Session III: Measurement & Monitoring

Laboratory and Field Procedures

Content

- Hydrocarbon-oxidizing Microbes
- Microbial Calamities
- Quantification Methods based on Case Studies
  - —Microscopy
  - —Gene Probes
  - —Culture Techniques
  - —Activity Measurement via ATP
  - —Immunological, Species-specific Detection
- Summary & Perspectives

Species Distribution

- Hydrocarbons are excellent energy- and carbon sources
- Hydrocarbons are widespread in nature, similar substances are vegetable and animal waxes, lipids, oils and fats

Microbial peculiarities

- induction of cell specific changes
- efficient uptake of hydrocarbons by cell membrane receptors, and/or (bio-)tensides
- special enzymes (oxygenases) which introduce molecular oxygen into the hydrocarbon molecule
- the production of metabolites enable to enter common catabolic pathways
- special survival strategies: individual adaptations (eg. higher phospholipid content)
- often, catabolic plasmids (eg. alk (C_5 to C_{12} n-alkanes*[1])) are responsible for special pathways.
- Plasmids can sometimes be exchanged between different species.
- gene-based detoxification program

*[1] **Sayler, G. S., et al:** Microbiol. Ecol. **19:**1–20 (1990)

Conclusion

- „Everything is everywhere, but the environment selects“ (Baas Becking/Beijerinck)
- Several hundreds of species are described (e.g. Zobell 1973, Atlas 1981, Beerstecher 1954, Gaylarde 1999, → DGMK 2010)
- Jones and Edington (1968): 1% to 10% of isolates out of non-contaminated soils are able to grow on hydrocarbons

Identification of Isolates

Gaylarde, C.C., Bento, F.M., Kelley, J.:
Microbial contamination of stored hydrocarbon fuels and its control
Revista de Microbiologia (1999) 30:01-10.

Ludzay, J., Weyandt, R.:
Diesel fuels with 5% (V/V) FAME and micro-organisms
DGMK Research Report 695 (2010), 139p.

Gaylarde et al	DGMK	
Stored liquid fuels	***only B5***	**Comparison**
28 bacteria	9 bacteria	→ No identical species
12 yeasts	5 yeasts	→ No identical species
83 moulds	19 moulds	→ 6 identical species

Conclusion (Questions)

- B5 is a „new fuel“, there is no comparability given to other fuels ?
- The amount of species involved is sometimes much higher than expected ?
- The detection techniques & principles differ ?

Selection & Growth Limitation

Working Assumption

The fact, that there are so many species involved, but that there are only sporadic calamities instead of a general, permanent & obvious contamination problem, is a signal for the existence of circumstances which restrict microbial growth.

Conclusion ⟶ **Growth limiting factors (early & far reaching)**

- oxygen, free water, major & minor bio-elements like N, S, P, Fe, pH, osmolarity...
- toxic substances or substance concentrations
- competitors and predators

As a consequence

- the introduction of new compounds (eg. FAME, ethanol)
- application of a new additive
- the change of milieu

can and will influence the microbial growth

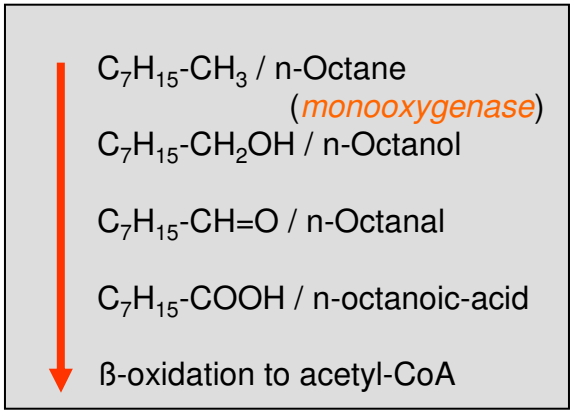
Detection

Primacy

There is a clear primacy for the ***quantification*** of different microbial groups before/instead of naming the species involved, but the knowledge of their ecological niches and their population dynamics can be very helpful for prevention.

