TECHNISCHE UNIVERSITÄT
BERGAKADEMIE FREIBERG
Die Ressourcenuniversität. Seit 1765.

Bioremediation and phytoremediation 2021/22

Methods in Biohydrometallurgy

Jun.-Prof. Dr. Sabrina Hedrich
TU Bergakademie Freiberg

Sampling

- Taking samples as sterile as possible

(sterile equipment, gloves, steril bottles,…)

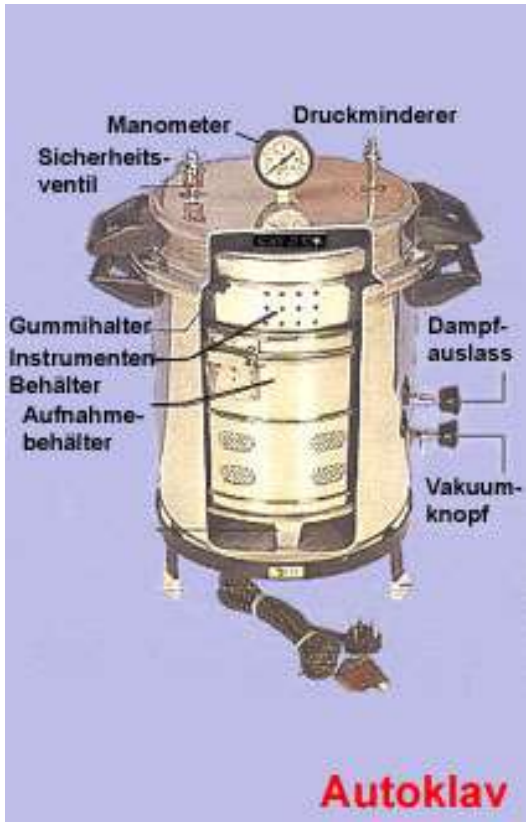
Killing of microorganisms (sterilisation)

Basically, the following methods are used:

- moist heat
- dry heat
- filtration
- radiation
- chemicals

1. Moist heat and pressure

- Bacteria and fungi are killed at 60°C within 5-10 minutes
- Yeast and fungal spores only above 120 ° C (15 min)
- Clinics are recommended for 5 minutes at 134 ° C to kill even heat-resistant spores (Bacillus stearothermophilus)
- To reach temperatures above the boiling point of water, so-called autoclaves are used. These are devices similar to a steam pressure pot in the kitchen.



2. Dry heat

Dry heat spores are killed only at higher temperatures. Therefore, sterilization at least 60 minutes at 180°C or 120 minutes at 160°C. Only then proteins denature irreversibly.

Bacteria	Incubation time 150°C	Incubation time 180°C
Bacillus antracis	60-120 min	3 min
Clostridium botulinum	25 min	5-10 min
Clostridium tetani	30 min	1 min
Soil bacteria	180 min	15 min

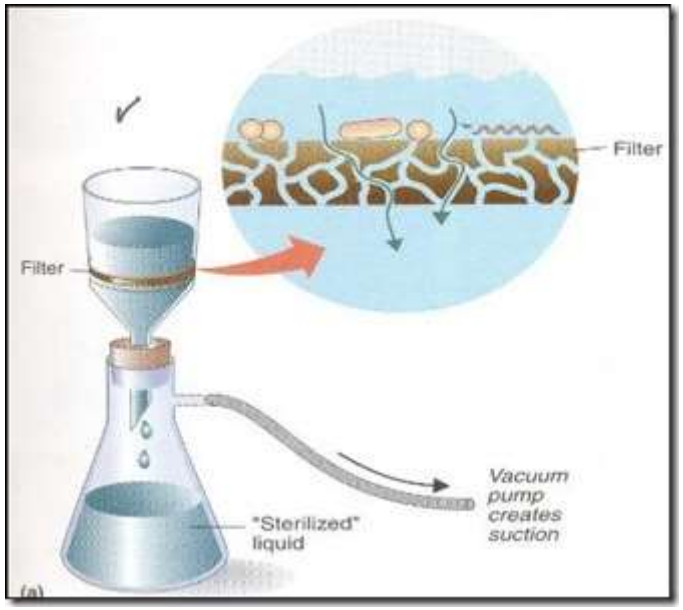
Pasteurization

Partial germination of heat-sensitive foods is e.g. used with milk, fruit juice, sauerkraut, meat and fish salads ....

Louis Pasteur (1822-1895)

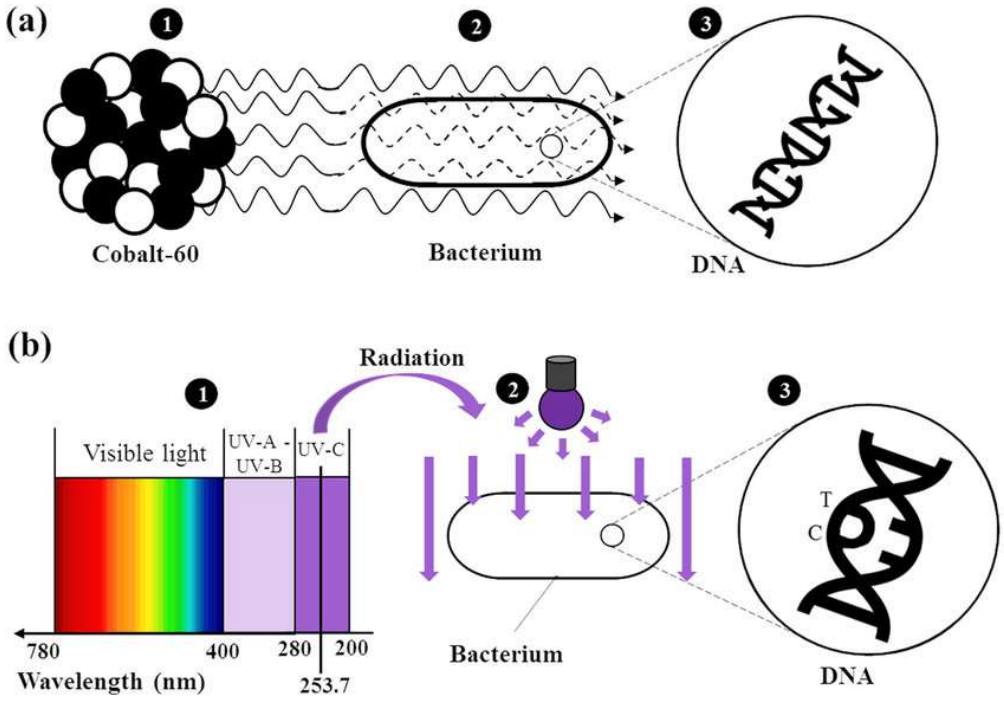
3. Filtration

- Heat-sensitive solutions
- sterilization by microfilter, e.g. in the drinking water disinfection filters made of diatomaceous earth or plastic with pore sizes of 0.22 to 0.45 µm
- However, bacterial toxins can not be removed that way



4. Radiation

- UV-, X-ray and gamma radiation are used



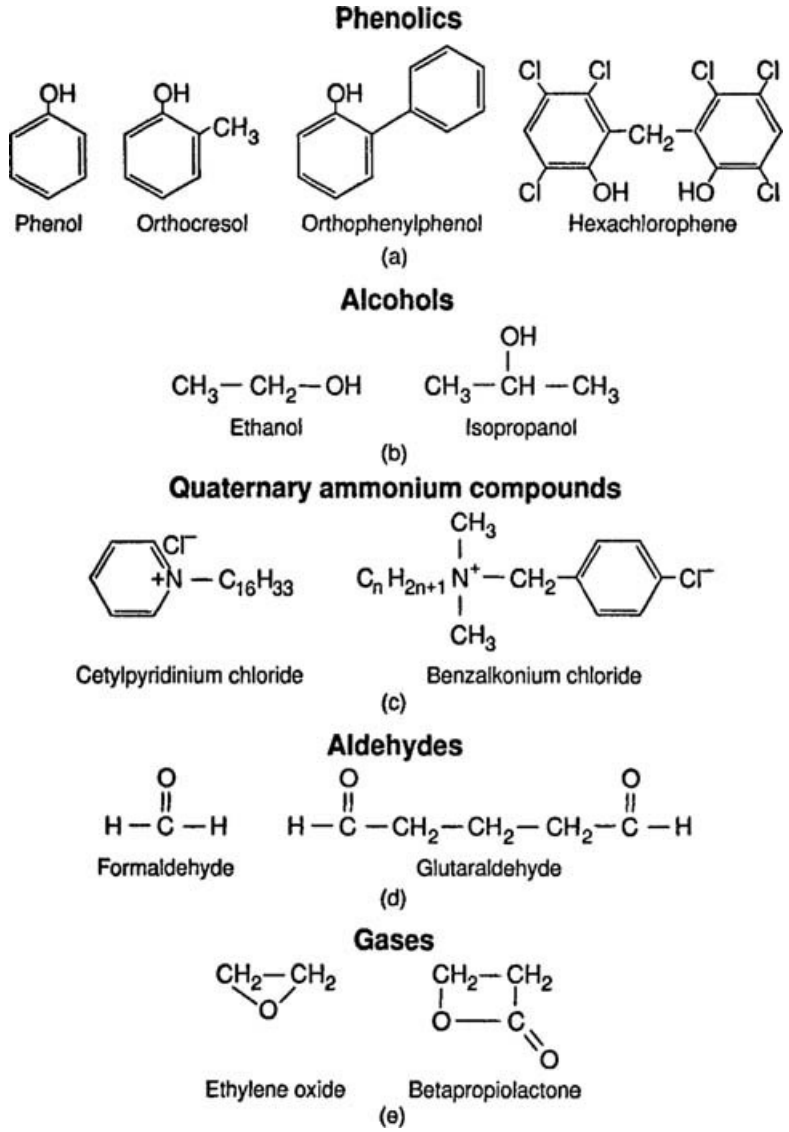
- generation of mutations and destruction of DNA
- UV light at 260 nm → absorbed by the DNA →death cells
- X-rays 10-8 -10-10 m, gamma radiation 10-10 -10-14 m (Co 60 or Cs 137)

→ ionizing effect on microbial structures, hydrogen bonds in proteins are destroyed and bases in the nucleic acids changed

5. Chemicals

- Thermosensitive devices or areas are sterilized with chemicals such as ethylene oxide (ethene oxide), ozone or formaldehyde vapor
- Ethylene oxide only works in the presence of water (5-10%)
- Ozone is e.g. used for sterilizing plants in poultry farming (Listeria) or improving sanitation in oil platforms and disinfecting water in the fish processing industry and swimming pools

Disinfectants



Sampling

- Taking samples as sterile as possible (sterile equipment, gloves, steril bottles,…)
- replicates
- diverse ecosystems → choice of sampling locations
- Measuring on site parameters

Field geochemical analysis

1. Composite probes,
   e.g.$_1$ YSI multiprobe: pH/ORP/T/Ec/DO

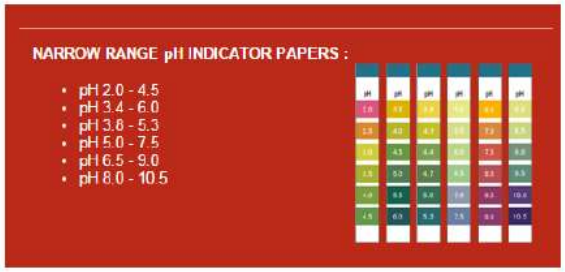
e.g.$_2$ Hanna water tester: pH/ORP/T/Ec

2. Single measurement meters

Field geochemical analysis

3. Indicator strips/paper

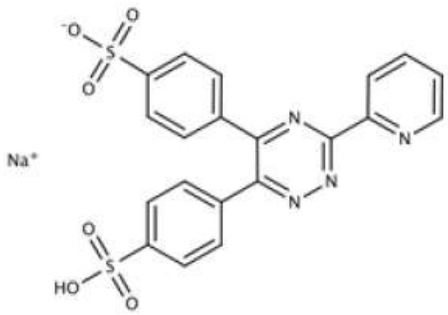
- narrow range pH papers

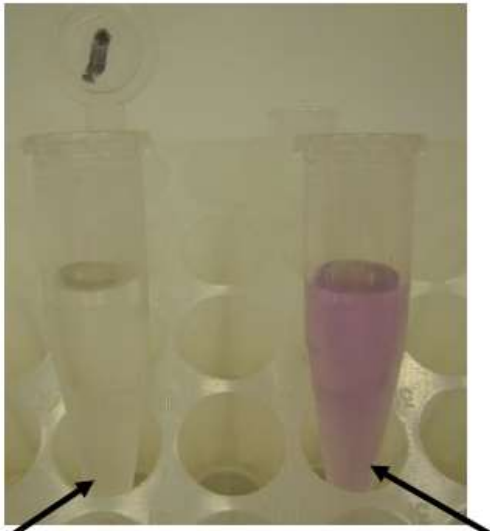


- Merkoquant test strips

Field geochemical analysis

Stookey, L. (1970) Ferrozine –a new spectrophotometric reagent for iron. Analytical Chemistry 42:779-781





950 µL of Ferrozine reagent

+50 µL test solution
(may require dilution;
max 1 mM (56 mg/L) Fe^{2+})

The coloured complex is stable for at least 24 h.
Samples can be taken back to the lab for spectro-photometric analysis

