Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

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English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

REPORT OF THE OECD/KEMI/EU WORKSHOP ON MICROBIAL PESTICIDES: ASSESSMENT AND MANAGEMENT OF RISKS - ANNEX 6 (PRESENTATIONS - PART 3/3)

Series on Pesticides
No. 76

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This document contains part 3/ 3 of the Annex 6 of the REPORT OF THE OECD/KEMI/EU WORKSHOP ON MICROBIAL PESTICIDES: ASSESSMENT AND MANAGEMENT OF RISKS. Annex 6 includes slides of all presentations made during the seminar.

The main part of the seminar report, as well as Annexes 1-5, is published under the reference ENV/JM/MONO(2014)2.

PART 3 OF 3

COMPILATION OF PRESENTATION SLIDES PRESENTED AT THE OECD/KemI/EU WORKSHOP on Microbial Pesticides: Assessment and Management of Risks

17-19 June 2013
Vår Gård, Saltsjöbaden, Sweden

Organised jointly by:
OECD (Organisation for Economic Cooperation and Development)
KemI (Swedish Chemicals Agency)
European Commission

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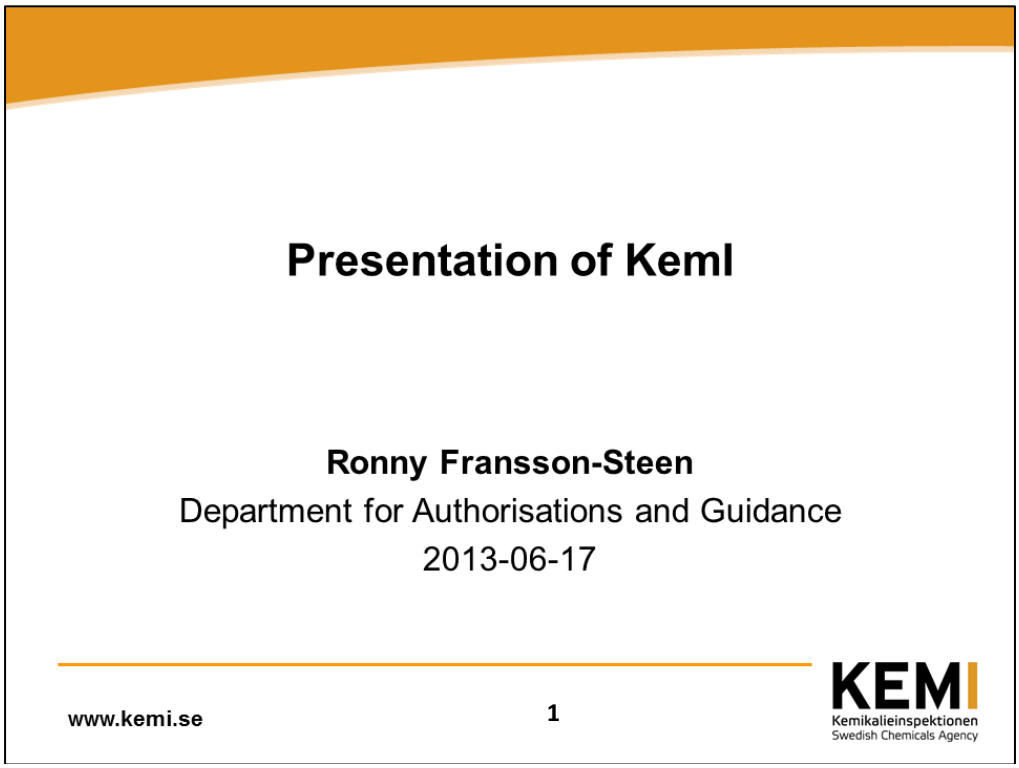
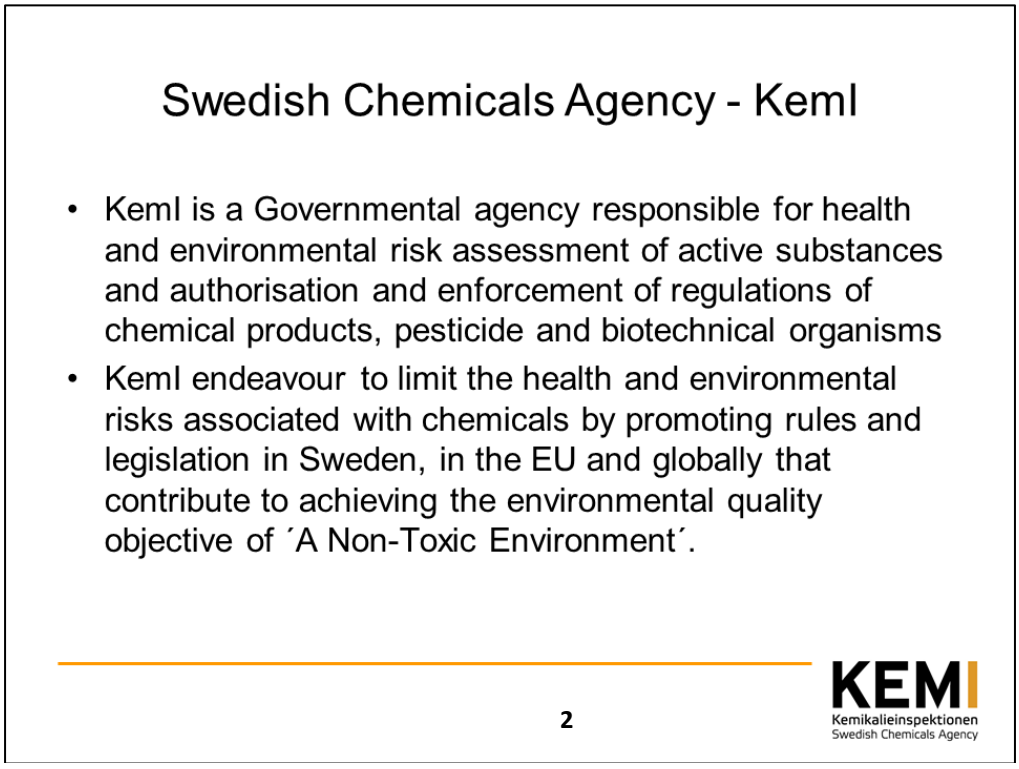
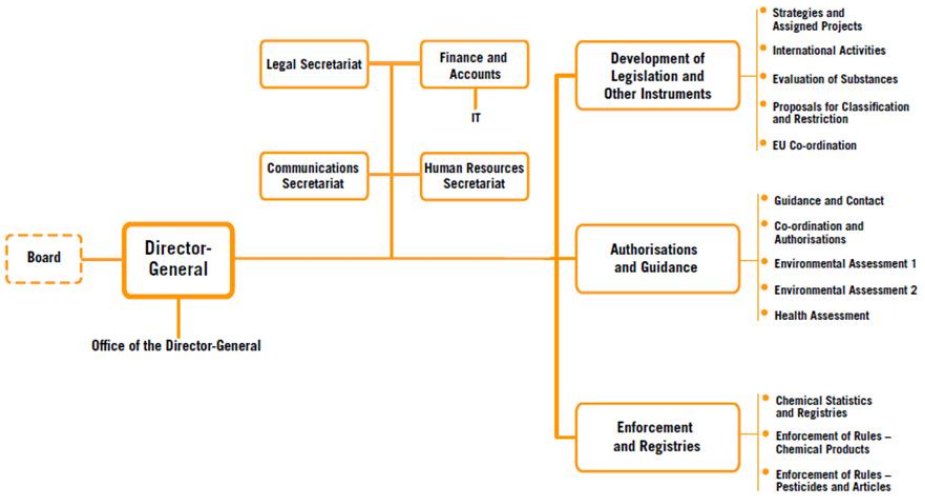
Presentation of Keml

By Ronny, Fransson-Steen
Director, Department for Authorisations and Guidance, Swedish Chemicals Agency





Organisation



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KEMI
Kemikalieinspektionen
Swedish Chemicals Agency

Preceeding meetings

- Saltsjöbaden, Sweden October **1991**
  - *"It is our hope that this meeting could in some aspects act as a catalyst for increased international efforts on co-operation and harmonization"* (Kerstin Niblaeus)
  - Main achievement → **OECD Working Group on Pesticides**
- Stockholm, Sweden October **1998**
  - *"Our hope is also that it will show that the workshop became a catalyst for increased harmonisation and co-operation in the field of microbial plant protection products between EU member states and OECD member countries"* (Vibeke Bernson)
  - Main achievement → **EU improved rules for data requirements and principles for product authorisation**

4

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Swedish Chemicals Agency

Preceeding meetings

- Arlington, USA April **2008**
  - The key-word was *Communication*
    - Communication between regulators, scientists, industry, consumer organisations, grower's organisations and NGO's should be encouraged
  - Achievement → For example **guidance document on environmental risk assessment**
  - The Arlington workshop recommended a **central website for biopesticides** to facilitate information exchange – still needs to be further prepared

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Urgent need

- To facilitate and streamlining the authorisation process of products containing micro-organisms it is necessary with more robust and harmonised rules and criteria
  - Rules and guidance for product authorisation and criteria for risk assessment are too indistinct
- Biological pesticides is still a small area, however increasing
- New EU regulation on plant protection products push for alternatives to the use of chemical pesticides, microbial pesticides is one alternative

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6

Present meeting

- Saltsjöbaden, Sweden June **2013**
  - Possible achievements
    - Establish **EU expert working group on microbial pesticides**
      - To harmonise and facilitate acceptance of risk assessment reports and product authorisations
      - An agency group on micro-organisms for use both in biocidal and in plant protection products.
    - Establish workshop series on **Microbial Pesticides: Assessment and Management of Risks** on a regular biannual basis to continue regulatory discussions like at this workshop.

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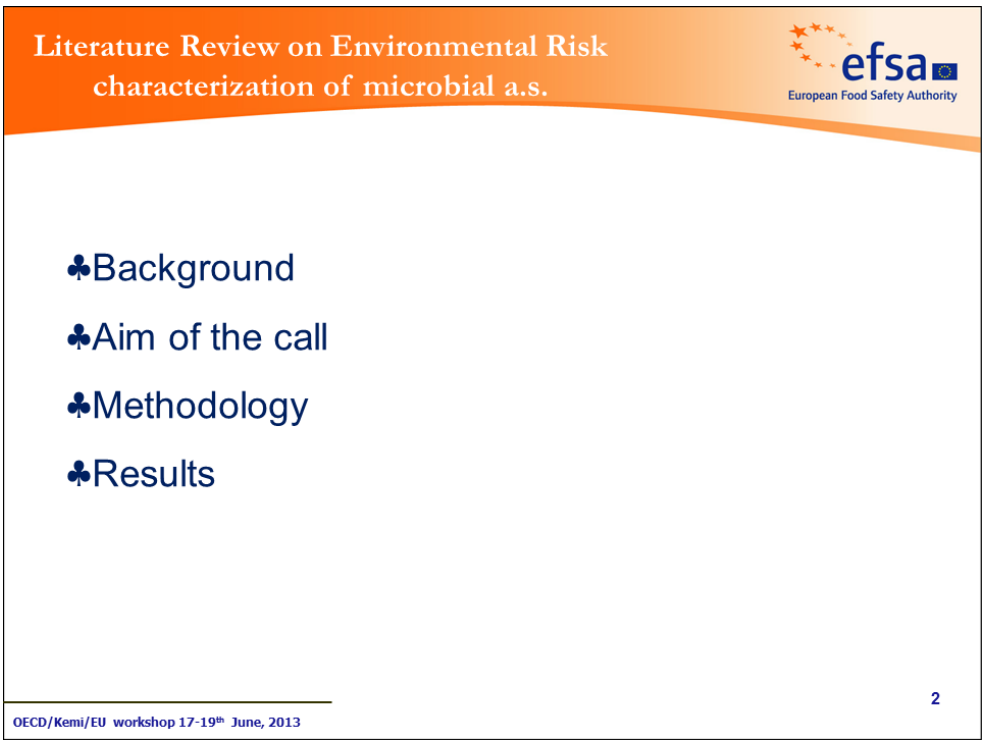
Science/Academia Views

Scientific support, literature review and data collection and analysis for risk assessment of microbial organisms used as active substance in plant protection products - Lot1-Environmental Risk characterization

By Arena Maria, Pesticides Unit, European Food Safety Authority



Background

Experience gained up to now during the peer review of active substances used in plant protection products (PPPs) has shown that risk assessment for microorganisms is indeed a complex task and differs from assessment of chemical pesticides. Guidance on their assessment is necessary to ensure also their consistent evaluation. Development of such guidance has also been identified as a priority by the Pesticide Steering Committee (PSC).

