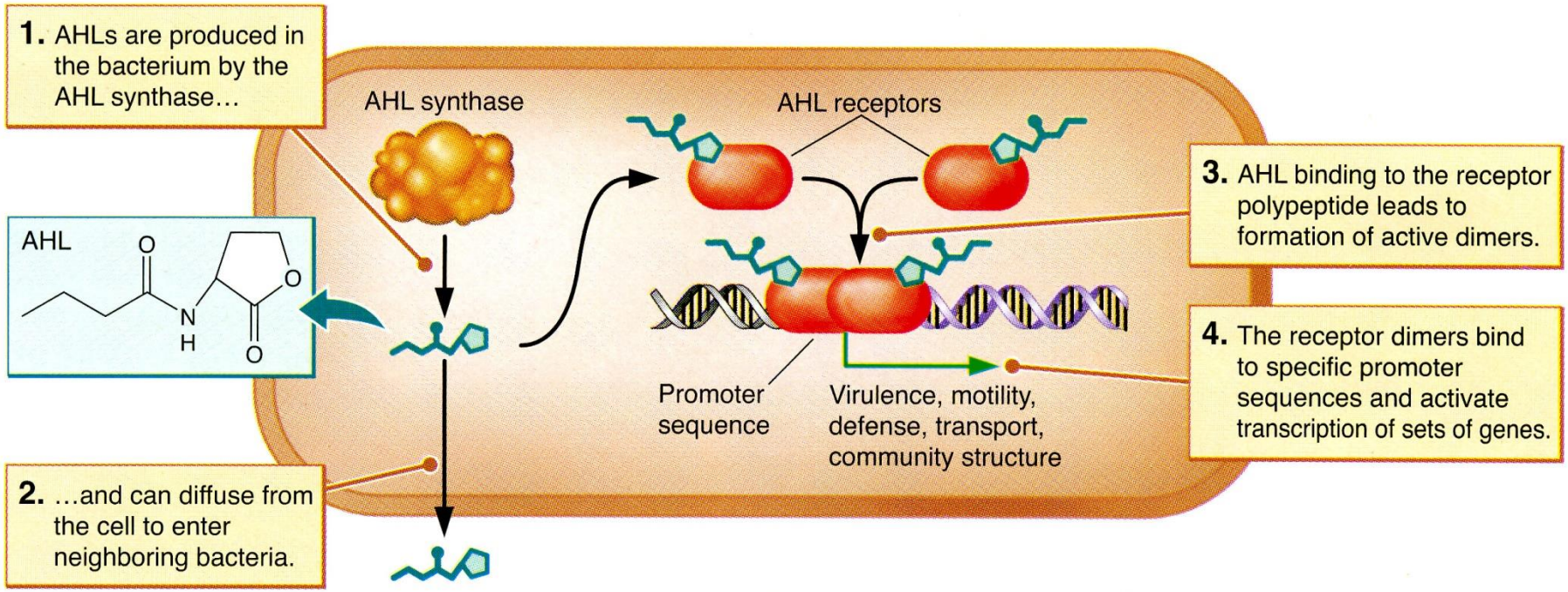


Exposed to copper-containig drinking water

- Cell elongation inhibited by copper,
- Numbers of copper-exposed metabolically active cells were similar to those of colony counts.

J. Wingender, M. Moritz, A. Dannehl, Biofilm Centre

Quorum sensing in VBNC?



Molecular mechanics of AHL-mediated QS. AHLs are produced in the bacterium by the AHL synthase and can diffuse from the cell to enter neighboring bacteria. AHL binding to the receptor polypeptide leads to formation of active dimers. The receptor dimers bind to specific promoter sequences in the bacterial genome and activate transcription of sets of genes.

Bauer et al., ASM News (2005) 71:129ff

- Quorum sensing is only active in viable cells
- In biofilms: large areas of VBNC or dead cells
- Anti-biofilm strategies using quorum sensing molecules of QS blockers will not address VBNC cells