Federal Institute for Occupational Safety and Health

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BAuA Unit 4.7 – Biological Agents

Biopesticide Exposure Measurement with Molecular and Microbiological Techniques

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Biopesticides

Pesticides - plant protection products (e.g. crop protection)

- biocides (e.g. mosquito/malaria containment)

Biopesticides - active substance is a microorganism (bacterium, fungus, virus) or other biological agent

Regulations (EC) 1107/2009 for PPP and (EU) 528/2012 for biocides require

- ➔ strong precautionary safety for human health and environment
- ➔ sufficient efficacy

Biological active substances

- ➔ are generally deemed safer for health and environment than synthetic active substances (specific, low side effects, biodegradable)
- ➔ may be applicable to only a few combinations of crop and pest

Approved active microorganisms (EU)

August 2016

➔ 483 approved active substances for plant protection products

thereof bacteria 10

fungi 25

viruses 7

➔ 802 rejected active substances for plant protection products

thereof bacteria 3

fungi 2

viruses 6

biocides ➔ 86 approved active substances, thereof 4 bacteria

European Commission, 2016, ECHA, 2016

Bacillus thuringiensis with insecticidal Cry proteins

Discovered 1901 (Ishitawa, Japan) ➔ sudden collapse silkworm disease

Description 1911 (Berliner, Germany) ➔ cells with crystal inclusions (Cry proteins)

First Application 1938 (France) ➔ „Sporine“ against flour moths, since 50s in USA

Bt-Plants 1996 ➔ corn, cotton

Global pesticide market and biopesticide perspective

Bacillus thuringiensis (~90 % of all biopesticides)	13 000 t (WHO, 1999)
Pesticides (biological and chemical) worldwide	2 300 Mio. t (EPA, 2007)

Rationales for biopesticide exposure measurement

46 approved microorganisms, 16 under review (PPP and biocides), more expected

➔ Diverse range of active substances with complex biochemistry

Registration ➔ exposure data for novel active substances

Post registration ➔ preventive risk assessment (Biological Agents Ordinance, 2013)

➔ acute cases, e.g. health effects – correlation with exposure?

No technique for bioaerosol exposure standardized at present

Precautionary product warning label ➔ microorganisms may cause sensitization

European Commission, 2016, ECHA, 2016

Microorganisms in bioaerosol

Bioaerosol

Airborne particles containing or composed of

Bacteria, fungi, viruses,

Pollen,

cell wall constituents (endotoxin, mycotoxins)

Natural bioaerosol background (onshore)

Viable bacteria and fungi in unpolluted outdoor air 10^2 - 10^4 cfu/m^3

Bacteria (n=216)

Fungi (n=665)

Kolk et al., 2009

Bioaerosol in agriculture

Sources	➔ livestock, crop, land, vectors (rodents), biopesticides
10^5-10^{10} cells/m^3	➔ Bacteria (Staphylococcus, Actinomycetes, gram-neg.), fungi, viruses
Health risks	➔ acute and chronic airway disorders (EAA, farmer´s lung),
	➔ (toxic effects, opportunistic and obligate infections)

Occupational diseases and biological agents

Germany 2014

➔ 75102 notifications

thereof infections	1796 (2,4 %)
thereof respiratory allergies	1976 (2,6 %)

➔ 16969 cases

thereof infections	814 (4,8 %)
thereof respiratory allergies	409 (2,4 %)

No standard technique for bioaerosol exposure at present

Precautionary product warning label ➔ microorganisms may cause sensitization

Correlation between exposure and health effects not well known

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Biopesticide aerosol sampling and analysis

Expectations for biopesticide exposure measurement (different to natural background)

- ➔ dominant and known biological agent(s)
- ➔ elevated concentrations above background levels
- ➔ spores are robust (vegetative active microorganisms may be more fragile)

Research targets determine sampling and analysis technique

Quantitative, e.g. workshift exposure

- ➔ microscopy, DNA quantification
  - ➔ retention through filter (dry) or impinger (liquid)
  - ➔ impaction, centrifugation, or precipitation on dry material or liquid

Qualitative, e.g. biopesticide spraying at high and undefined background

- ➔ cultivation, DNA sequence analysis
  - ➔ filter (draught stress!) or impinger
  - ➔ impaction or centrifugation, or precipitation on cultivation medium