Collection and evaluation of relevant information is needed to support the preparation of a guidance on how to conduct risk assessments for microbial pesticides within the frame of EU peer review of active substances in pesticides also with a view to gauge the extent of uncertainties associated with the use of such pesticides which will be important in regard to potential precautionary elements to be introduced in a future guidance.

Literature Search on microbials used as a.s

Launching date: March 2012

The call was split into 2 lots:

1. Environmental Risk Characterizaton
2. Toxicology

After the evaluation of the offers, only lot 1 could be awarded.

Contractor: Bio Intelligence service, a French consultancy company supported by 2 subcontractors:

- The University of Warwick, UK
- The University of Helsinki, UHEL

The activities started in October 2012.

The scheduled deadline for the draft final report is July 2013

Aim of the literature review microbial a.s.

efsa European Food Safety Authority

This call for tenders was explicitly launched to support the Panel on Plant Protection Products and their residues in developing a future guidance on risk assessment of microorganisms used as a.s. in PPPs focusing on topics like:

- Methodology on the extrapolation or read across among data from a strain to another
- Waiving for experimental data on non-target organisms
- Review of the test guidelines.

Methodology-Literature review microbial a.s.

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Objectives of the call for tender

- To perform a systematic literature search and review all available relevant scientific information for each area as specified below
- To present the scientific information in a complete, systematic, clear and concise report written in English.
- To include in the search not only all the publicly available (peer-reviewed) literature but also grey literature (guidelines and government reports) and other relevant information.

The search focused on all the microorganisms used or to be used as active substances in PPPs in the EU which are listed in the Regulation 540/2011 or for which a decision on completeness has been taken in accordance with Article 6(3) of the Directive 91/414/EEC.

Methodology-Literature review microbial a.s.

	Systematic Reviews	Narrative Reviews
Study Question	Focused and explicit	Often broad in scope
Eligibility criteria for inclusion or exclusion of studies	Pre-defined and documented; objectively applied	Not always explicitly stated
Description of the review method	Reported and also predefined in a protocol	Seldom reported
Literature search	Structured to identify as many relevant studies as possible	Not always extensive
Methodological quality assessment of included studies	Included, typically using a quality assessment tool	Variable
Reporting of study results	Full reporting of relevant results (numerical results)	Selective reporting; often of study author interpretation
Synthesis	Quantitative synthesis (meta-analysis) when possible	Usually narrative, sometimes selective

Methodology-Literature review microbial a.s.

Lot1- Environmental Risk Characterization
A systematic review should be performed to collect:

- Information on transfer of genetic material from the microorganism to other microorganisms that may lead to unacceptable effects on the environment
- The potential interference with the analytical system for the control of the quality of drinking water as provided for in Council Directive 98/83/EC
- Colonisation, mobility and persistence in different environmental compartments compared to the natural background level
- The mechanisms of toxin/metabolite production, conditions for the stability outside the microorganism and related study designs
- The appropriateness of existing test guidelines for the effect assessment on non target organisms

Methodology-Literature review microbial a.s.

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Lot1- Environmental Risk Characterization

A systematic review should be performed to collect:

- Different abiotic and biotic factors/parameters relevant for the evaluation of pathogenicity, infectivity of microorganisms and toxicity of toxins/metabolites to non-target organisms, including specificity of the host. This will support the evaluation of:
  - Whether or not extrapolation or read across between data obtained for one species/strain/isolate to another species/strain/isolate in regard to infectivity, pathogenicity and the different ecotoxicological endpoints/adverse effects (as described in Regulation 544/2011) is possible or scientifically valid for microorganisms used or intended for use in the EU as active substances in PPPs
  - Whether and which conditions could be set for waiving experimental data on non-target organisms

Methodology-Literature review microbial a.s.

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According to the aim of the call 6 different topics were identified and for each of them a number of review questions formulated:

1. Genetic stability and transfer (Can transfer of genetic material between MCA under study and other microorganism occur?)
2. Interference with the system for drinking water quality control (Can the MCA under study be present in drinking water?)
3. Fate and behaviour in the environment (What is the persistence of the MCA under study in the environmental compartments of relevance?)
4. Production of metabolites (especially toxins) and potential toxic effect on non-target organisms (Is the MCA under study capable of producing toxic metabolites and can these metabolites affect non-target organisms?
5. Host specificity range and potential effect of the MCA on non-target organisms (What is the host range of the MCA? Is it broad or narrow? Is the range variable under specific conditions?)
6. Existing test guidelines

Methodology-Literature review microbial a.s.

Topic	Associated key words
1. Genetic stability and transfer	"genetic stability" OR "gene stability" OR "genetic transfer" OR "gene transfer" OR "genome stability" OR "genetic uptake" OR "DNA stability" OR "DNA transfer" OR "DNA uptake" OR "natural competence" OR mutation
2. Interference with the system for drinking water quality control	("drinking water") AND (quality OR control OR analysis)
3. Fate and behaviour in the environment	(fate OR behaviour OR mobility OR persistence OR interaction OR colonization OR dispersal OR dispersion OR multiplication OR spread OR survival OR ecophysiology) AND (environment OR air OR water OR "aquatic environment" OR soil OR rhizosphere OR field OR crop OR plant)
4. Production of metabolites (especially toxins) and potential toxic effect on non-target organisms	metabolite OR toxin OR toxic OR "non target organism"
5. Host specificity range and potential effect of the MCA on non-target organisms	specificity OR pathogenicity OR pathogenic OR infectivity OR virulence OR lethality

Methodology-Literature review microbial a.s.

Eligibility criteria

- The papers are within the scope of the study / of the topic / of the research questions;
- The material covers the right geographic region (EU as a priority).

Data sources

Search engines and databases

- PubMed: www.ncbi.nlm.nih.gov/pubmed
- Web of Science: www.isiknowledge.com
- Science Direct: www.sciencedirect.com
- Cat. inist: cat.inist.fr
- Agricola: http://agricola.nal.usda.gov/
- CAB Abstacts: http://www.cabdirect.org/
- BIOSIS
- Scopus: www.scopus.com
- Google scholar
- Google

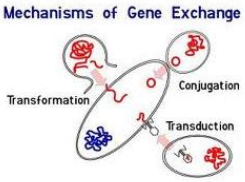
Grey Literature-Databases

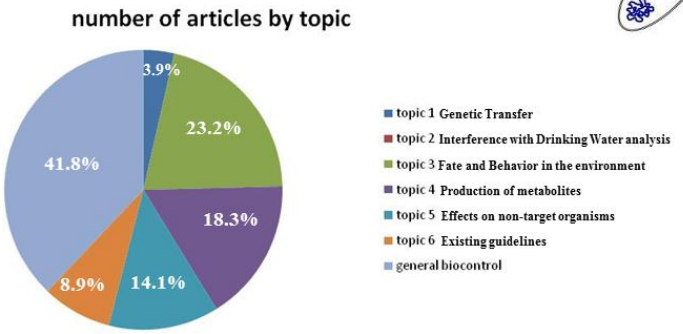
OECD
US-EPA
REBECA Project
EPPO

Preliminary Results

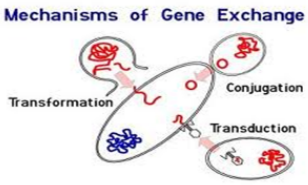
Number of Hits combining all the search terms in a single string:

- PubMed : 339
- Web of knowledge: 1 577
- Science Direct: 7251





Preliminary Results



Topic 1: genetic stability and transfer

The conclusions of EFSA often report data gaps related to this point.

Conclusion on the peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis subsp. kurstaki (strains ABTS 351, PB 54, SA 11, SA 12, EG 2348)*, EFSA Journal, 2012;10(2):2540.

In the literature is reported that transfer of genes between introduced *Bacillus thuringiensis subsp kurstaki* and indigenous Bacillus spp. can occur in soil under field conditions (Donnaruma et al., Soil Biology&Biochemistry42(2010)1329e1337).

Authors reported that isolates of *B. mycoides* acquired part of the sequence of the **cry1A** gene from *Bacillus thuringiensis subsp kurstaki*. No cells of *Bacillus thuringiensis subsp kurstaki* or *B. mycoides* carrying the238-bp fragment of the cry1Ab9 gene were isolated from samples of unsprayed control soil.

Preliminary Results

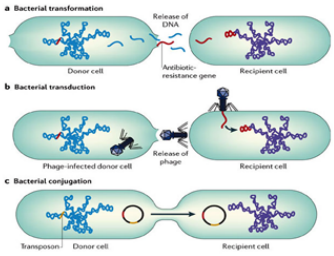
Preliminary results:

Topic 1: genetic stability and transfer

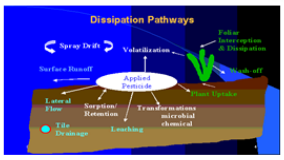
Conclusion on the peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis israelensis AM65-52.* EFSA Journal 2013;11(4):3054

A data gap has been identified with regard to the potential for gene transfer

Ankarloo et al., Current Microbiology; 40 (2000), pp. 51–56 reported that the Bt *israelensis* mosquitocidal crystal toxins are plasmid encoded and may be transferred by conjugation as showed among different soil isolates of *Bt israeliensis*.



Preliminary Results



Preliminary results:

Topic 3: Fate and behaviour in the environment

Conclusion on the peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis subsp. kurstaki (strains ABTS 351, PB 54, SA 11, SA 12, EG 2348)*, EFSA Journal, 2012;10(2):2540.

No studies on persistence and multiplication in soil and aquatic environment of *Bacillus thuringiensis* subsp. *kurstaki strains PB54, ABTS-351, SA-11, SA-12 and EG-2348* were provided and then a data gap has been identified.

Vettori et al., Soil Biology & Biochemistry 35 (2003) 1635–1642 indicated that *Bacillus thuringiensis* subsp. *kurstaki* is able to survive in soil in which it is not an indigenous bacterium, but they also showed, that *Bacillus thuringiensis* subsp. *kurstaki* could be detected more than 7 yr (the longest time studied) after introduction to natural soils in sprays. Its toxin was detected 28 months after spraying by immunological assay, but at a reduced concentration while the larvicidal activity decreased essentially linearly to 14 months and then decreased markedly between 14 and 28 months.

Conclusions

- The preliminary results highlight the importance of a systematic review of the literature for a complete Risk Assessment of the microbial a.s. A guidance has been provided by EFSA on how to identify and select "scientific peer-reviewed open literature" as required by Article 8(5) of Regulation (EC) No 1107/2009 on the placing of plant protection products on the market and how to report it in a dossier.