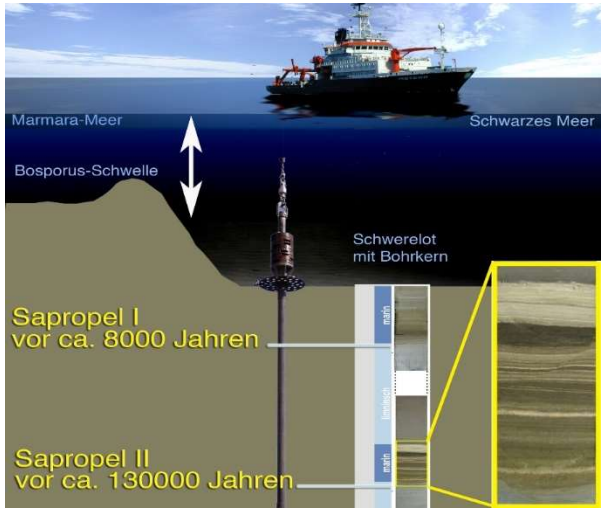
Sampling

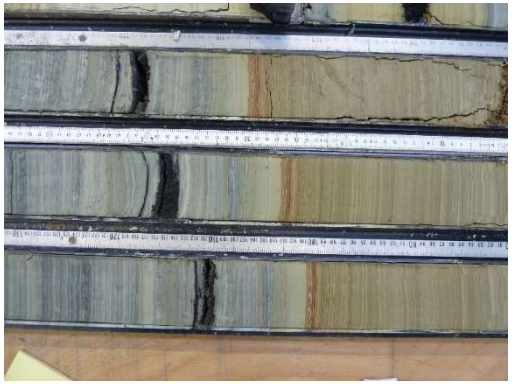
- Taking samples as sterile as possible (sterile equipment, gloves, steril bottles,…) replicates
- diverse ecosystems → choice of sampling locations
- Measuring on site parameters

- Cooling and oxygen supply of samples
- Fast sample transport
- Storage of samples depends on analyses (cold, frozen, room temperature)

Sampling - Terrestrial sites

Terestric samples: drill cores, flushing, tracers,…





Water samples

Water sampling

- Niskin Bottle

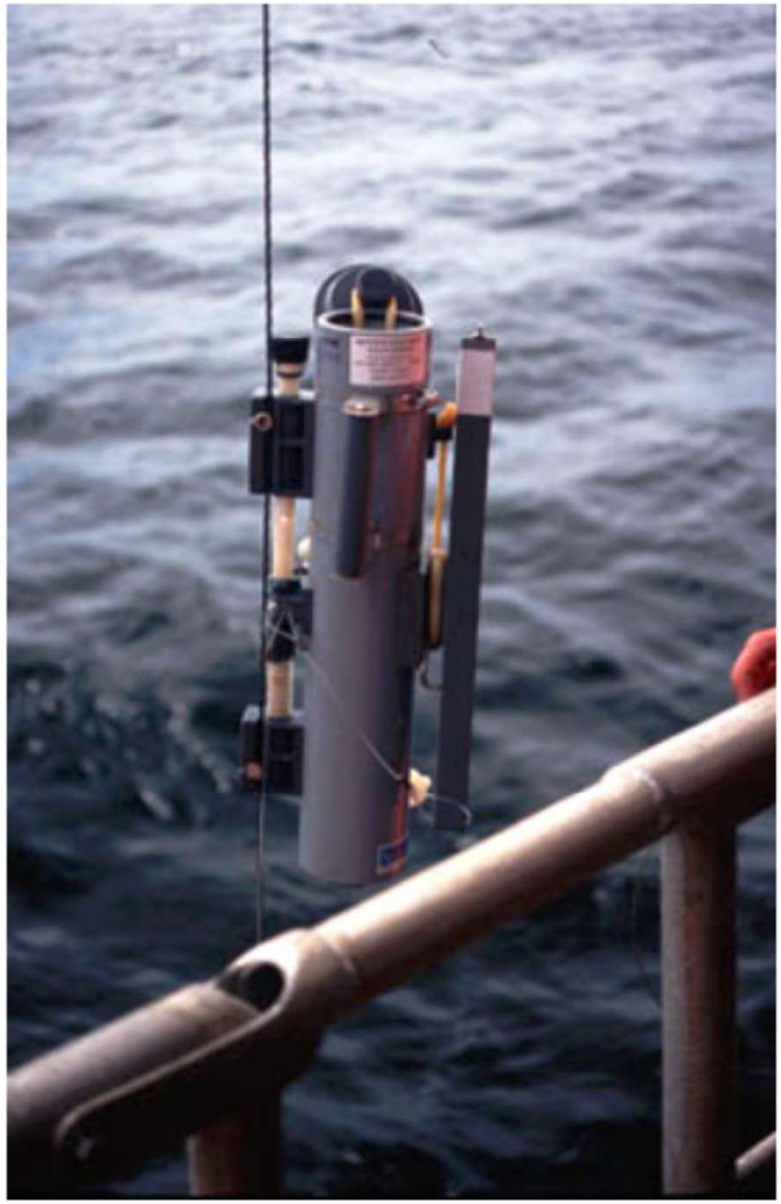
  -used for greater depths.

  -lids open or ‘cocked’ as the instrument is put into the seawater

  -At the desired depth the lids are triggered to close

  -Bottles are sealed airtight to avoid contamination

  -Water analysis conducted on board



Sample transport/ storage

(i) Solids

→ sealed polythene bags

(ii) Liquids → sterile containers (for enumeration/isolation): keep COOL (not frozen!)

→ filter through 0.2 µm (pore size) membranes

→ biomass (for DNA; can be preserved by freezing)

→ sterile water sample (for lab analyses; DOC sulfate etc))

→ add small amount of conc. HNO_3 to some sterile water sample to maintain metals in solution for lab analyses

Microbial characterization methods

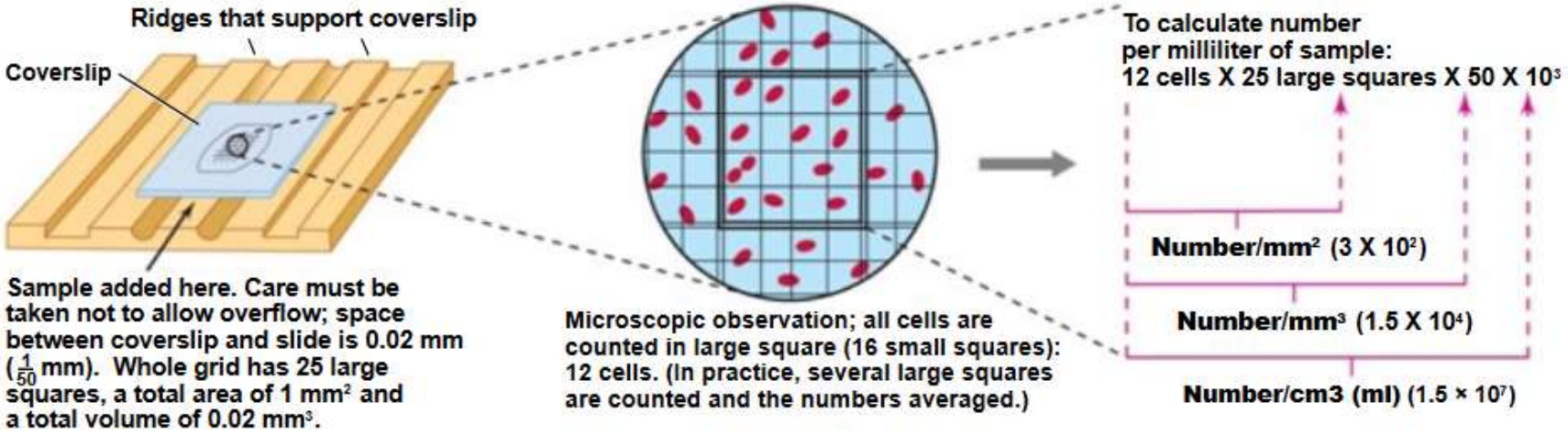
- ***In situ* methods**
  - light and electron microscopy

- **Cultivation-dependent methods**
  - growth on nutrient medium
  - conditions often unknown
  - only 1% of all organisms can be cultivated

- **Culture-independent methods**
  - extraction of nucleic acids, proteins, etc.

Total cell counts using microscopy

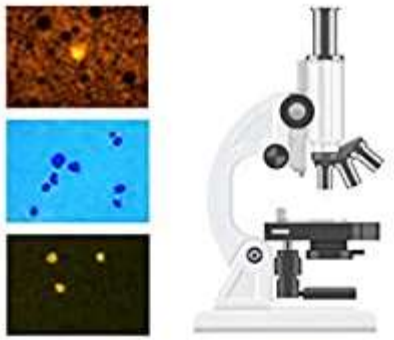
- Microbial cells are enumerated by microscopic observations (Figure 5.15)



Direct cell counts under the microscope

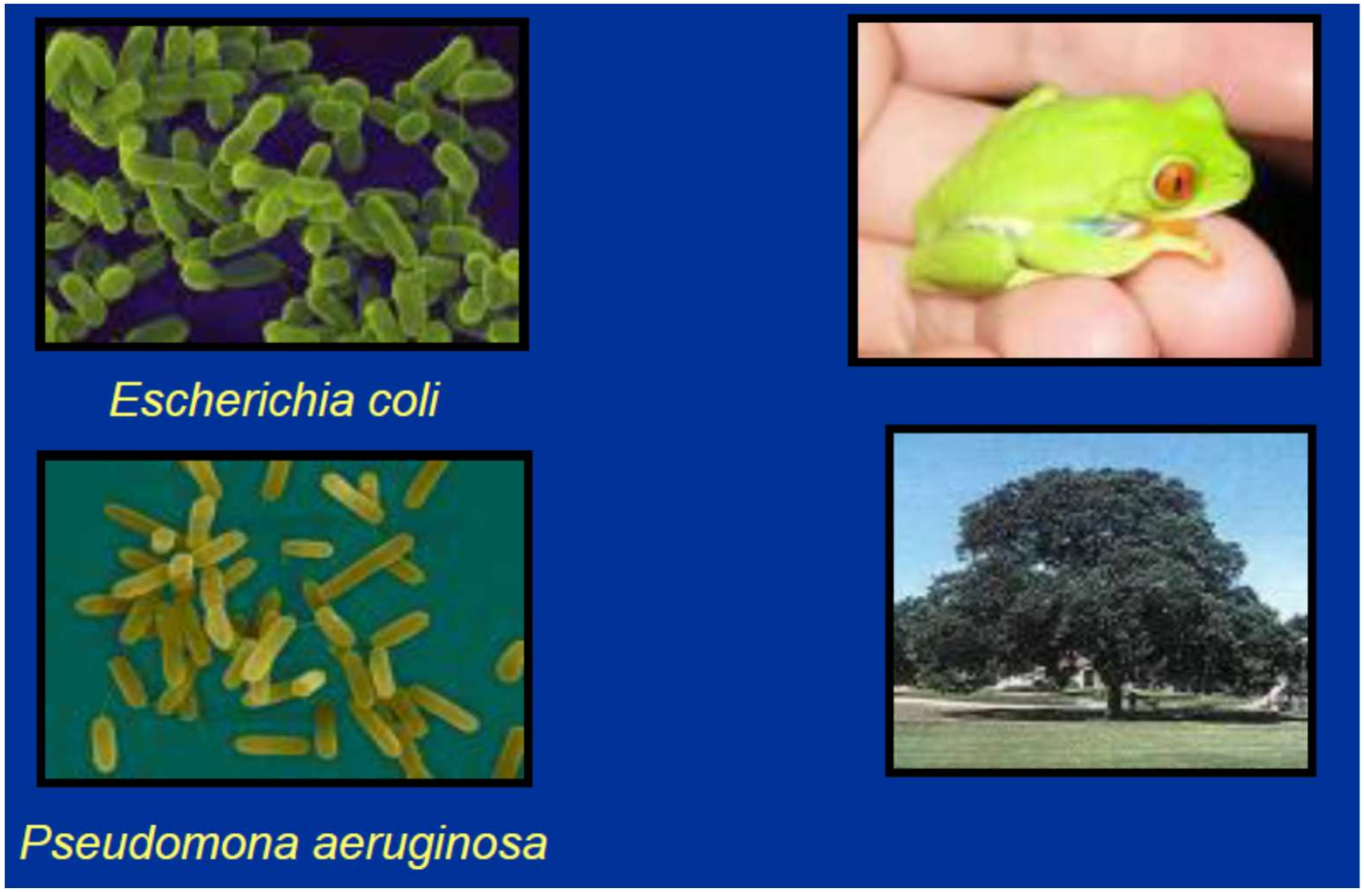
- Highest expected cell number
- Minimal equipment
- Quick and easy

- No discrimination between dead and living cells
- Background interference
- Pretreatment to remove e.g. iron minerals
- Suitable for protozoa, eukaryots…. Difficult for bacteria



Why is it so difficult to distinguish bacteria by micrsocopy?