Microbial groups and species of relevance

- aerobic bacteria
- anaerobic bacteria
- yeasts
- moulds (hyphae-forming~)
- algae



Microbial Calamities

We can distinguish between different calamities/inducements which are correlated to microbial growth

- blocked filters and valves
- fluid turbidity
- material corrosion
- high water content
- reduced performance
- formation of local depositions and sludge
- inconvenient odour

- routine check on-site

Conclusion

There can not exist a single & simple quantification (detection) method for all purposes

→ **General overview & Case studies**

Methods / Tools to detect & to quantify microbes

Direct approach

Microscopy
- total counts, live / dead
- gene probes, eg. FISH

Indirect approach

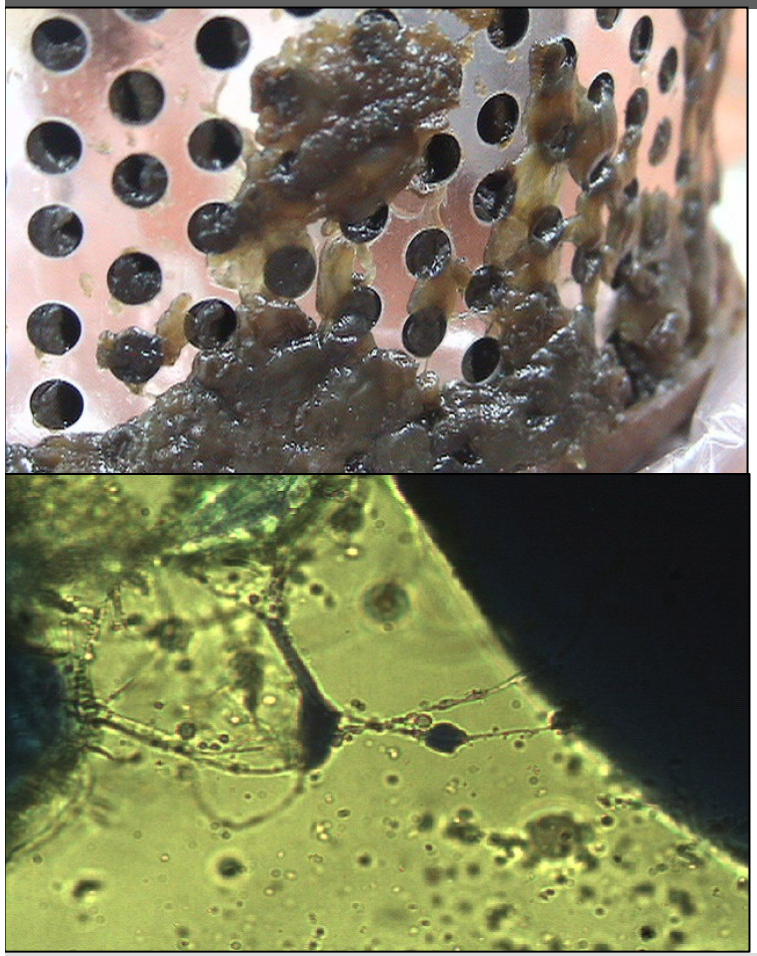
Culture techniques
- membrane filter method
- most probable number
- spread plate method
- pour plate method

Biomarkers
- ATP (Adenosin triphosphate)
- cell wall / membrane components
- proteins
- nucleic acids

Enzymatic activities
- dehydrogenases
- catalase

Molecular Probes
- PCR-based techniques

Case Study 1



Petrol pump blocked

Causes (microbial) supposed
- bacteria / yeasts / moulds ?
- viable / dead ?

Methods
- on-site: ATP for living microbes
- lab: →**Microscopy**; bacteria and/or fungi
- further investigations

Remarks
- no quantification possible
- identification possible only for predominant species
- no differentiation between cause and effect
- in rare cases: specific-species detection possible (cannot be recommended as stand alone solution due to specific risks)

Case Study 2

(Bio-) Corrosion (metal, plastics, paints..)

Causes (microbial) supposed

- aerobic bacteria *(production of acids...)*
- anaerobic bacteria *(SRB, Clostridia...)*

Methods

- → **ATP**
- only laboratory:
  - special cultural techniques for quantification *(special media, special techniques („Hungate"), MPN)*
  - gene probes
- simulation tests
- further investigations

Case Study 3

Routine check: Fluid sample

Quantification of microbes

- aerobic bacteria + yeasts + moulds

Methods

- in the field: ATP
- in the lab:
  - → **Cultural Techniques** for quantification
  - — microbial activity (eg. ATP)
  - — biomass (eg. Bio-markers)

- further investigations (eg identification)
- in well known systems: specific detection of eg ***Hormoconis resinae*** → **Immuno-Assay**

Summary & Perspectives

- The sampling as well as the handling and the preparation before testing belongs to the general problems to quantify microbes.
- It could be shown, that there are many different methods applicable to detect microbes & biomass in fuels.
- The methods differ regarding their practicalness and their diagnostic validity. There is no single method available to satisfy all the needs.
- The fixation on guide or threshold values for microbial quantities is therefore critical.
- Most often, a combination of different diagnostic tools would be the best approach.
- The introduction of new fluids (eg. ***B5)*** can cause microbial damages. If the microbial growing process itself can not be deduced from an ecological insight, the generation of data turns out to be precise and helpful.
- There is still a great necessity to better understand the behaviour and the specific ecological coherence of microbes in technical habitats/biotopes.
- Promising methods and techniques actually wait to be adopted to the fuel surveillance and can help closing the gap.