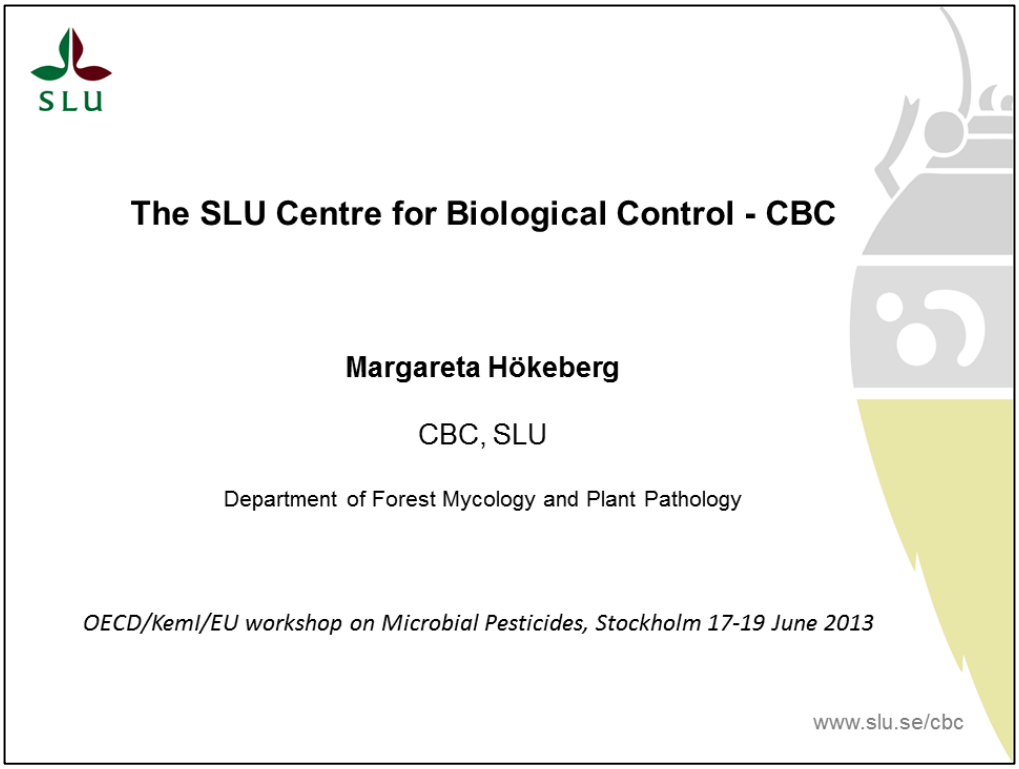
- GD on how to do the Risk Assessment for these active substances.
  This need has also been identifed by the PSC.

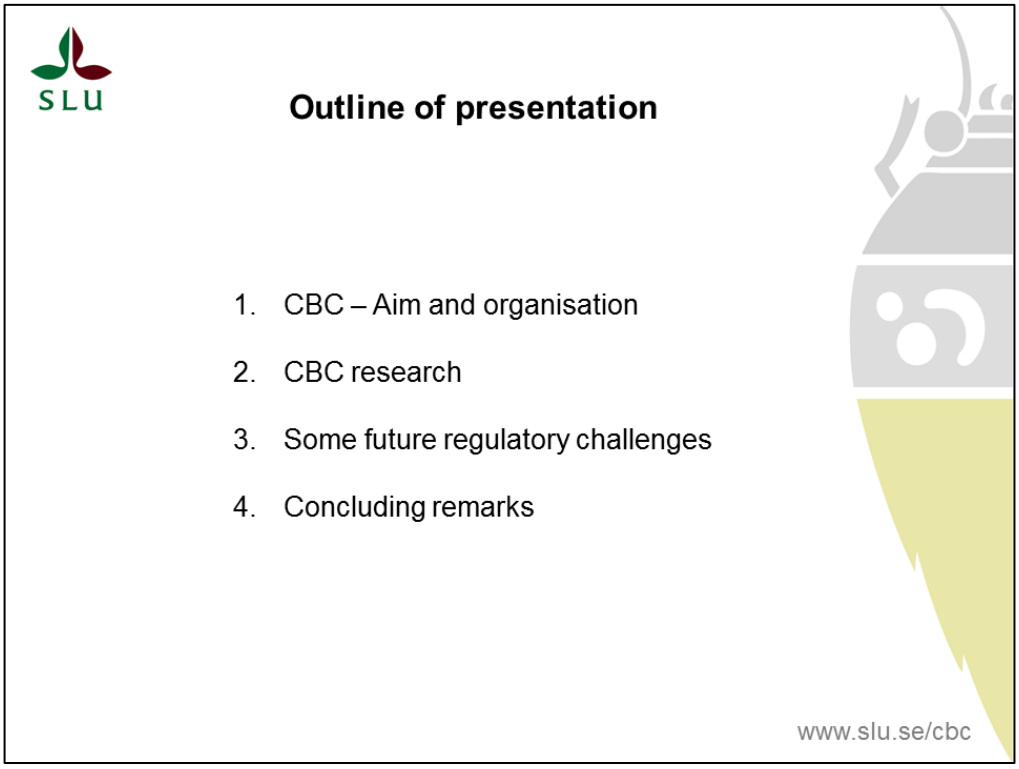
Literature review microbial a.s.

Thanks for the attention!

The SLU Center for Biological Control

By Margareta Hökeberg
Swedish Agricultural University





CBC

Finansed by the Swedish Ministry for Rural Affairs

- Focus is on the use of living organisms to control or restrict damages caused by harmful organisms.
- Research and development in different areas of biocontrol.
- Promote biocontrol research and research collaboration.
- Cooperation with stakeholders – companies, authorities, extension, growers…
- Steering group + Advisory group
- Annual stakeholder meeting on biological control, seminars.

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CBC research

- Fundamental and applied research on biological control to strengthen the knowledge base;
- Facilitate the development and implementation of new biocontrol products and approaches.
- Five research areas:
  insects/arachnides, fungi and bacteria for biocontrol;
  microbial stabilisation/formulation; safety and regulation.

Current focus: biocontrol in agricultural and horticultural crops with both augmentation/application and conservation biological control strategies. Biological control in IPM.

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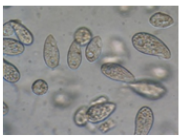
Ingvar Sundh: Safety and regulation

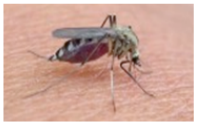
Better methodology assessing human safety:

In vitro toxicity tests of model compounds with cell lysates/extracts from microbes



Tetrahymena pyriformis

***Ecology of biocontrol agents**: Fate in the environment; strain specific SCAR markers*



Aedes sticticus
Photo: T. Vinnersten

- Pseudomonad against snow mould in wheat
- Two *Trichoderma* used against plant diseases
- Population ecology of Bti in periodically flooded riverine meadows.

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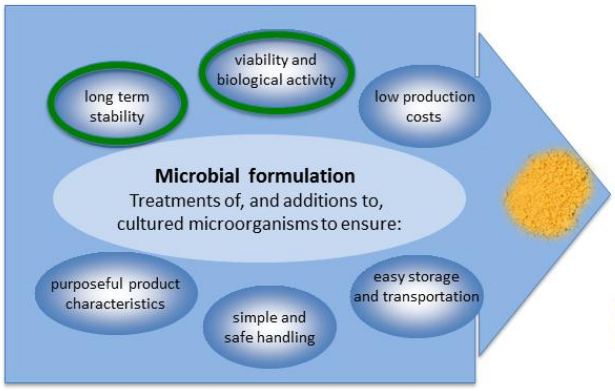
Sebastian Håkansson: Microbial Stabilization/Formulation



Process Engineering
- Thermodynamics

Physical chemistry
- Pharmaceutical galenics

Biology
- Stress metabolism
- Bioengineering

Interdisciplinary field

Example: Pre- and postformulation mixtures of BCAs, fungi + bacteria

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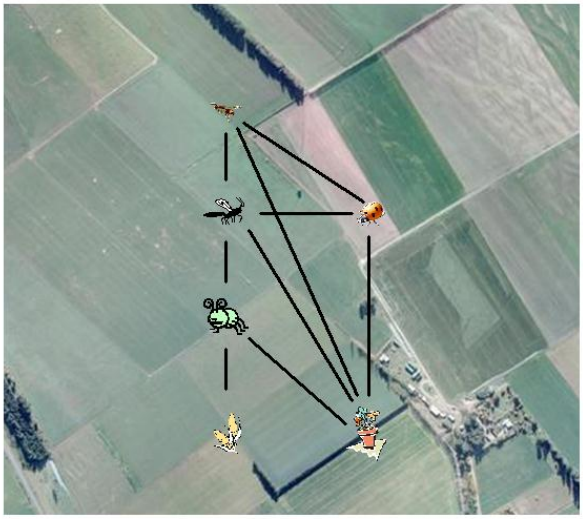
Mattias Jonsson – Insects and arachnides

Conservation biological control in a food web and landscape perspective



Biological control as an ecosystem service

Hanna Friberg: Fungi for biological control

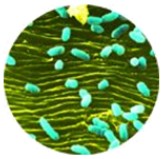
Ecological aspects – interactions between crop plant pathogens and their antagonists in their environment

Stimulation of naturally occurring antagonistic organisms – crop rotation, cultural practices etc.

How can introduced organisms be enhanced?

Ex. *F. graminearum* – Survival on plant residues in different tillage systems, succession av fungi, etablishment of antagonists.

(Future: Antagonism as an eco-system services?)



Margareta H: Bacteria for biological control

Biological seed treatment – P. chlororaphis, P. azotoformans
(Products Cedomon, Cerall, Cedress, AMASE; Lantmannen BioAgri)

BCA mixtures – Could mixtures improve disease controlling effects?
Compatibility, interactions, root colonising ability, efficacy spectrum.

Combination of control measures, crop perspective: Augmention and conservation biocontrol, seed and planting material, crop rotation, cultivation practices, chemical control, etc.
Interactions insect and disease biological control.

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Some future regulatory challenges – plant beneficial microorganisms

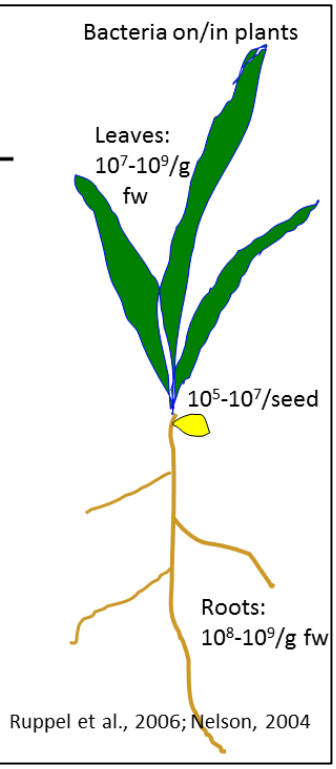
Plant strengthernes/stimulators vs MBCAs

Microbial consortia

Endophytic microbes

Plant and microbe integrated breeding

Closing gap conservation – augmentation biocontrol



Ruppel et al., 2006; Nelson, 2004

Concluding remarks

Biological control is an on-going, competitive processes in organism interaction.

Its efficiency can be increased in various ways, e.g. by promoting beneficial organisms *in situ,* or by adding them to the system after mass-production.

Current regulation for microbial pesticides is mainly based on a single strain concept. How should microbial consortia be evaluated?

MBCAs often have additional plant beneficial traits to antagonism. Will authorisation as plant stimulants be used as a fast track to the market?

How to make regulatory systems safe and reliable, but still flexible enough to handle new product concepts?

Thank you!

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Evaluation of non-viable residues and relevant metabolites

By Ingvar Sundh
Swedish University of Agricultural Sciences

SLU

Evaluation of non-viable residues and relevant metabolites

Ingvar Sundh

Centre for Biological Control (CBC), SLU

(Department of Microbiology, SLU)

OECD/KemI/EU workshop on Microbial Pesticides, Stockholm 17-19 June 2013

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SLU

Contents of presentation

1. EU data requirements/uniform principles: examples
   - Relevant metabolites
   - Non-viable residues
2. The 'fate' of microbial organic matter/detritus (metabolites/residues) in the environment
3. EFSAs pesticide peer reviews
   Usually identify several 'data gaps', why?
4. Conclusions and recommendations

Focus on EU regulation

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EU data requirements (DR)/uniform principles (UP) 1

• 'Relevant metabolites (i.e. if expected to be of concern to human health and/or the environment)….' (DR 1.4.2)

1107/2009: 'A metabolite is deemed relevant if there is a reason to assume that it has intrinsic properties comparable to *the parent substance*…..'

• 'Member states shall evaluate the possibility of exposure of humans or animals to non-viable residues …. the following information should be taken into account:

- the stage of development of the micro-organism at which non-viable residues are produced,…' (UP 2.6.2.1)

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EU data requirements (DR)/uniform principles (UP) 2

• 'Methods to determine and quantify residues (viable or non-viable) of:

- the active micro-organism(s)
- relevant metabolites (especially toxins)' (DR 4.2)

on and/or in crop, food and feedstuffs, animals and humans, soil, water

and in air where relevant.