- Exist on earth since many years (much longer than higher organisms)→ higher diversity
- Limited morphology (cocci, rods, spirilli, vibri)



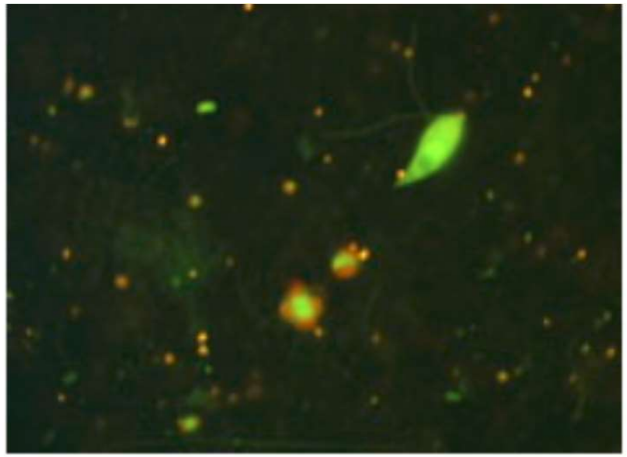
Fluorescence microscopy

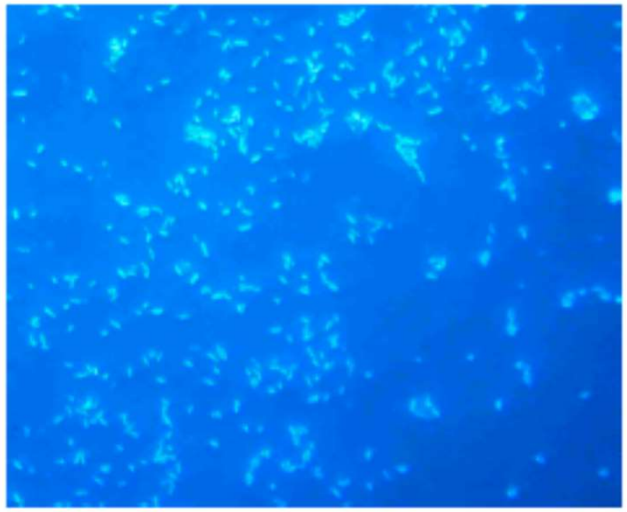
Direct counts:

Fluorescent dyes & epifluorescence microscopy

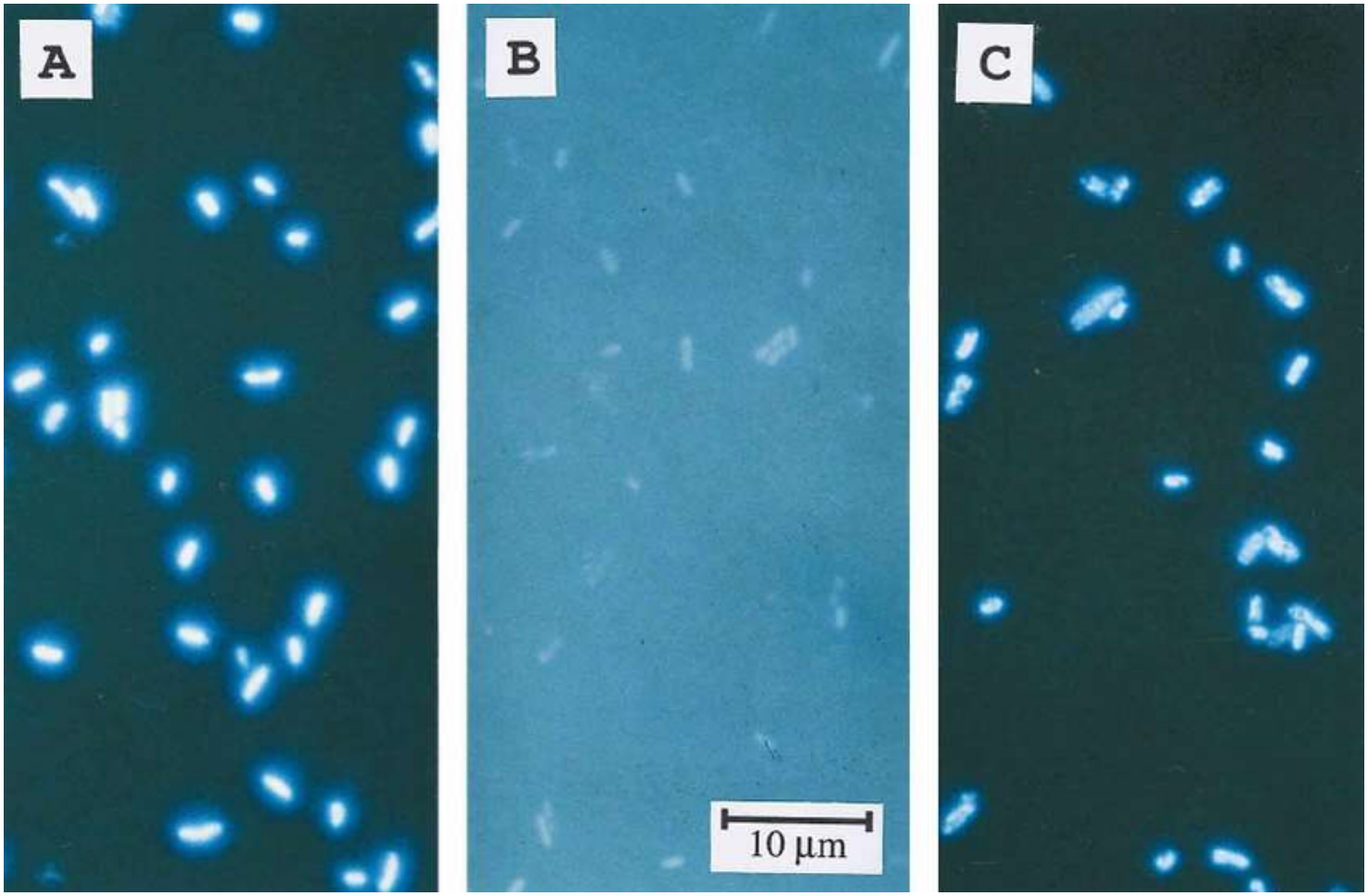
-Acridine Orange Direct Counts (AODC) general nucleic acid stain, fluoresces green or red

-DAPI – 4,6-diamidino-2-phenylindole AT-specific DNA stain, fluoresces blue or white



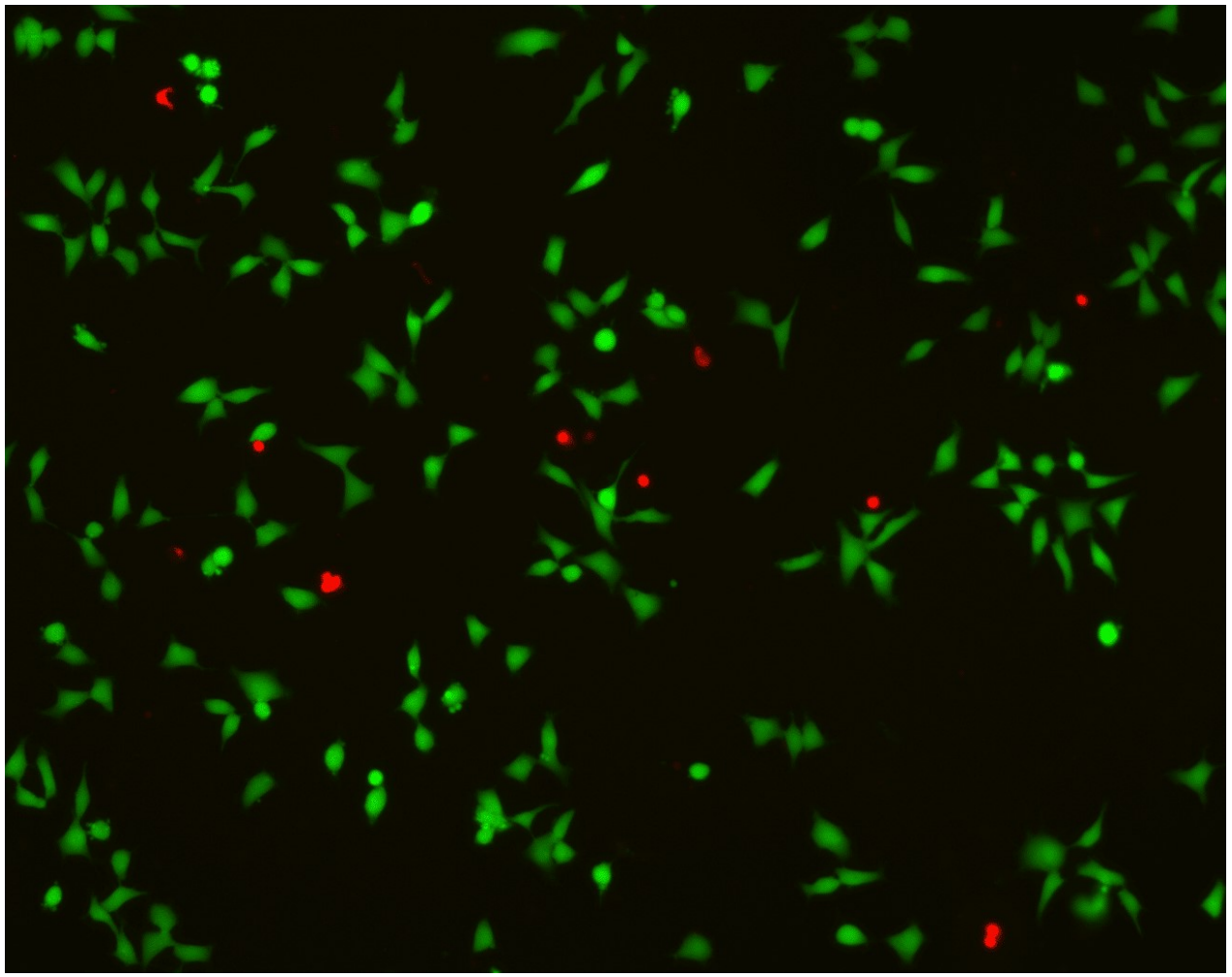


Fluorescence microscopy



Cells of E. coli stained with DNA stain (DAPI) to determine total cell number

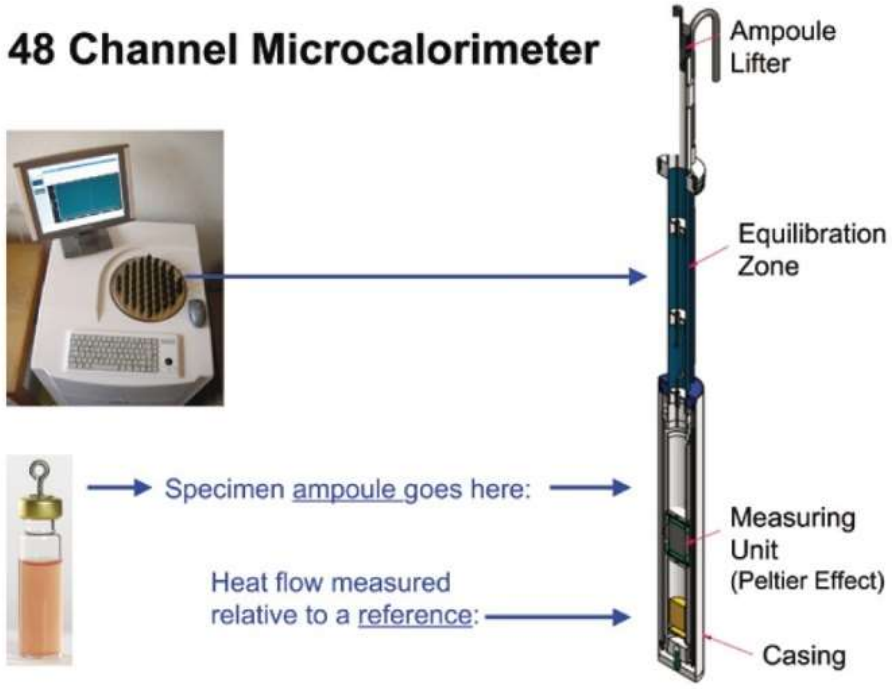
Live/dead staining

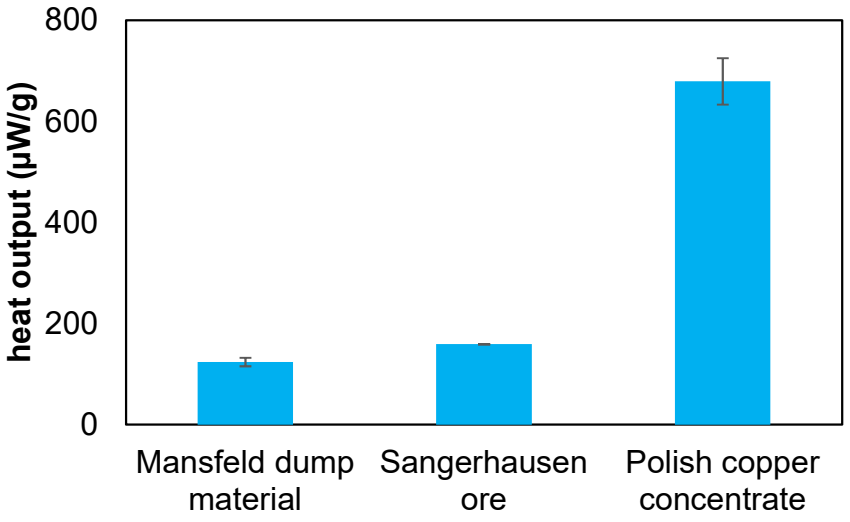


Viability staining to distinguish living (green) and dead (red) cells using the LIVE/DEAD Bac Light™ kit

Microcalorimetry

- performance of active culture on substrate (ore)
- determination of cell activity by heat release

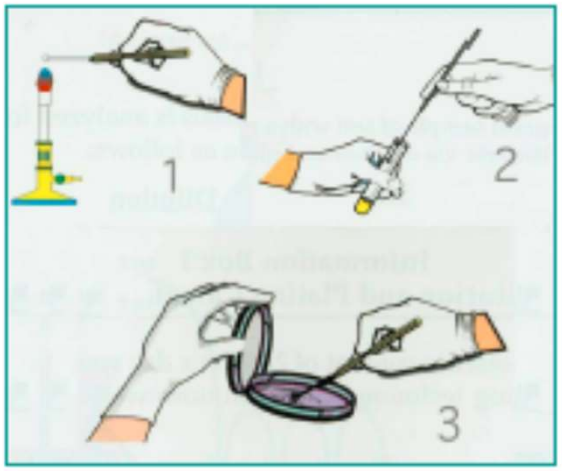




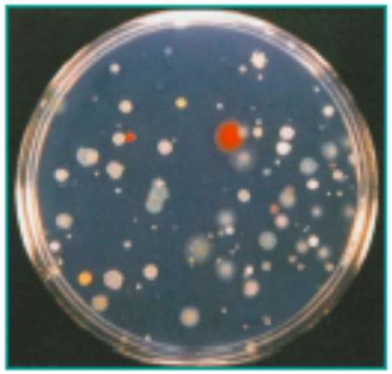
Cultivation-dependent techniques

- Enumeration and isolation using selective media
- Target specific organisms
- Gives indication of activity
- Gives model organisms for laboratory studies
- But will underestimate diversity