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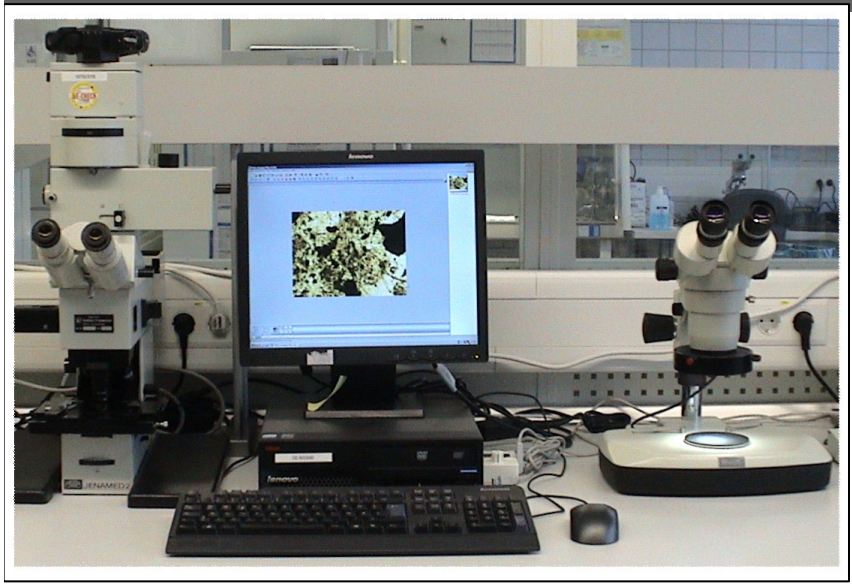
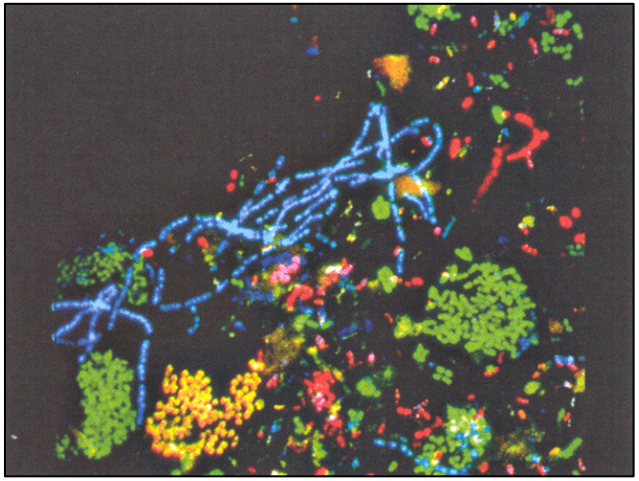
Microscopy



- live / dead via special dyes
- total counts independent from culture
- complexicity of biofilms, debris, deposits, precipitations
- structure of complexe particles
- quantification / identification ⟶ FISH

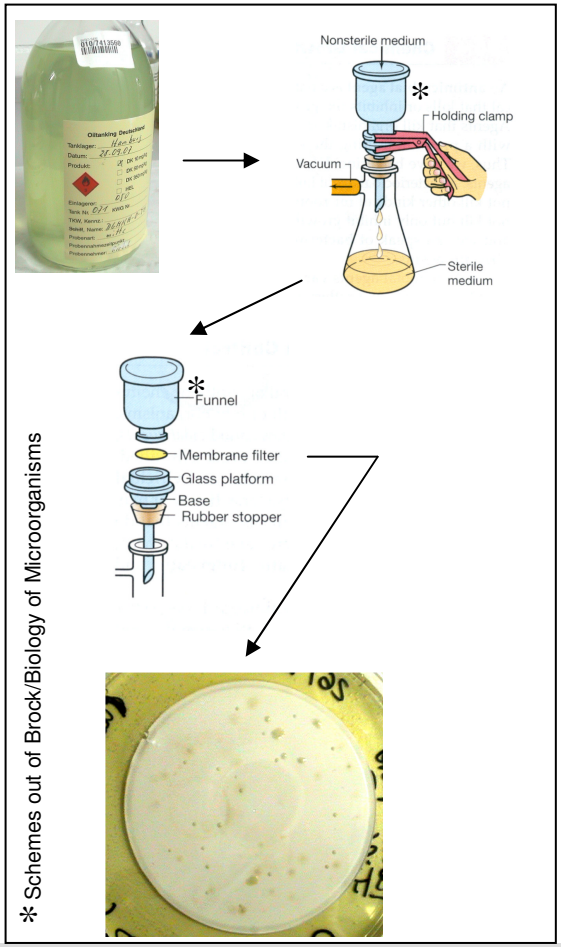
FISH = Fluorescence In Situ Hybridisation