• Mobility in the environment (DR 7.2): 'The possible spread of the micro-organism *and its degradation products* in relevant environmental compartments has to be evaluated….'

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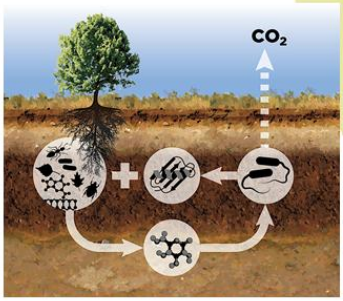
Environmental fate of metabolites/non-viable residues of microbes

• Recycling: Production new microbial biomass balanced by loss processes

• Dead microbes
- enter the pool of "microbial detritus"/humus
- contribute to the input of organic matter
- substrates for heterotrophic organisms in the detrital degrader/consumer food web

• Very high background of organic matter/residues from other organisms

• Degradation pathways for residues are present!



Graphics: Oak Ridge National Laboratory, US

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Examples of difficulties and problems

EFSAs conclusions on pesticide peer reviews of microbial active 'substances'

1. Data gaps with respect to information that testify that the organism do not produce metabolites that might fulfil the criteria (DR 7 iv):
   - Stable outside the microorganism
   - Biologically active independently of the presence of the microorganism
   - Intended to be applied at levels above background levels


efsa

Scientific Opinion on the report of the FOCUS groundwater working group (FOCUS, 2009): assessment of lower-tier

2. Effects of organism/residues on analytical systems for control drinking water. How likely? Examples?

3. Potential contamination of groundwater
   How likely? Examples?

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Conclusions and recommendations

- Microbials are legally pesticides, but must be evaluated as microbes.
- Less attention to requirements for xenobiotic chemicals, more to requirements for microbes in other areas of utilisation.
- Environment: Focus should be on the living organism, not what it produces. Non-viable residues are highly unlikely to poison the environment!
- Updated data requirements/guidance for non-viable residues/metabolites are urgently needed.
- Best way forward? a) produce guidance for requirements no longer in line with current microbial safety assessment/ecology knowledge?; or b) develop more relevant data requirements?
- How far can the requirement to demonstrate *absence* of 'relevant metabolite' production be drawn? Microbials are "guilty until proven innocent", but 100% proof of absence is not possible.

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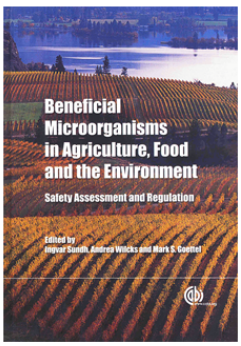
Publications

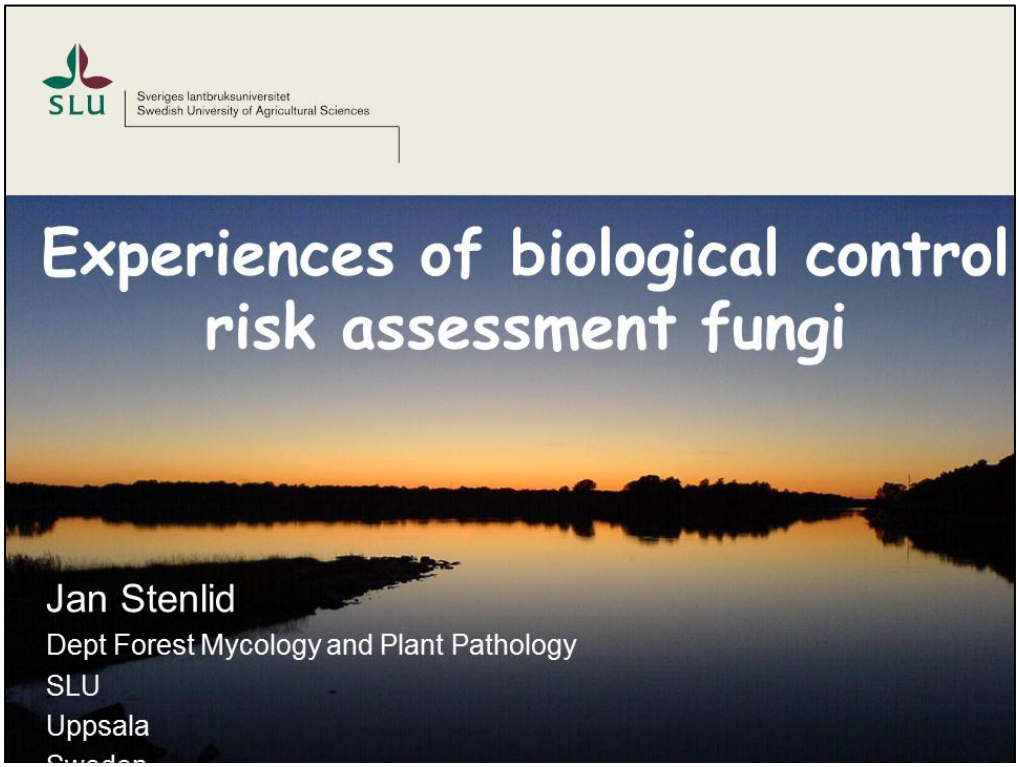
- 'Safety and regulation of yeasts used for biocontrol or biopreservation in the food or feed chain'
  (Antonie van Leeuwenhoek, 99: 113-119, 2011)
- 'Regulating biocontrol agents: a historical perspective and a critical examination comparing microbial and macrobial agents'
  (BioControl 2013, DOI, on-line first)
- 'Beneficial Microorganisms in Agriculture, Food and the Environment: Safety Assessment and Regulation'
  (Book on CABI Publishing, 2012)
- 'Harnessing the value of beneficial microorganisms: role of regulatory landscapes'
  (CAB Reviews 2013, 8: No. 013)

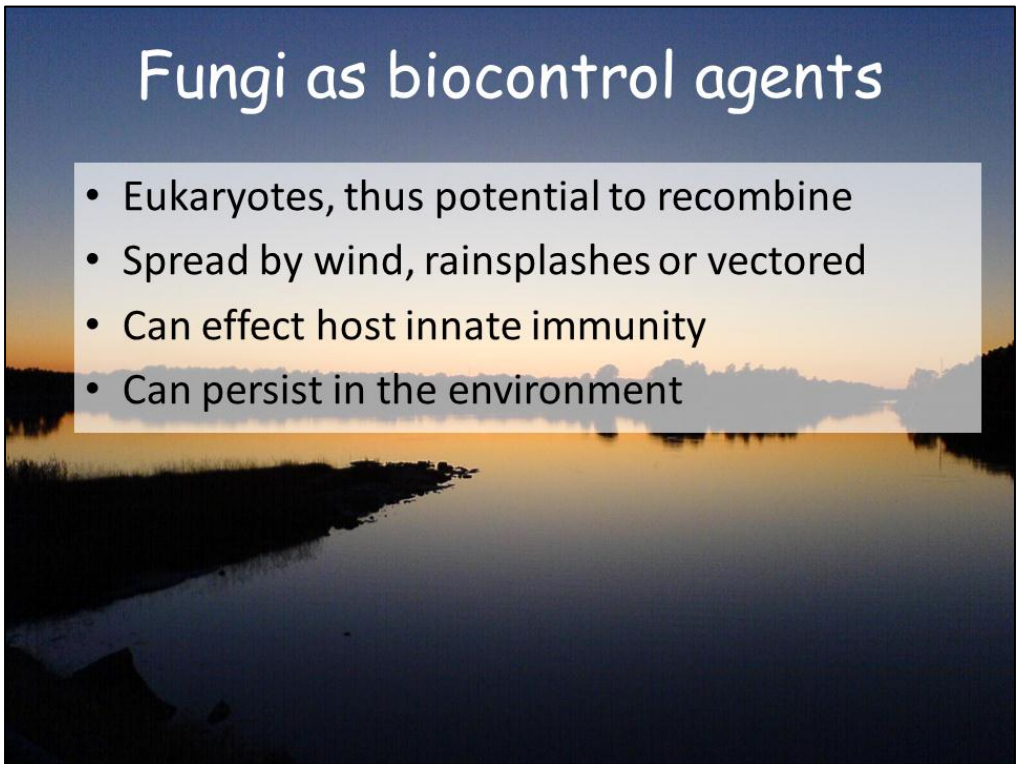


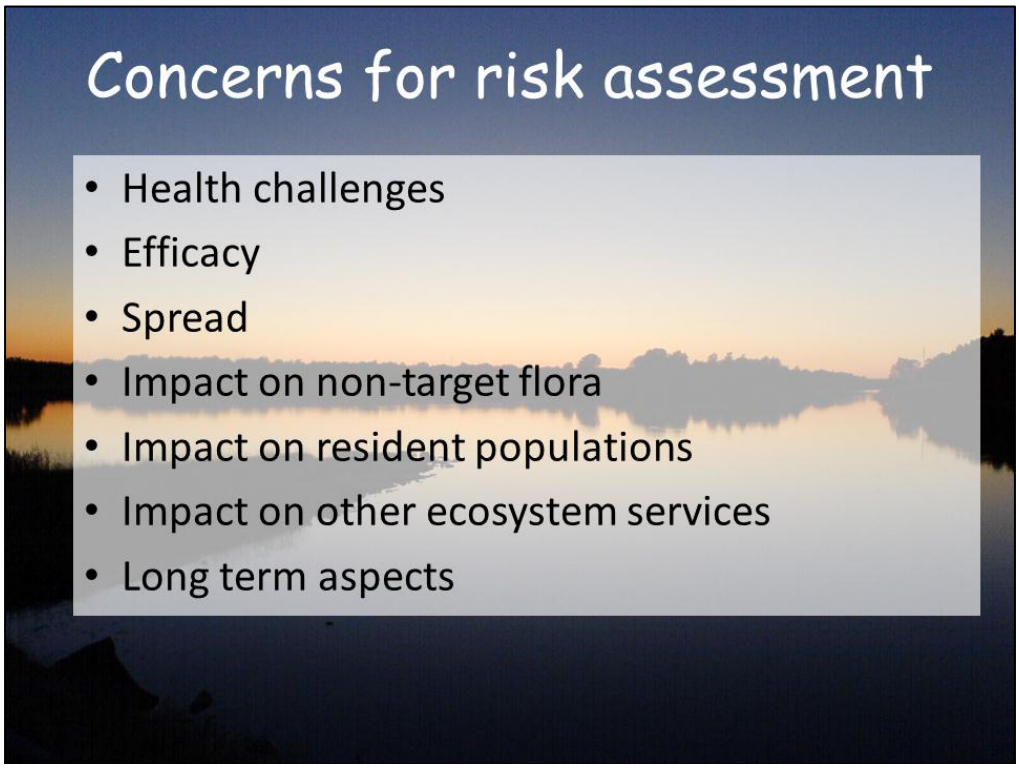
www.slu.se/cbc

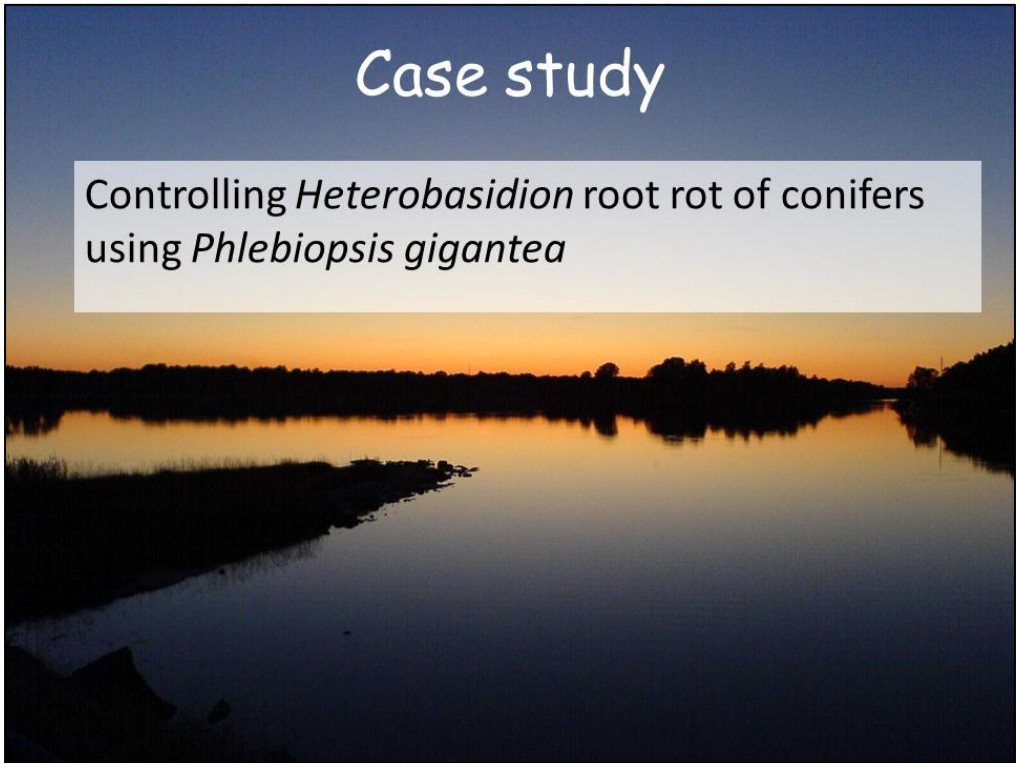
Experiences of biological Experiences of biological control, risk assessment, fungi.

