Organisation de Coopération et de Développement Économiques
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English - Or. English

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

ANNEX TO THE REPORT OF THE 6TH BIOPESTICIDES STEERING GROUP SEMINAR ON HAZARD AND RISK ASSESSMENT OF SECONDARY METABOLITES PRODUCED BY MICROBIAL PESTICIDES

Series on Pesticides
No. 89

Please note that this document is available on OLIS in PDF format only.

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Unclassified
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This document only contains Annex 4 of the report of the BPSG Seminar. Annex 4 includes slides of all presentations made during the seminar. The main part of the seminar report, as well as Annexes 1-3, is published under the reference ENV/JM/MONO(2017)5.

ANNEX 4

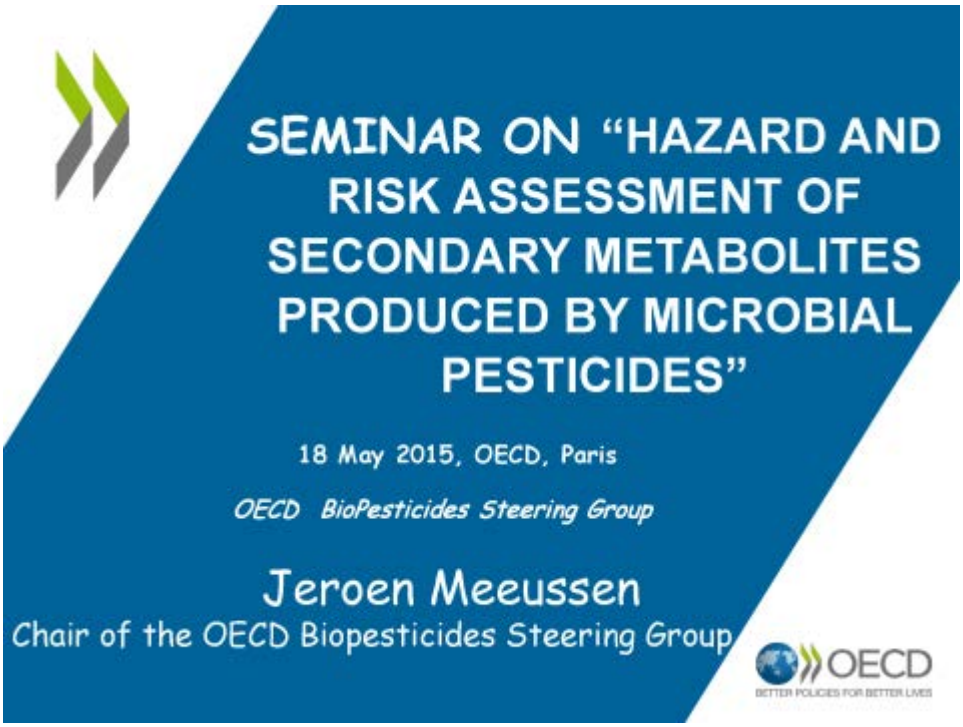
List of presentations

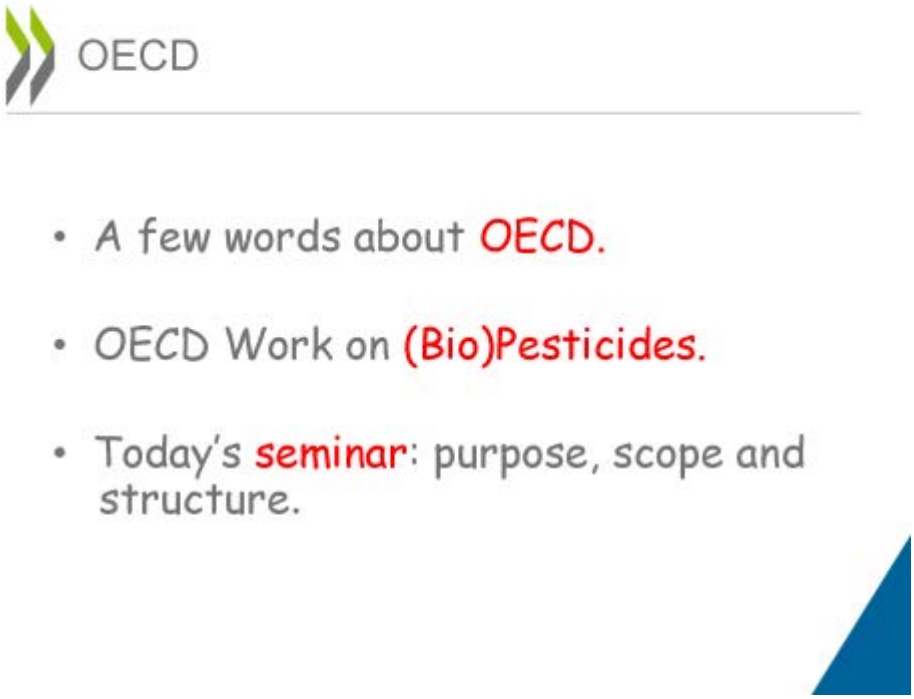
6th BioPesticides Seminar on Hazard and Risk Assessment of Secondary Metabolites Produced by Microbial Pesticides
18 May 2015, OECD, Paris, France

Presentation 1

Presentation on the OECD, the work of OECD BPSG and general introduction to the Seminar

Jeroen Meeussen, BPSG Chair, European Commission





A few words about OECD

OECD: The Organisation for Economic Co-operation and Development



OECD

- Started after World War II;
- Transformed in 1961 into the Organisation for Economic Co-operation and Development with trans-Atlantic and then global reach;
- Today the OECD has 34 member countries;
- More than 70 developing and transition economies are engaged in working relationships with the OECD (Brazil, China and India).

OECD

What is OECD?

A forum in which governments work together to:

- Co-ordinate and harmonise policies;
- Discuss issues of mutual concern;
- Work together to respond to international problems.

A provider of comparative statistics and economic and social data with more than 250 publications per year.

OECD

How do pesticides fit in all this?

One of the fields in which OECD is actively involved is the sustainability of agriculture.





OECD-WGP

OECD - Working Group on Pesticides

The OECD work on agricultural pesticides aims to help member countries:

- improve the efficiency of pesticide control;
- share the work of pesticide registration and re-registration;
- minimise non-tariff trade barriers;
- reduce risks to human health and the environment.



OECD-BPSG

- The BioPesticides Steering Group (BPSG) was established by the WGP in 1999 to help member countries to harmonise the methods and approaches used to assess biological pesticides.

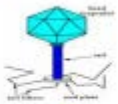
OECD
BETTER POLICIES FOR BETTER LIVES

OECD-BPSG

Biological Pesticides:

- Macro-organisms
- Microbial biopesticides
- Semiochemicals
- Plant extracts/Botanicals





OECD-BPSG

The first tasks of the BPSG consisted of:

(i) reviewing regulatory **data requirements** for three categories of biopesticides; and
(ii) developing **formats for dossiers and monographs** for microbials, and pheromones and other semio-chemicals.



OECD-Publications (I)

Registration requirements:

- for pheromones (Series on Pesticides, No. 12, 2001); ⟹ under revision
- for microbial pesticides (Series on Pesticides, No. 18, 2003);
- for invertebrate biocontrol agents/IBCAs (Series on Pesticides, No. 21, 2004).

OECD-BPSG

The BPSG then decided to concentrate its efforts on **science issues** that remain as barriers to harmonisation and work-sharing.

OECD-Publications (II)

- Working Document on the Evaluation of Microbials for Pest Control (Series on Pesticides No. 43, 2008).

This document is essentially a set of examples/case studies aimed at helping the regulatory authorities to deal with these issues in the assessment of (microbial) biopesticides.

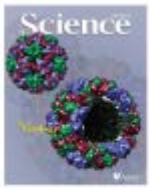
OECD-Publications (III)

- Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products (Series on Pesticides No. 65, 2011);
- Guidance to the Environmental Safety Evaluation of Microbial Biocontrol Agents (Series on Pesticides No. 67, 2012).

Workplan 2013-2016

- Promote **communication** and **exchange of information** among regulatory authorities of participating countries.

- Organise **seminars** and **workshops** on topics of common interest.



OECD-BPSG workshops

- Workshop on the Regulation of Biopesticides: **Registration and Communication Issues**; 15-17 April 2008, EPA, Arlington, USA.

- Workshop on Microbial Pesticides: **Risk Assessment and Risk Management**; 17-19 June 2013, Saltsjöbaden, Sweden

OECD-Seminars (I)

- Report of Seminar on "Identity and Characterisation of micro-organisms", OECD Series on Pesticides No. 53, 2010);
- Report of Seminar on "The fate in the environment of microbial control agents and their effect on non-target organisms", OECD Series on Pesticides No. 64, 2011);



OECD-Seminars (II)

- Report of Seminar on "Characterisation and Analyses of Botanicals for the use in Plant protection Products", OECD Series on Pesticides No. 72, 2012);
- Report on Seminar on " *Trichoderma* spp. for the use in Plant Protection Products: similarities and differences" OECD Series on Pesticides No. 74, 2013).




OECD-Seminars (III)

- Report of Seminar on "Application Techniques for Microbial Pest Control Products and Semiochemicals: Use Scenarios and Associated Risks", in publication.



Seminar on secondary metabolites

Why was this topic selected?

This was one of the main issues discussed at the June 2013 OECD/KemI/EU Workshop on Microbial Pesticides with a clear recommendation to develop a guidance document on secondary metabolites.

Seminar - Scope

Discussion on:

- Microorganisms can potentially produce **a wide array of secondary metabolites under different conditions. What to assess?**
- **When** are metabolites **formed** (after application)**?**;
- **What** are **relevant** metabolites;
- **Stability** of the metabolite, and **potential (adverse) effects**.
- Microorganisms' **biology** is crucial,
- ....etc.

Seminar - Structure

Presentations on:

- **government**, **research** and **stakeholder** experience and perspectives,

followed by discussion after each set of presentations.



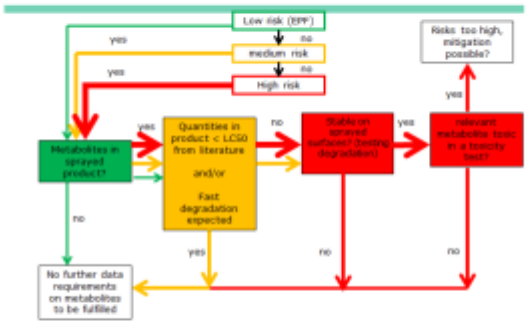
Seminar - Results

With the focus on "hazard and risk assessment of secondary metabolites produced by microbial pesticides", the goals of this seminar are

1. for participants to share information and to promote a dialogue, and

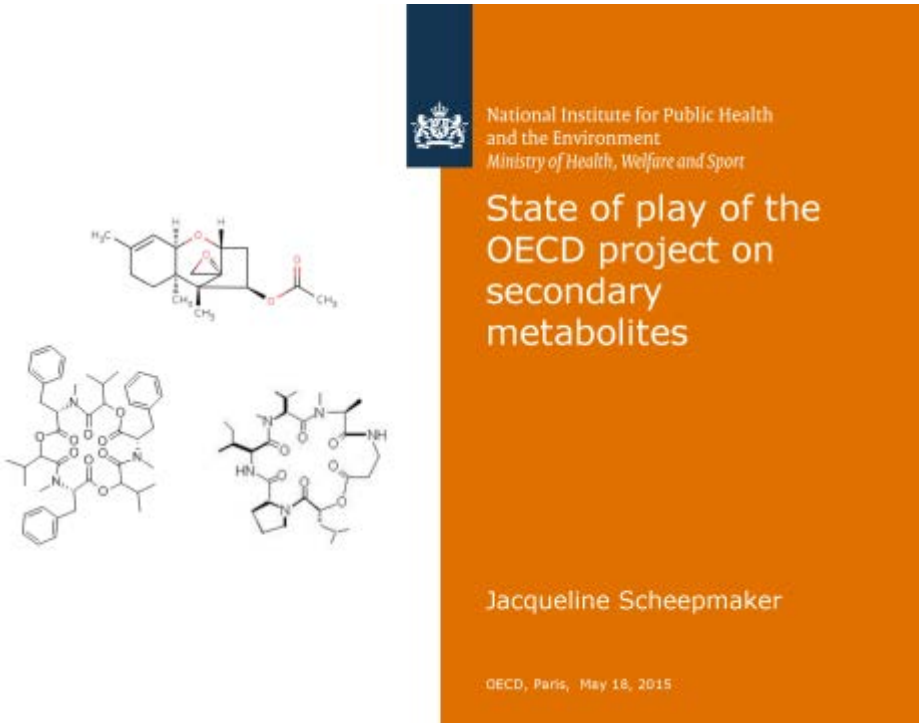
2. to initiate a process to make recommendations for improvements to the draft OECD Guidance Document on Hazard and Risk Assessment of Secondary Metabolites produced by Microbial Pesticides.

Seminar on application techniques



I wish you an interesting and useful seminar!

Presentation 2
State of play of the OECD project on secondary metabolites
Jacqueline Scheepmaker (RIVM, Bilthoven; The Netherlands)



Incentive of this project

- This issue of secondary metabolites was recognized as a problem in the risk assessment of biological control agents by Biopesticide Steering Group
- Data gaps caused by secondary metabolites

- RAFBCA and REBECA have addressed this topic earlier

Members of the Advisory Group

Adi Cornelese (Ctgb, the Netherlands)
Bilgin Karaoglan (UBA, Germany)
Ruediger Hauschild (GAB Consulting, Germany)
Flora Limache (Novozymes Biologicals FR S.A., France)
Vera Ritz (BfR, Germany)
Denis Rochon (PRMA, Health Canadia, Canada)
Chishio Sasaki (ACIS-FAMIC, Japan)

Origin of data requirement

Chemical pesticides ⟷ **Biological pesticides**

Parent → Metabolite(s)

Risk assessment >10% parent

Primary metabolites → Secondary metabolites

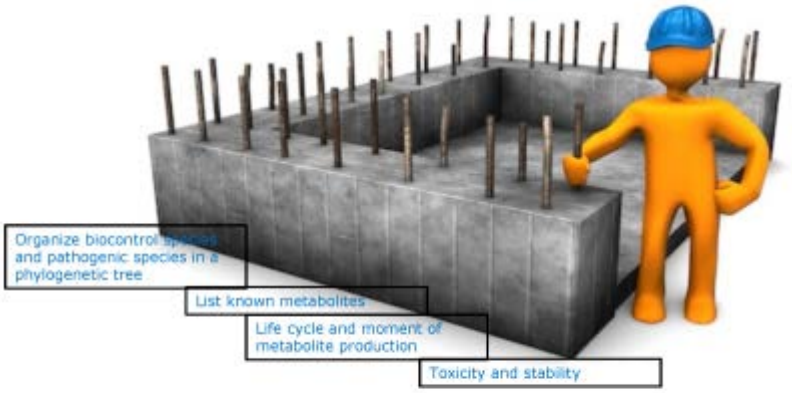
alcohols ethanol, lactic acid, amino acids

Mycotoxins are included!

Risk assessment When,how, which ones????

Statements to sustain qualitative risk assessment

Contents of the project



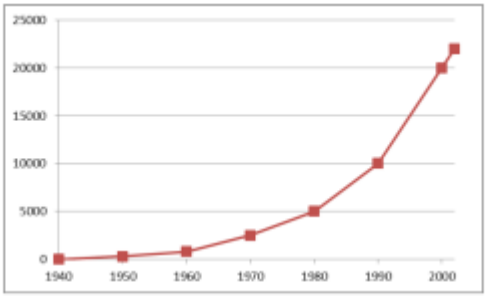
Literature included

- EFSA report: Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products - Lot 1 Environmental
- Selected reviews on individual biocontrol genera/species
- Relevant references mentioned therein

Published last month and not included:

- EFSA report: Literature search and data collection on RA for human health for microorganisms used as plant protection products risk characterisation

Discovery of new bioactive secondary metabolites



Bérdy J. 2005. Bioactive microbial metabolites,
A personal view. J. Antibiot. 58: 1-26.

Reviewed species

First part of the project (2014):

Entomopathogenic fungal species

- *Metarhizium, Beauveria, Isaria, Paecilomyces, Verticillium lecanii*

Second part of the project (2015):

Trichoderma,

Pseudomonas, Bacillus, Burkholderia, Streptomyces, Serratia

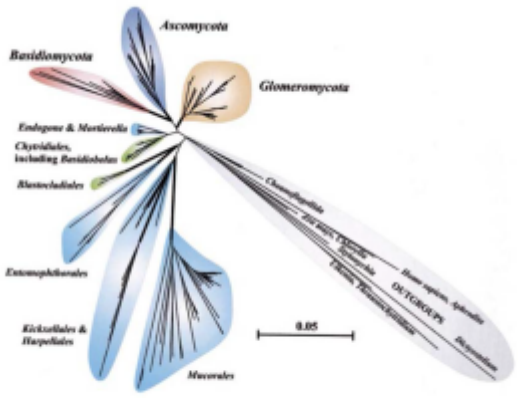
BUT: Considering the amount of literature, the data and information presented in the chapters cannot be exhaustive!!

Results

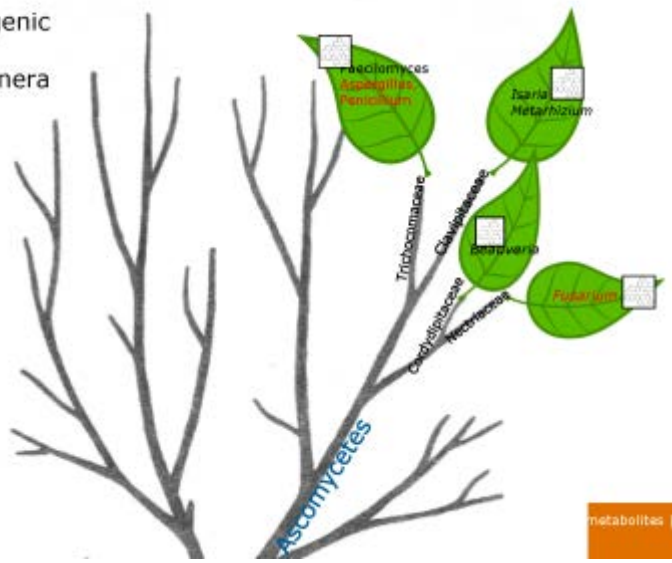
1. **Overview phylogeny biocontrol species**
2. Life cycle and moment of SM production
3. Toxicity
4. Stability

Relatedness

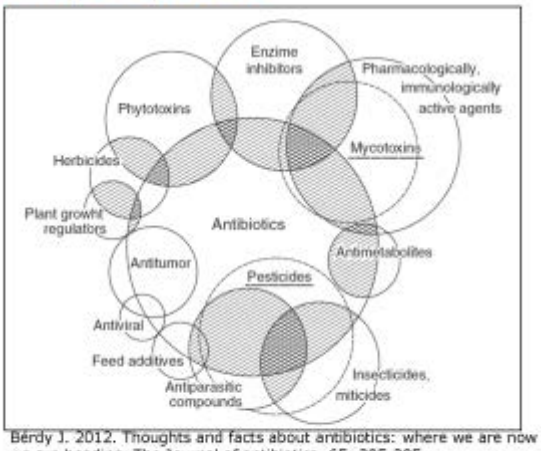
Is the biocontrol species closely related to pathogenic species producing mycotoxins?