Dilution series for SRBs

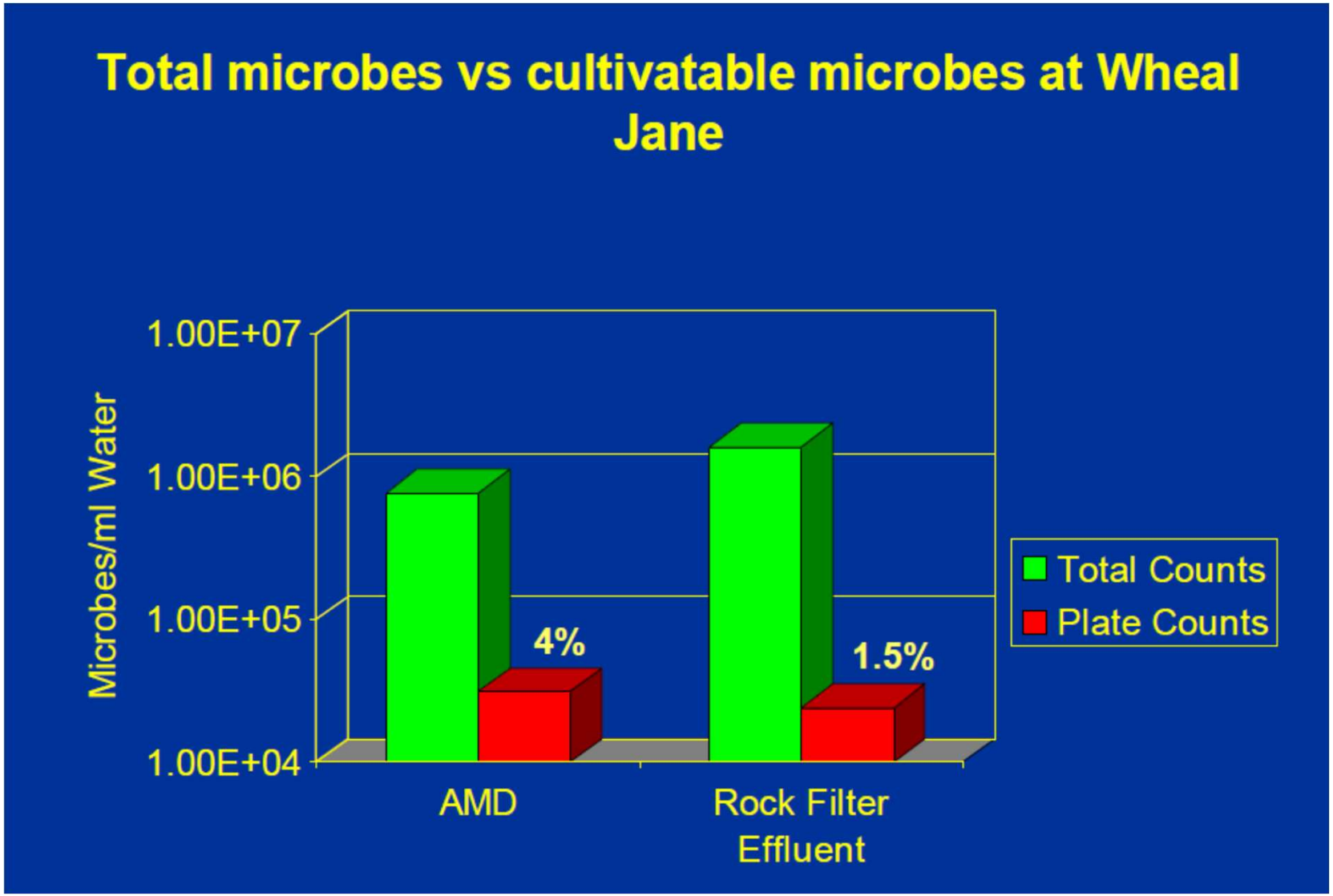


Isolation techniques



Agar plate + colonies

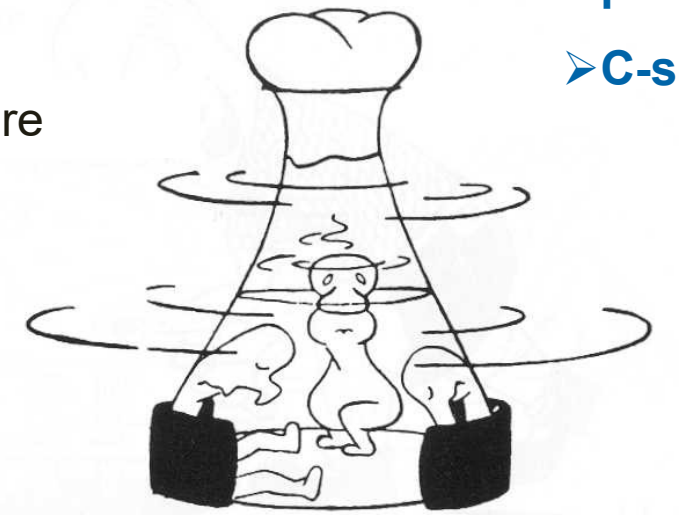
Anaerobic cabinet


Total microbes vs cultivatable microbes at Wheal Jane
Microbes/ml Water
1.00E+07
1.00E+06
1.00E+05
1.00E+04
4%
1.5%
AMD
Rock Filter Effluent
Total Counts
Plate Counts

Selction by variation of growth medium:

- salts
- pH- value
- C-source

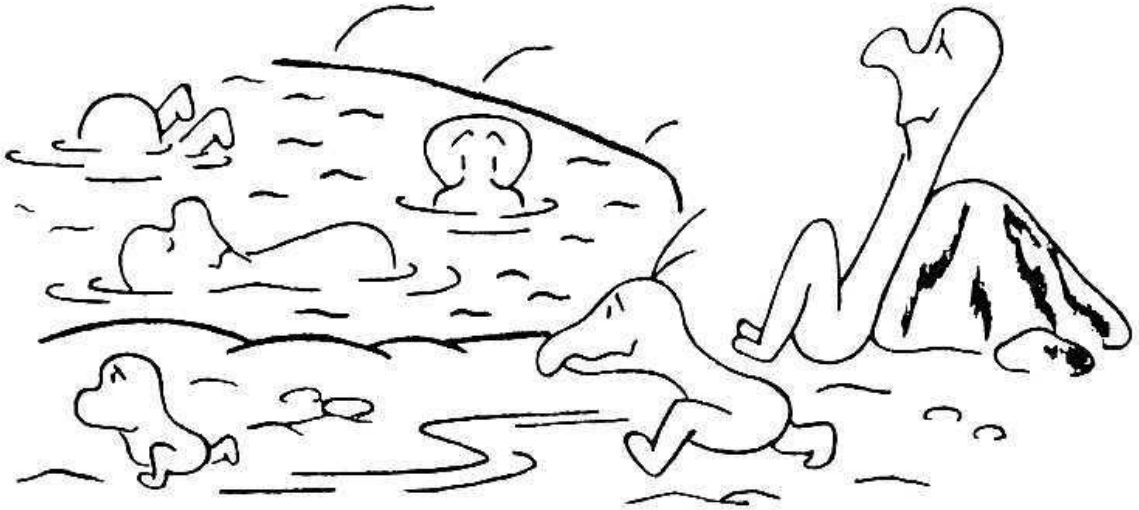
Enrichment culture



Die Anzucht unter speziellen Laborbedingungen wird nur von wenigen überstanden.

Von Joachim Czichos: What's so Funny about Microbiology?

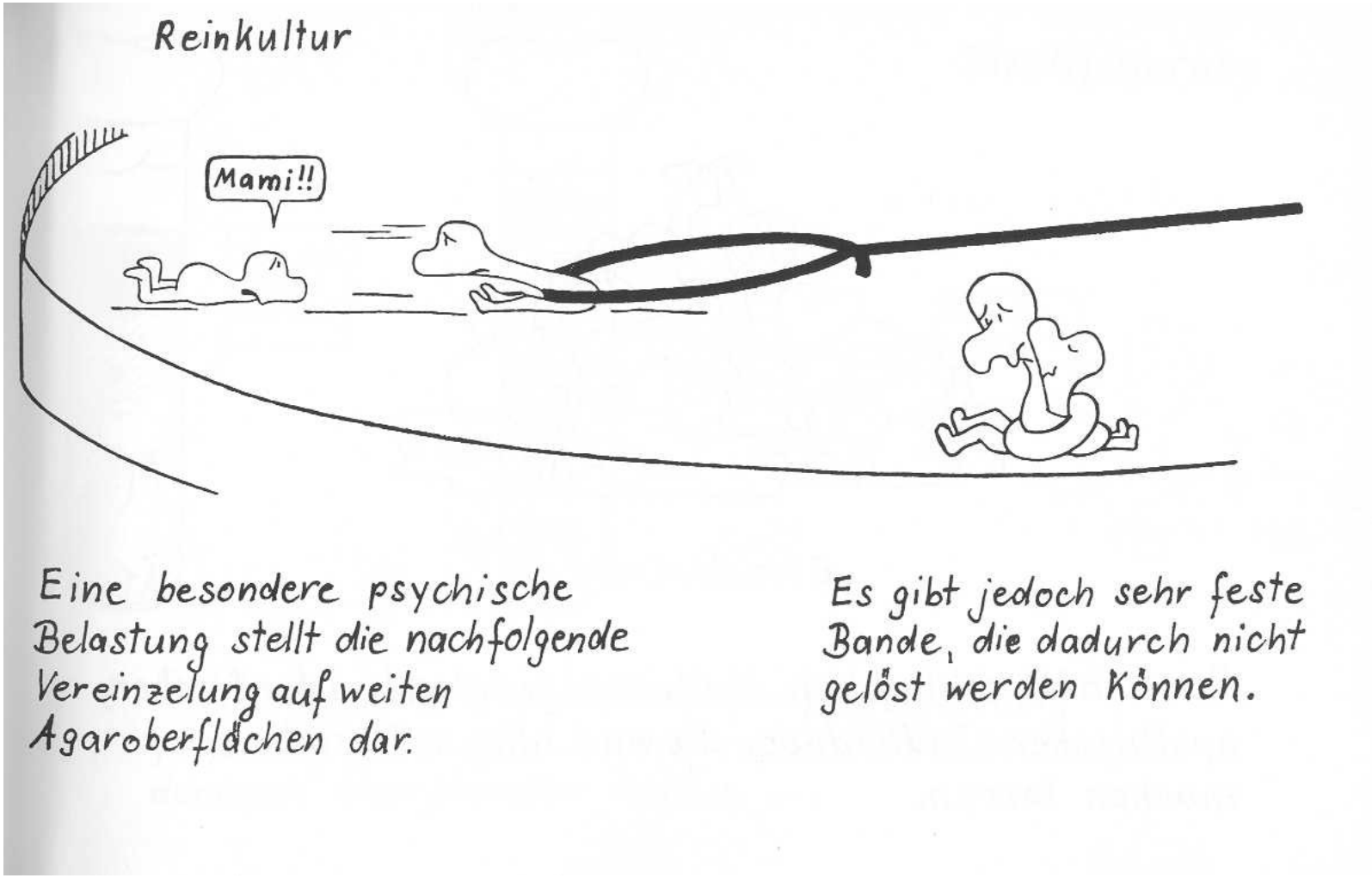
Mixed culture



leben die Bakterien in
Mischkultur...

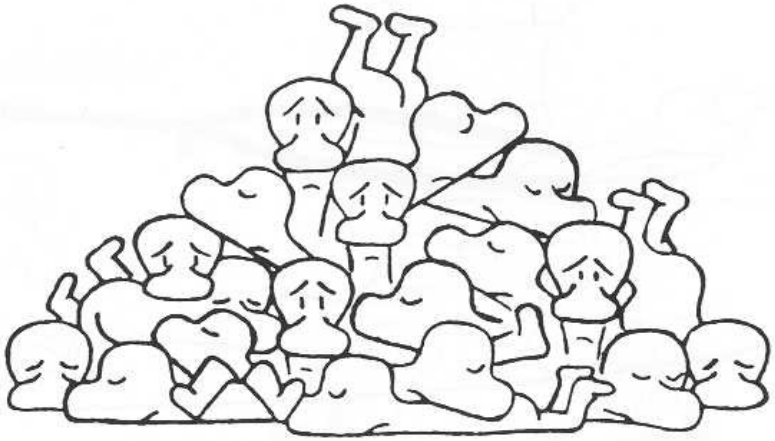
Von Joachim Czichos: What's so Funny about Microbiology?

Isolation using agar plates



Von Joachim Czichos: What‘s so Funny about Microbiology?

Pure culture

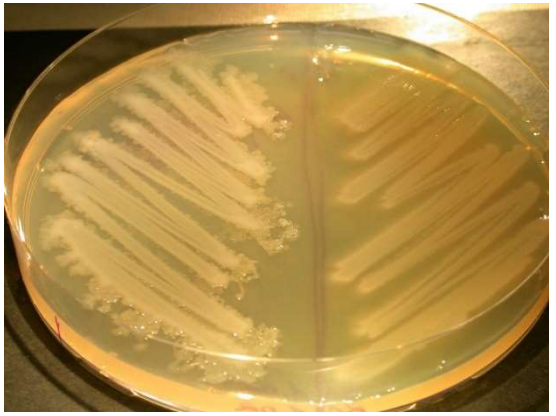


Einzelkolonie

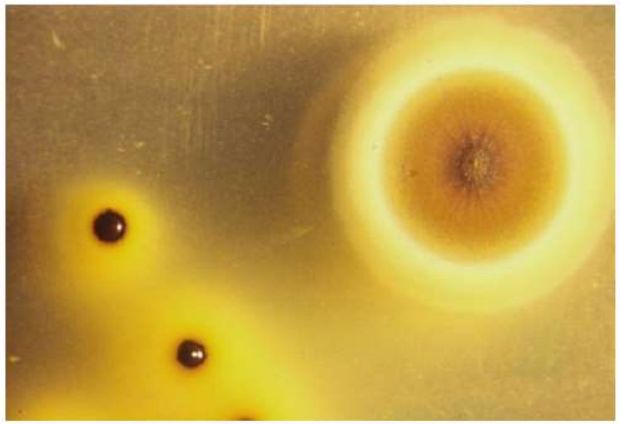
Man erhält schließlich Kulturen genetisch identischer, apathischer Individuen, die nun alles mit sich machen lassen.