By Jan Stenlid
Swedish University of Agricultural Sciences

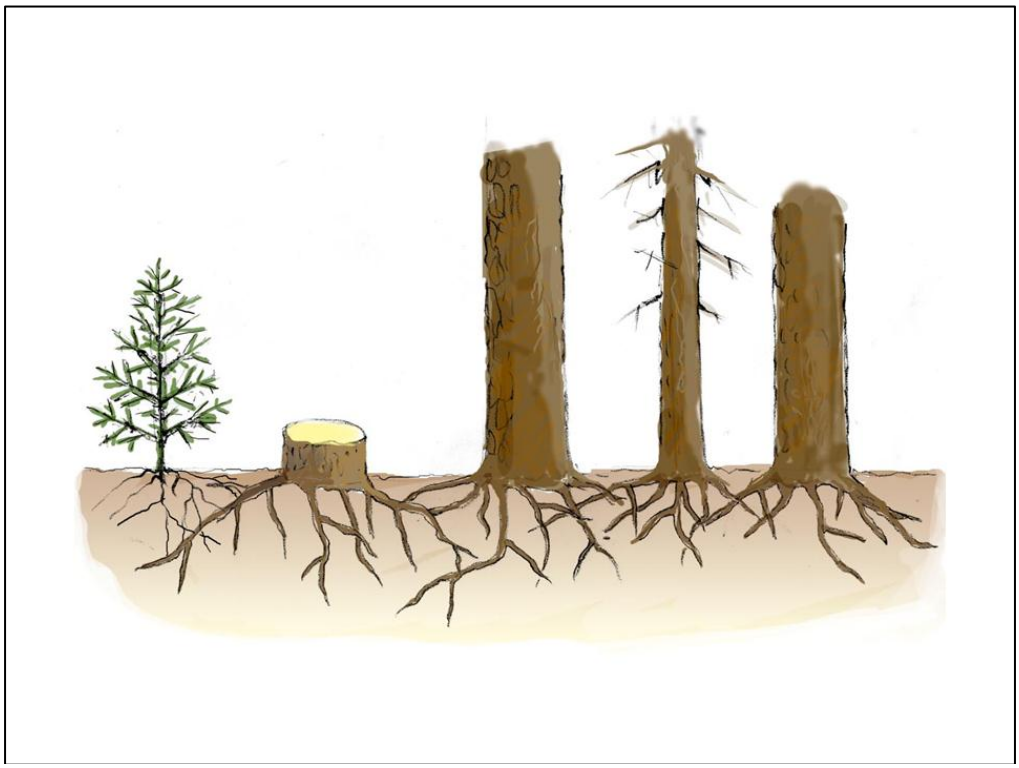


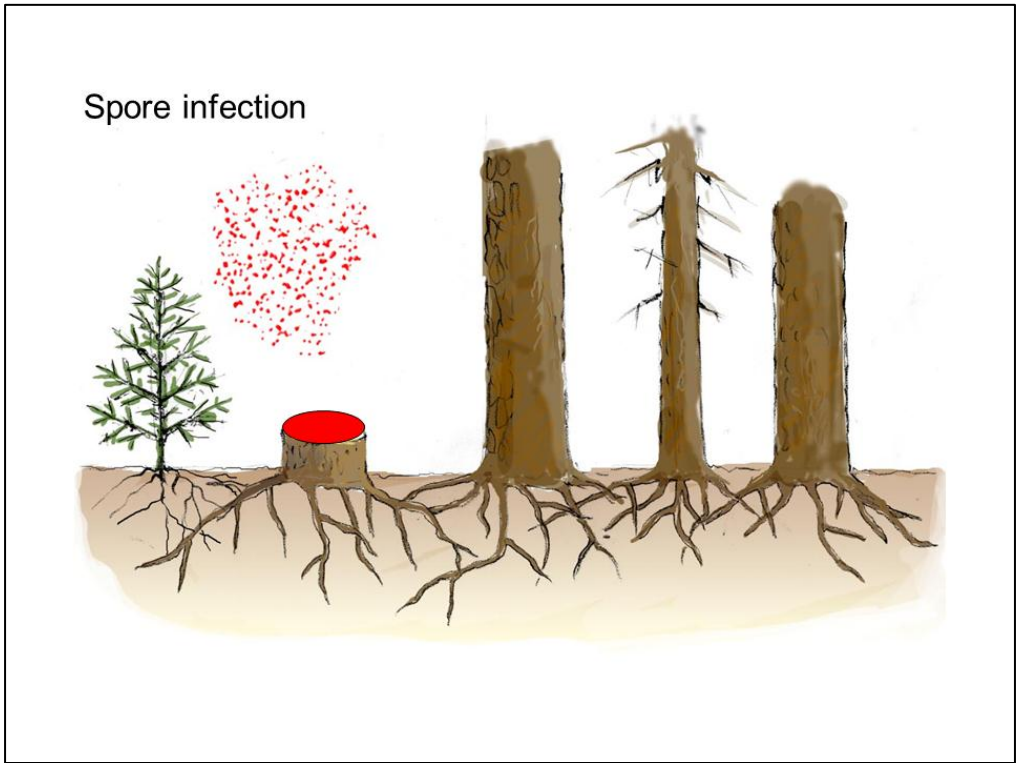


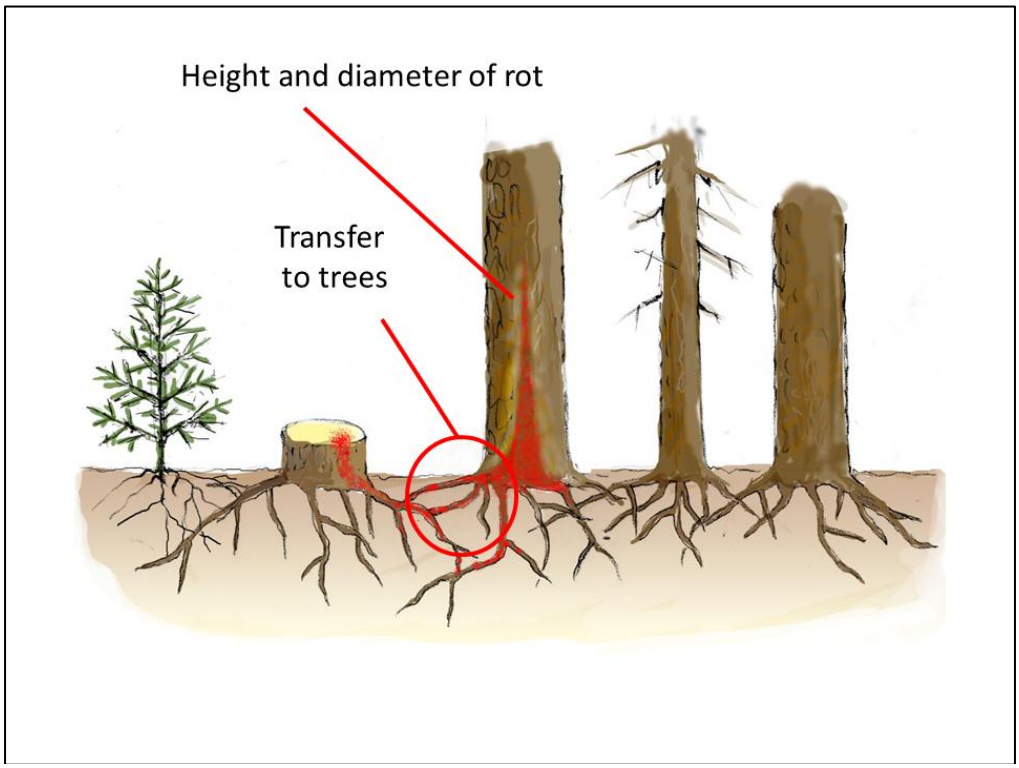

Concerns for risk assessment
• Health challenges
• Efficacy
• Spread
• Impact on non-target flora
• Impact on resident populations
• Impact on other ecosystem services
• Long term aspects

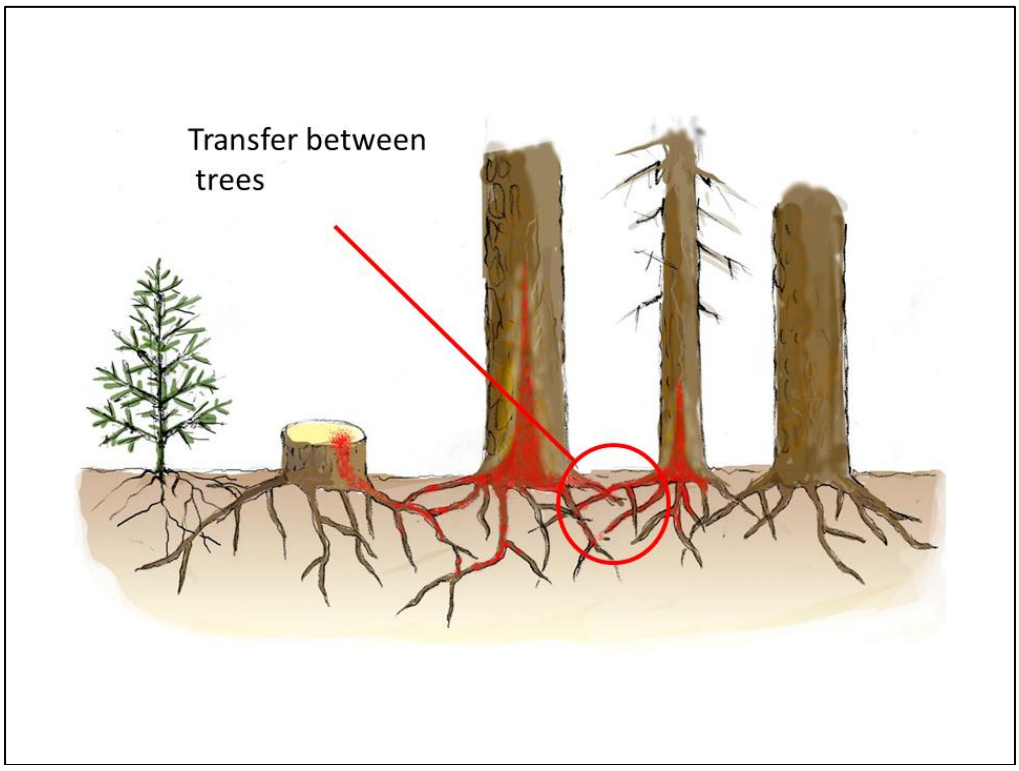

Case study
Controlling *Heterobasidion* root rot of conifers using *Phlebiopsis gigantea*