Relatedness entomopathogenic fungi and pathogenic genera



Metabolites in perspective



Berdy J. 2012. Thoughts and facts about antibiotics: where we are now and where we are heading. The Journal of antibiotics. 65: 385-395.

List mycotoxins in Appendix of the guidance

PART of Table 2. List fungal genera producing mycotoxins

Genera	mycotoxin	Species
Acremonium	Crotocin	*Acremonium crotocinigenum*
Alternaria	Altenuic acid	*Alternaria alternata*
	Alternariol	*Alternaria alternata*
	Gliotoxin	*Alternaria, Aspergillus fumigatus, Penicillium*
Aspergillus	Aflatoxin	*Aspergillus flavus, A. parasiticus*
	Aflatrem	*Aspergillus flavus*
Fusarium	Beauvericin	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Butenolide	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*

Mycotoxins also produced by biocontrol agents

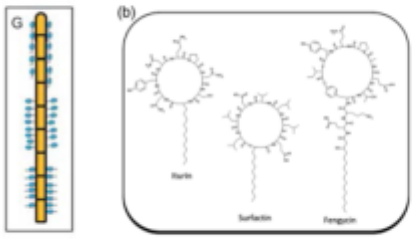
Metabolite	On the list	Biocontrol agent
beauvericin	*Fusarium*	*Beauveria, Isaria, Paecilomyces*
gliotoxin	*Alternaria, Aspergillus, Penicillium*	*Gliocladium, Trichoderma*
trichodermin		*Trichoderma*
destruxin B	*Alternaria, Aspergillus*	*Metarhizium*

Results

1. Overview phylogeny
2. **Life cycle and moment of secondary metabolite production**
3. Toxicity
4. Stability

Bacillus

- *Bacillus* closely associated with soil (pathogenic) fungi.
- *Bacillus* makes biofilms on the surface of fungi.
- In these biofilms iturins, surfactins and fengycin are produced
- The expression of genes to form secondary metabolites is under the control of quorum sensing



The hydrocarbon tail penetrates pathogen cell membranes, while the amino acid end stays in the soil solution. This action creates openings in cell membranes, inhibiting the growth of the pathogen

Statements

Metabolites that play a role in hyperparasitism or competitions for space or nutrients pose no hazards that need to be addressed.

Results

1. Overview phylogeny
2. Life cycle and moment of SM production
3. **Toxicity**
4. Stability

Toxicity tests

- Research goal of many toxicity tests is the identification of a link between a metabolite(s) and the suppression of a particular disease. To show fungicidal or antimicrobial properties.
- Tests with purified metabolite(s).
- Endpoints are hardly ever useful for a quantitative risk assessment!

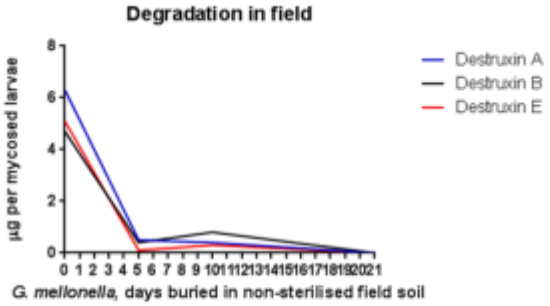
PART OF Table 1: Toxicity data of Meterhizium metabolites

Metabolite	Test organism	Endpoint	In vivo/ vitro	Test system	Level of effect	Ref.
AQUATIC						
Destruxin A	*Artemia salina*	LC50	In vivo	bioassay	2.92 µg/mL, 36 h 9.78 µg/mL, 24 h	[Pavela, 2006]
Destruxin A	*Daphnia magna*	LC50	In vivo		0.16 µg/mL, 36 h 0.20 µg/mL, 24 h	[Pavela, 2006]
Crude extract V245	*Daphnia magna*	LC50	In vitro	Wells in test plate	0.30 µg/mL, 24 h	[Skrobek et al., 2006]
Crude extract V275	*Daphnia magna*	LC50	In vitro	Wells in test plate	0.04 µg/mL, 48 h 0.06 µg/mL, 24 h	[Skrobek et al., 2006]
TERRESTRIAL						
Mammals						
Destruxin A	Mice	LD50	In vivo	intraperitoneally	1–1.35 mg/kg	[Kodama, 1961]
Destruxin B	Mice	LD50	In vivo	intraperitoneally	13.2–16.9 mg/kg	[Kodama, 1961]
Insects						
Destruxin A	Silkworm larvae	LC50	In vivo		0.015–0.030 mg/g, 24 h	[Kodama, 1961]

Results

1. Overview phylogeny
2. Life cycle and moment of SM production
3. Toxicity
4. **Stability**

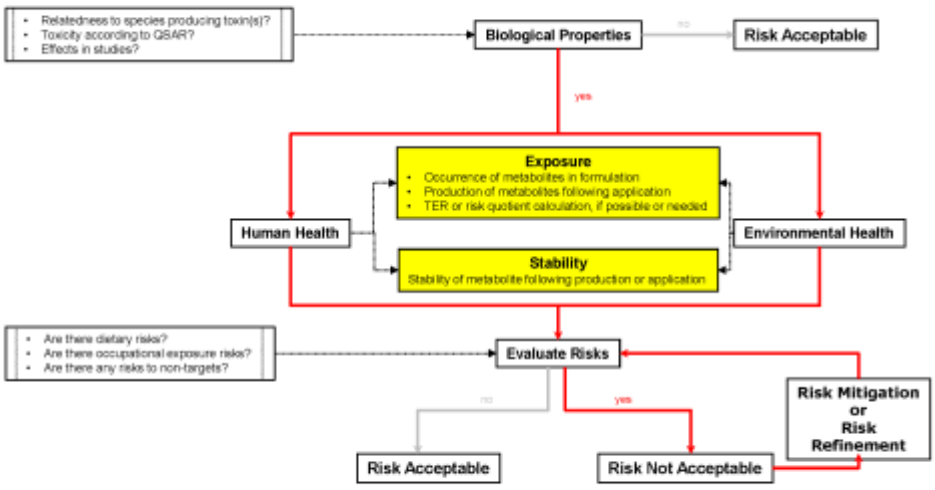
Example stability



Levels of destruxins A, B and E by *Metarhizium anisopliae* V245 in *G. mellonella* cadavers measured in the field (modified from Skrobek et al. (2008))

Draft Guidance

Simple construction, with independent elements



Statement

Statement made in report of the OECD/KemI Workshop Sweden:

We must recall that the phytopathogenic microorganisms that we are aiming to control also produce secondary metabolites and toxins, which are tolerated at low levels in feed and food. We should not be more restrictive on a biological control agent than we are for the pathogenic microorganisms it controls.

Maximum levels in food/feed

MLs are set according to ALARA principle
= As Low As Reasonably Achievable

- Aflatoxin (B_1) : 2-12 µg/kg;
- Aflatoxin (sum of B_1, B_2, G_1 and G_2) : 4-15 µg/kg;
- Ochratoxin A : 2-80 µg/kg;
- Patulin : 10 -50 µg/kg;
- Deoxynivalenol : 500-1750 µg/kg;
- Zearalenone : 50-400 µg/kg;
- Fumonisins : 800-4000 µg/kg;
- Citrinin : 2000 µg/kg

No Maximum Levels determined yet for most mycotoxins and none is produced by a biocontrol species

Organizations involved in setting MLs:
- CONTAM Panel EFSA (Contaminants in the Food Chain)
- Joint FAO/WHO Expert Committee on Food Additives
- Scientific Committee on Food
- US FDA

Quantities Beauvericin

Fusarium:	in plants up to	3400 mg/kg
Paecilomyces fumosoroseus:	in insects	1.6 mg/kg

Presentation Kersti Kustafsson, OECD seminar 2014

- Rather reasoning than further testing
- Analyse the exposure situation before further testing

Qualitative rather than
quantitative risk assessment

Problems identified…

- Few toxicity and stability data available and difficult to find
- *In situ* testing of toxicity not possible
  - Secondary metabolite production depends on too many factors that cannot be controlled.
  - Impossible to develop a standardised test
- No ready-to-use lists available to compare toxicities

Development draft guidance

Secondary metabolites:
A myriad of forms, activities, modes of action
A myriad of research performed
A myriad of solutions?

"What you need to do, you have to picture it as something easy. By doing that, it becomes easy"

Emile Coué, French psychologist
1857-1926

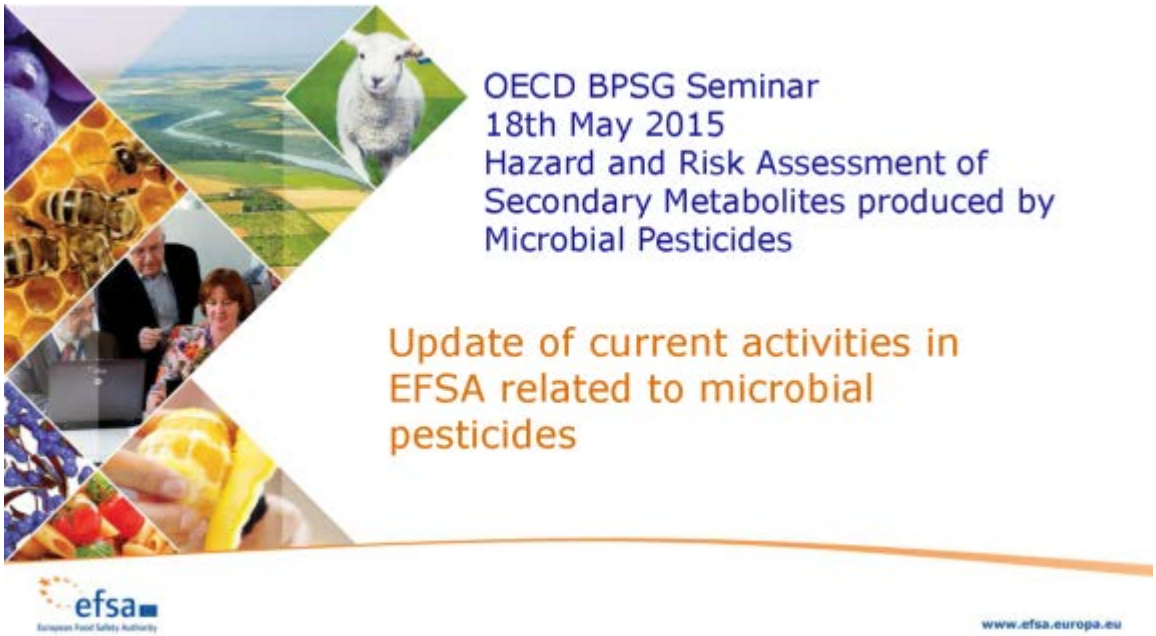
Acknowledgements

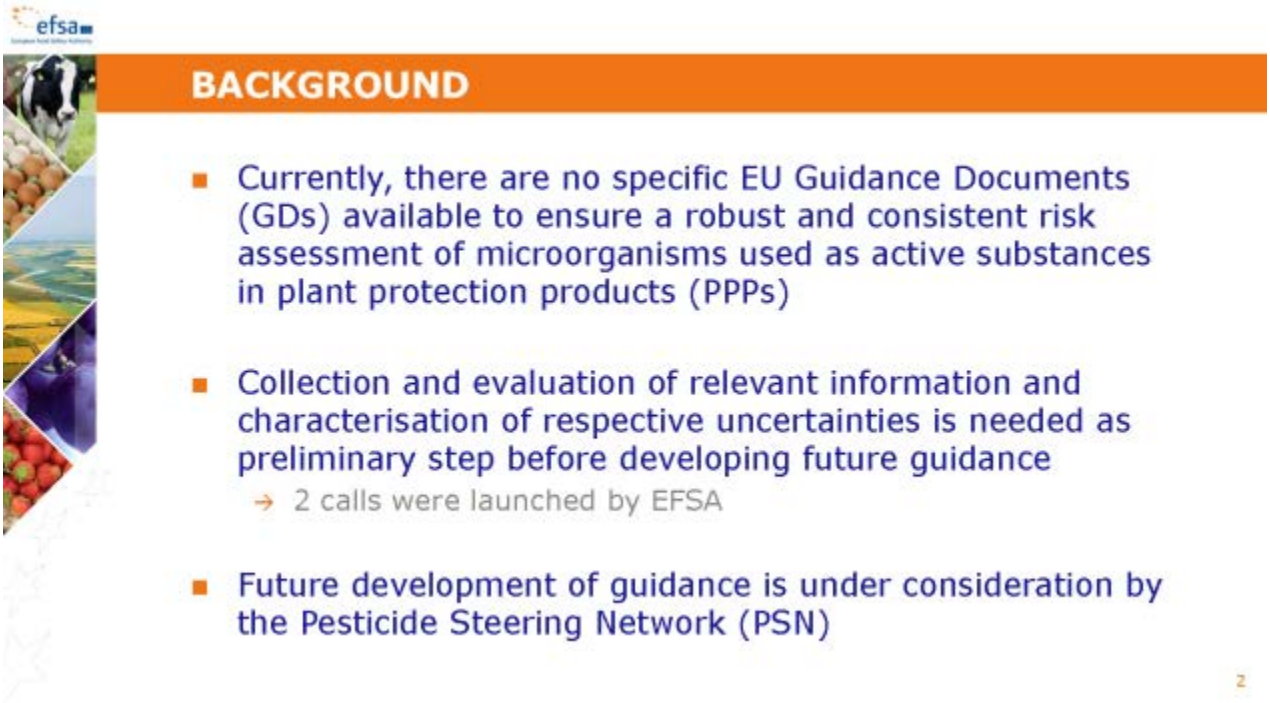
OECD to facilitate and finance this project

Everybody who gave support by reading and commenting on the chapters and draft guidance.

Ministry of Infrastructure and the Environment of the Netherlands for giving additional financial support

Presentation 3
Update of current activities in EFSA related to microbial pesticides
Frédérique Istace (European Food Safety Authority, Parma; Italy)





RECENTLY PUBLISHED ON EFSA WEBSITE:

- External Scientific Reports: Literature review and data collection on microorganisms used in plant protection products
  - 1. Environmental Risk characterisation
  - 2. Risk assessment for human health
- 3. Scientific Opinion: Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition
- 4. Future activities

3

1. ENVIRONMENTAL RISK CHARACTERISATION

Published in December, 2013

http://www.efsa.europa.eu/it/supporting/doc/518e.pdf

EXTERNAL SCIENTIFIC REPORT

Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products –Lot 1 Environmental Risk characterisation

4

1. ENVIRONMENTAL RISK CHARACTERISATION

Topics: Risks and hazards linked to

1) genetic stability and transfer (99)
2) interference with the system for drinking water quality control (2)
3) fate and behaviour in the environment (462)
4) production of metabolites/toxins and potential toxic effect on non-target organisms (NTOs) (400)
5) host specificity range and potential effect on NTOs (418)
6) appropriateness of existing test guidelines for effect assessment on NTO (123)

5

1. ENVIRONMENTAL RISK CHARACTERISATION

Results

1) Cases of gene acquisition from bacteria by conjugation and transformation, and transfers among fungal species have been reported
2) Microbials used as PPPs could potentially interfere with the system of quality control of drinking water
3) Most agro-ecosystems are heavily disturbed and do not represent a natural situation for assessment of background levels.

Most of the studies addressing growth, survival and virulence have been carried out for only one set of environmental conditions.

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1. ENVIRONMENTAL RISK CHARACTERISATION

Results

4) Toxin production:

Among bacteria, the major producers of bioactive compounds are

- Streptomyces species (7630)
- Bacillus species (860)
- Pseudomonas species (795)

Among fungi, the major producers of bioactive compounds are

- Trichoderma species
- Beauveria species

Non-target effects of secondary metabolites are still poorly studied and reported in the available literature

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1. ENVIRONMENTAL RISK CHARACTERISATION

Suggestions

For the future, a powerful tool could be sequencing the genomes of a wide range of strains of microorganisms used as PPPs, and conducting studies to identify the genetic basis for traits linked to risk assessment, including ecotoxicology, fate and behaviour in the environment, host range etc.



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RECENTLY PUBLISHED ON EFSA WEBSITE:

- External Scientific Reports: Literature review and data collection on microorganisms used in plant protection products
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- 4. Future activities

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Published in April, 2015

http://www.efsa.europa.eu/en/supporting/pub/801e.htm

EXTERNAL SCIENTIFIC REPORT

Literature search and data collection on RA for human health for microorganisms used as plant protection products

Reference: OC/EFSA/PRAS/2013/02

Corporate authors

Abstract

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Methodology

- The main body of the report was divided in three parts:
  1. Extensive literature search including a database and mini-reviews discussing the information assembled in the database
  2. Application of read-across for evaluating risk to human health, using the literature search for evaluating pathogenicity, infectivity and toxicity of closely related microorganisms using *bacillus* and *pseudomonas* as models
  3. The literature search and the read-across analyses were discussed among experts

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Results part 1 – Mini reviews

- **Minireview 1**: factors relevant for addressing pathogenicity of microorganisms used as PPPs
- **Minireview 2**: assessing potential colonisation behaviour and adverse effects of microorganisms used as PPPs
- **Minireview 3**: evaluating genetic stability of microorganisms used as PPPs
- **Minireview 4:** evaluating microbial PPPs regarding **toxin production and toxicity** of the produced metabolites/toxins
- **Minireview 5 to 10:** reviewing publications from EFSA, WHO/FAO, OECD, EC, non-EU regulatory authorities, research projects

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Results part 1: mini review 4

- Toxin production is described for bacterial species within the *Bacillus* and *Streptomyces* genus
- Secondary metabolites are described for fungal species including *Aureobasidium*, *Beauveria*, *Clonostachys (Gliocladium)*, *Isaria* (*Paecilomyces*), *Metarhizium*, *Purpureocillium* (*Paecilomyces*) , *Trichoderma*, *Yarrowia* (*Candida*)
- Methods for the identification and testing of toxic compounds are described

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Results part 2 – Exploring possibilities and limits of read across:

Using database (part 1) together with gene and genome information (from NCBI databases), comparative analyses are provided for the genetic determinants of pathogenicity from *Bacillus* and *Pseudomonas*

- Improve understanding of the genetic basis underlying pathogenicity traits
- Still lack of information about gene-function relationships especially in an environmental context, and complex physiological traits cannot be easily inferred from gene sequence data
- For the majority of environmental microorganisms, insufficient sequence information available to yield meaningful information regarding pathogenicity traits through comparative genome analysis

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Results part 3 - Recommendations

- Based on experts' discussions in workshops
- First topic related to data requirements for the risk assessment of microorganisms used as PPPs:
  - Key risk factors (regarding human health)
  - Determinants of pathogenicity/infectivity/toxicity
  - Data requirements that cannot be properly addressed based on the scientific evidence and for which further research efforts are needed
- Second topic related to the methodologies that are available to identify pathogenicity factors and determinants (including toxicity of metabolites)
  - Confirmatory tests should be requested because pathogenicity traits may differ from strain to strain
  - Possible tiered approach towards toxicology testing was discussed
  - Suggestions of using modern molecular methods: solid phylogenetic analysis and whole genome sequencing, metabolomics, genetic markers and quantitative PCR, LC-MS/MS data

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Conclusions

- Considering the biological properties of individual PPP strains when evaluating pathogenicity, infectivity, and toxicity
  - Unambiguous identification at the strain level is needed
    - Full genome sequence information should be provided (even though there are still major gaps of knowledge regarding gene-phenotype associations)
  - RA of microbial PPPs should be tailored according to the microorganisms's specific characteristics (e.g. phylogeny)
  - A catalogue of recommended test methods for pathogenicity, infectivity, and toxicity could be provided

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Conclusions

- How testing for potential adverse effects may relate to actual exposure
  - Guidance is needed on how tests should be designed, taking into consideration realistic exposure scenarios and a natural background of environmental microorganisms
- Addressing potential transfer of antibiotic resistance genes
  - Guidance is needed on how to specifically address testing for antibiotic resistance of PPP strains submitted for authorisation

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Conclusions

- How to test for potential toxicity of microbial metabolites
  - Toxin production should be addressed at strain level
  - Virtually all microorganisms produce secondary metabolites
  - It is impossible that testing will account for the production of all possible secondary metabolites under all possible environmental conditions
  - It has to be verified that there are no toxic compounds persisting in the edible part of the crop that is being protected against pests
  - Starting point could be to test for all major known toxins (under various defined conditions), and to provide guidance regarding appropriate test methods tailored to specific (phylogenetic) groups of microorganisms

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Conclusions

- How to use previous knowledge and read across
  - Will depend on the quality and quantity of strain-specific data available
  - As a general rule, pathogenicity determinants must be regarded as strain specific
  - Applying read-across for inferring risks regarding pathogenicity, infectivity, and toxicity from known microorganisms to unknown ones will only provide probabilities
  - Read-across within species, genera, or families is not feasible.