Von Joachim Czichos: What's so Funny about Microbiology?

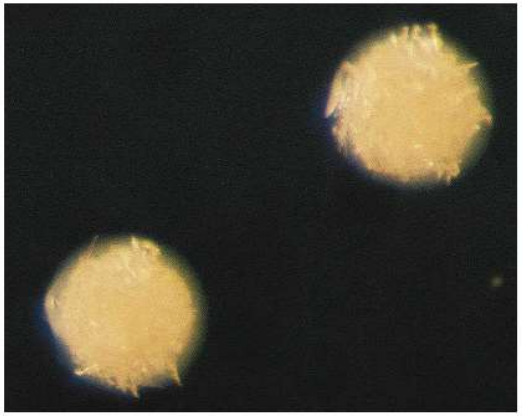
Isolation of pure cultures on agar plates



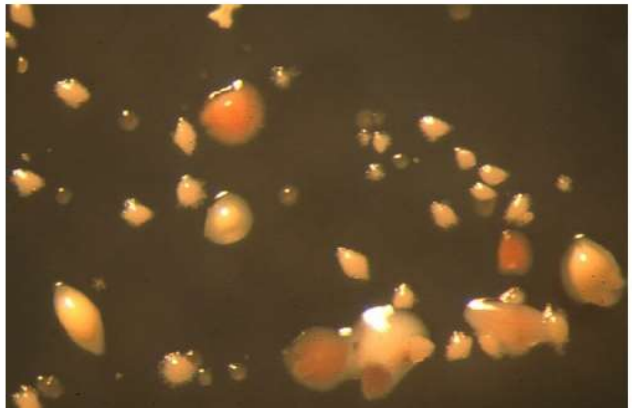
Single colonies on agar plates



Colonies of Fe-oxidising bacteria on iFeo medium



Colonies of S-oxidising bacteria on FeSo medium

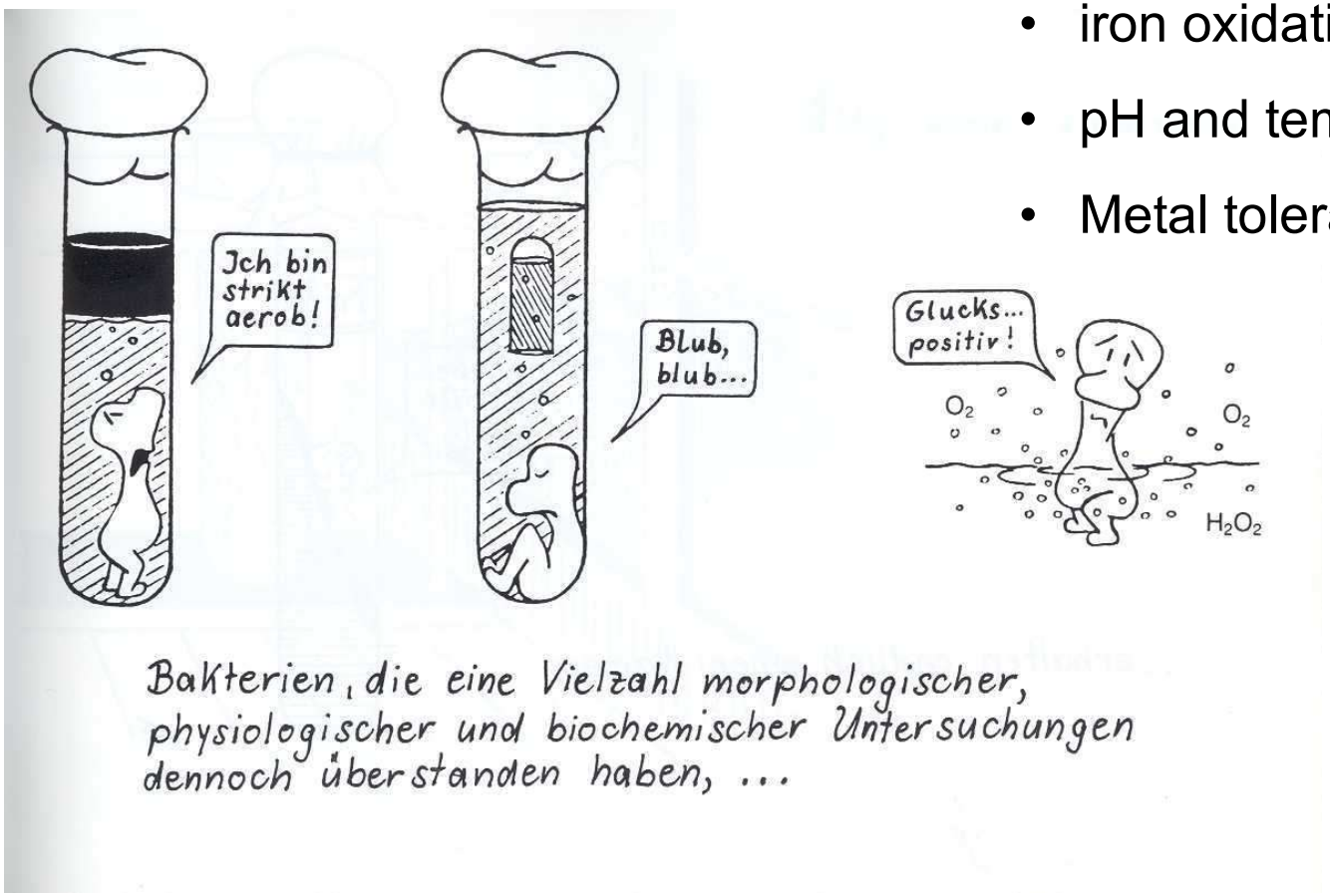


Colonies of heterotrophic acidophiles onYE3o medium

Naming a novel microorganisms

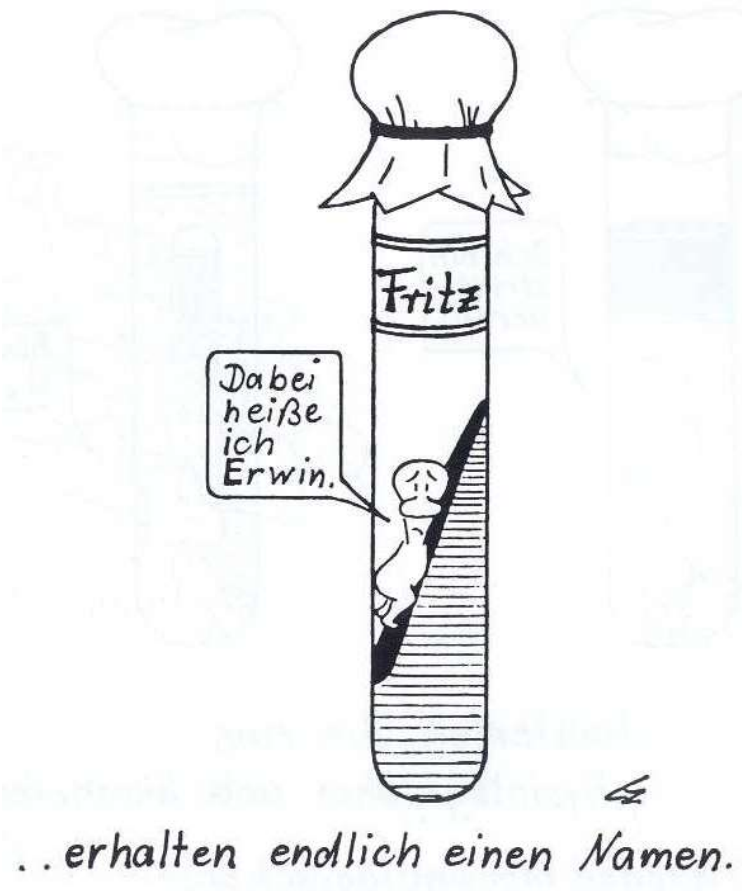
Test physiological properties:

- iron oxidation
- pH and temperature limits
- Metal tolerance



Von Joachim Czichos: What's so Funny about Microbiology?

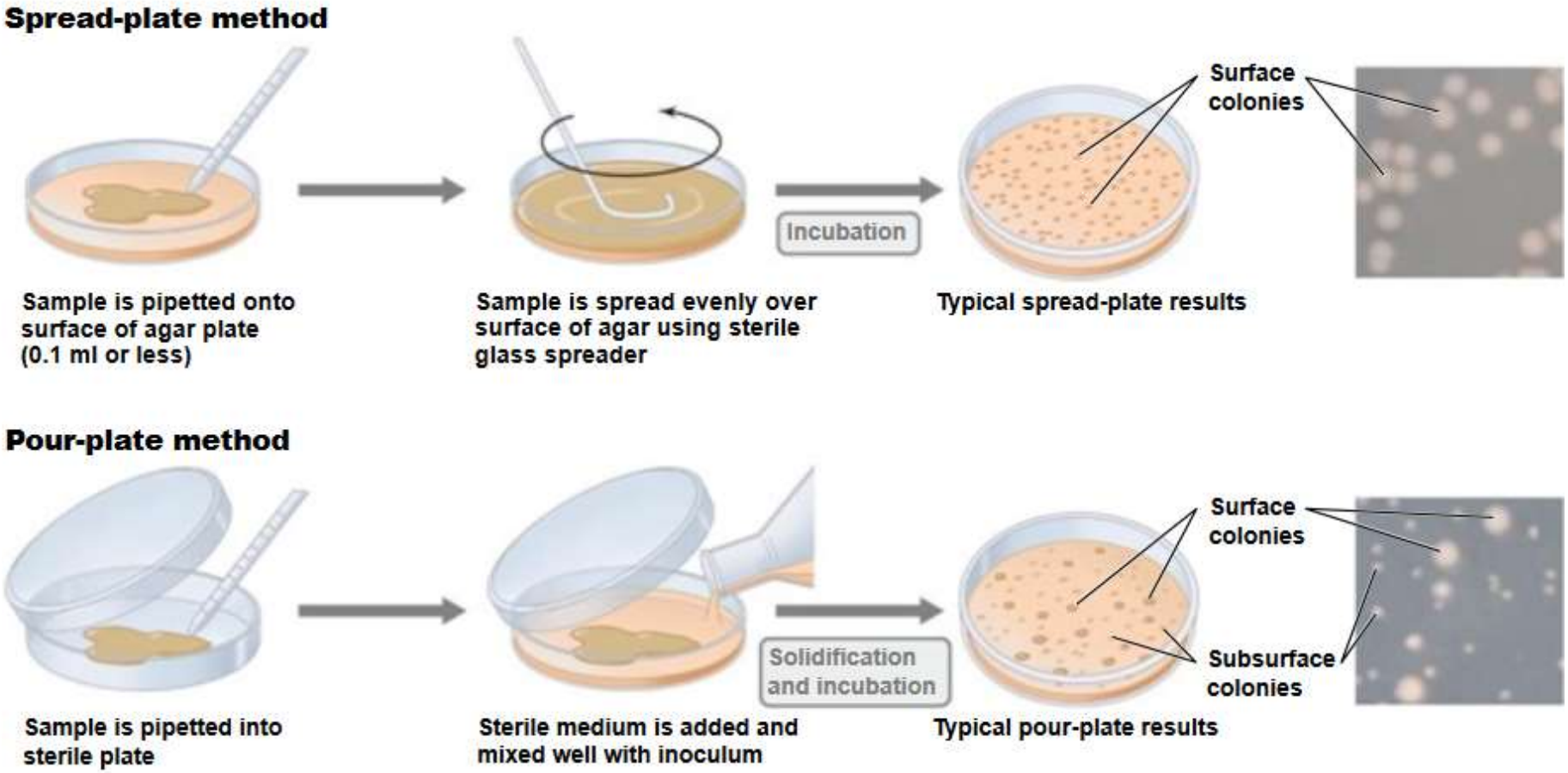
Naming a novel microorganisms



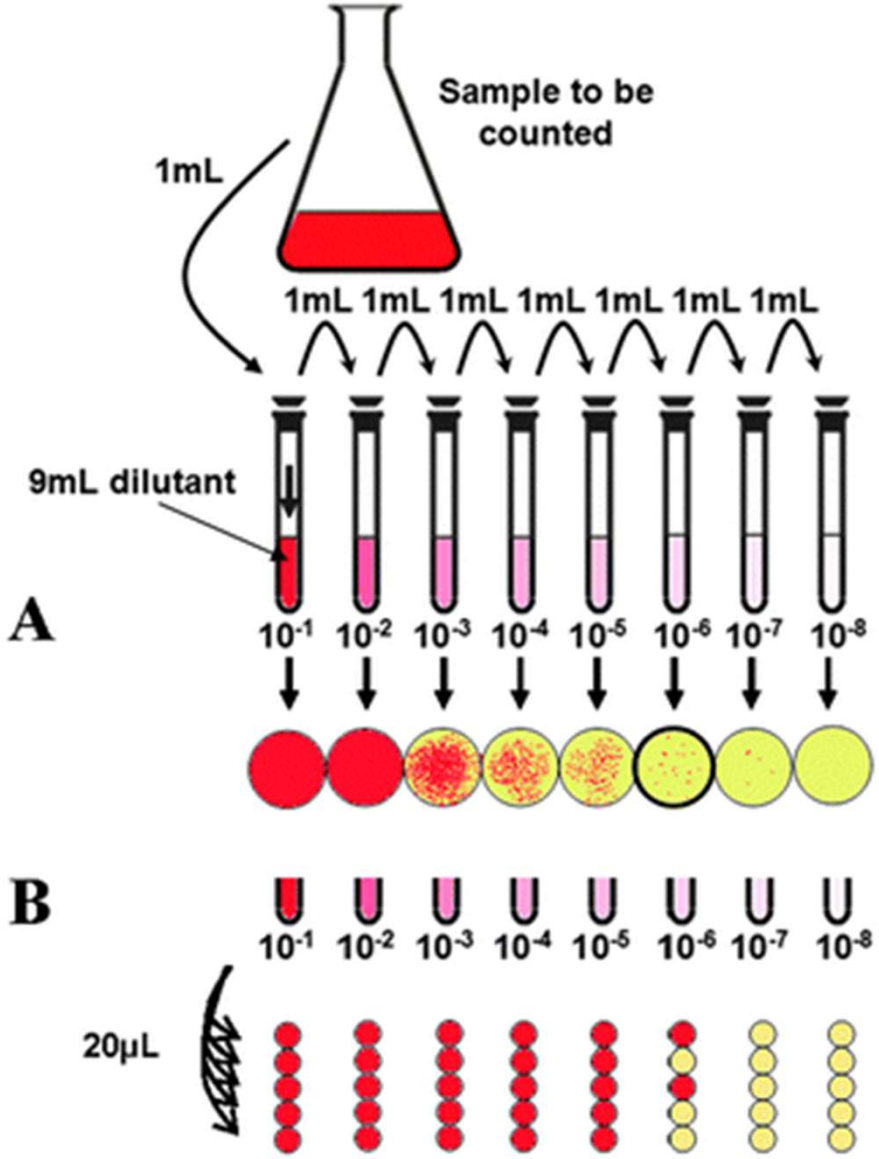
Von Joachim Czichos: What‘s so Funny about Microbiology?