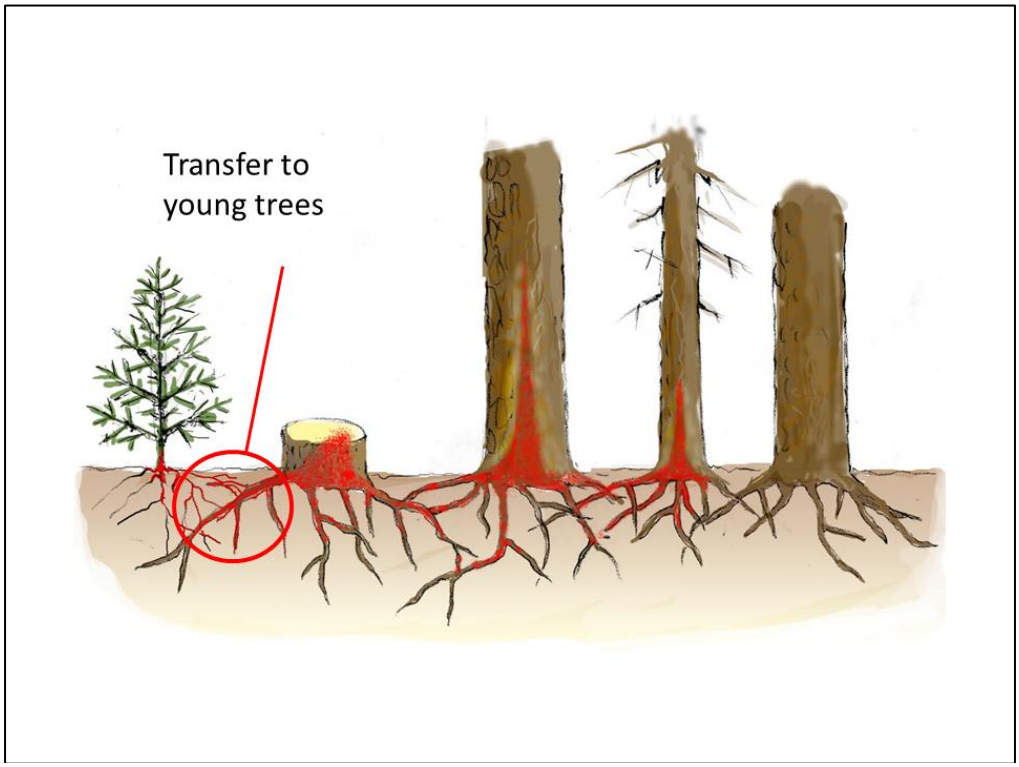
Heterobasidion annosum

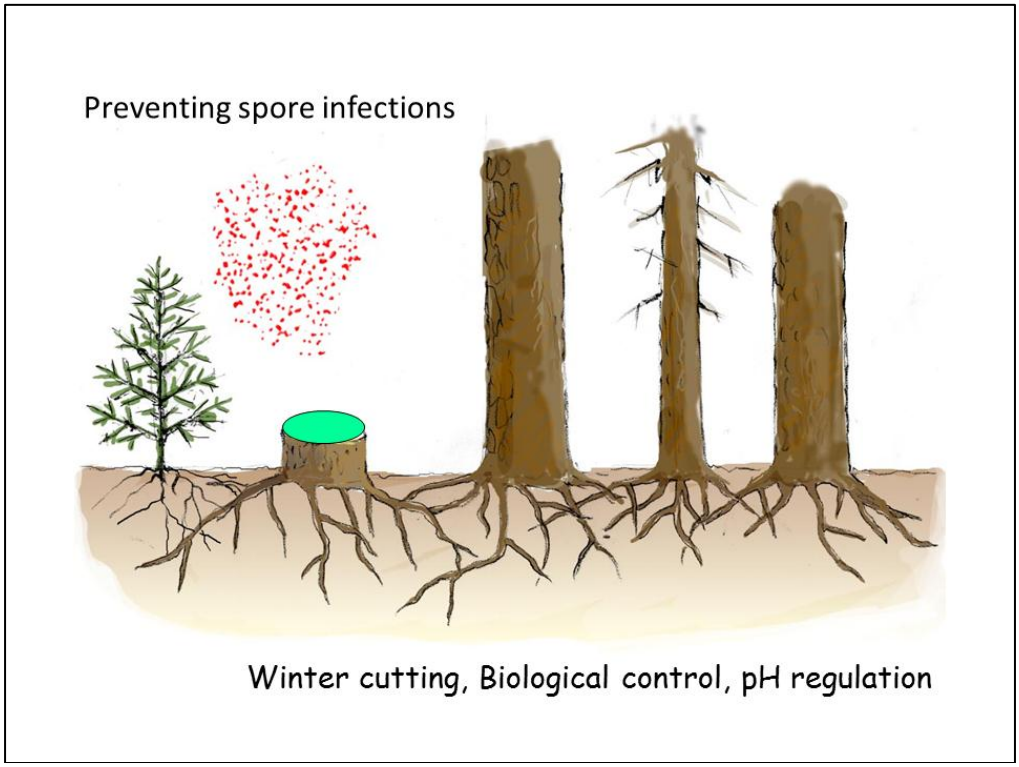



Spore infection


Height and diameter of rot
Transfer
to trees


Transfer between
trees

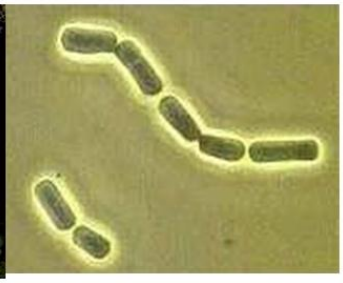

Transfer to
young trees


Preventing spore infections
Winter cutting, Biological control, pH regulation

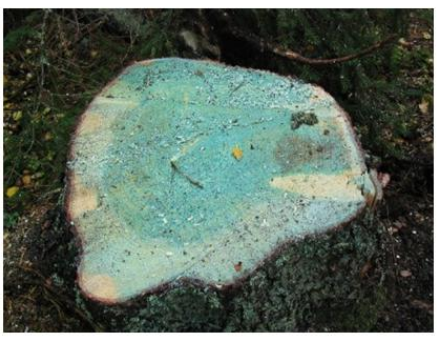
Application of spores of a competing fungus – *Phlebiopsis gigantea*

- Manual treatment of stumps at felling using pine adapted isolates from 1960. Large scale in spruce since 1992.







Concerns for risk assessments

- Health challenges – No major, no harmful metabolites, not allergenic
- Impact on non-target flora – Minor
- Impact on resident *Phlebiopsis* - Minor
- Impact on other ecosystem services – None known
- Spread – Little effect
- Long term persistance – Low
- Efficacy - Good also long lasting effects
- Long term effects on target populations - Possibly

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Biological Control 31 (2004) 405–413

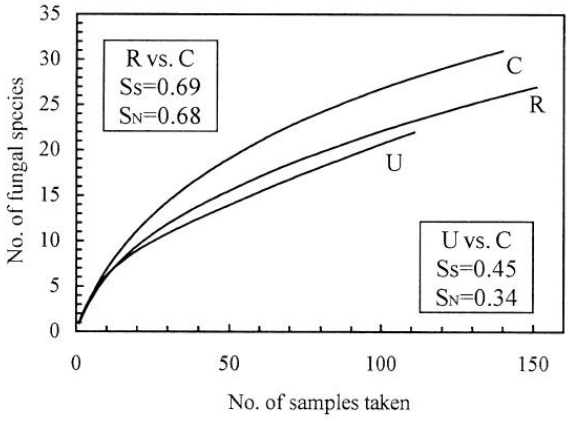
Biological Control
www.elsevier.com/locate/ybcon

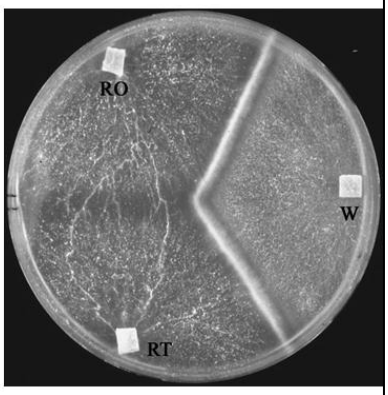
Impact of biological (Rotstop) and chemical (urea) treatments on fungal community structure in freshly cut *Picea abies* stumps

R. Vasiliauskas,[a,*] V. Lygis,[b] M. Thor,[c] and J. Stenlid[a]

[a] *Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 7026, SE-750 07 Uppsala, Sweden*
[b] *Department of Forest Protection and Wildlife Management, Lithuanian Forest Research Institute, Girionys, LT-4312 Kaunas, Lithuania*
[c] *The Forestry Research Institute of Sweden (Skogforsk), Uppsala Science Park, SE-751 83 Uppsala, Sweden*

Received 24 March 2004; accepted 24 May 2004
Available online 8 July 2004





Most common fungal species 7 weeks after Rotstop and urea treatments

Species	Control %	Rotstop %	Urea %
Morteriella isdell	71	91	29
Morteriella ram	62	33	10**
Sistotrema brinkmanii	48	33	5***
Nectria fuckeliana	43	43	76
Hypholoma capnoides	24	43	5
Giberella avenacea	19	10	71***
Heterobasidion parviporum	19	5	0
Bjerkandera adusta	19	0	0
Cylindrocarpon did	14	19	28
Epicoccum niger	5	14	43**
Nectria	5	14	29
Phlebiopsis gigantea	5	62***	0

Biocontrol Science and Technology,
Vol. 19, No. 3, March 2009, 263–276

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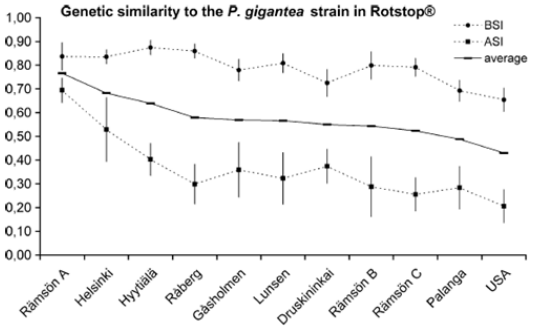
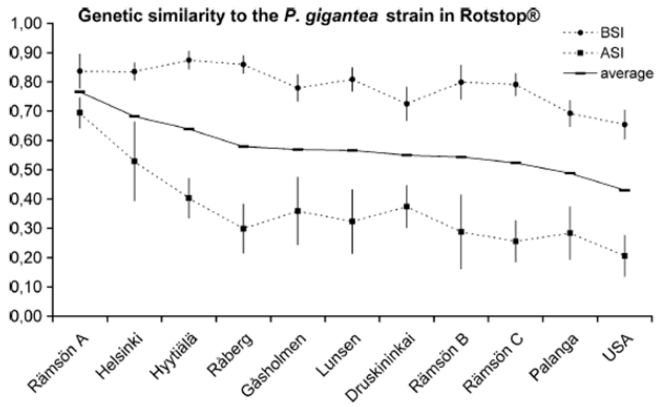
RESEARCH ARTICLE

Impact of the biological control agent *Phlebiopsis gigantea* on its resident genetic structure in the Baltic Sea area

Nicklas Samils*, Rimvydas Vasaitis, and Jan Stenlid





- A) Traps in treated area after 7 years
- D + E) From Rotstop original area
- F) Swedish 4 yrears after treatment 2 km away
- G+H) Resident Swedish before treatment
- I+J) Resident Lithuanian
- B) Traps 200 m from treated area after 7 years
- C) Resident population 200 m from treated area

Table 3. Observed (H_O) and expected (H_E) heterozygosities and *P*-values from Chi^2 calculations in the different populations and groups of *P. gigantea*.