Filtration

Portable or stationary pumps with filterheads

➔ adjustable air-flow, e.g. respiratory rate

➔ filterheads in proximity to nose and mouth

➔ sampling of most relevant inhalative aerosol

Parts can be disassembled

➔ Filter mounting and removal

➔ Cleaning and heat decontamination

➔ Variability, e.g. inlet cones with varying hole diameter

Filtration – limitations and alternatives

- Draught stress for microorganisms on filter
  - limited use for analysis of viable, fragile microorganisms
  - ➔ use shorter sampling intervals
- Filters may clog at high aerosol concentrations and high humidity
  - suitable for low aerosol concentrations (background and below)
- Incomplete release of collected microorganisms from filter

Impingement with Impinger

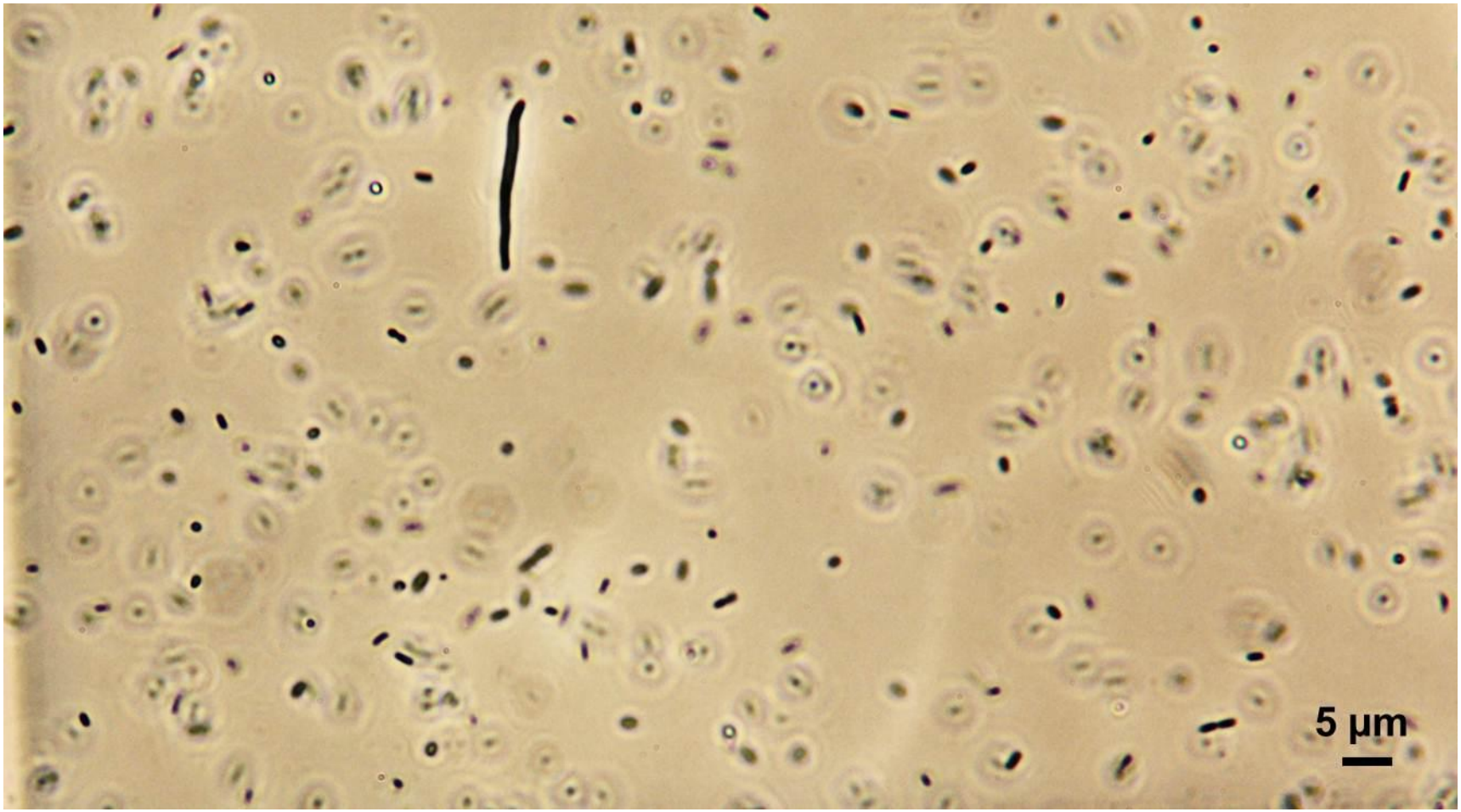
- Retention in liquid
- Sampling of microorganisms which are sensitive to draught stress
- Robust at elevated aerosol concentrations