– species-specific gene marker
–15.000 Ribosomes / Cell
– 65 % RNA, 35 % Protein
– Diameter 18 nm



Brock/Biology of Microorganisms

Cultural Techniques 1



* Schemes out of Brock/Biology of Microorganisms

IP 385: 99

Determination of the viable aerobic microbial content of fuels & fuel components boiling below 390°C, Filtration & Culture method

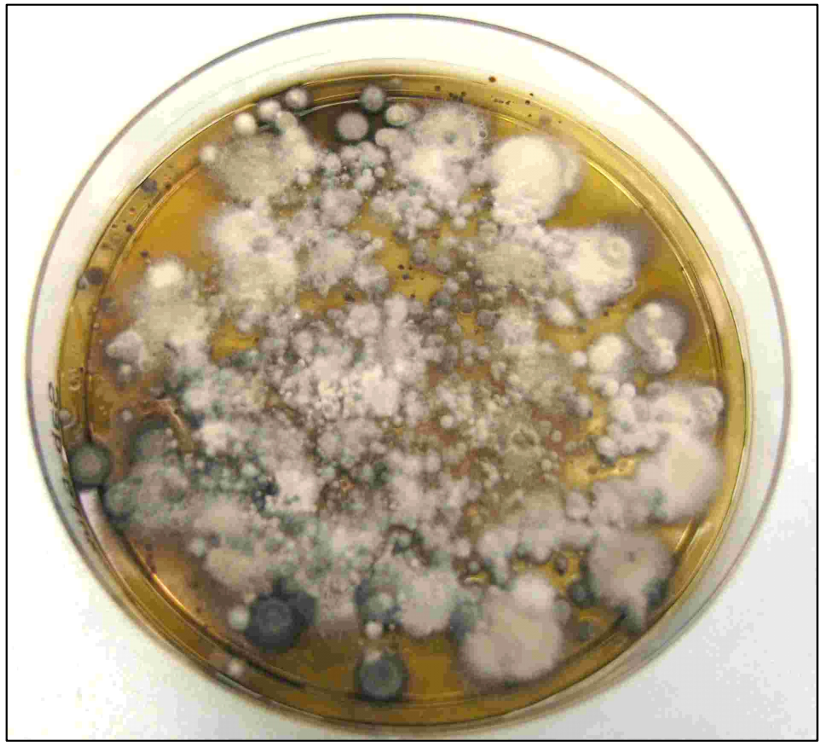
ASTM 6974-03

Enumeration of viable bacteria and fungi in liquid fuels - Filtration & Culture procedure

Sample

Membrane Filtration
(0.45µm/50ml)
Removing of fuel
(10 ml detergent solution)
Washing with Ringer solution
(3x10ml)
Placing the membrane on agar growth media
TSA --> bacteria
MEA --> yeasts & fungi
Incubation
(3-5d, 25°C)
Calculation/Quantification

Cultural Techniques 2

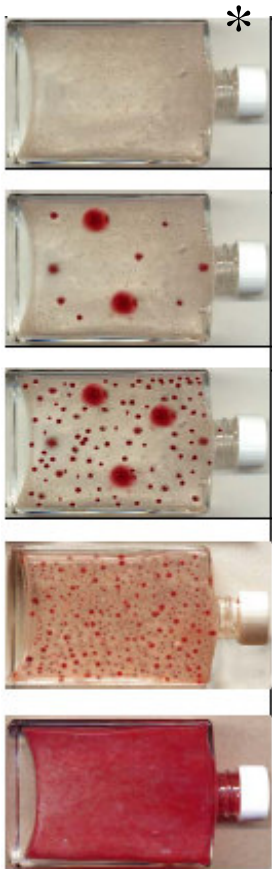


DIN 51441:2007

Testing of mineral products - Determination of the microbial colony number in petroleum products with a boiling range below 400°C.