	Pg7			Cov2			S18		
ID	H_E	H_O	*P*-value	H_E	H_O	*P*-value	H_E	H_O	*P*-value
Rämsön A	0.414	0.224	0.00711	0.480	0.327	0.03153	0.507	0.277	0.00187
Rämsön B	0.722	0.625	0.28942	0.435	0.042	0.00010	0.776	0.792	0.84955
Rämsön C	0.698	0.444	0.09720	0.459	0.556	0.56180	0.765	0.556	0.13761
Helsinki	0.700	0.667	0.80106	0.000	0.000	–	0.818	0.833	0.89176
Hyytiälä	0.665	0.818	0.28175	0.375	0.091	0.05163	0.828	0.455	0.00102
Gåsholmen	0.643	0.400	0.10898	0.490	0.400	0.57001	0.765	0.889	0.37482
Lunsen	0.612	0.400	0.16835	0.278	0.200	0.58292	0.805	0.300	0.00006
Råberg	0.747	0.636	0.39906	0.444	0.364	0.58964	0.720	0.364	0.00848
Palanga	0.717	0.867	0.19775	0.332	0.267	0.58884	0.809	0.643	0.12105
Druskininkai	0.737	0.867	0.25476	0.231	0.000	0.03372	0.733	0.867	0.10485
USA	0.729	0.231	0.00005	0.298	0.077	0.08190	0.852	0.333	0.00003

Values are based on three microsatellite loci.

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Biological Control 32 (2005) 295–304
Biological Control
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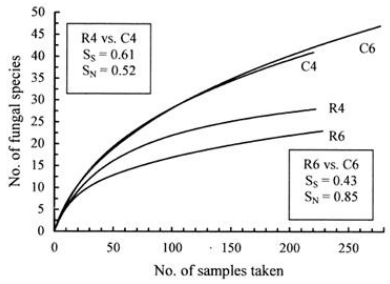
Persistence and long-term impact of Rotstop biological control agent on mycodiversity in *Picea abies* stumps

R. Vasiliauskas[a,*], E. Larsson[b], K.-H. Larsson[b], J. Stenlid[a]



Phlebiopsis not present in 6-year-old stumps

Most common fungal species 4-6 years after stump treatment with Rotstop

Species	Av frequency	Significant changes after treatment
Phialocephala sp.	33.1	NS
Sistotrema brinkmanii	30.0	NS
Heterobasidion parviporum	29.2	Lower
Recinicium bicolor	27.7	NS
Trichoderma polysporium	20.0	NS
Phlebiopsis gigantea	16.2	Higher
Ascocoryne sarcocides	13.1	Lower
Ascocoryne cylichium	13.1	NS
Hypholoma capnoides	12.3	Higher
Stereum sanguinolentum	10.8	NS
Lecytophora sp.	10.8	NS
Phialophora fastigiata	10.8	Lower

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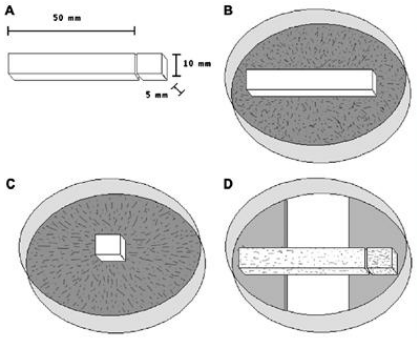
Biological Control 45 (2008) 419–426
Biological Control
www.elsevier.com/locate/ybcon

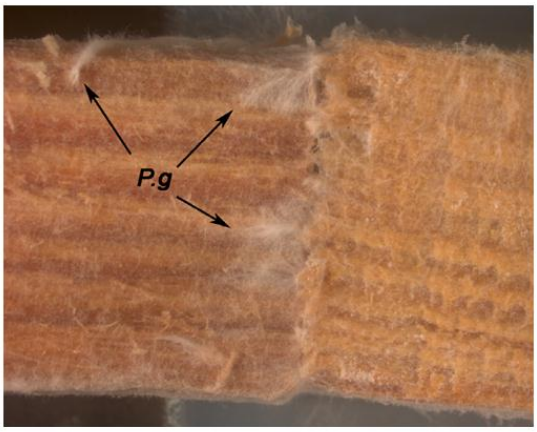
The capacity in *Heterobasidion annosum* s.l. to resist overgrowth by the biocontrol agent *Phlebiopsis gigantea* is a heritable trait

N. Samils *, Å. Olson, J. Stenlid

Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 7026, SE-750 07 Uppsala, Sweden

Received 18 October 2007; accepted 21 March 2008
Available online 29 March 2008





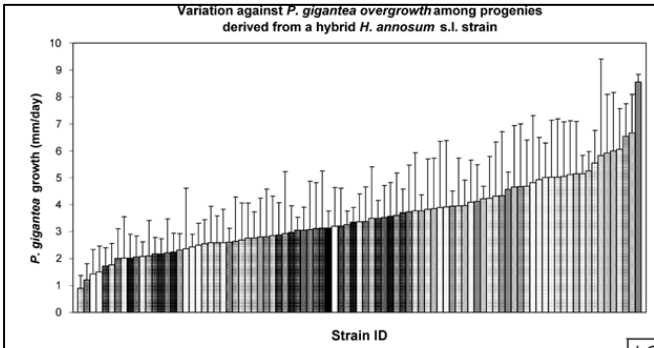
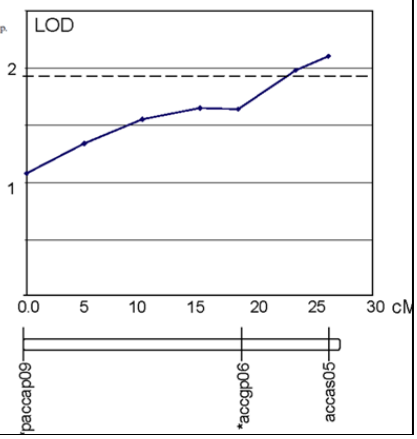


Fig. 3. The variation among 91 *H. annosum* s.l. hybrid progenies to resist overgrowth by the *P. gigantea* strain in the biocontrol agent Rotstop. show the standard deviation. The average growth rate of the *P. gigantea* strain over the 91 progenies was 3.6 mm/day.

Broad sense heritability = 0.336

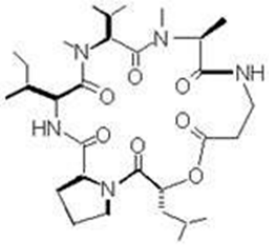



Thanks!
• Nicklas Samils
• Rimvydas Vasaitis
• Magnus Thor
• Ellen Larsson
• Karl Henrik Larsson
• Åke Olson
• Vaidotas Lygis

Secondary metabolites: a literature study - Key stones for risk assessment methodology

By Jacqueline Scheepmaker
the Netherlands

National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport



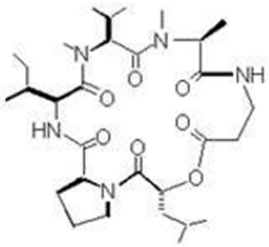
Destruxin A

Secondary metabolites
Results of a literature study

1

Jacqueline Scheepmaker, June 18, 2013

National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport



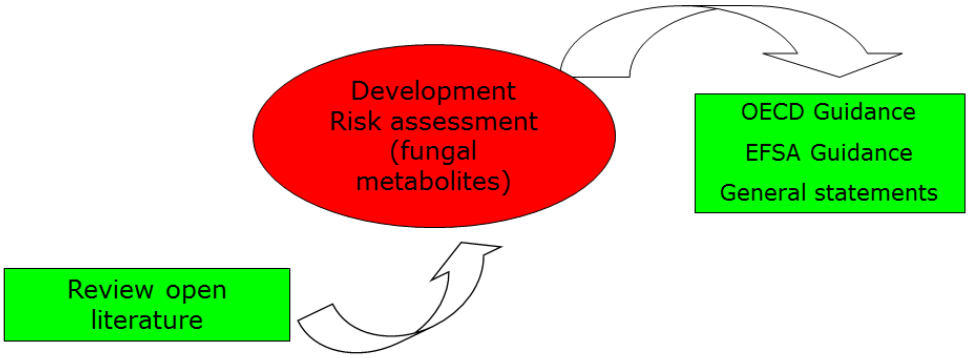
Destruxin A

Short introduction
Setup literature review
6 representative graphs
Conclusions
How to proceed?

2

Jacqueline Scheepmaker, June 18, 2013

Role literature review



Relevant recent work

Data requirement of persistence:

Scheepmaker, J.W.A. & Butt, T.M. (2010)
Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment and in accordance with EU regulations. Biocontrol Science and Technology 20: 503-552.

Purpose of this literature study

1. Provide basic information on secondary metabolites for registration purposes
2. Make a review of the range of metabolites produced by entomopathogenic fungi (EPF)
3. Determine factors influencing the production of key metabolites

Importance of metabolites

Opportunities:

1. pharmaceuticals
2. taxonomic markers
3. biopesticides (spinosad: metabolite of soil bacterium *Saccharopolyspora spinosa*
   abamectin: metabolite of soil bacterium *Streptomyces avermitilis*)

Reasons for data requirements:

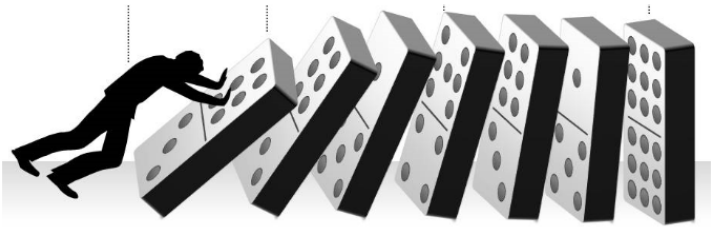
Fears:

- Secondary metabolites can be mycotoxins (aflatoxin by *Aspergillus*)
- Metabolites present in formulated product and stable after application
- Uncontrolled production of metabolites
- Secondary poisoning

7. Fate and behaviour in the environment

Data requirements and the corresponding risk assessment needs to be fulfilled if all the following conditions are met:

1. The relevant metabolite is stable outside the microorganism
2. A toxic effect of the relevant metabolite is independent of the presence of the microorganism
3. The relevant metabolite is expected to occur in the environment in concentrations considerably higher than under natural conditions



Description relevant metabolite in regulation 544/2011? implementing Regulation no. 1107/2009

"where they are of significance
for human health and/or the environment"

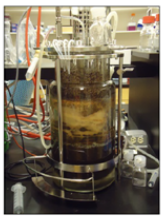
depends on the combination of
- quantities produced and
- their toxicity

If both are not known beforehand this would need investigation

Problem for industry, risk assessment ánd registration

Contents database

- 78 references with measured quantities (120 others used for manuscript)
- Different EPF species and strains
- Different culture methods (solid state, liquid fermentation, insects)
- Variety of growing media,
- Different conditons (pH and temperature)
- Fermentation periods/timing of sampling
- Different extraction methods: 1-butanol, acetone, acetonitrile, dichloromethane, ethyl acetate, methanol, methylene dichloride



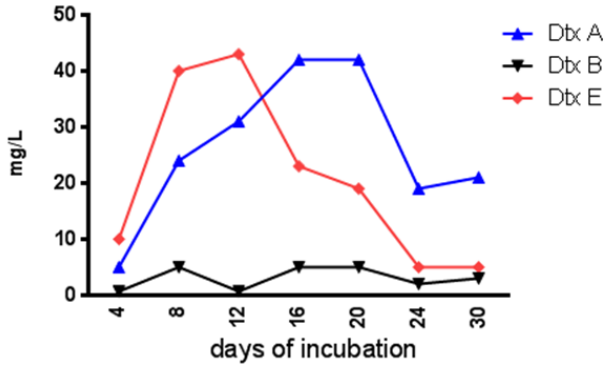
10L Liquid fermenter

Restrictions

- Focus on environment only
- Only well known entomopathogenic fungi (EPF)
  (*Metarhizium, Beauveria, Isaria, Lecanicillium)*
  - non-EPF microbials are included in case they produce the same metabolites
  - Maximum quantities + days of incubation
- Literature search on EPF was not exhaustive