- Low flow-through, unsuited for low aerosol concentrations
- Fragile equipment, not portable, limited use under harsh field conditions

Microscopy

- Total cell count (viable and non-viable) ➔ sensitization is not dependent on viability
- No or very limited identification ➔ low morphological diversity of microorganisms

Phase contrast (picture) ➔ viable (movements), but debris may look like cells



Microscopy with DAPI stain of DNA

Fixation and staining of sample – only DNA is stained

Distinction between DNA-containing cells and debris of similar size (no cells)

Quantitative and qualitative DNA analysis

DNA extraction from sampled cells ➔ chemical/mechanical procedure

DNA amplification ➔ specific regions (species-specific or conserved) are amplified (a, b, or c)

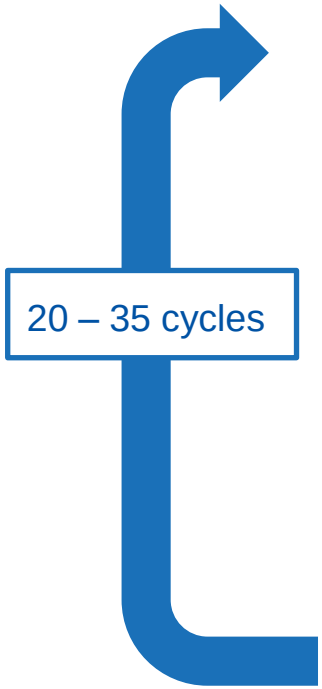
Length of bacterial genome ~10^6 – 10^7 bp (base pairs)

Length of universal 16S rRNA gene ~1900 bp

PCR product length	for quantification (qPCR)	150 - 250 bp
	for sequence analysis (16S clone libraries)	~1500 bp

PCR - Polymerase Chain Reaction

Reaction volumes 25 – 100 µl per sample, thermocycler



Denaturation of
double strand DNA
1 min at 95 °C

Annealing of short synthetic single strand
oligonucleotides with complementary
sequence „primers“ 1 min at 54 °C

Elongation with thermostable DNA
polymerase ➔ doubling of DNA
2 min at 72 °C

qPCR for quantification of *Saccharopolyspora rectivirgula*

- ➔ major causative of extrinsic allergic alveolitis (EAA, farmer´s lung) in agriculture and compost plants
- ➔ Bioaerosol analysis of compost plants (5700 – 6000 workers in Germany)

Schäfer et al., 2011, 2013

S. rectivirgula - DAPI total cell count and qPCR

Aerosol sampling by stationary filtration in a compost plant

➔ delivery ➔ compost heaps ➔ sieving machine ➔ wheel loader

Schäfer et al., 2013

Critical parameters of PCR and qPCR

- ➔ DNA extraction efficiency (*S. rectivirgula* 7 – 55 %)
- ➔ biopesticide spores may be recalcitrant to DNA extraction
- ➔ amplification efficiency (*S. rectivirgula* primer system 98 %)
- ➔ more than one rRNA operon in one genome, *i.e*. more target DNA copies than targeted microorganisms (overestimate)

Schäfer et al., 2011, 2013

16S rRNA clone libraries

Identification method when species identity of analysed biological agents is not known or to be verified

➔ e.g. when biopesticides are used under high bioaerosol background

➔ when contaminants are suspected

Bioaerosol sampling – impaction and centrifugation

Collection through mass inertia on cultivation medium or other material (dry or liquid)

Mass-sensitive fractionation is possible

Cut-off of smaller fractions

- Direct collection of viable bioaerosol
- Portable devices available (cyclone)
- Overload at elevated aerosol levels (with direct cultivation)

Bioaerosol sampling – precipitation (thermal or electric)

Collection through thermal gradient or by ionisation and electrical charge gradient on cultivation medium or other material

- Mild collection of fragile aerosol
- Highly sensitive detection of very low quantities
- Differential deposition of charged particles

Outlook

➔ Different techniques for sampling and analysis of biopesticide exposure are available

➔ For standardized risk assessment in registration and regular application

- robust and reproducible methods and affordable equipment

➔ Choice of optimal method may depend on specific active microorganism and specific application parameters

Thank you very much for your attention!