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2. RISK ASSESSMENT FOR HUMAN HEALTH

Conclusions

- Proposed steps forward
  - Multiple stakeholder involvement is essential
  - Joint stakeholder workshop organised by the Pesticide Unit /PPR Panel to develop the key topics presented above, with experts from academia and industry and participants from regulatory bodies
  - This could provide the foundation for the preparation of a guidance document on how to conduct the regulatory risk assessments for microbial pesticides and how to characterise the respective uncertainties

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RECENTLY PUBLISHED ON EFSA WEBSITE:

- External Scientific Reports: Literature review and data collection on microorganisms used in plant protection products
  - 1. Environmental Risk characterisation
  - 2. Risk assessment for human health
- 3. Scientific Opinion: Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition
- 4. Future activities

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3. GUIDANCE ON BACILLUS SAFETY

Published in May, 2014

Main safety concern : capacity for toxin production potentially related to foodborne diseases

Objective: provide guidance on how to conduct the safety assessment of *Bacillus*-based products.

http://www.efsa.europa.eu/en/efsajournal/pub/3665.htm

efsa

European Food Safety Authority

EFSA Journal 2014;12(3):3665

SCIENTIFIC OPINION

Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Bacillus species are used in animal production directly as microbial feed additives or as the source of other feed additives, notably enzymes. The principal safety concern for consumers and, to a lesser extent livestock, associated with *Bacillus* is a capacity for toxin production. However, the capacity for toxin production and the nature of the toxins produced is unevenly distributed over the genus, occurring frequently in some species and more rarely in others. In principle, the selection of strains belonging to the *B. cereus* taxonomic group for direct use in animal production is considered inadvisable. If, however, they are proposed then the full genome should be sequenced and a bioinformatic analysis made to search for genes coding for enterotoxins and cereulide synthase. If there is evidence of homology, the non-functionality of the genes (e.g. mutation, deletion) must be demonstrated. For other species, concern appear to be associated to the production of surfactin like-lipopeptides, although the relation between the presence of these compounds and/or other toxic factors and the risk of illness in humans has not yet been established. In the absence of animal models shown to be able to distinguish hazardous from non hazardous strains, the FEEDAP Panel relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect. Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal feed products. If the strain proves to be cytotoxic it is not recommended for use.

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KEY WORDS

Bacillus species, enterotoxin production, emetic toxin, cereulide, surfactin-like lipopeptides

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3. GUIDANCE ON BACILLUS SAFETY

Strategy

- For the assessment of *Bacillus* species other than *cereus*:
  - *in vitro* cell-based methods (cytotoxicity)
- For the assessment of species belonging to the *Bacillus cereus* group:
  - full genome (including chromosome and plasmids) should be sequenced and analysed to search for genes coding for enterotoxins and cereulide synthase
  - If there is evidence of homology, the non-functionality of the genes should be demonstrated

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RECENTLY PUBLISHED ON EFSA WEBSITE:

- External Scientific Reports: Literature review and data collection on microorganisms used in plant protection products
  - 1. Environmental Risk characterisation
  - 2. Risk assessment for human health
- 3. Scientific Opinion: Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition
- 4. Future activities

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4. FUTURE ACTIVITIES

New call for proposals

- New approaches in identifying and characterizing microbiological and chemical hazards
  - A. Making use of molecular approaches to identify and characterise microbial foodborne pathogens, specifically using whole genome sequence analysis
  - B. Development and application of read across methodologies to the hazard assessment of chemicals in the food safety area
- Reference: GP/EFSA/AFSCO/2015/01 (30/04/2015)

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4. FUTURE ACTIVITIES

Request of the European Commission

- Concerning the risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp, including *Bacillus thuringiensis* in foodstuffs
- Asking also for an update of the opinion of the BIOHAZ Panel on *Bacillus cereus* and other *Bacillus* spp in foodstuffs (EFSA Journal (2005) 175, 1-48)

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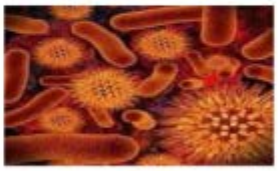
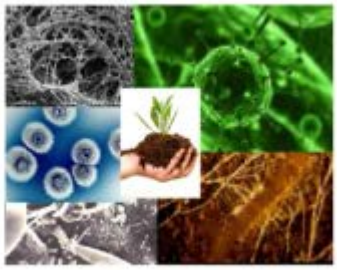
Thank you for your attention !

Istace Frederique
Pesticide Unit
Regulated Products Directorate – EFSA
E-mail: frederique.istace@efsa.europa.eu





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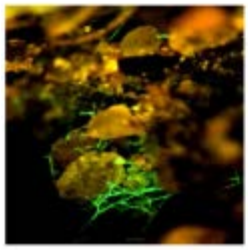
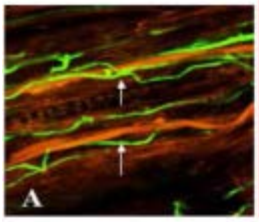
Presentation 4
***Trichoderma* secondary metabolites: how to identify the main compound and mycotoxins**
Matteo Lorito (Università di Napoli Frederico II, Napoli; Italy)

Food security and safety depend on soil and plant microbiomes

ASM study: "How Microbes can Feed the World" being used i.e. by USA governement to define new agriculture policies

Some *Trichodermas* are beneficial microbes that affect positively soil/leaf microbiomes

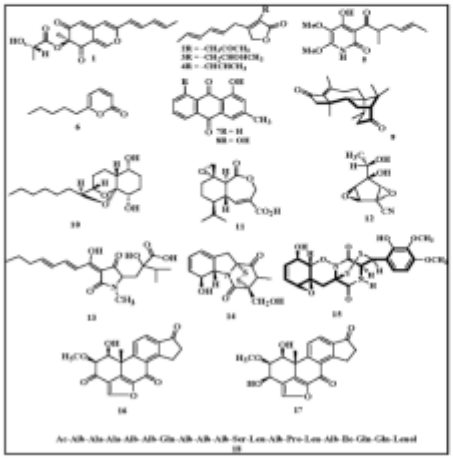




Trichoderma secondary metabolites (SMs)

About two hundred identified :

- **volatile antibiotics,** i.e. 6-pentyl-a-pyrone and most of the isocyanide derivates;
- **water-soluble compounds**, i.e. heptelidic acid or koningic acid;
- **water un-soluble compounds**, i.e. gliotoxin and trichorzianines;
- **peptaibols**, linear oligopeptides of 12-22 AA rich in a-aminoisobutyric acid, N-acetylated at the N-terminus and containing an amino alcohol at the C-terminus.



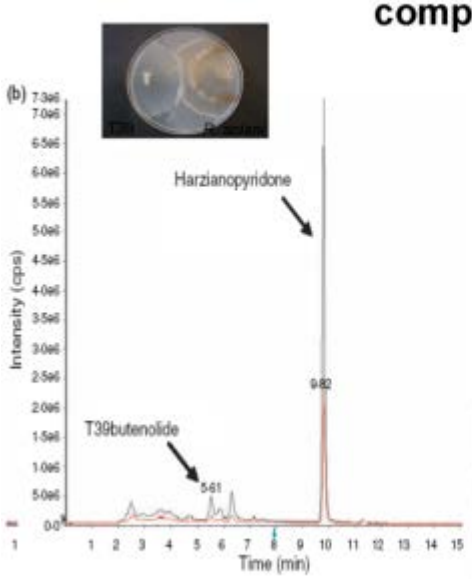
1: T22azaphilone;
2: T39butenolide;
3: harzianolide;
4: dehydro harzianolide;
5: harzianopyridone;
6: 6-pentyl-α-pyrone;
7: 1-hydroxy-3-methyl-anthraquinone;
8: 1,8-dihydroxy-3-methyl-anthraquinone;
9: harziandione;
10: koninginin A;
11: heptelidic acid;
12: trichoviridin;
13: harzianic acid;
14: gliotoxin;
15: gliovirin;
16: viridin;
17: viridiol;
18: trichorzianines.

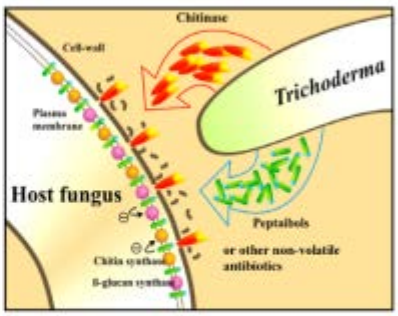
Production of *Trichoderma* SMs

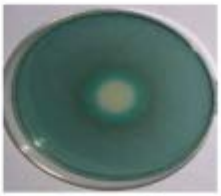
The quality and/or the quantity of SMs produced by *Trichoderma* depends on:

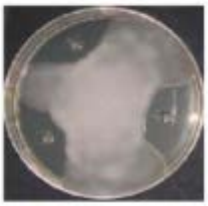
- **the compound considered**
- **the species and the strain**
- **the microbiome composition or the presence of a host or of plant tissues**
- **the balance between elicited biosynthesis and biotransformation rate**
- **In vitro: the growth condition**

Trichoderma SMs in microbial parasitism and competition

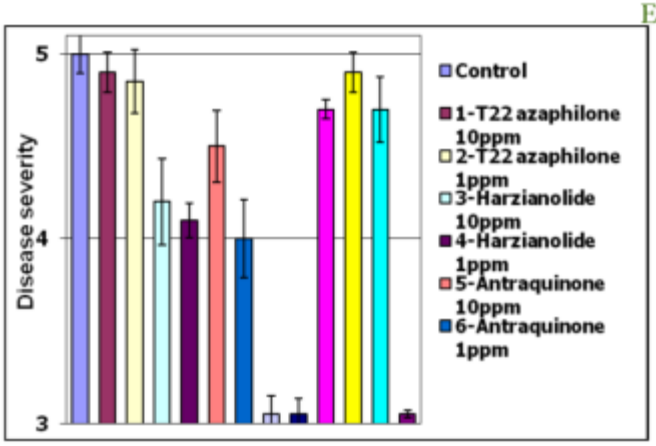


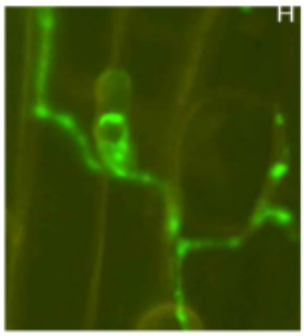


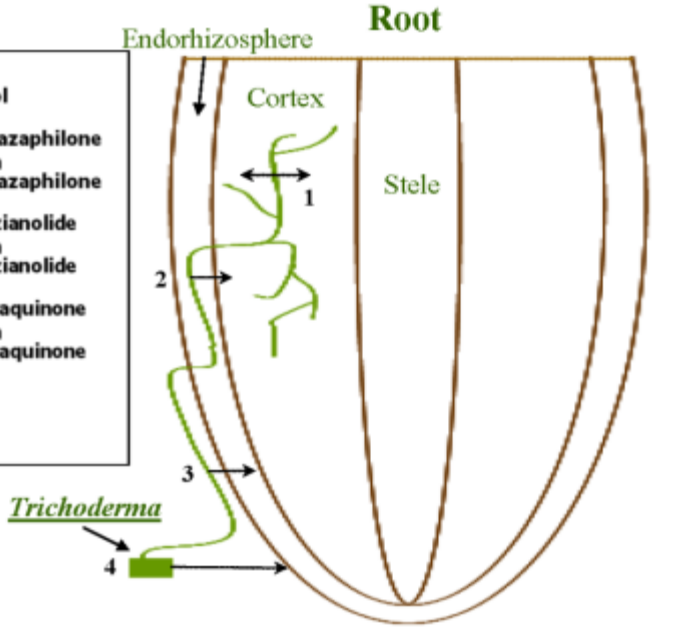




Induction of disease resistance

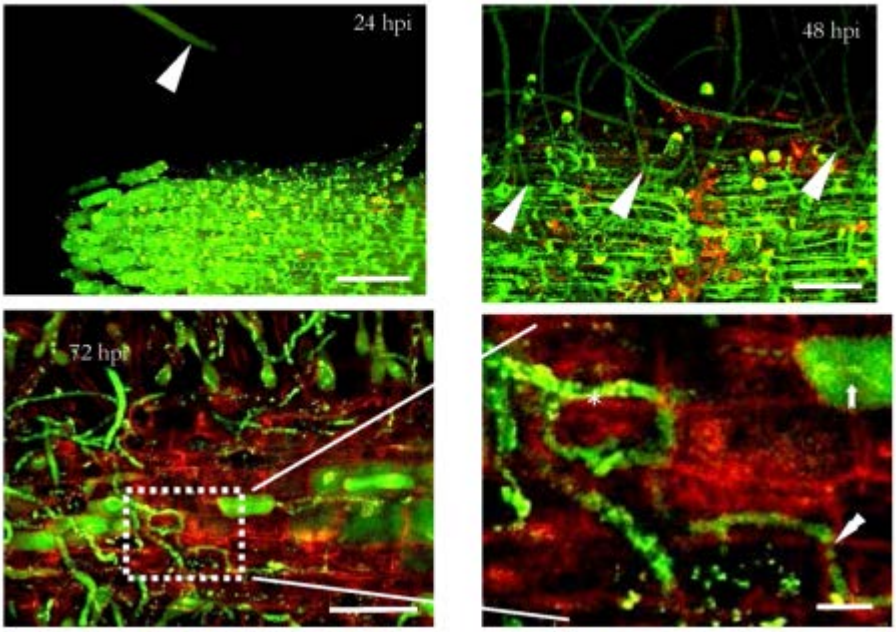




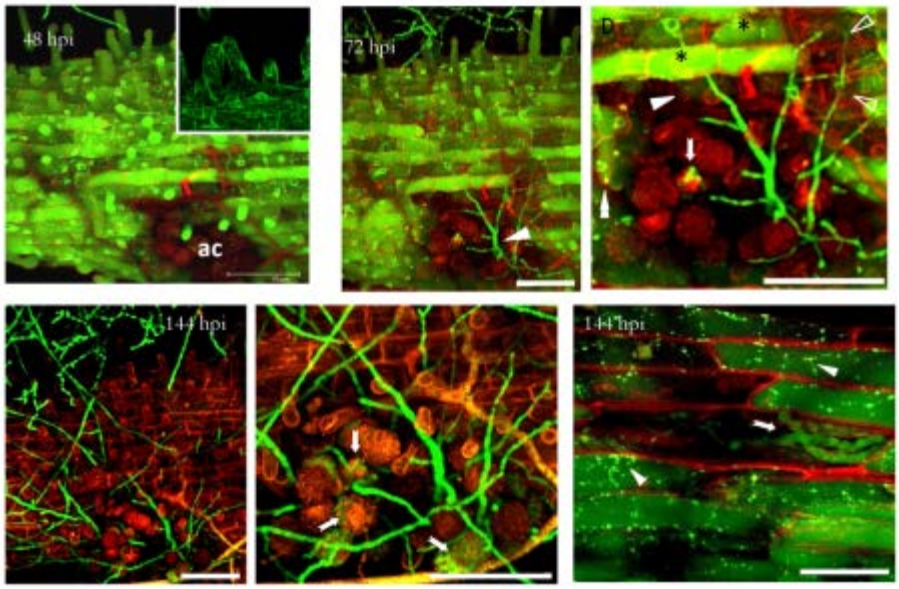


Potential sites of production of ***Trichoderma*** metabolites that can affect plant host metabolism. ●Metabolites produced within live cortical cells. ●Metabolites produced in the root surface and within dead cortical cells. ●Metabolites produced in the rhizosphere. ●Metabolites produced in soil organic matter, although in low amounts and possibly diffused, may still be adequate to function as signals for inducers of host defence against invading pathogens

Effect of *T. atroviride* on Medicago root epidermis cells

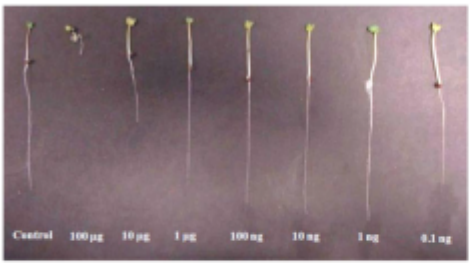


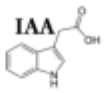
Three-way interaction *Medicago–Glomus –Trichoderma P1*



T. atroviride acted as an endophytic root colonizer causing cell death, but did not activate pre-penetration cell responses found for symbiotic and pathogenic interaction.