**Frightening messes
of stray yarns and swatches
all entangled together into
fabulously gruesome disasters**

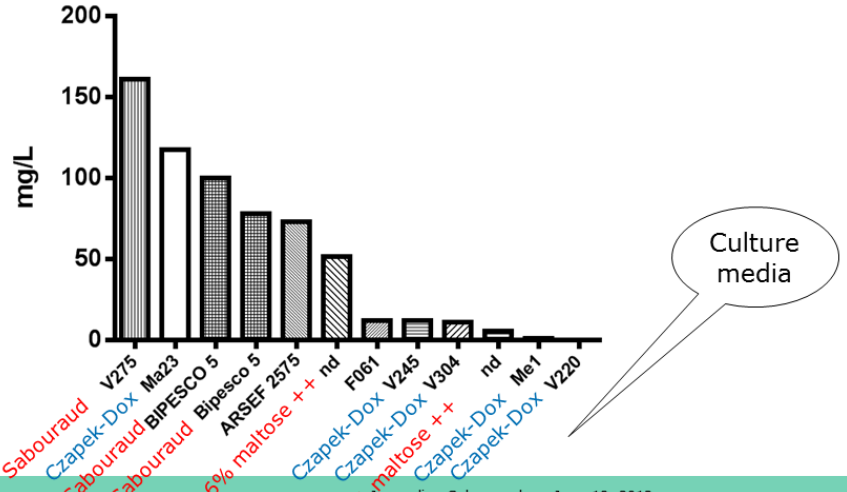
Time course for production of destruxins by *M. anisopliae* V245 in liquid medium



From: Amiri-Besheli et al., 2000

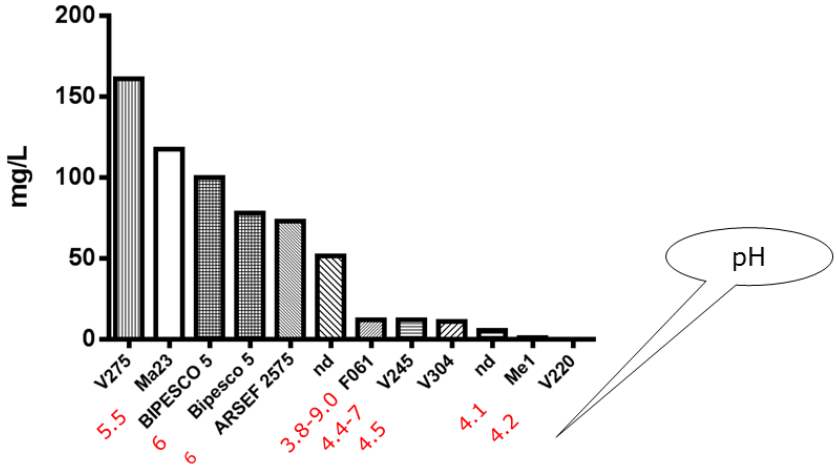
Different quantities by different strains

Destruxin A production by different strains of *Metarhizium anisopliae var. anisopliae*



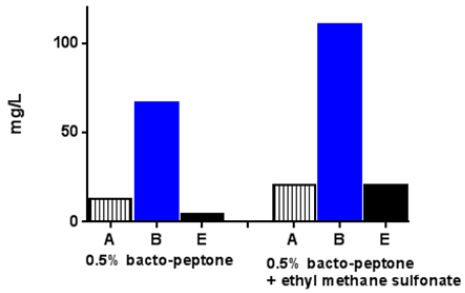
Different quantities by different strains

Destruxin A production by different strains of *Metarhizium anisopliae var. anisopliae*



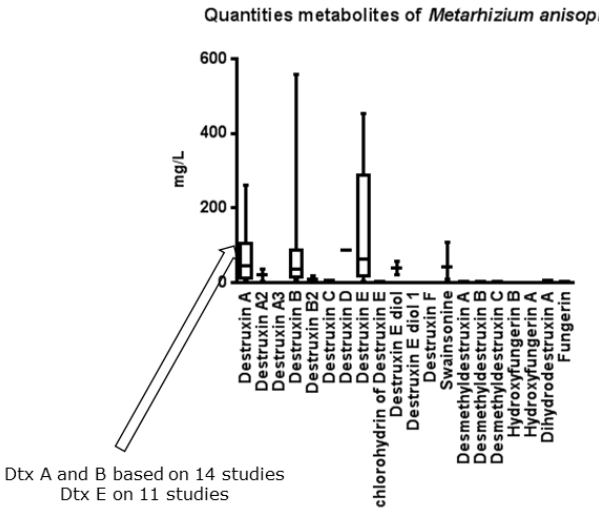
Yield of metabolites depends on the content of the medium

Destruxin production by *Metarhizium* in two different Czapek Dox Broth media

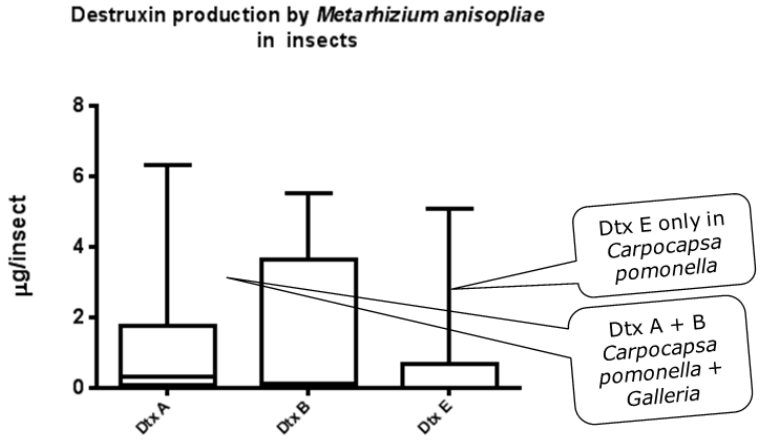


From: Hsiao et Ko, 2001

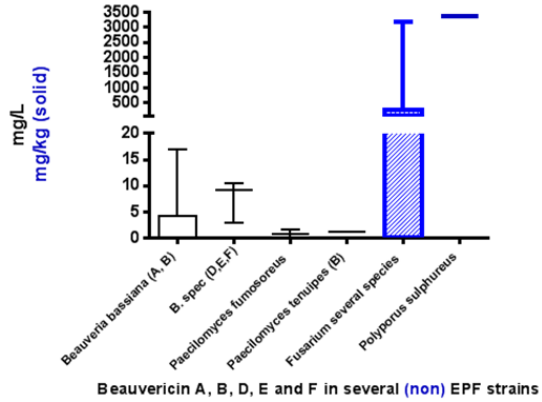
In liquid fermentors, Destruxin A, B and E major metabolites



In insects: only Destruxin A, B and E



Beauvericin produced by several species



Beauvericin A, B, D, E and F in several (non) EPF strains

Conclusions of this literature search

- Range and quantities depend on contents of fermentation medium and vary between species/strains
  - Only three metabolites of Metarhizium in insects, Destruxins A, B and E
  - These three are dominant in liquid fermentation
  - Destruxin E is not always present
  - All others are incidental and at low concentrations

⇒ Data requirement for whole range of metabolites is not possible, useful

The same metabolite can be produced in different genera

  - Quantities of Beauvericin: In Fusarium >>> EPF

- Data from literature are incoherent and incomplete:
  - Studies differ in all parameters : difficult to create coherent subsets
  - Incomplete descriptions of culture methods

Standard Test Method??

Development of a standard test method seems impossible due to different responses of EPF

Development of a standard test method is not useful: the obtained metabolite profile may differ from the profile obtained in the commercial fermentation method.

How to continue?

- We need to think out of the box and invent other strategies
- Construct simple risk assessment schemes for low, medium and high risk groups
- Identify key questions and perform literature studies where needed
  - Are metabolites present in the formulated product?
  - Low and high yielding fermentation media?
- Identify the most important route of exposure

Fate of metabolites in product application on plant surface

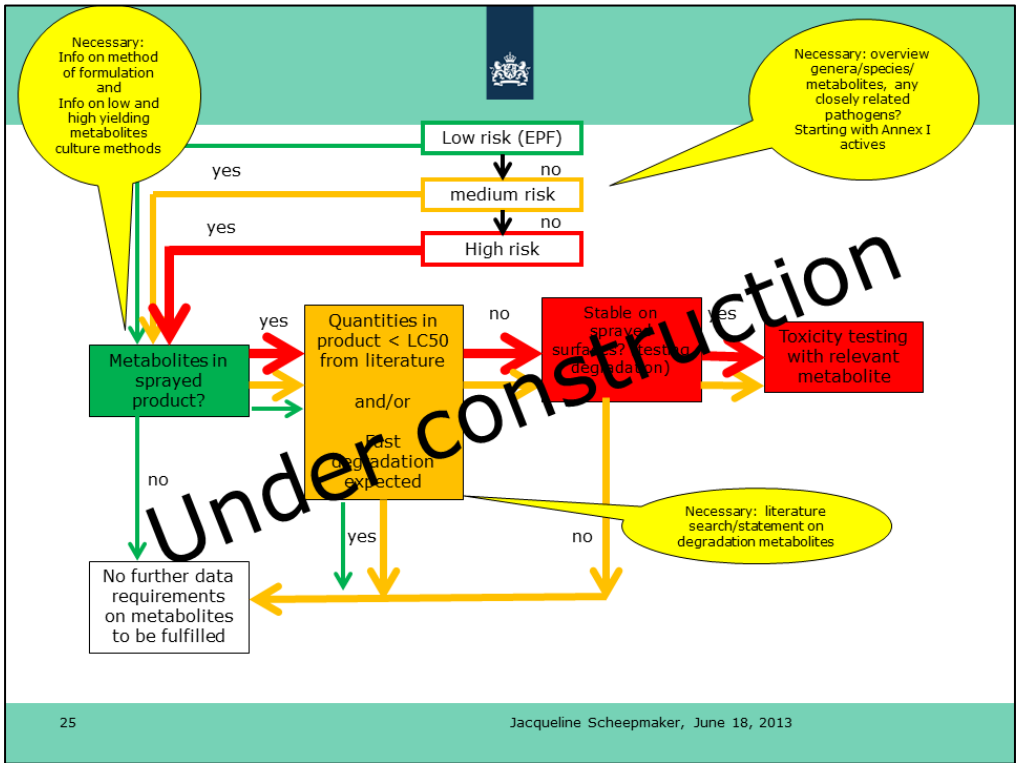
Metabolites in the sprayed EPF product?

If so, relevant in comparison with known L(E)C50 values?

Toxicity (in microgram/L)

Route of exposure	Parameter	endpoint	organisms	Stage	Crude extract	Destruxin A	Destruxin B	Destruxin E	Authors
Contact	Mortality	LC50	*Bemisia tabaci*	2nd nymph		89.8	96.5	-	Hu et al. 2009
Contact	Mortality	LC50	*Empoasca vitis*	?				38.2	Poprawski et al., 1994
Contact	Mortality	LC50	*Phaedon cochleariae*	larval		87	>500	50	Amiri & Ibrahim, 1999
Contact	Mortality	LC50	*Plutella xylostella*	larval		56	376	53	Amiri & Ibrahim, 1999
Contact	Mortality	LC50	*Serangium Japonicum*	4th larval		165.4			Hu et al. 2009
Oral	Antifeedant activity	AI50	*Phaedon cochleariae*	larval		5	104	90	Amiri & Ibrahim, 1999
Oral	Antifeedant activity	AI50	*Plutella xylostella*	larval		10	102	35	Amiri & Ibrahim, 1999
Cell substrate	Alterations cell behaviour	IC50	*Spodoptera frugiperda*			138	12	1187	Male et al., 2009
Cell substrate	Cell viablity	LC50	*Spodoptera frugiperda*		>500	5	>500	>500	Skrobek & Butt, 2005

LC = Lethal dose
AI = Antifeedent Index
IC = Inhibitory Concentration
TCID = Tissue Culture Toxic Dose



Under construction, your input is necessary!

Statement	Lacking info	In what way simplifying data requirements
Makes sense to differentiate into low, medium and high risk micro-organisms (EPF = low risk)	Prepare general overview genera/species/metabolites	No further data requirements for low risk microorganisms
Many commercial microbial products only contain spores or biomass	General overview. Contribution Industry?	Tool to differentiate into low, medium and high risk
Culture methods can be made either low or high yielding in metabolites	Determine what is low yielding. Comparison with LC50 values if present in literature?	If low yielding no further data requirements on metabolites
Secondary metabolites are degraded rapidly outside the micro-organism, therefore the condition "The relevant metabolite is stable outside the microorganism" is never met	Literature search necessary	No further data requirements on metabolites for fate and behaviour. Domino effect for other data points?

26 Jacqueline Scheepmaker, June 18, 2013

Thank you
for you attention!