Viable cell counts (plate counts)

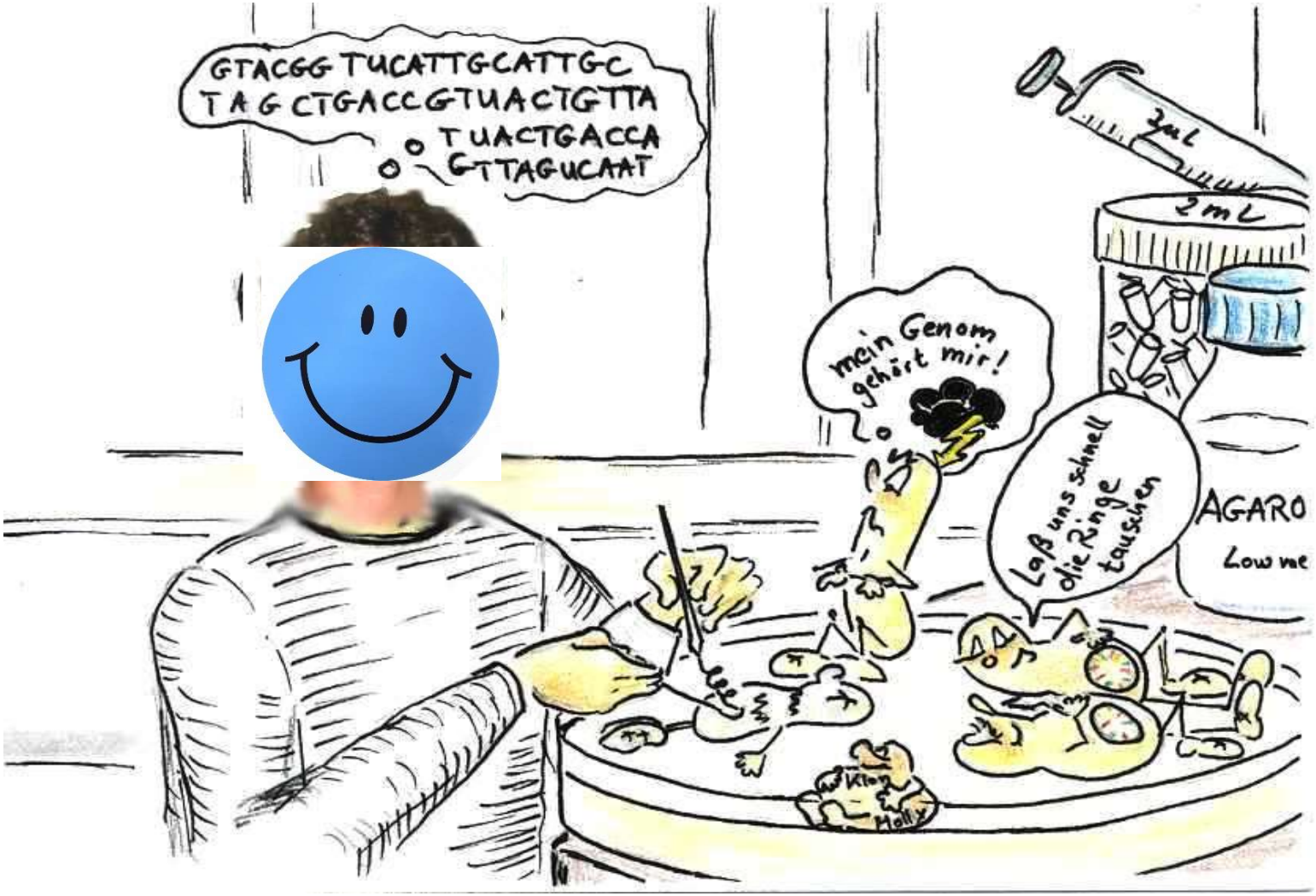
→ Measurement of living, reproducing population



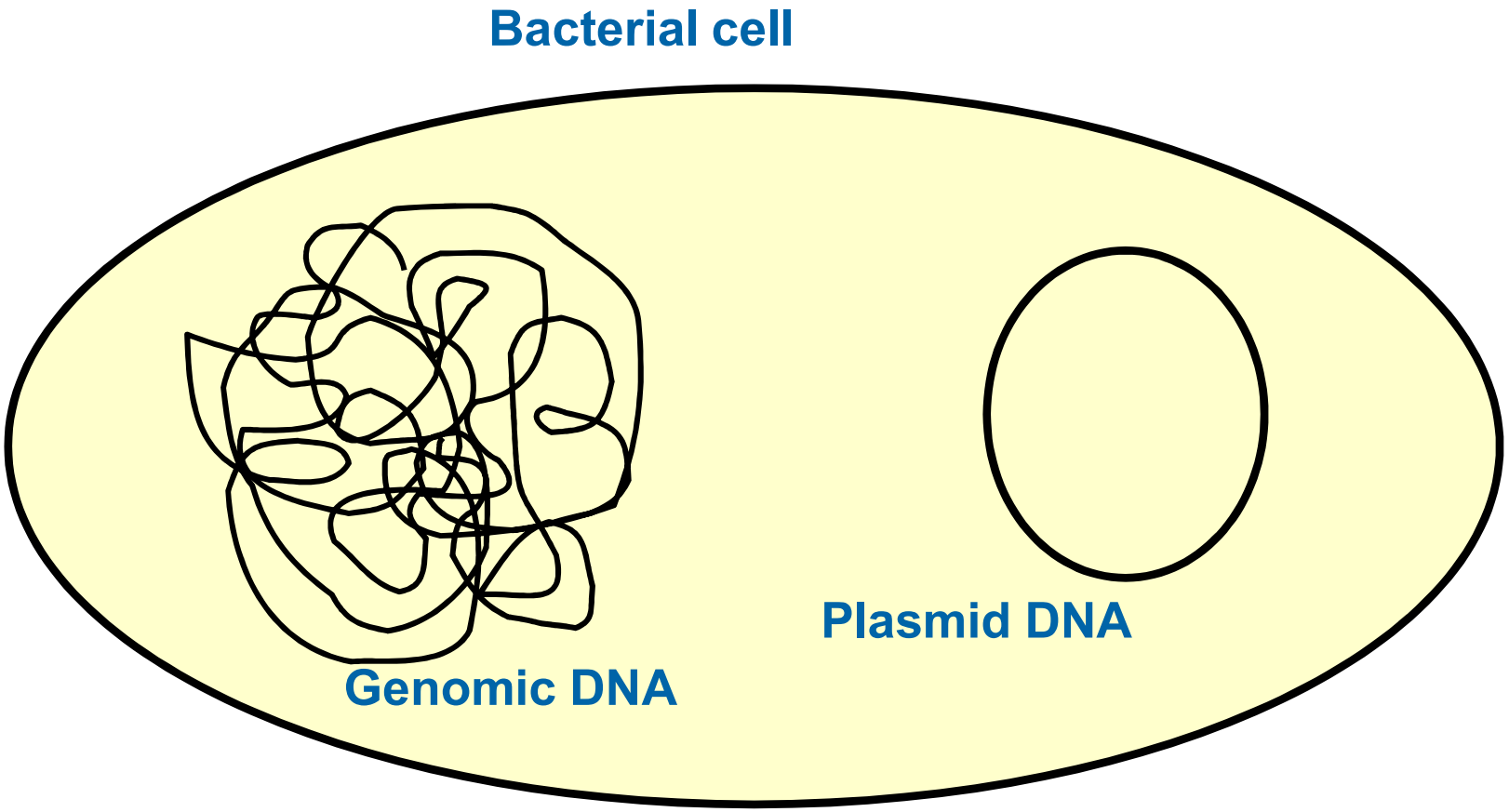
MPN (most probable number) counts



Molecular methods



Molecular methods



Molecular methods

Aim: Quantification of different groups of microorganisms in different geosystems

- Conventional cultivation methods are time consuming and capture only a small portion of the microorganisms
- Molecular biological methods are based on the specific detection of nucleic acids (DNA, RNA) and detect all microorganisms

The 16S rRNA gene (16S rRNA)

- a subunit of the ribosomal RNA responsible for translation of a gene into a protein
- present in all organisms and highly conserved since it performs the same function in all organisms (16S rRNA in prokaryotes, 18S rRNA in eukaryotes)
- easily isolated and detected since it is a relatively abundant molecule in cell
- most importantly, the sequence of 16S rRNA gene has been determined from over **30,000 microbes**

Role of rRNA in the cell

- All functions of a cell are encoded as DNA as **genes** (including the rRNA)
- Genes do nothing but store information
- The information in a gene is transcribed into **mRNA** (messenger RNA) based on the nucleotide sequence of the gene
- mRNA transfers the information in the gene to ribosomes
- **Ribosomes** produce proteins that carry out cellular functions (or synthesize molecules that do) from amino acids based on the nucleotide sequence in the mRNA

Genes for specific metabolic groups

Gene	Enzyme activity	Microbe function
N-cycle		
amo	ammonia monooxygenase	ammonia oxidation
nirK	nitrite reductase	nitrite reduction
narG	nitrate reductase	nitrate reduction
S-cycle		
apsA, *dsrAB*	sulfate reduction	sulfate reduction
*dsrAB**	sulfur oxidation	sulfur oxidation
C-cycle		
nahA	napthalene dioxygenase	aromatic degradation
tfdA	2,4-D dioxygenase	herbicide degradation

DNA extraction

DNA- enrichment:

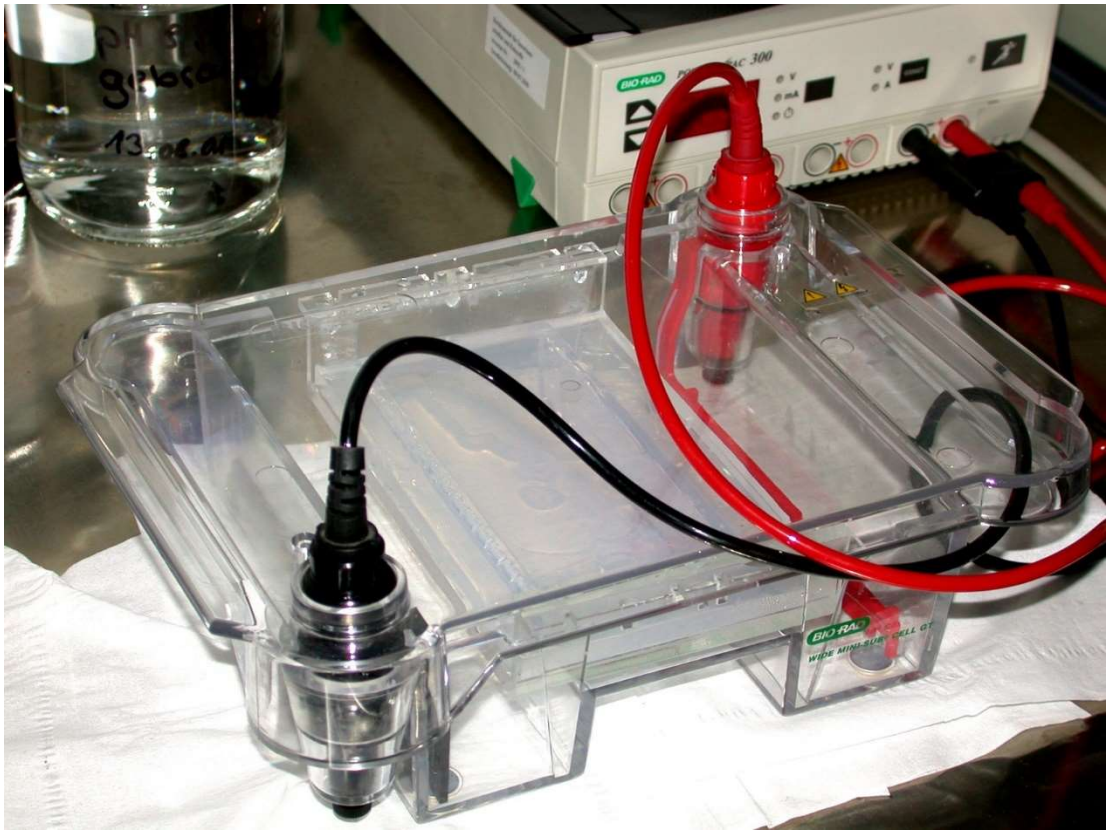
- Cultivation of organisms
- Concentration of biomass by e.g. centrifugation, filtration

DNA- extraction:

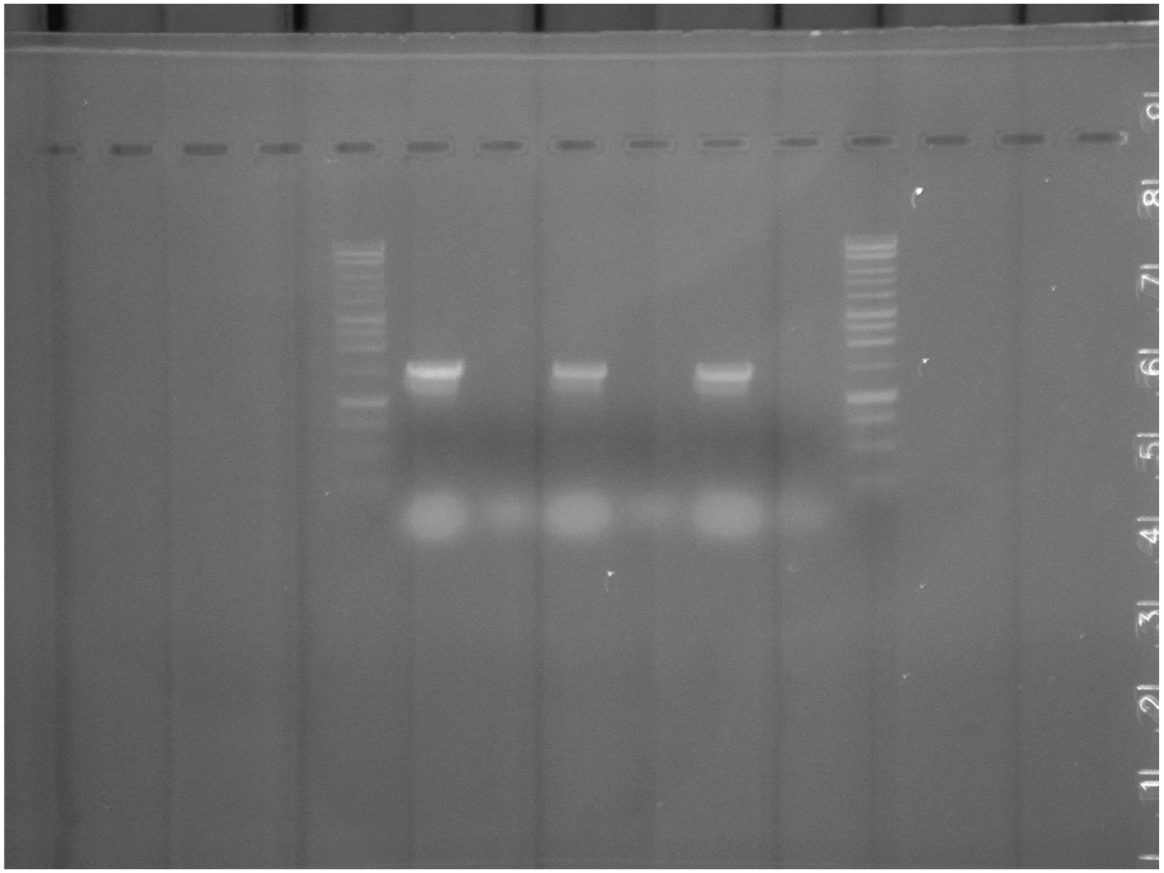
- cell lysis (enzymatically, mechanically, chemically)
- protein precipitation
- washing of DNA

Polymerase chain reaction

Gel electrophoreses



Agarose gel with DNA bands

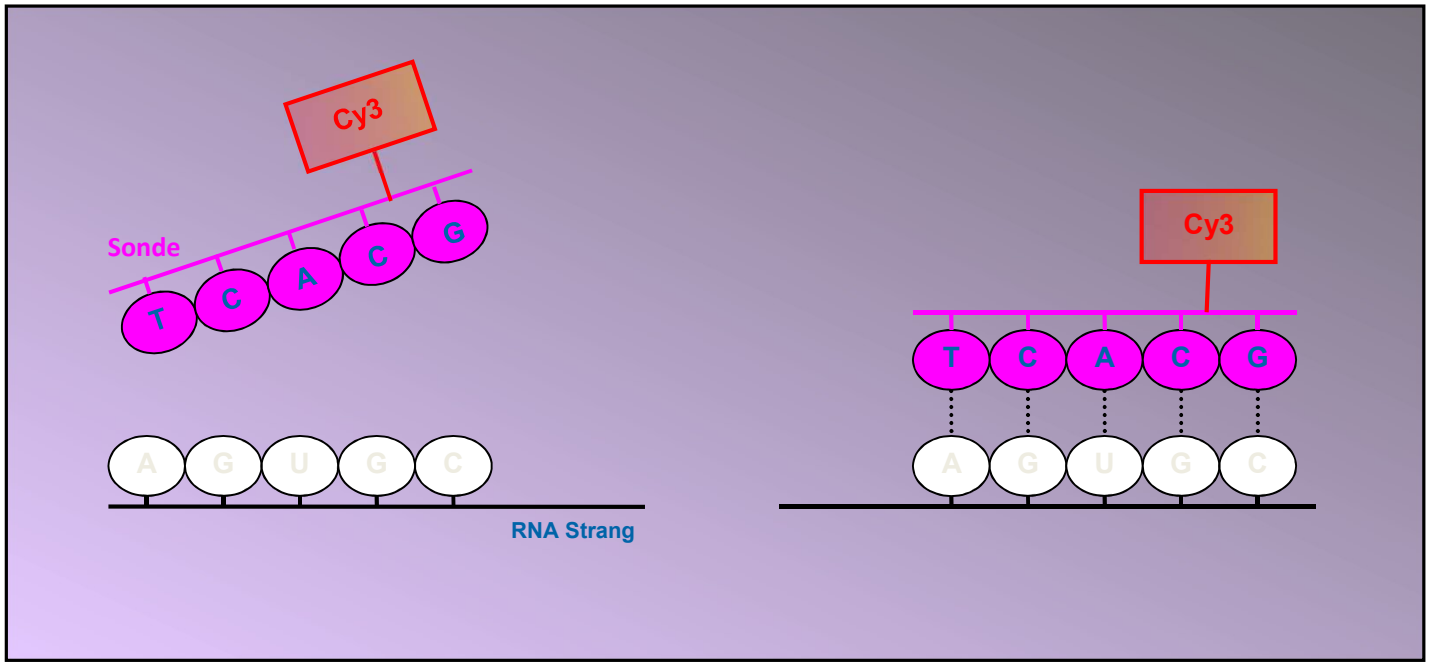


Fluorescence In Situ Hybridization (FISH oder CARD-FISH)

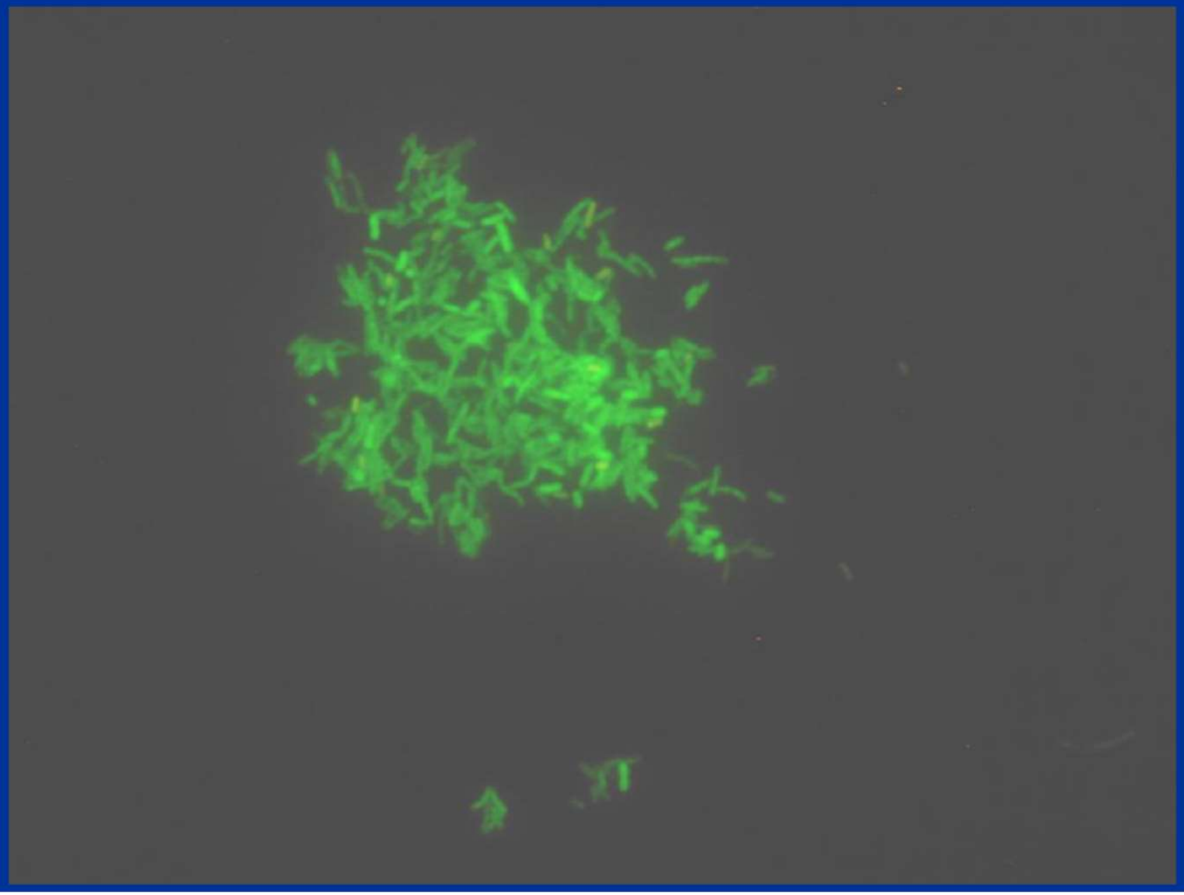
- Samples are chemically fixed to formaldehyde.
- Genetically relevant groups of microorganism-specific gene probes hybridize to a target sequence of ribosomal RNA found only in living cells.
- The gene probes emit a fluorescent signal, causing the microorganisms to shine.
- By counting on the fluorescence microscope, the number of living microorganisms in the sample is determined.
- Analysis time: 2 days

FISH

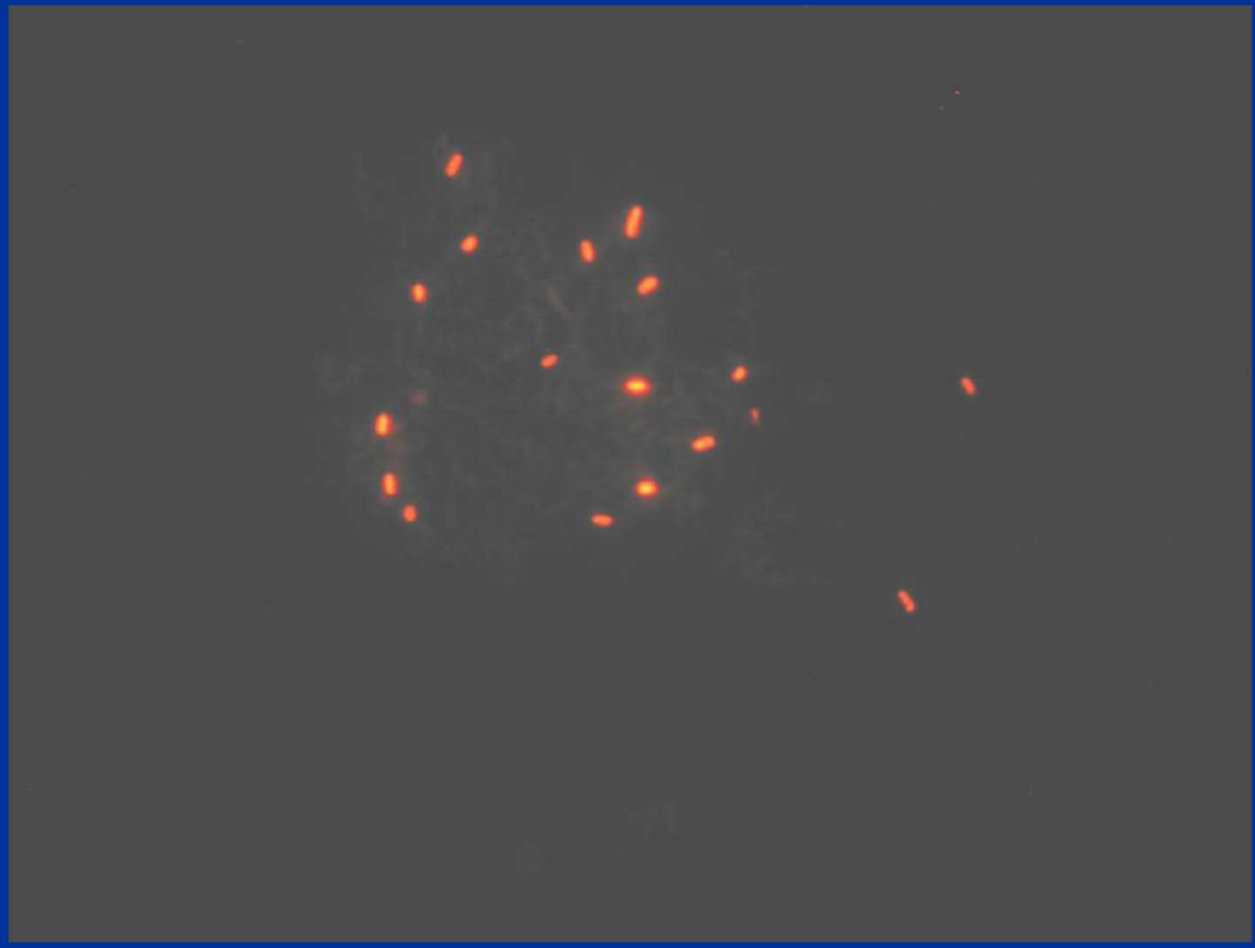
(Fluorescence In Situ Hybridization)