Trichoderma SMs affect plant growth



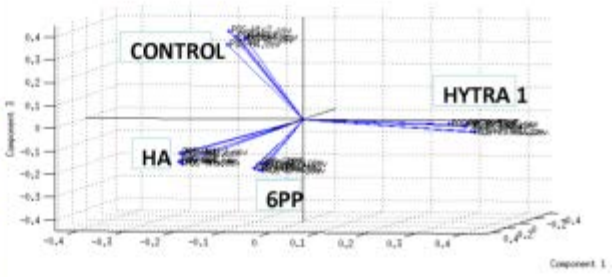


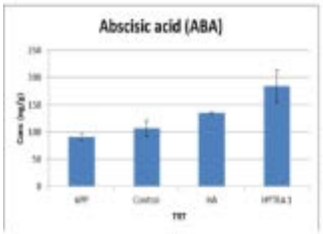
indole-3-acetic acid (IAA) or auxin analogues.

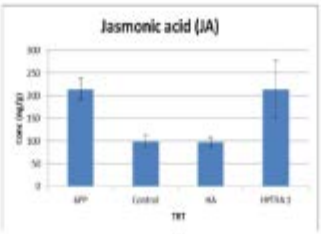
Siderophores - different families
Produced under iron deficiency : coprogen (B) fusarinine C (aka fusigen) ferricrocin

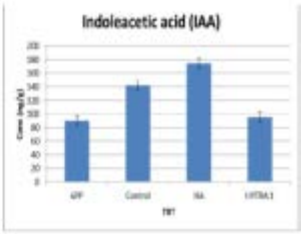
- Pyrone 6-pentyl-2H-pyran-2-one (6PP)- *T. atroviride, T. viride, T. harzianum* and *T. koningii*
- koninginins A–E and G - complex pyranes - *T. koningii, T. harzianum, T. aureoviride*
- Viridiol - Steroidal antibiotic of the viridin series - *T. virens*
- Harzianopyridone , Harzianopyridone, Harzianic acid - Nitrogen heterocyclic compounds - *Trichoderma* spp.
- Cerinolactone - Lactones derivatives -*T. cerinum*
- Harzianolide and T39butenolide – Butenolides - *Trichoderma* spp.
- Trichocaranes A - D - *Trichoderma virens*
- Peptaibols

Effects of *Trichoderma* SMs on *Arabidopsis* Metabolome

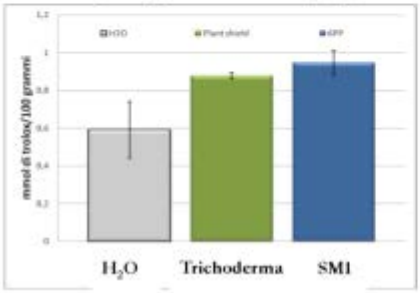


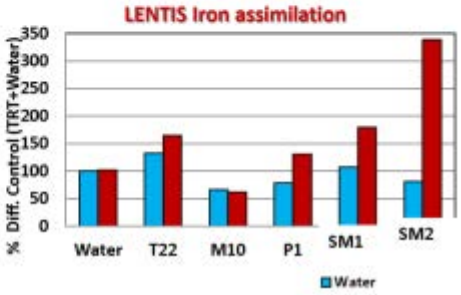






Grape Polyphenol content (grape)

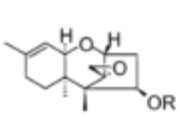




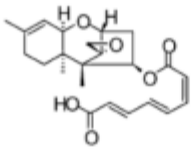
Trichoderma toxins

Mycotoxins are secondary metabolites with adverse effects on humans and animals that result in illnesses and economic losses. Aflatoxins, ochratoxins, trichothecenes, zearelenone, fumonisins, tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance.

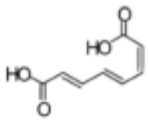
Mycotoxins reported from *Trichoderma* include trichothecenes and gliotoxin.



Trichodermin R = Acetate
Trichodermol R = H

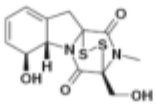


Harzianum A



(2*Z*,4*E*,6*E*)-octa-2,4,6-trienedioic acid

Harzianum A made by esterification of trichodermol with octa-2Z,4E,6E-trienedioic acid



Gliotoxin (from *T. virens*)

Trichoderma mycotoxins (trichothecenes)

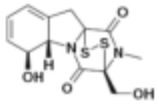
Trichodermas typically used for biocontrol purposes do not produce trichothecenes (Nielsen et al., 2005)

Table 2. Trichothecene Production from Selected *Trichoderma* Strains on Semisolid and Shaken Media, Incubated for 10 Days in Darkness

medium		YES[a]		PDA		OA		PDliq		YESliq		RTliq	
strain number	species	tric	harz A	tric	harz A	tric	harz A	tric	harz A	tric	harz A	tric	harz A
IBT 40841	*T. brevicompactum*	++[b]	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
IBT 40840	*T. brevicompactum*	++	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
IBT 40839	*T. brevicompactum*	++	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
IBT 40838	*T. brevicompactum*	+	ND	+++	ND	++	ND	++++	ND	+++	ND	+++	ND
LEO ND8	*T. brevicompactum*	++	ND	+++	ND	++	ND	not performed		not performed		not performed	
IBT 40837	*T. brevicompactum*	+	+++	+	+++	ND	+++	ND	++++	ND	++++	ND	+++
IBT 40836	*T. brevicompactum*	ND	++++	ND	++++	+	+++	ND	++++	ND	++++	ND	+++
IBT 40842	*T. brevicompactum*	ND	+++	+	++++	ND	+++	ND	++++	ND	++++	ND	+++
ATCC 90237	*T. brevicompactum*	+	+++	+	+++	+	+++	ND	++++	ND	++++	ND	+++
IBT8866	*T. viride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT8212	*T. viride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT8850	*T. harzianum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9153	*T. harzianum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9134	*T. harzianum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT8965	*T. atroviride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT8964	*T. atroviride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9155	*T. longibrachiatum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9126	*T. longibrachiatum*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9135	*T. citrinoviride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IBT9149	*T. citrinoviride*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND, not detected; YES, yeast extract sucrose agar; OA, oat meal agar; PDA, potato dextrose agar; YESliq, shaken yeast extract sucrose medium; PDliq, shaken potato dextrose medium; RTliq, shaken Raulin–Thom medium. [b] +, peak area of 5–100 counts; ++, peak area of 100–500 counts; +++, peak area of 500–3000 counts; ++++, peak area of 3000–30 000. The trichodermin peak area was determined from the *m/z* 293.18 (ESI^+) and harzianum A from *m/z* 399.18 (ESI^-).

The case of the Gliotoxin



- **A mycotoxin (epipolythiodioxopiperazine) antimicrobial but also immunosuppressive, apoptotic on mammals, inhibitory on the transcription factor NF-kappaB, capable of damage DNA.**
- **Involved in biocontrol, found in the substrate of BCA**
- **Does the toxin translocate through the plant and/or accumulates in edible portions ? Thus, glyotoxin-producing BCAs pose a significant risk for animal and human health?**

We have demonstrated that:

- **concentration** in the soil after inundative application ranges from 0 to 0.3 micrograms/cm^3 (sandy, clay, peat, various pH)

- **secreted locally** *in vitro* during the replicative growth and **rapidly degraded** *in vivo* (very sensitive to oxidation and instable in aqueous solutions) - concentration is strongly reduced 1 week after application especially in neutral and alkaline soils

Not transfered to edible parts

- **Absorption reduced by suberin endodermis and exodermis of root cells because of relatively large MW**
- **soluble in DSMO, ethanol, methanol, DMF, but essentially insoluble in water**
- **is very toxic for the plant, inhibit growth and germination (inhibit cell development; branched-chain AA production, thiol-requiring enzyme activities, damage DNA by ROS, induces apoptosis)**
- **mass spectrometry-generated profile of strawberry, lettuce and potato treated with a gliotoxin-producer strain or a non-producer mutant is not different in terms of newly formed (or accumulated) molecules of non-plant origin**

Methods to identify the main *Trichoderma* SMs

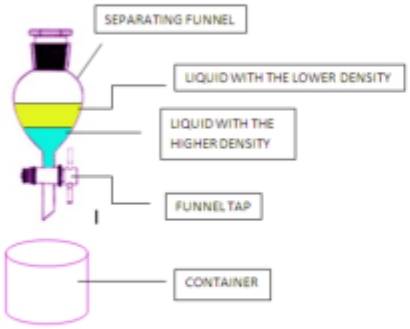
IF THE BIOLOGICALLY ACTIVE SM IS NOT KNOWN

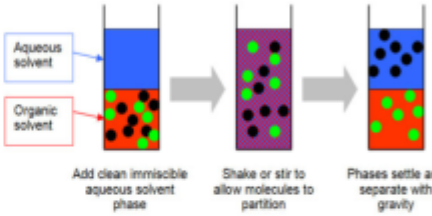
- **determine the suitable bioassay both in vitro and in vivo to detect the biological activity: i.e. brine shrimp lethality test (*Artemia salina*); antibiotic; insecticidal; antiviral; cytotoxicity etc.**
- **obtain extracts in adequate amount from at least one rich and one poor medium, containing simple or complex C and N sources (usually static culture gives better yield)**
- **perform a bioassay-guided fractionation and test all fractions for biological activity**

EXTRACTION

Separation of a substance from a matrix

Liquid–liquid extraction, also known as solvent extraction and partitioning



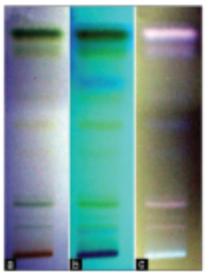


In choosing a solvent for extraction, its ability to extract the known components has to be considered

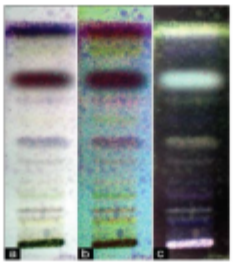
ISOLATION (TLC)

•**The separation to obtain a pure compound often requires several separation steps involving different chromatographic techniques.**

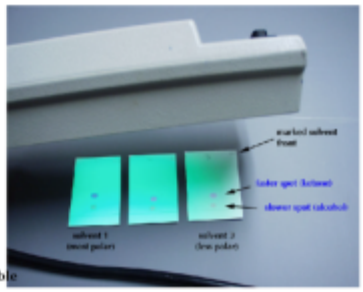
•**<u>Thin layer chromatography (TLC)</u> the movement of the compounds to be separated is the result of the driving force of the mobile phase and the retarding action of the stationary phase. Silica gel is the most used layer material**



a) Thin layer chromatography (TLC) of leaf ethanolic extract in visible light. (b) TLC of leaf ethanolic in UV-Long wavelength (254 nm). (c) TLC of leaf ethanolic extract in UV-short wavelength (365 nm)



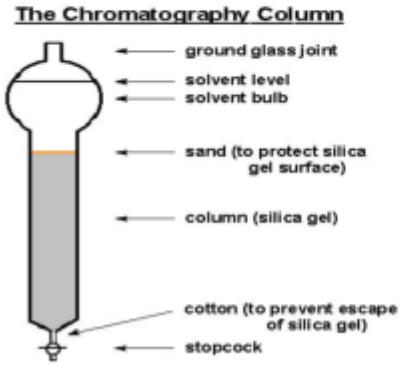
a) TLC of leaf ethanolic extract in visible light after spraying with anisaldehyde-sulphuric acid reagent. (b) TLC of leaf ethanolic extract in UV-Long wavelength after spraying with anisaldehyde-sulphuric acid reagent. (c) TLC of leaf ethanolic extract in UV-short wavelength after spraying with asnisaldehyde-sulfuric acid reagent

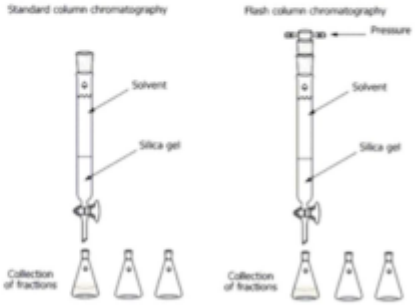


ISOLATION (Chromatography)

Column Chromatography

Samples at ratios of 1:10 to 1:300 can be used depending on the difficulty of separation of the components of the mixture. Silica of different particle size (10–200 μm) and porosity (50 nm) is mostly used





ISOLATION (VLC)

Vacuum Liquid Chromatography

Flow of the solvent is maintained by vacuum. The column is prepared in a sintered glass funnel using TLC grade packing (aluminium oxide, silica gel, or reverse-phase supports). The sample is applied uniformly at the top of the support. Step gradient elution is used and the column can be allowed to run dry after collection of each fraction.

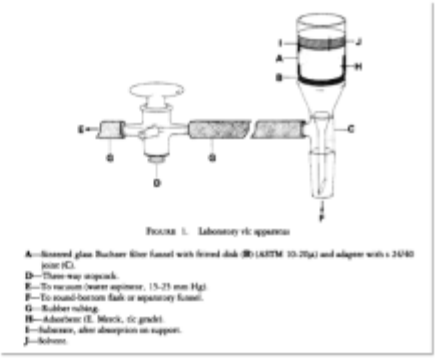


FIGURE 1. Laboratory vlc apparatus

A—Sintered glass Buchner filter funnel with fritted disk (Ⓡ) (ASTM 10-20(μ)) and adapter with ± 24/40 joint (C).
D—Three-way stopcock.
E—To vacuum (water aspirator, 15-25 mm Hg).
F—To round-bottom flask or separatory funnel.
G—Rubber tubing.
H—Adsorbent (E. Merck, tlc grade).
I—Substrate, after absorption on support.
J—Solvent.

ISOLATION (HPLC)

Preparative Pressure Liquid Chromatography
HPLC High-Performance Liquid Chromatography

CHARACTERIZATION (LC-MS and NMR)

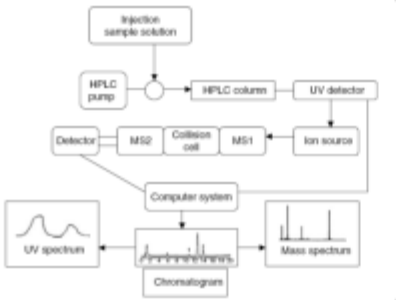


FIGURE 9.1 Diagrammatic representation of an HPLC-UV-MS/MS system.

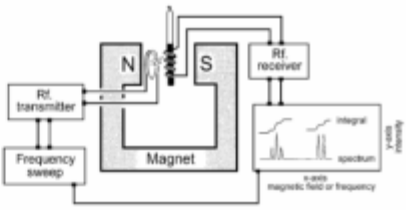
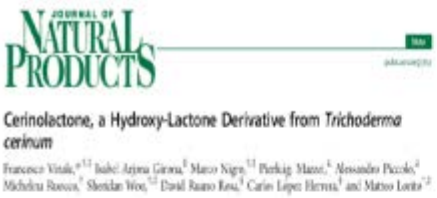
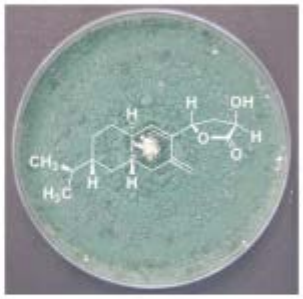


Figure 5.2 Schematic Representation of a CW NMR Spectrometer

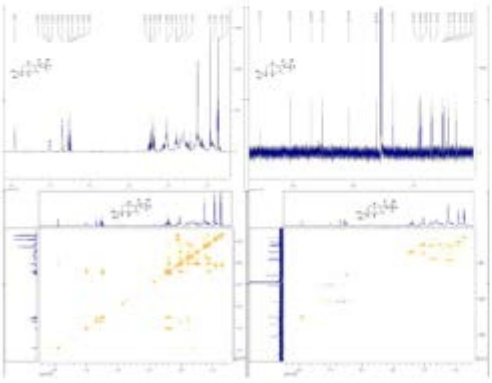
- **ULTRAVIOLET (UV) AND INFRARED (IR) SPECTROSCOPY**
- **MASS SPECTROMETRY**
- **NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY**
- **13C NMR SPECTROSCOPY**
- **TWO DIMENSIONAL NMR**

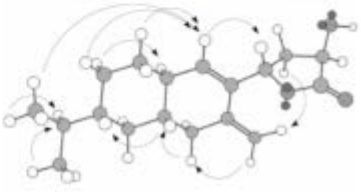


Cerinolactone from *T. cerinum*



The structure and absolute configuration determined by NMR





The most used *Trichoderma* strains produce only a few main SMs (i.e. *T. harzianum* T39)

EXTRACTION

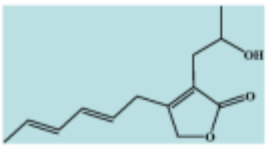
10 fractions

Screened for antibiotic activity against *G. graminis* var. *tritici*

Fraction N. 6

known compound

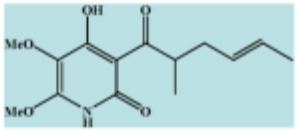
Harzianolide (22 mg)



[3-(2-hydroxypropyl)-4-(hexa-2E, 4E-dien-6yl) furan-2 (5H)-one]

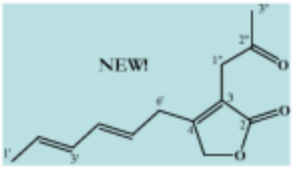
TLC purification of fractions N. 3-4

Harzianopyridone (11 mg)

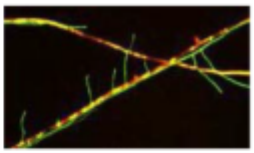


known compound

New butenolide (9 mg)



Final considerations

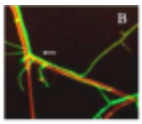


•Even though many SMs are known, most used strains produce only a few main SMs (detectable in substrates)

•New strains properly characterized at secondary metabolite levels, and some species eventually avoided

•SMs may be present in the formulation, but technique such as pure spore isolation avoid the problem

Final considerations



•*Trichodema* SMs make contact with plant cells in the interaction zone, but do not accumulate within the plant (also for endophytic) nor in the edible parts

•Accumulation to a detectable level in natural soil has not been reported so far, while at least in one case LC-MS demonstrates no accumulation nor translocation

•The risk of exposure at field level should be considered minimal or null. Natural soils may contain high level of Trichoderma !

Sheridan Woo
Michelina Ruocco
Francesco Vinale
Rosaria Varlese
Roberta Marra
Stefania Lanzuise
Nadia Lombardi
Alberto Pascale
Gelsomina Manganiel
Roberta Panza
Roberta Quarto
Federica Lacatena
Pasquale Lombari
Tonia Aliberti
Luca Iovine
David Stellitano
Luigi De Vito
Valeria Manzo
Maria Alaia
Matteo Lorito

UNIVERSITY OF NAPLES
Department of Agricultural Sciences

CNR Institute
for Sustainable Plant Protection

Presentation 5

Evaluation of relevant metabolites from microbial control agents: What do we need to know?

Ingvar Sundh (Swedish University of Agricultural Sciences, Uppsala; Sweden)

SLU

Evaluation of relevant metabolites from microbial control agents: What do we need to know?