90 ml Sample + 10 ml Ringer solution
Shaking period
(30 sec.)
Separation phase
(10 min.)
Aliquotes of the water phase → spread on
TSA --> bacteria
MEA --> yeasts & fungi
Incubation
3-5d, 25°C
Calculation/Quantification

Cultural Techniques 3



* *ECHA Microbiology Limited*

MicrobMonitor^2 *

1-Step-Quantification Tool for Aerobes (Bacteria + yeasts + moulds) based on growth-dependent, respiratory DH-activity (⟶ red colour of colonies)

Sample --> growth medium / Gel
(0.5 ml fuel resp. 0.1 ml water)
Shaking period
(30 sec vigorously)
Incubation
(*3-7d, 25° to 30°C)*
Calculation/Quantification

Remarks

- sample Material itself is carbon- & energy source
- ~ comparable to the standards cited
- suitable for small labs
- no possibility for further investigations on cultured microbes
- decontamination and disposal ?

Cultural Techniques 4

Possible Weak Points

- **Influence of carbon sources**
  Normally the growth media used do not contain any hydrocarbon source. There is a strong selection towards protein- and sugar-consuming microbes. No differentiation between autochthonous and allochthonous organisms
- **Detection of Aerobes**
  There is a complete neglect of anaerobes (eg. SRB)
- **Numbers of cultured organisms**
  The generated CFUs do not allow to project the total amount of living microbes
- **Detection of viable organisms**
  Sometimes dead biomass is a cause for calamities
- **Quantification Findings**
  Validity is given only for the sample itself; no deduction possible from fluid results to surfaces etc.
- **Recommendation of Methods**
  It is not clear which method is qualified under certain conditions

More complex alternatives

- **Quantification of hydrocarbon oxidizing microbes**

→ **selective nutrient media**

liquid media → MPN-Method
agar plates → emulsification of hydrocarbons
agar plates → silica gel technique (BARUAH et al 1967)

→
→ **detection of co-metabilization**
analyses of the behaviour of pure and mixed
→ **cultures**
→ **effects of additives**
→ **long-time assays**
...

Detection of Microbial Activity in Fuels

Adenosine-5'-triphosphate (ATP) is well known as the intracellular energy conducter molecule of each living organism. The ATP-concentration depends eg. on species & activity status.

Collection of 1000 ml fuel sample

Addition of 5 ml capture solution
(water based; contains tensides, salts, dye)
Shaking Period
(10 sec.)
Stand Still Phase
(10 min.)
Retrieve of capture solution

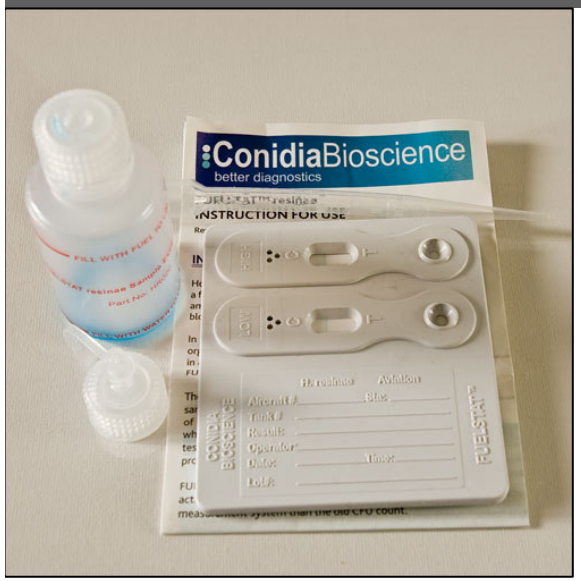
Loading the detection pen
Starting of the biochemical reaction

Measurement of bioluminescence
Calculation/Quantification

Conclusions

- no correlation to CFUs, no detection of dead biomass
- very fast detection and independent from lab equipment ⟶ on-site

Species-specific Detection



Detection of ***Hormoconis resinae*** via immuno-assay

Collection of eg. 50ml fuel sample

Extraction solution is within the vessel
(water based; contains salts, dye)
Shaking Period
(5 sec.)
Transfer of some droplets of the blue watery aliquote to the sample paddle
Incubation period (antigen-antibody-reaction, kind of chromatography)
(10 min.)
Detection / Read-out
Calculation

Remarks

- adopted to water, fuel, and mixtures of both
- only detects ***H. resinae***
  → ***H. resinae*** is known to be an indicator for microbial contamination of aviation fuels
- great risk for miss-interpretation / oversight of other severe microbial contaminants