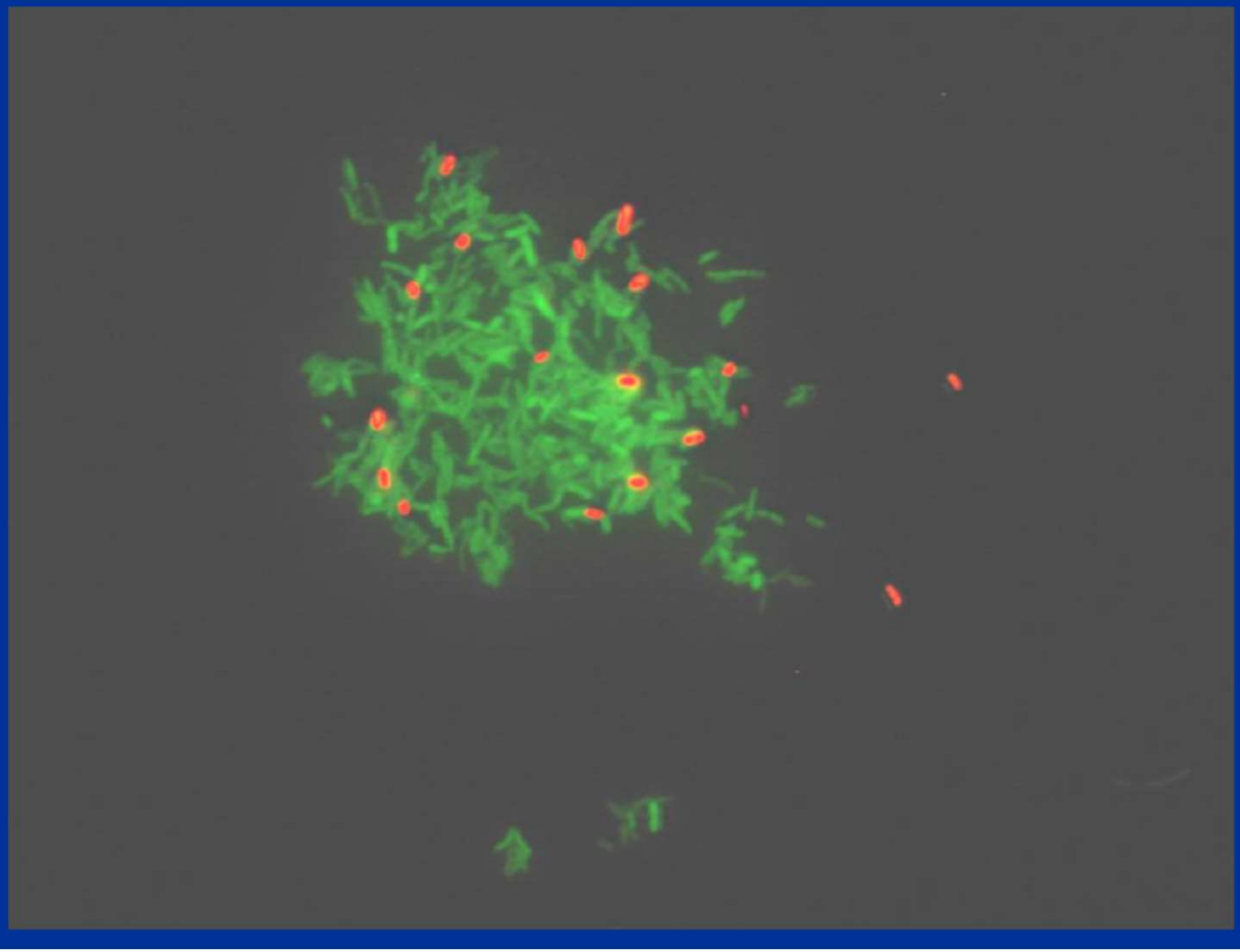
Biofilm probed with bacterial specific oligonucleotide



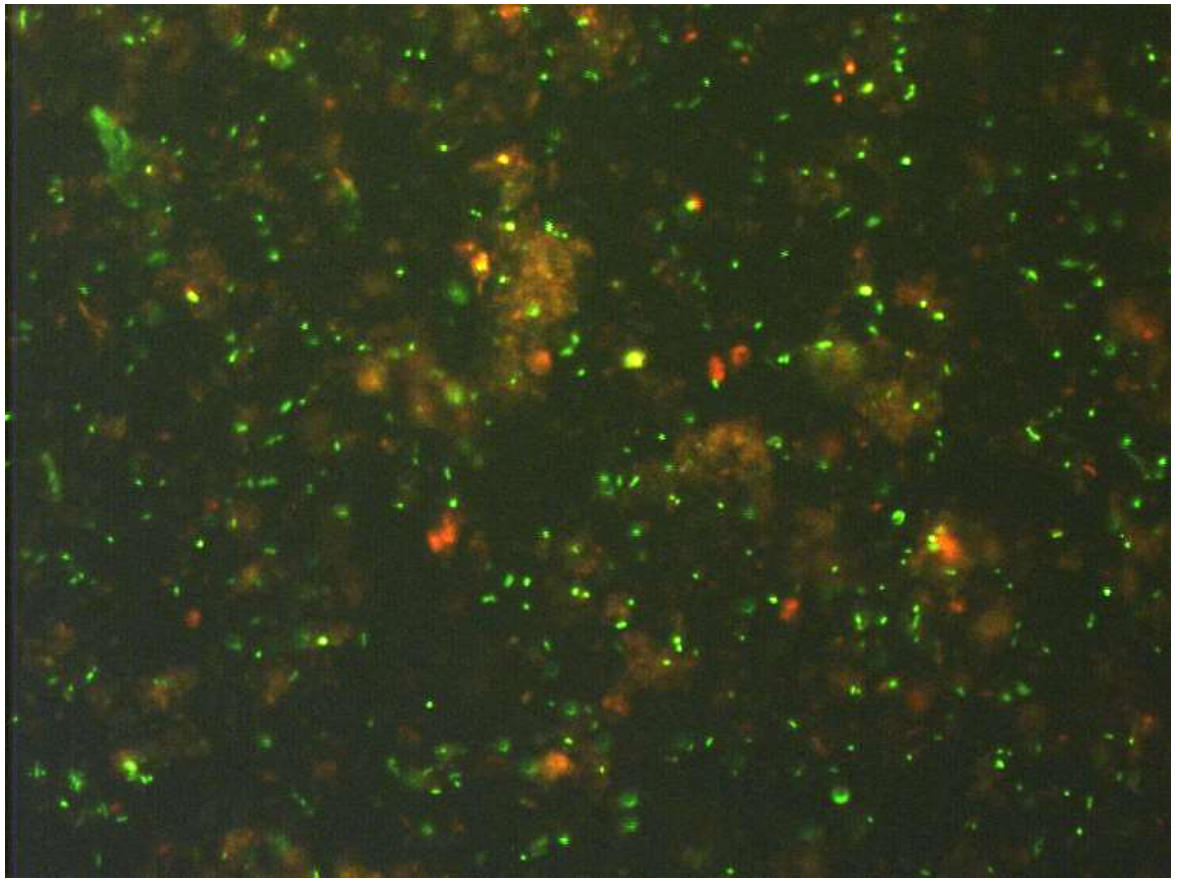
Same sample with *Acidithiobacillus ferrooxidans* probe



Combined images



Microbial cells in tailing sample



Omics studies

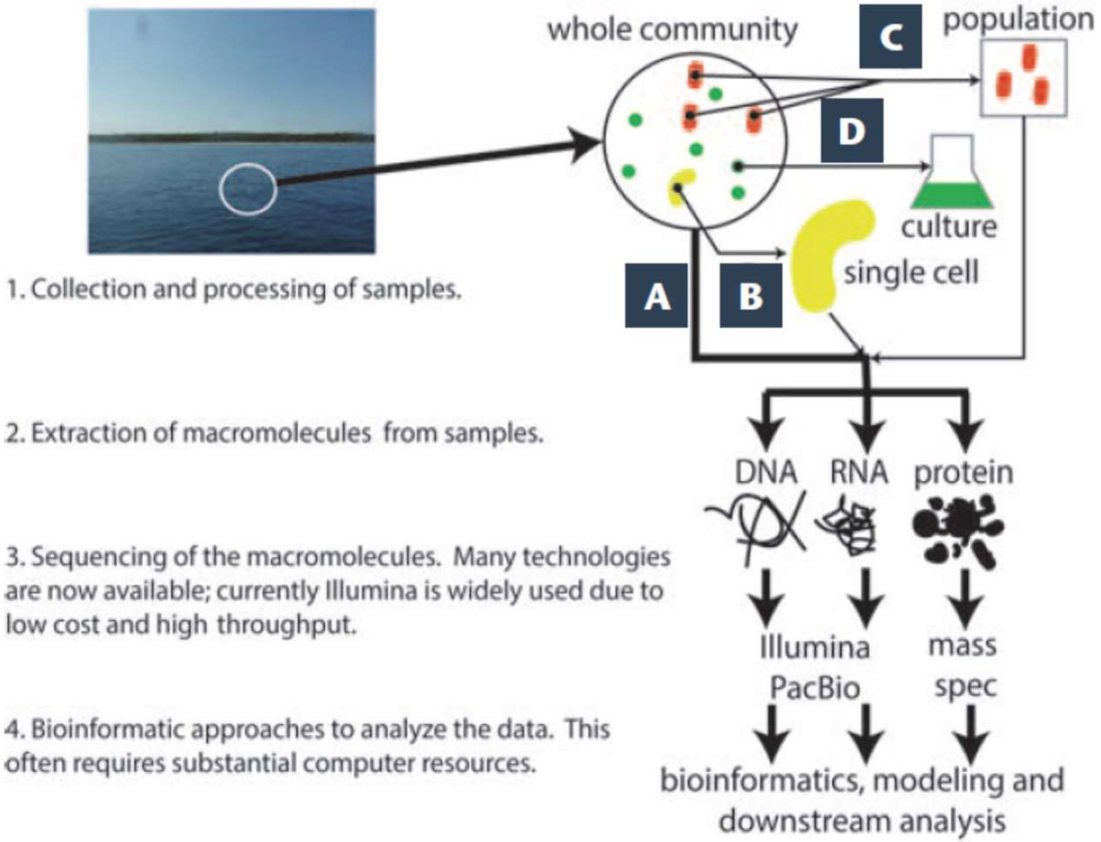
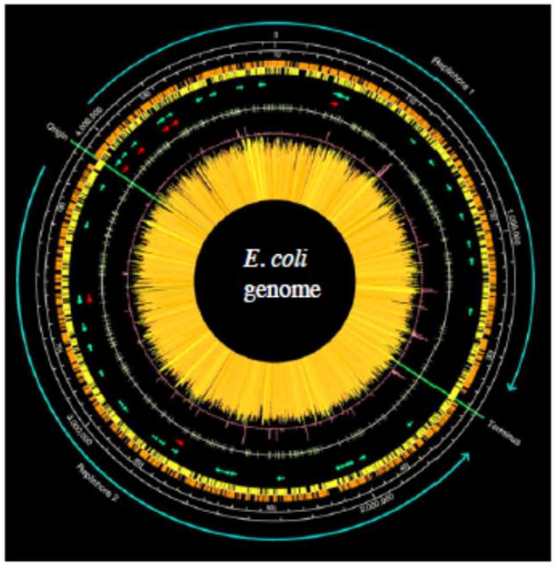
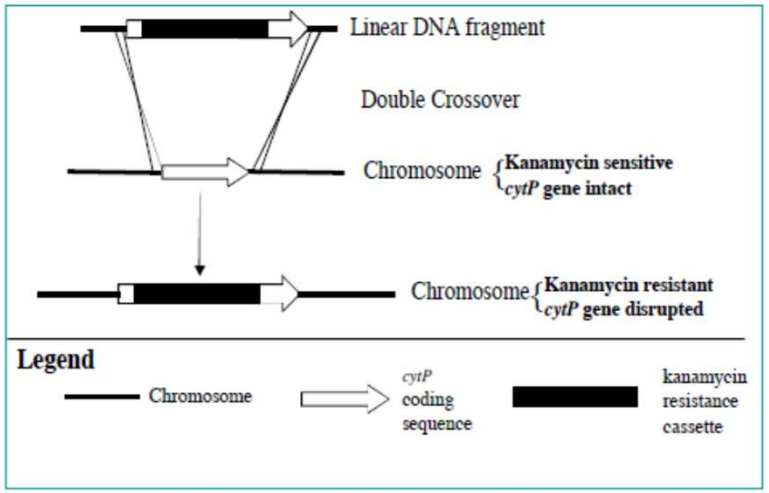


FIGURE 2 Processing samples for omics approaches. Samples may be from the field, (e.g. seawater, soil, or rock), experiments, or laboratory cultures, and can be analyzed directly in four ways: (**A**) as whole communities ("metagenomics"); (**B**) processed to select specific cells (single cell genomics); (**C**) as populations; (**D**) as cultures.

The genomics revolution

- Genomes sequenced and available for many subsurface microbes
- Tools available for genetic analysis via WWW
- Genetic systems available for many subsurface microorganisms
- Gene arrays and proteomics tools





Omics studies

- Computational power for analysis, assembly, annotation, and comparative studies
- Data storage and accessibility
- Adoption of worldwide standards (standardized genome, metagenome reports)
  - The Genomic Standards Consortium
  - Standards in Genomic Sciences journal

Molecular methods to determine diversity and function of microorganisms

- Statement about type of microorganisms available
- Selective quantification of different microorganism groups
- Determination (and quantification) of existing metabolic processes
- Depending on the efficiency of the extraction of nucleic acids / proteins
- often not enough comparison data available
- more complex methods have lower resolution
- expensive

Summary

- total microscopic cell count good for overview,
- BUT difficult to define bacteria types and physiology
- Cultivation-dependent methods easy to handle,
- BUT difficult to cultivate all organisms
- Overall picture about microbiology of a sample achieved only with molecular biological methods
- Molecular biological methods use DNA, RNA or proteins
- Each method has its drawbacks, so more than one method should be used to characterize a sample
- (Cultivation-dependent methods supplement molecular biological data)

Thank you for your attention!

Glück auf!