Ingvar Sundh

Centre for Biological Control (CBC), SLU

(Department of Microbiology, SLU)

OECD/BPSG seminar on Secondary Metabolites of Microbial Pesticides, 18 May 2015

www.slu.se/cbc

SLU

Contents of presentation

1. Secondary metabolites from microorganisms:
   - Numbers and compounds
   - Functions in ecosystems
   - In medicine and biotechnology
   - Degradation in ecosystems
2. Their relation to "relevant" metabolites/degradation products of chemicals
3. Human vs. environmental hazards and risks
   - Exposure a key
4. Conclusions and recommendations

Focus on EU regulation

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1. Secondary metabolites of microorganisms (I)

- Produced in all major groups, and particularly fungi and actinobacteria
- Various bioactives, including e.g. antibiotics, signalling molecules, specialised lipids, and toxins
- Critical role for ecological fitness and in competitive interactions!
- Source of new antibiotics for human and veterinarian use

Example 1

Number (modelled) of antimicrobial compounds produced by genus *Streptomyces* (actinobacteria) is in the order of 100 000

[Watwe et al. 2001; Archives of Microbiology 176, 386-390].

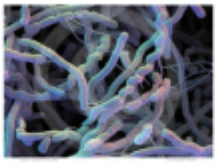

Photo: www.scharfphoto.com

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Secondary metabolites of microorganisms (II)

Example 2

- Three strains N_2-fixing root nodule genus *Frankia* (actinobacteria):
  - 65 secondary metabolite biosynthetic gene clusters
  - 25 secondary metabolite structures predicted
  - Secondary metabolic activity expressed in lab cultures

[Udwary et al. 2011; Applied and Environmental Microbiology 77, 3617-3625]

Photo: www.mokka.hu/drupal/

Frankia nodules on alder roots

Photo: www.biolib.cz/

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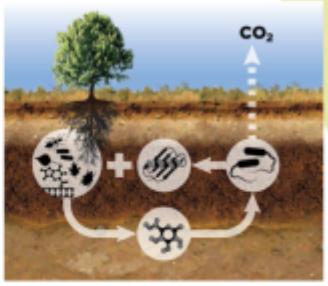
Secondary metabolites of microorganisms (III)

Biodegradation of microbial metabolites in ecosystems

- Recycling: Production new microbial biomass balanced by loss processes
- Secreted compounds and dead microbial organic matter
  - enter the pool of "microbial detritus"
  - contribute to new production of organic matter/humus
  - substrates for heterotrophic organisms in the detrital degrader/consumer food web
- Very high background of organic matter/substances from other organisms
- Degradation/mineralisation pathways for secondary metabolites are present!



Graphics: Oak Ridge National Laboratory, US

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2. Relation to "relevant" metabolites/degradation products of chemicals (I)

Concept/term "relevant metabolite" originates from chemical pesticides

Data requirements (DR) and uniform principles (UP) microorganisms:

- '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*…..'
- '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.
- Fate and behaviour environment (UP 2.7): 'An assessment shall be made of the fate and behaviour of *any known relevant metabolite* that is produced by the microorganism. ….for each environmental compartment…'

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Relation to "relevant" metabolites/degradation products of chemicals (II)

- The "relevance" of a metabolite/degradation product of a chemical determined by comparing its toxicity to that of the parent substance
- But can this concept be transferred in a meaningful way to the fact that microorganisms produce secondary metabolites?!

Main concern is not whether a microbe produces (relevant) secondary metabolites, but whether it produces *ANY* toxic compounds *of potential concern*.

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3. Human vs. environmental hazards and risks (I)

Human exposure is a concern

- Human safety critical: If toxins produced, can humans (producers, handlers, users, bystanderds) be exposed at levels of concern?
- Human exposure should be evaluated in relation to "background" exposure to the same or similar compounds (and to live microbes).
  But what is the usual load/exposure?
  More research needed!
- Critical question: How much attention to production of *unknown* vs. *known* (for the microbial group/species) toxins?
  How much effort is needed to look for unknowns in well described genera/species?

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Human vs. environmental hazards and risks (II)

Exposure to toxins of non-target organisms in the environment?

- Environmental safety critical?? Specific toxins with known effects on e.g. other mammals? But can exposure reach levels of concern?
- Environmental exposure should be evaluated in relation to "background" exposure to the same or similar compounds (and to live microbes). But what is the usual load/exposure? Is more research needed?!
- As a rule, particularly microbes and plants produce secondary metabolites. But they are recycled in the detrital food-web.

Amanita virosa

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Photo: http://vetamix.net/

4. Conclusions and recommendations (I)

- The concept of "relevant metabolite" is highly unsuitable for safety assessment of microbial control agents. Avoid term?
- With respect to potential toxin production in microbial control agents, main concern is likely human, rather than environmental, risk.
- Exposure is a key, but determining *total* exposure including "background" is a big challenge!
- Environmental safety: Secondary metabolites enter detritus and are degraded, thus highly unlikely to poison the environment!

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Conclusions and recommendations (II)

- Knowledge and framework for generating more appropriate toxicity evaluations of microbial control agents is in place. E.g. *recent systematic reviews of EFSA*.
- Updated data requirements/guidance for assessment of toxin production in microbial control agents are urgently needed.
- The low risk concept of Regulation 1107/2009 quite suitable for microbes.

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Acknowledgements to CBC:

Centre for Biological Control

Special grant to SLU (Swedish University of Agricultural Sciences)

- Bacteria for biological control
- Fungi for biological control
- Insects and arachnids for biological control
- Formulation and stabilisation
- Safety and regulatory issues

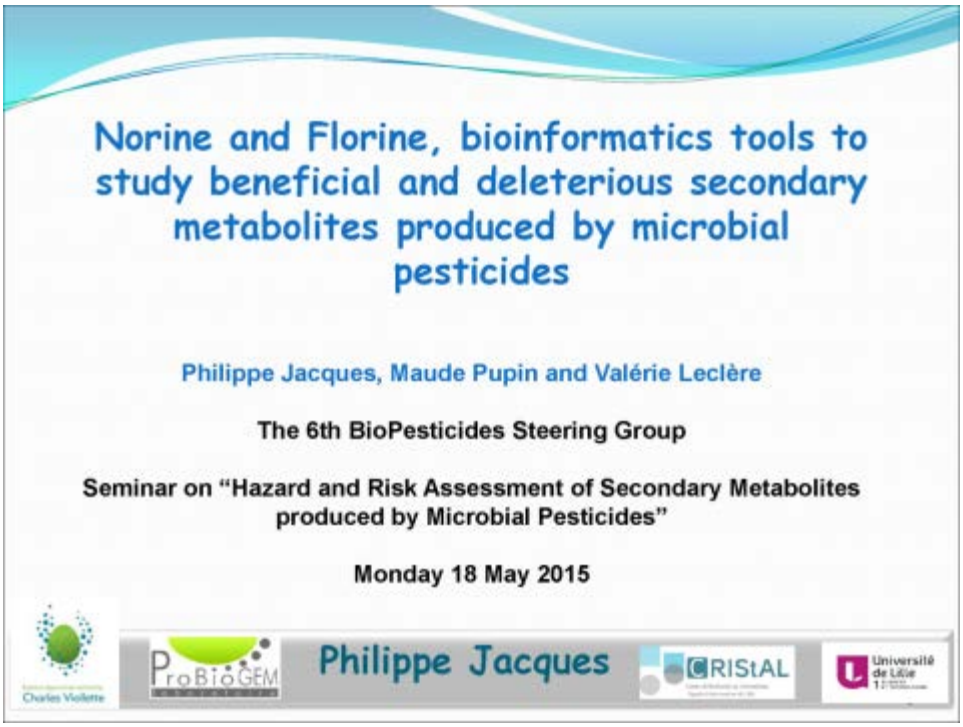
Mainly research but also information and extension services.

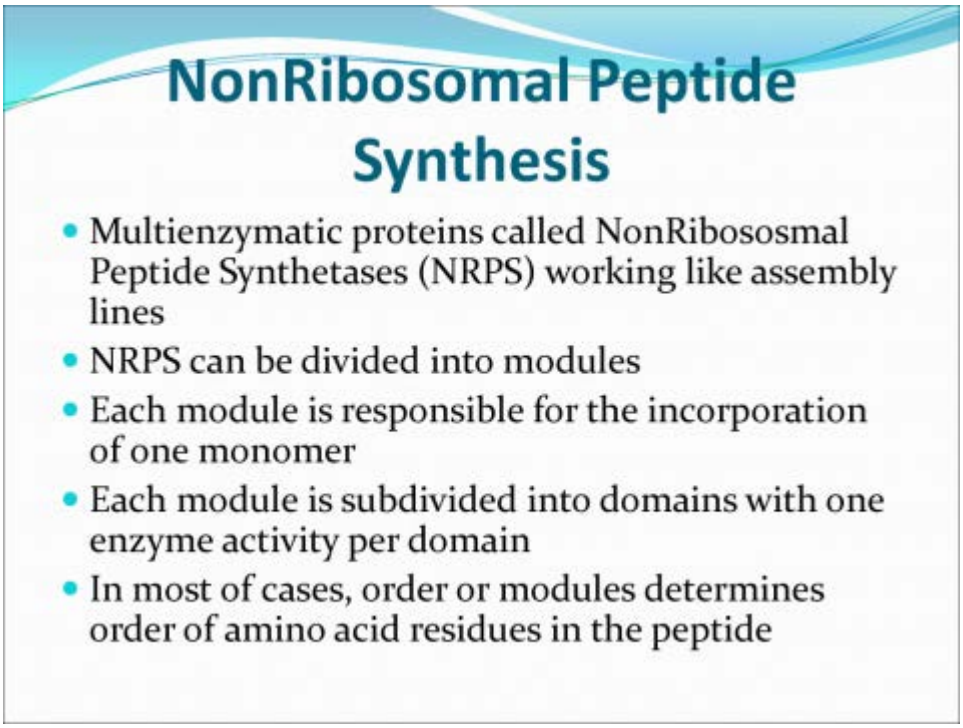
Homepage: www.slu.se/cbc

Presentation 6

Norine and Florine, bioinformatics tools to study beneficial and deleterious secondary metabolites produced by microbial pesticides

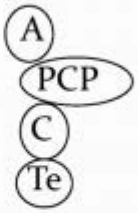
Philippe Jacques (Université Lille, Villeneuve d'Ascq Cedex; France)



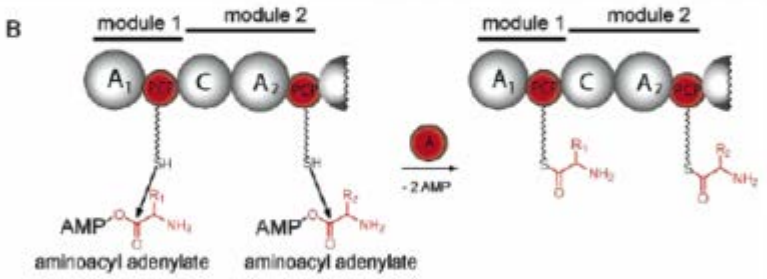


Main domains

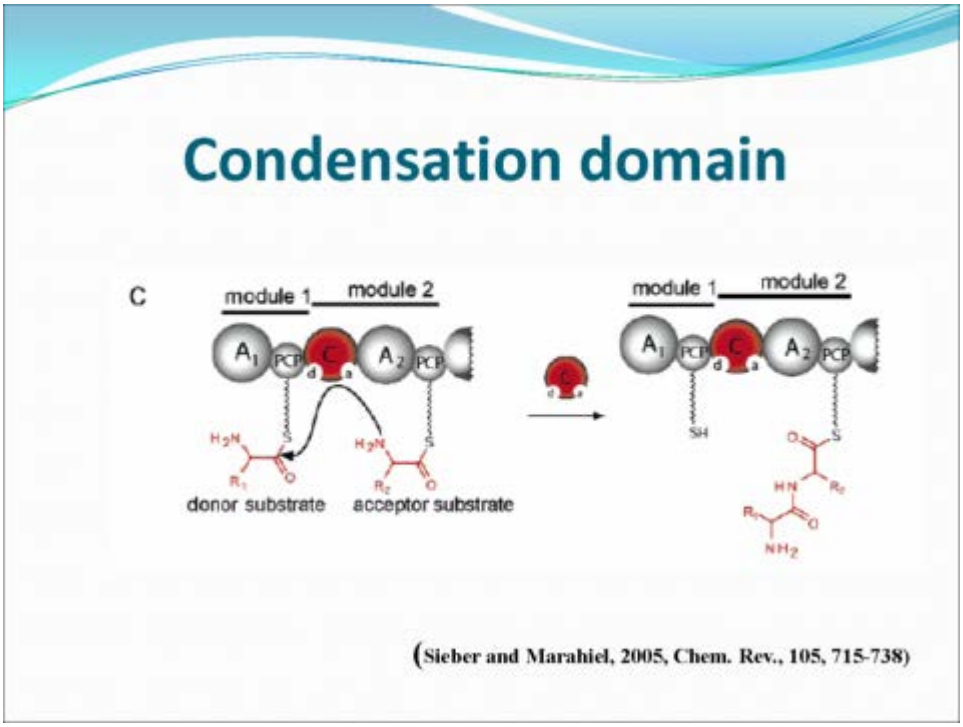
- Adenylation domain
- Peptidyl Carrier Protein
- Condensation domain
- Thioesterase domain

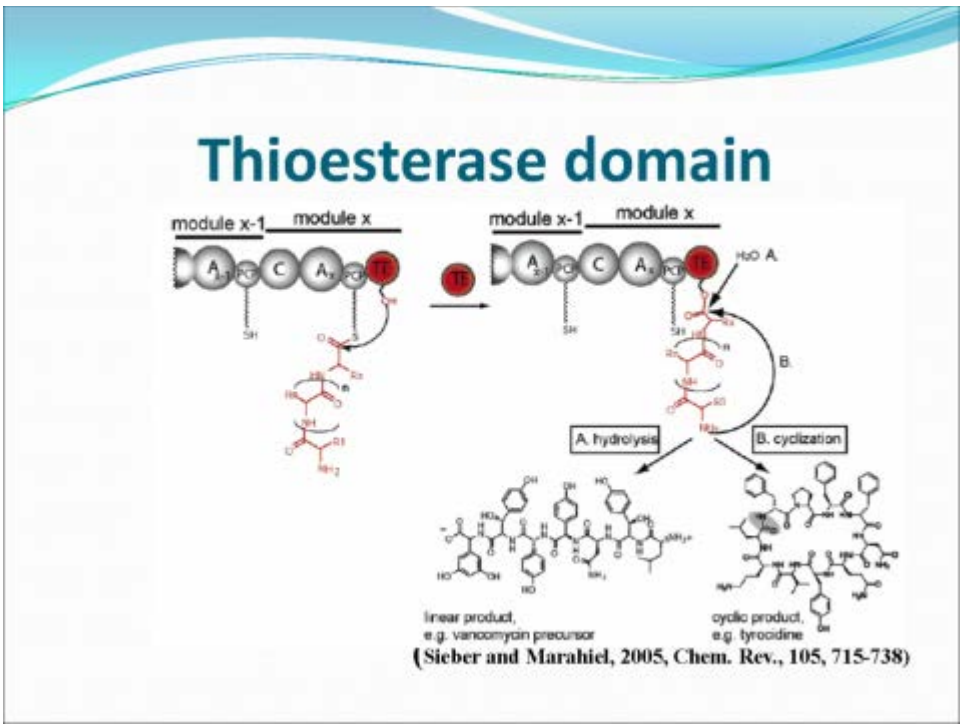


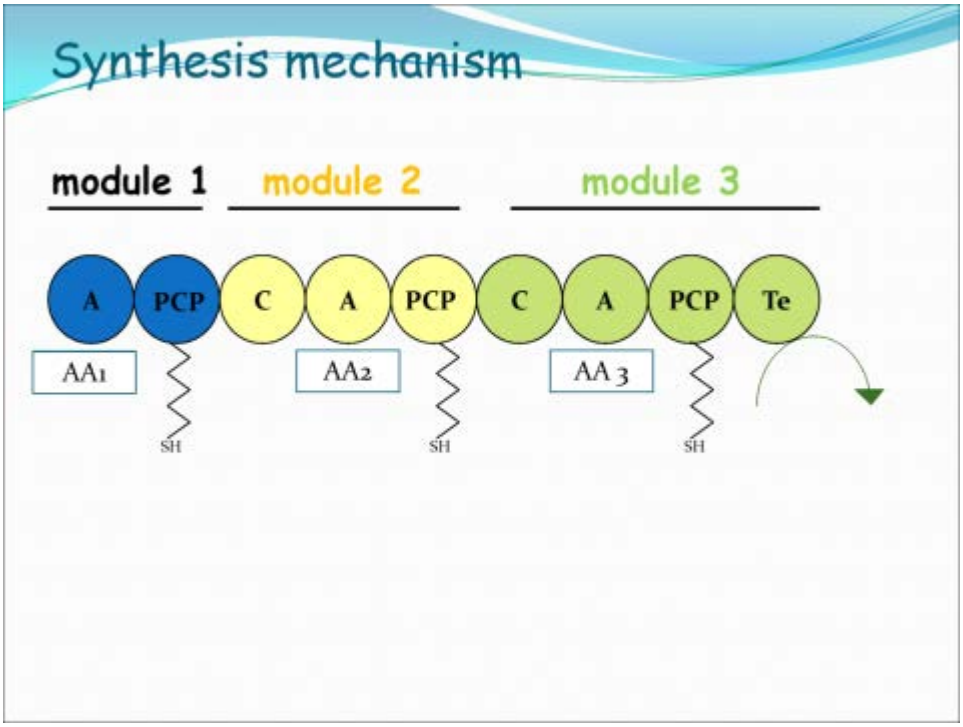
Peptidyl carrier protein (PCP)

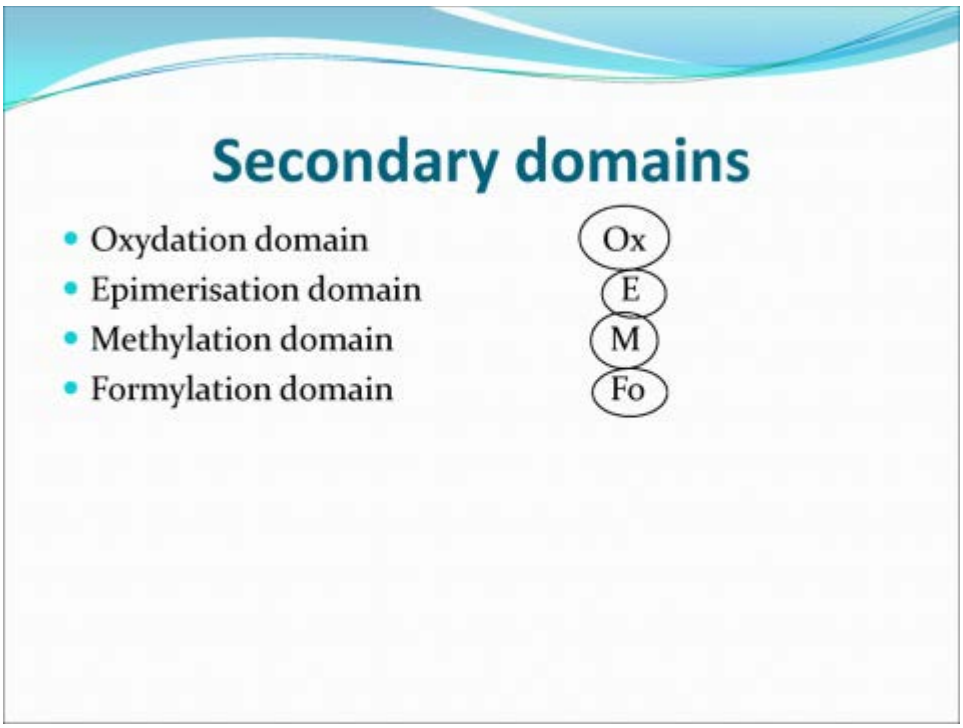


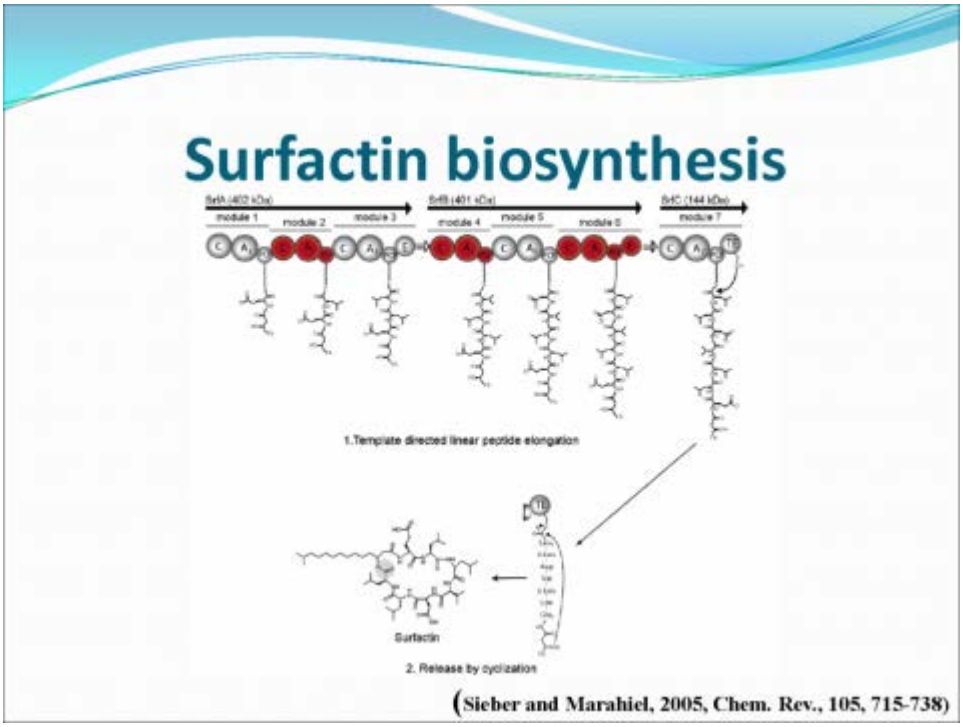
(Sieber and Marahiel, 2005, Chem. Rev., 105, 715-738)

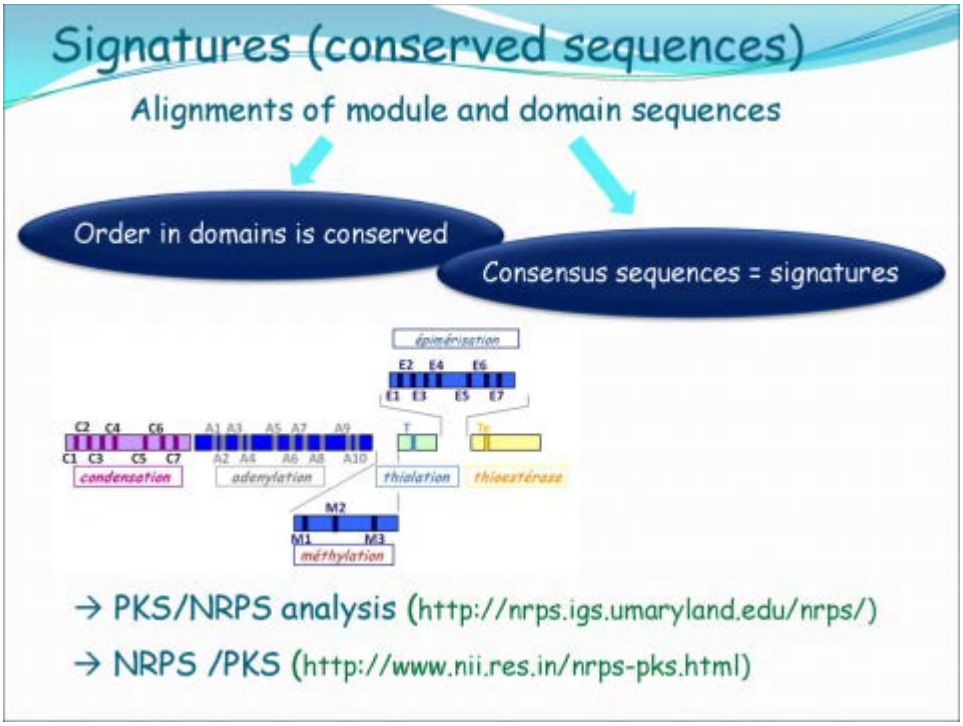

Condensation domain
C
module 1
module 2
donor substrate
acceptor substrate
(Sieber and Marahiel, 2005, Chem. Rev., 105, 715-738)

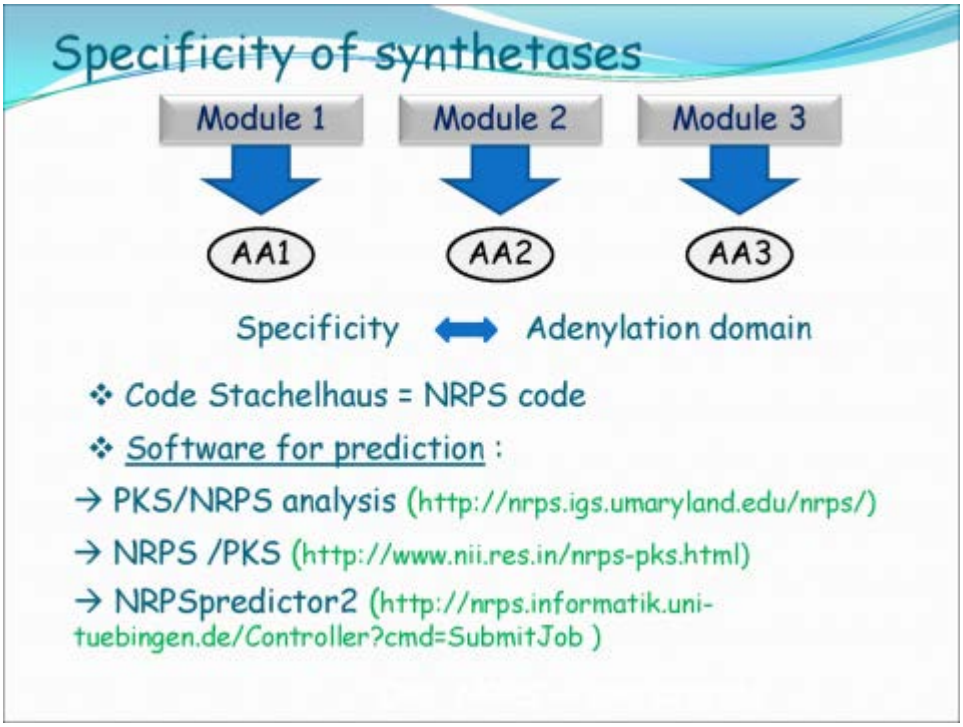

Thioesterase domain
module x-1
module x
A. hydrolysis
B. cyclization
linear product,
e.g. vancomycin precursor
cyclic product,
e.g. tyrocidine
(Sieber and Marahiel, 2005, Chem. Rev., 105, 715-738)

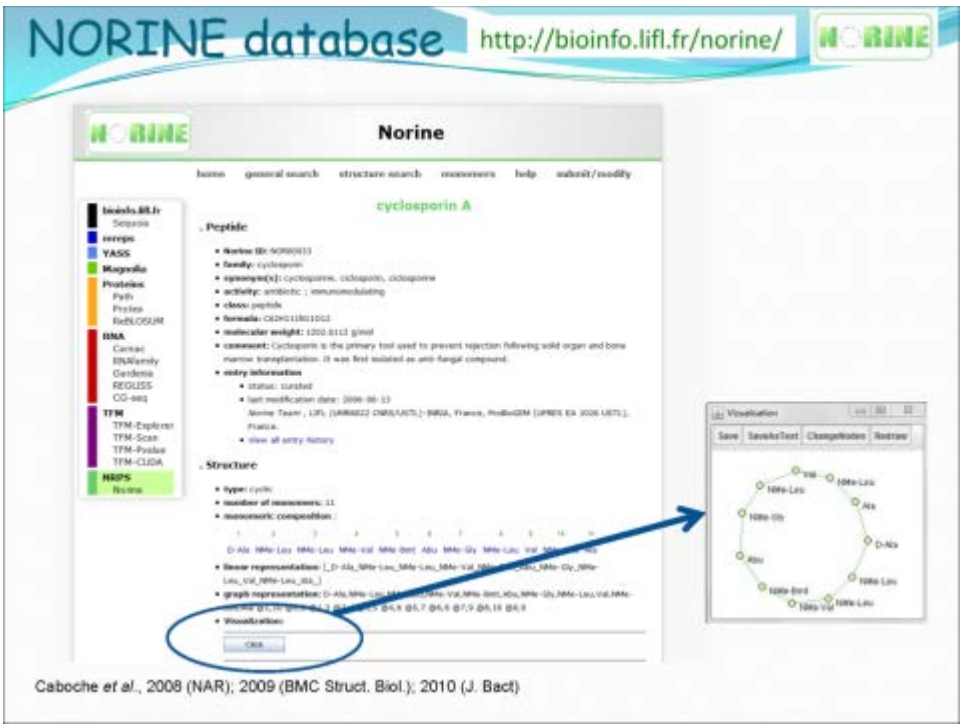

Synthesis mechanism
module 1
module 2
module 3
A
PCP
C
A
PCP
C
A
PCP
Te
AA1
AA2
AA 3
SH
SH
SH

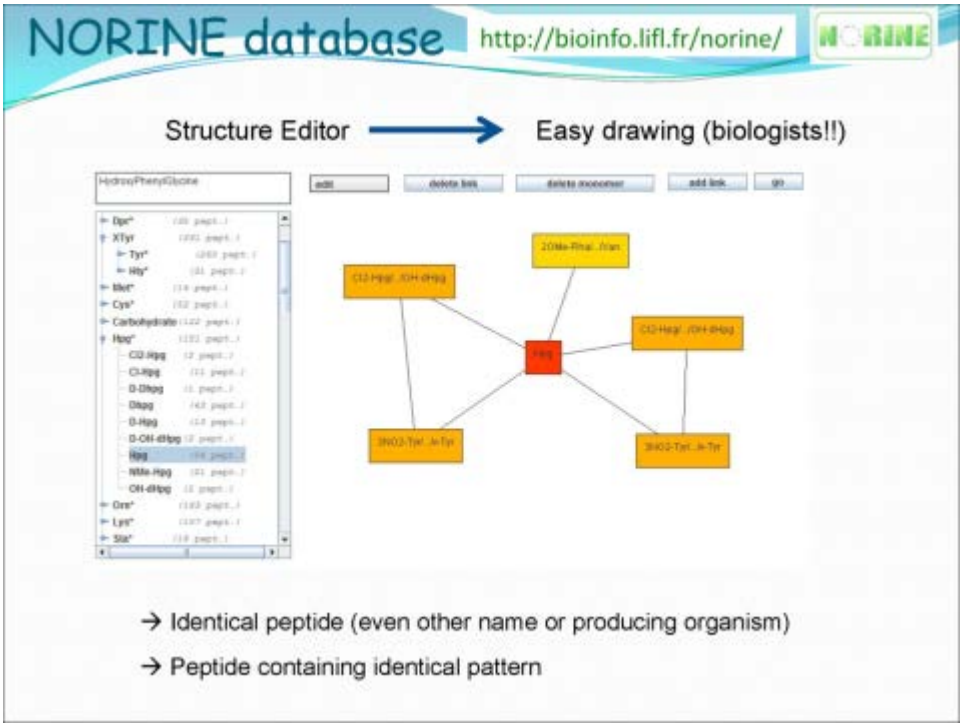

Secondary domains
Oxydation domain
Epimerisation domain
Methylation domain
Formylation domain
Ox
E
M
Fo

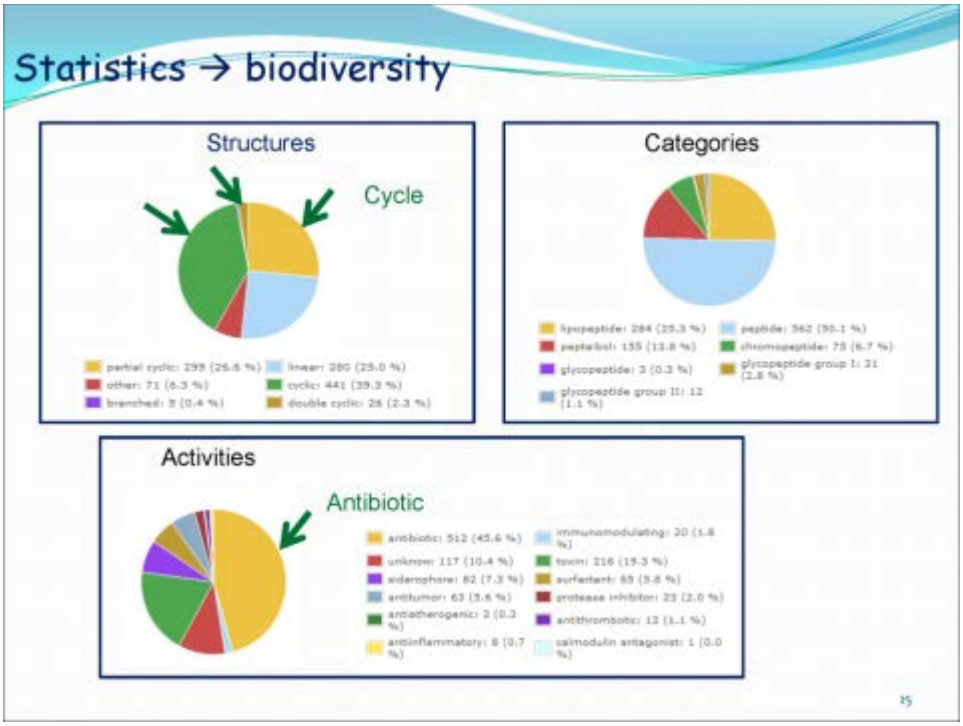

Surfactin biosynthesis
module 1
module 2
module 3
module 4
module 5
module 6
module 7
1.Template directed linear peptide elongation
Surfactin
2. Release by cyclization
(Sieber and Marahiel, 2005, Chem. Rev., 105, 715-738)


Signatures (conserved sequences)
Alignments of module and domain sequences
Order in domains is conserved
Consensus sequences = signatures
condensation
adenylation
thiolation
thioestérase
méthylation
→ PKS/NRPS analysis (http://nrps.igs.umaryland.edu/nrps/)
→ NRPS /PKS (http://www.nii.res.in/nrps-pks.html)


Specificity of synthetases
Module 1
Module 2
Module 3
AA1
AA2
AA3
Specificity
Adenylation domain
Code Stachelhaus = NRPS code
Software for prediction :
→ PKS/NRPS analysis (http://nrps.igs.umaryland.edu/nrps/)
→ NRPS /PKS (http://www.nii.res.in/nrps-pks.html)
→ NRPSpredictor2 (http://nrps.informatik.uni-tuebingen.de/Controller?cmd=SubmitJob)


NORINE database
http://bioinfo.lifl.fr/norine/
Norine
cyclosporin A
Caboche et al., 2008 (NAR); 2009 (BMC Struct. Biol.); 2010 (J. Bact)


NORINE database
http://bioinfo.lifl.fr/norine/
Structure Editor
Easy drawing (biologists!!)
→ Identical peptide (even other name or producing organism)
→ Peptide containing identical pattern

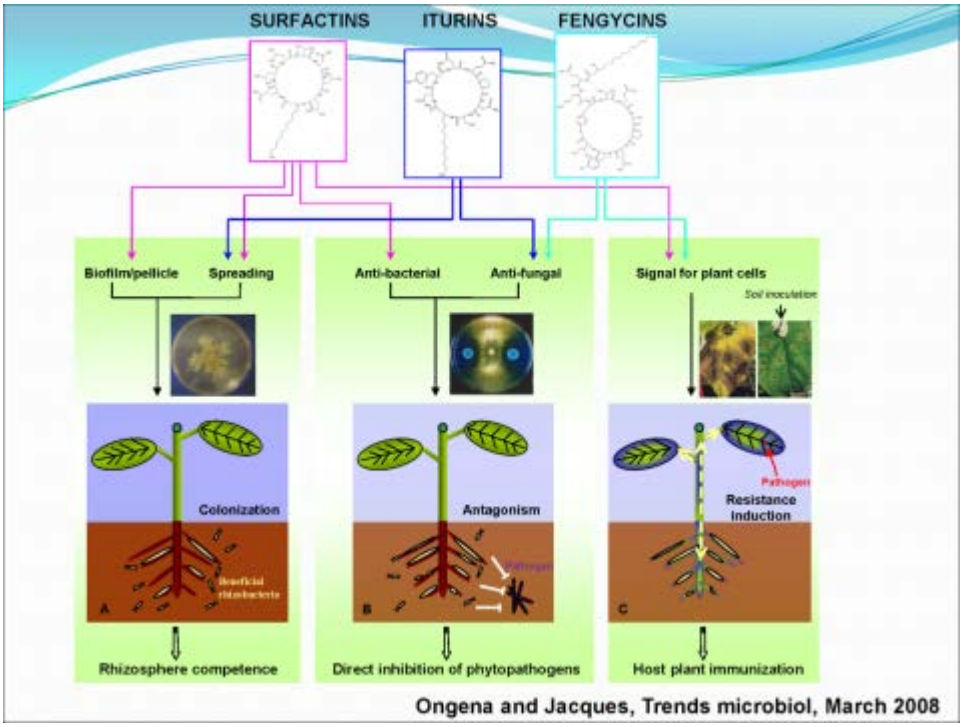

Statistics → biodiversity
Structures
Cycle
Categories
Activities
Antibiotic

Use of Norine to detect NRPs produced by biocontrol agents

- *Bacillus*
  - 155 different NonRibosomal Peptides (NRPs)
  - 20 families
- *Pseudomonas*
  - 140 different NonRibosomal Peptides (NRPs)
  - 17 families
- *Trichoderma*
  - 72 different NonRibosomal Peptides (NRPs)
  - 9 families

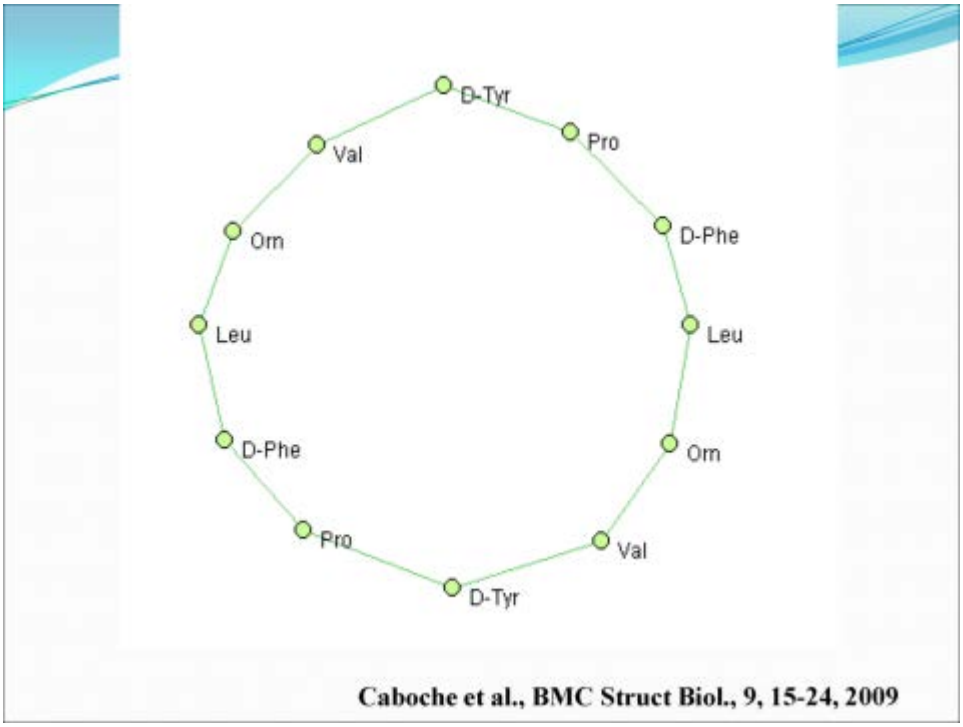
Main properties

Siderophore	Bacillibactin
Immunomodulatory compounds	Fengycin, edeine
Biosurfactant	Surfactin, lichenysin, fengycin, iturin
Toxin	Cereulide
Antimicrobial compounds	Bacilysin, bacitracin, edeine, fengycin, gramicidin, gratisin, iturin, polymyxin, tyrocidine



Main properties

• Siderophores	• Bacillibactin
• Immunomodulatory compounds	• Fengycin, edeine
• Biosurfactant	• Surfactin, lichenysin, fengycin, iturin
• Toxin	• Cereulide
• Antimicrobial compounds	• Bacilysin, bacitracin, edeine, fengycin, gramicidine, **gratisin**, iturin, polymixine, tyrocidine

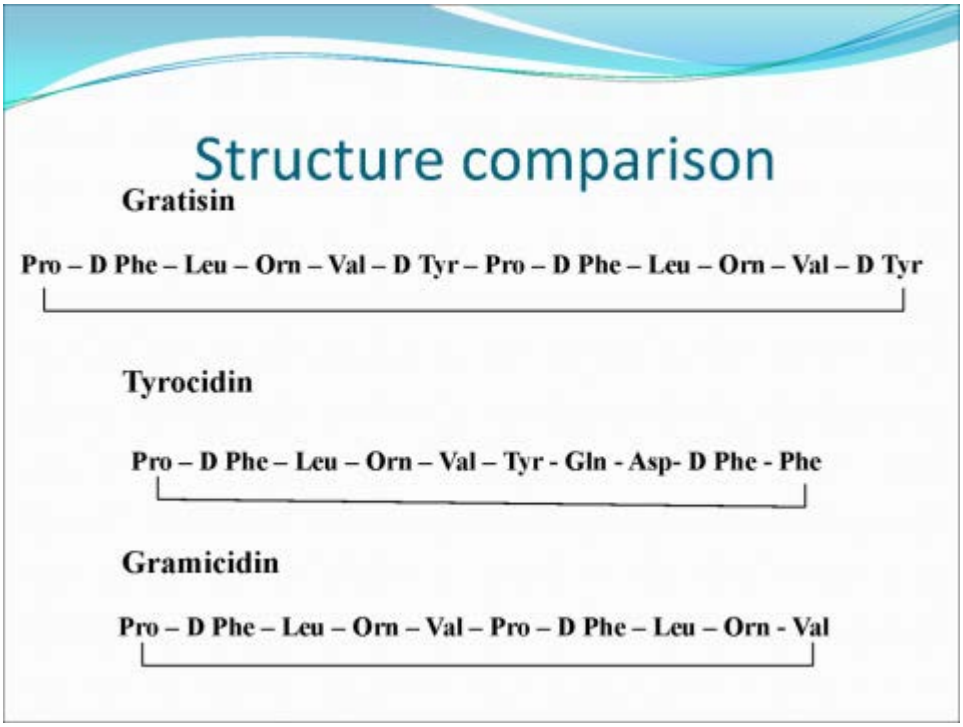


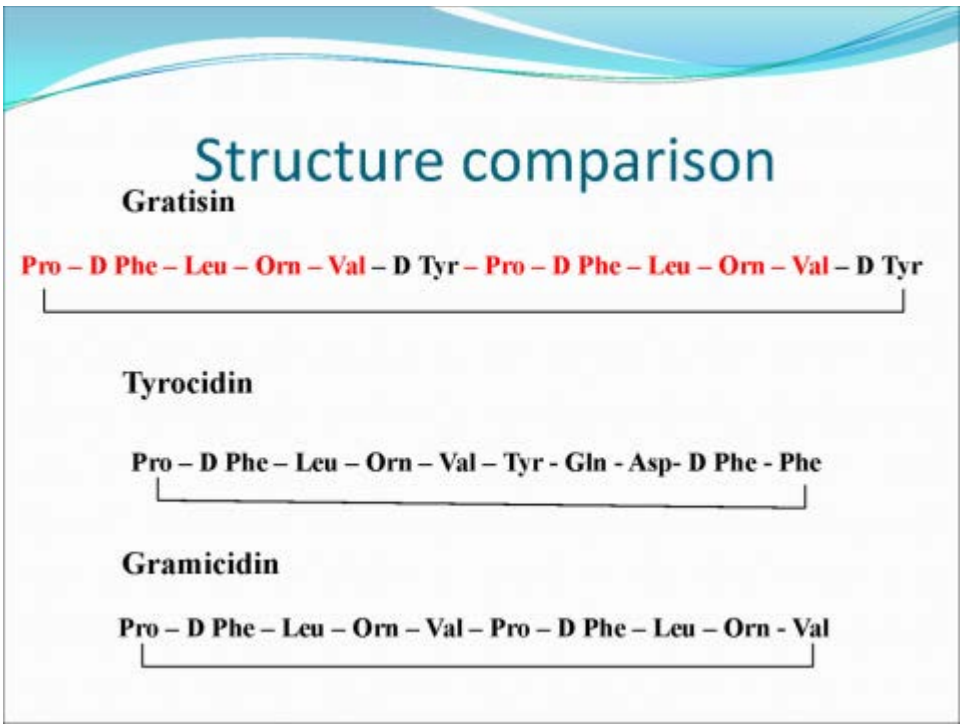
Caboche et al., BMC Struct Biol., 9, 15-24, 2009

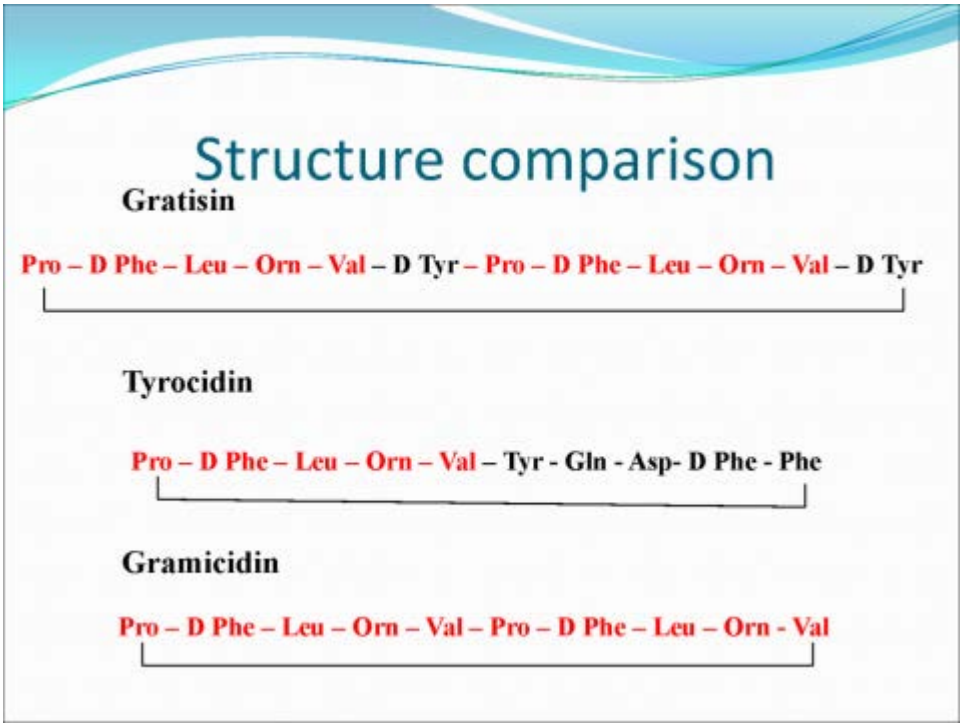
Structure comparison

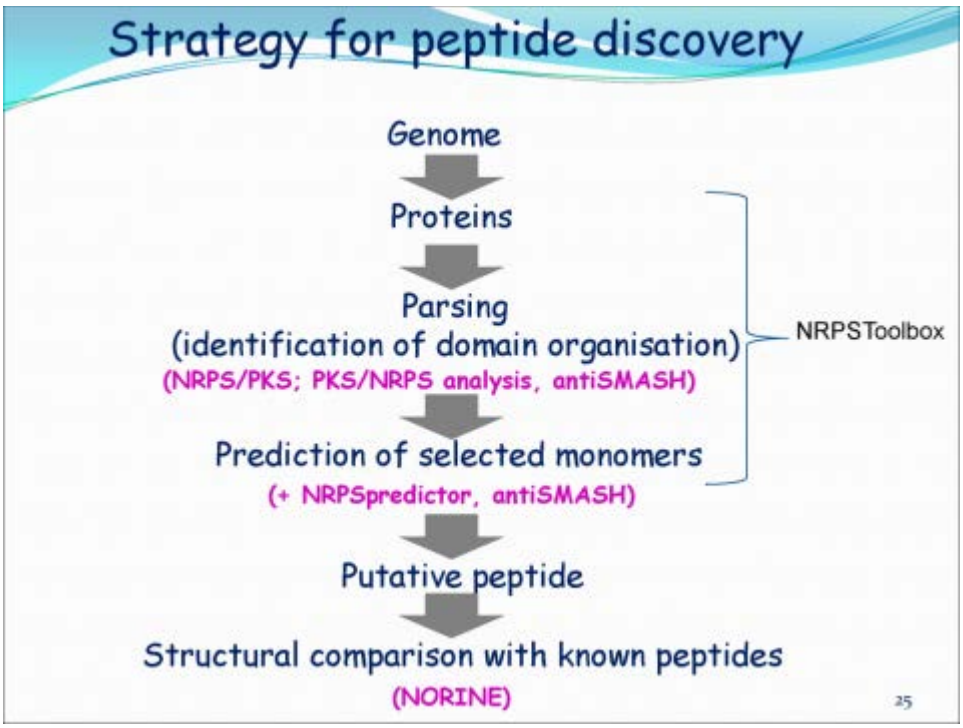
Gratisin

Pro – D Phe – Leu – Orn – Val – D Tyr – Pro – D Phe – Leu – Orn – Val – D Tyr

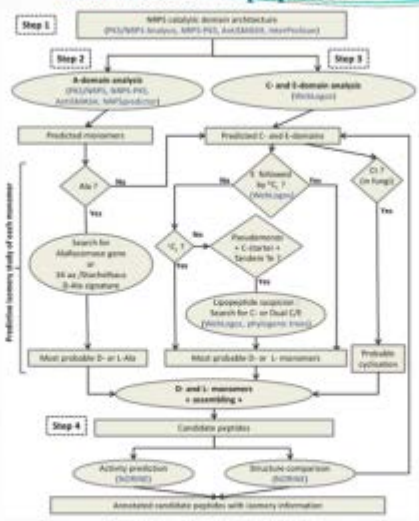

Structure comparison
Gratisin
Pro – D Phe – Leu – Orn – Val – D Tyr – Pro – D Phe – Leu – Orn – Val – D Tyr
Tyrocidin
Pro – D Phe – Leu – Orn – Val – Tyr - Gln - Asp- D Phe - Phe
Gramicidin
Pro – D Phe – Leu – Orn – Val – Pro – D Phe – Leu – Orn - Val


Structure comparison
Gratisin
Pro – D Phe – Leu – Orn – Val – D Tyr – Pro – D Phe – Leu – Orn – Val – D Tyr
Tyrocidin
Pro – D Phe – Leu – Orn – Val – Tyr - Gln - Asp- D Phe - Phe
Gramicidin
Pro – D Phe – Leu – Orn – Val – Pro – D Phe – Leu – Orn - Val


Structure comparison
Gratisin
Pro – D Phe – Leu – Orn – Val – D Tyr – Pro – D Phe – Leu – Orn – Val – D Tyr
Tyrocidin
Pro – D Phe – Leu – Orn – Val – Tyr - Gln - Asp- D Phe - Phe
Gramicidin
Pro – D Phe – Leu – Orn – Val – Pro – D Phe – Leu – Orn - Val


Strategy for peptide discovery
Genome
Proteins
Parsing
(identification of domain organisation)
(NRPS/PKS; PKS/NRPS analysis, antiSMASH)
Prediction of selected monomers
(+ NRPSpredictor, antiSMASH)
NRPSToolbox
Putative peptide
Structural comparison with known peptides
(NORINE)
25

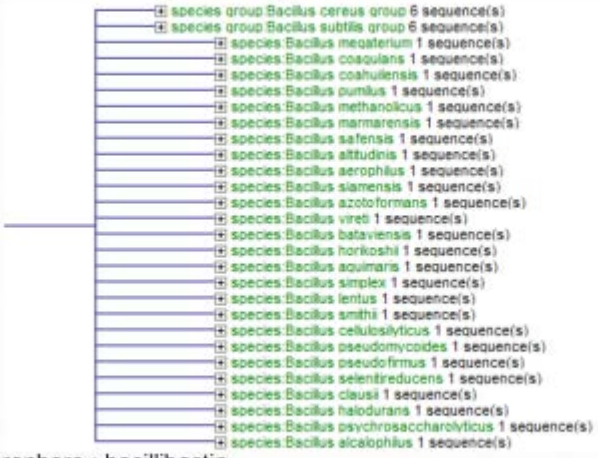
Florine : a workflow dedicated to structure prediction of nonribosomal peptides.



Caradec T, Pupin M, Vanvlassenbroeck A, Devignes MD, Smail-Tabbone M, et al. (2014) Prediction of Monomer Isomery in Florine: A Workflow Dedicated to Nonribosomal Peptide Discovery. PLoS ONE 9(1): e85667. doi:10.1371/journal.pone.0085667

Application 1 : NRPS in *Bacillus*

NCBI → 38 sequenced genomes



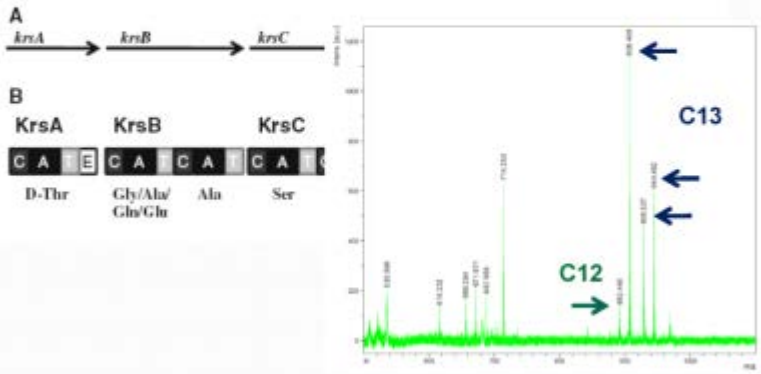
Siderophore : bacillibactin

Known antibiotics : gramicidin, tyrocidin

Lipopeptides: Fengycins, iturines, surfactins

Application 1 : NRPS in *Bacillus*

❖ Identification of the complete operon of kurstakin



❖ Lipopeptide → spreading

Abderrahmani *et al.*, 2011 Appl. Microbiol. Biotechnol.

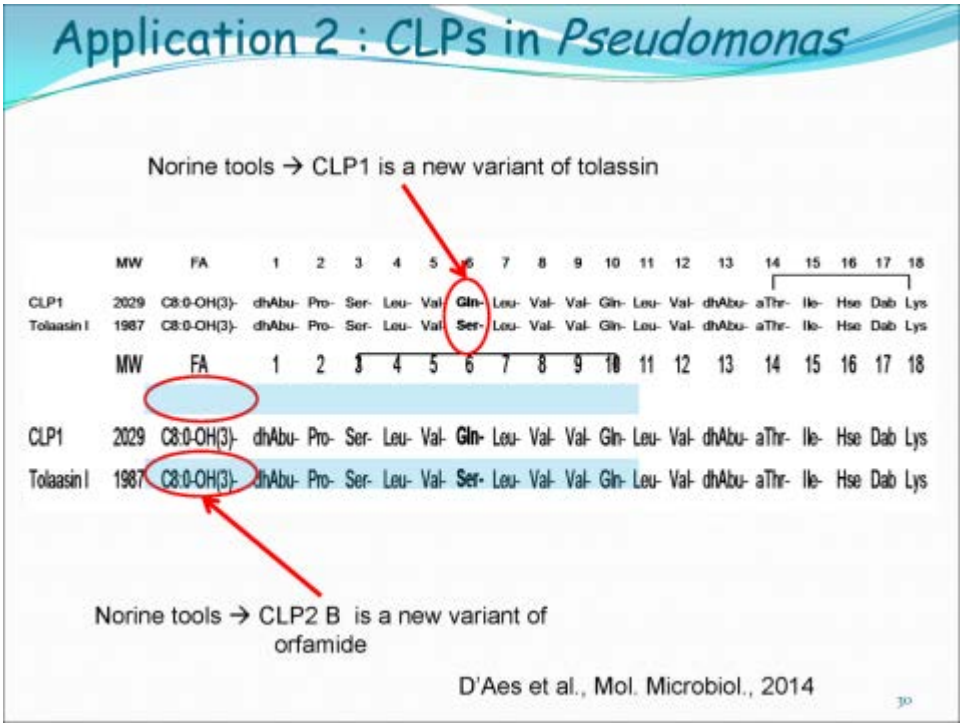
Application 2 : NRPS in *Pseudomonas*

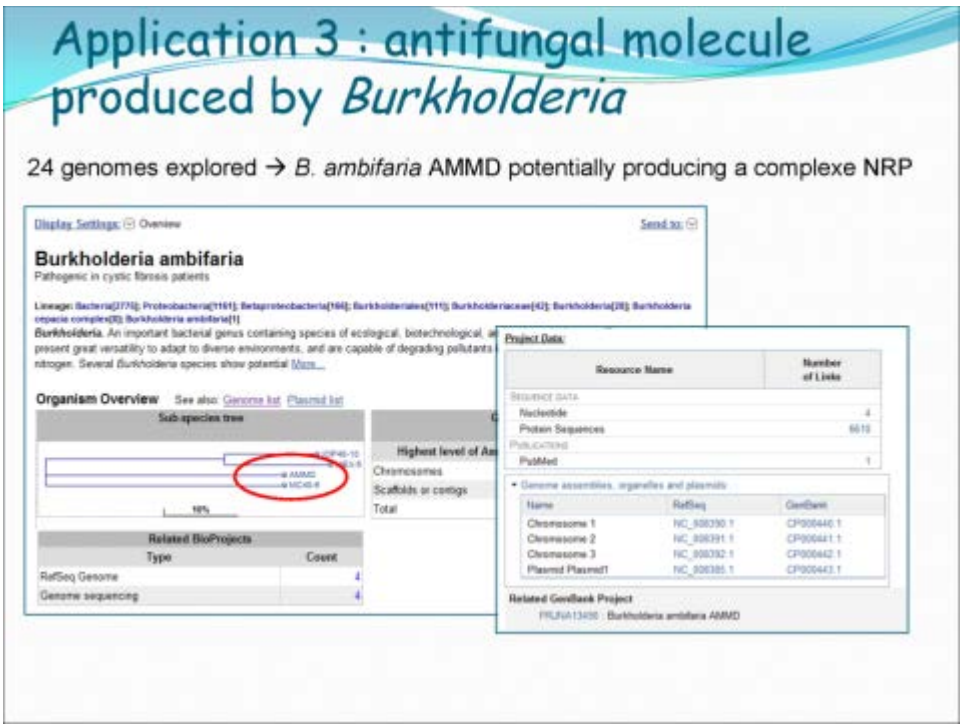
❖Overview on genomic data from NCBI → identification of pyoverdin variants and LP

❖Identification of 2 strains producing lipopeptides : CMR12a, *P. cichori* (collaboration with the University of Ghent and the University of Liege)

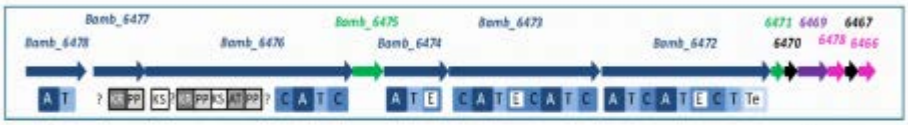
Genomes sequenced ➡ Identification lipopeptide synthetases

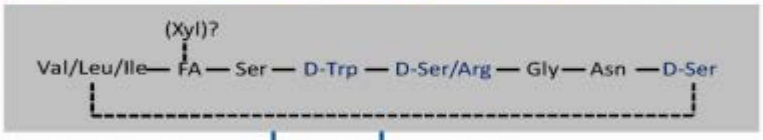
Pauwelyn et al., MPMI, 2013; D'Aes et al., Mol. Microbiol., 2014

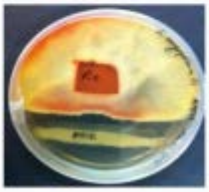

Application 2 : CLPs in Pseudomonas
Norine tools → CLP1 is a new variant of tolassin
MW FA 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
CLP1 2029 C8:0-OH(3)- dhAbu- Pro- Ser- Leu- Val- Gln- Leu- Val- Val- Gln- Leu- Val- dhAbu- aThr- Ile- Hse Dab Lys
Tolaasin I 1987 C8:0-OH(3)- dhAbu- Pro- Ser- Leu- Val- Ser- Leu- Val- Val- Gln- Leu- Val- dhAbu- aThr- Ile- Hse Dab Lys
Norine tools → CLP2 B is a new variant of orfamide
D'Aes et al., Mol. Microbiol., 2014


Application 3 : antifungal molecule produced by Burkholderia
24 genomes explored → B. ambifaria AMMD potentially producing a complexe NRP
Burkholderia ambifaria
Pathogenic in cystic fibrosis patients
Organism Overview
Related BioProjects
Type Count
RefSeq Genome 1
Genome sequencing 1
Project Data
Resource Name Number of Links
Nucleotide 4
Protein Sequences 6610
PubMed 1
Chromosome 1 NC_008390.1 CP000440.1
Chromosome 2 NC_008391.1 CP000441.1
Chromosome 3 NC_008392.1 CP000442.1
Plasmid Plasmid1 NC_008385.1 CP000443.1
Related GenBank Project
PRJNA13408 : Burkholderia ambifaria AMMD

Application 3 : antifungal molecule produced by *Burkholderia*







Occidiofungin family

Antifungal activity

Take home messages

- You can easily access to bioinformatics tools to discover new NRPs from sequence data
- Complete predictions still need expert analysis
- Predictions are no more than predictions → results have to be confirmed by further experiments (structural analysis)

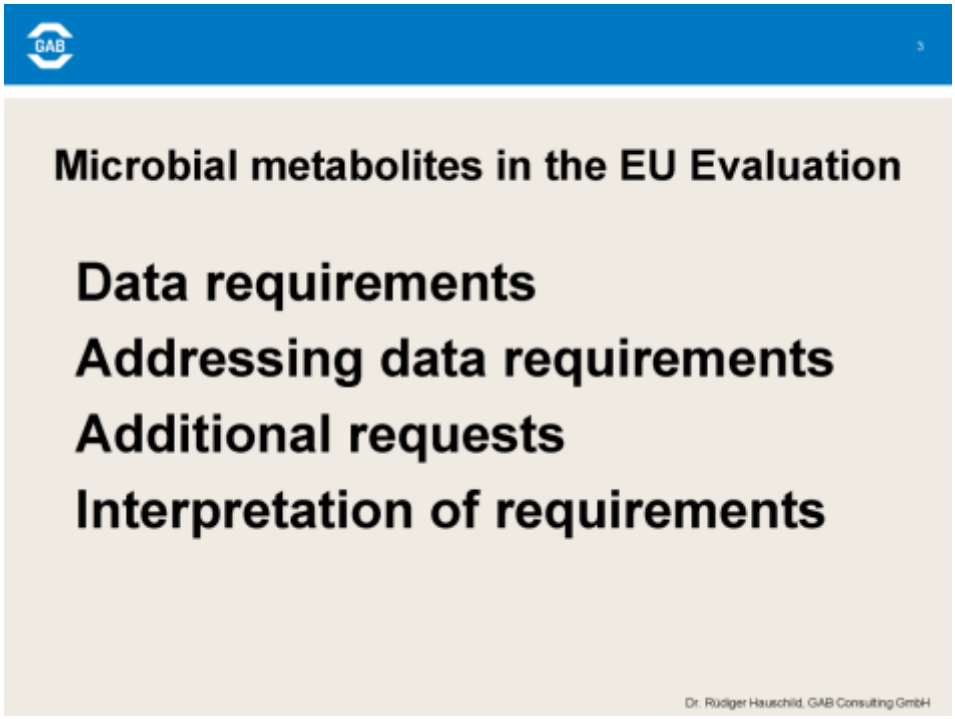
VERY USEFUL

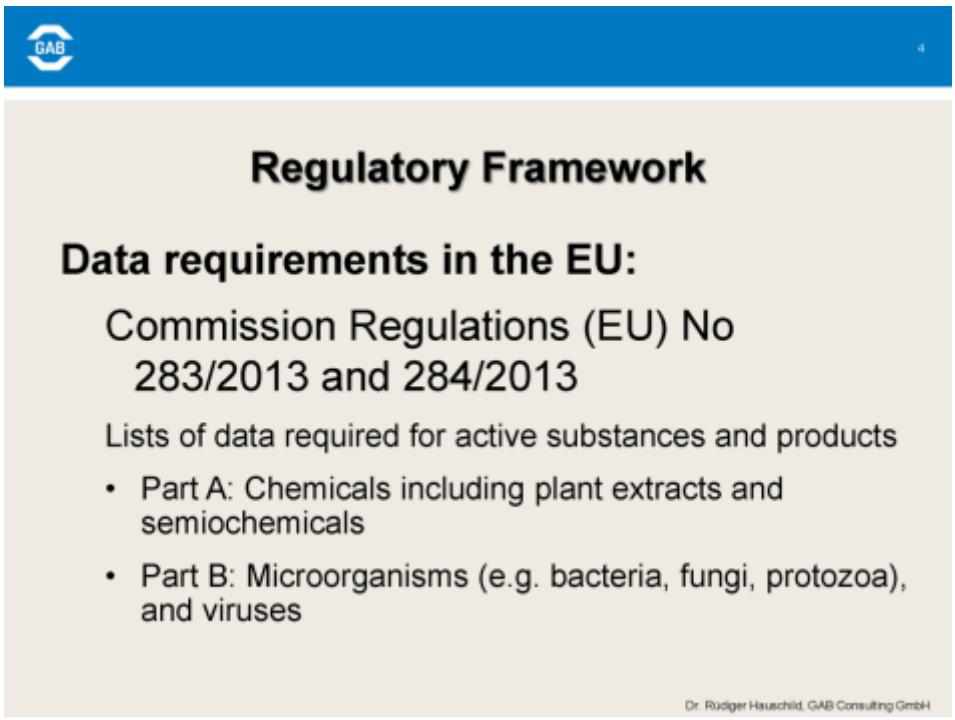
→Focuse on what you are expecting
→ time saving

Presentation 7

Experiences from industry in the EU in the risk assessment of secondary metabolites produced by microbial pesticides

Rüdiger Hauschild (GAB Consulting GmbH, Lamstedt; Germany)


GAB
3
Microbial metabolites in the EU Evaluation
Data requirements
Addressing data requirements
Additional requests
Interpretation of requirements
Dr. Rüdiger Hauschild, GAB Consulting GmbH


GAB
4
Regulatory Framework
Data requirements in the EU:
Commission Regulations (EU) No 283/2013 and 284/2013
Lists of data required for active substances and products
• Part A: Chemicals including plant extracts and semiochemicals
• Part B: Microorganisms (e.g. bacteria, fungi, protozoa), and viruses
Dr. Rüdiger Hauschild, GAB Consulting GmbH

Experience with data requirements

Data requirements are justified to exclude negative effects on humans, non-target organisms or the environment

Differences exist in the interpretation of data requirements

Which requirements need to be addressed by strain-specific studies and which can be covered using published literature?

Dr. Rüdiger Hauschild, GAB Consulting

Data requirements: Metabolites

Secondary metabolites

- Discussion of possible metabolites from published literature
- Potential for accumulation and exposure
- Determination of "relevant" metabolites in the product
- Determination of production capacity for the particular strain

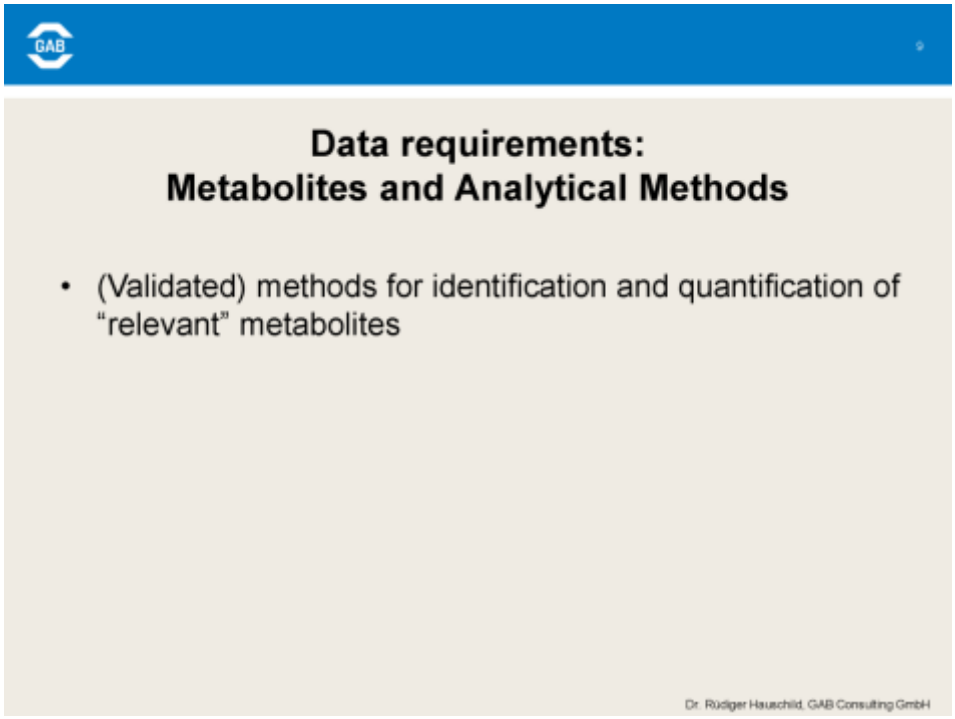
Dr. Rüdiger Hauschild, GAB Consulting GmbH

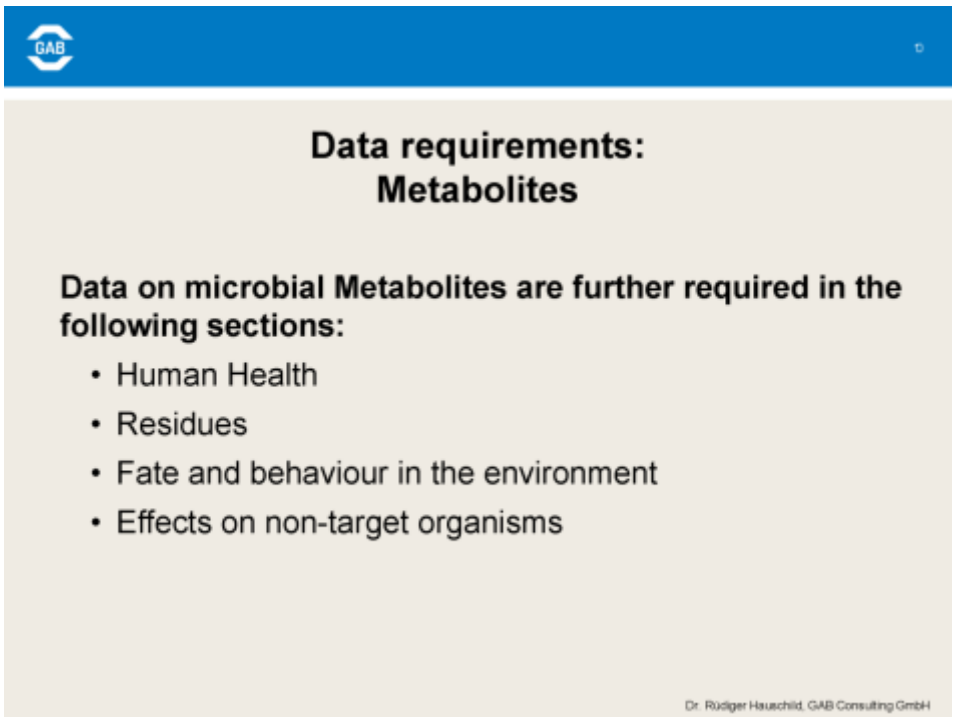
Data requirements: Metabolites

Metabolites with unacceptable effects on human health and/or the environment need to be described:

- Properties (nature, structure, stability, cellular localization)
- Role in mode of action
- Biosynthesis (external conditions, physiology of regulation)
- Effects on humans, animals or other non-target-organisms

Dr. Rüdiger Hauschild, GAB Consulting GmbH


GAB
9
Data requirements:
Metabolites and Analytical Methods
• (Validated) methods for identification and quantification of "relevant" metabolites
Dr. Rüdiger Hauschild, GAB Consulting GmbH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10
Data requirements:
Metabolites
Data on microbial Metabolites are further required in the following sections:
• Human Health
• Residues
• Fate and behaviour in the environment
• Effects on non-target organisms
Dr. Rüdiger Hauschild, GAB Consulting GmbH

Addressing Data Requirements on microbial metabolites: Sources of Information

"Well known species"

- Search open literature for metabolites which are described for the species and the genus
- Identify which metabolites might be harmful for humans or other non-target organisms
- If such metabolites occur, determine whether the strain can produce them
  - Genetics: presence of genes involved in their biosynthesis
  - Biochemistry: Identification and quantification of metabolites in the product
- Appropriate toxicology and ecotoxicology studies will reveal effects of metabolites
- Assessment of risk through metabolites

Dr. Rüdiger Hauschild, GAB Consulting GmbH

Addressing Data Requirements on microbial metabolites: Sources of Information

"New species"

- No or very little information available in the literature
- Consider information from development experiments on mode of action
- Appropriate testing of the product using standard test species
- If toxicity through metabolites is detected, chemical characterization of these metabolites
- Identification and quantification of metabolites in the product
- Assessment of risk through metabolites

Dr. Rüdiger Hauschild, GAB Consulting GmbH

Data Requirements on microbial metabolites: Responses from evaluators (selected)

The approach is widely accepted

Request for additional data:

1. Validation of methods for metabolite determination
2. Toxicological data to define the toxicological profile of toxins/secondary metabolites
3. Identification and quantification of toxins/secondary metabolites formed on plants or in soil

Dr. Rüdiger Hauschild, GAB Consulting GmbH

Data Requirements on microbial metabolites: Additional data requests (selected)

1. Validation of methods for metabolite determination
   In most cases feasible, sometimes difficult to perform within the given timeline or if no analytical standards are available

2. Toxicological data to define the toxicological profile of toxins/secondary metabolites
   Feasible from published literature, at least for major metabolite groups

Dr. Rüdiger Hauschild, GAB Consulting GmbH

Data Requirements on microbial metabolites: Additional data requests (selected)

3. Identification and quantification of toxins/secondary metabolites formed on plants or in soil

Technically and economically not feasible and not justified!

Metabolite production depends on

- Substrate
- Presence of the target organism
- Physiological parameters and host plant
- Abiotic factors

Potential unknown toxins/secondary metabolites cannot be detected by biochemical means

Dr. Rüdiger Hauschild, GAB Consulting GmbH

Data Requirements on microbial metabolites: Additional data requests (selected)

"Identification and quantification of toxins/secondary metabolites formed on plants or in soil" is not justified if the biology of the microorganism is considered:

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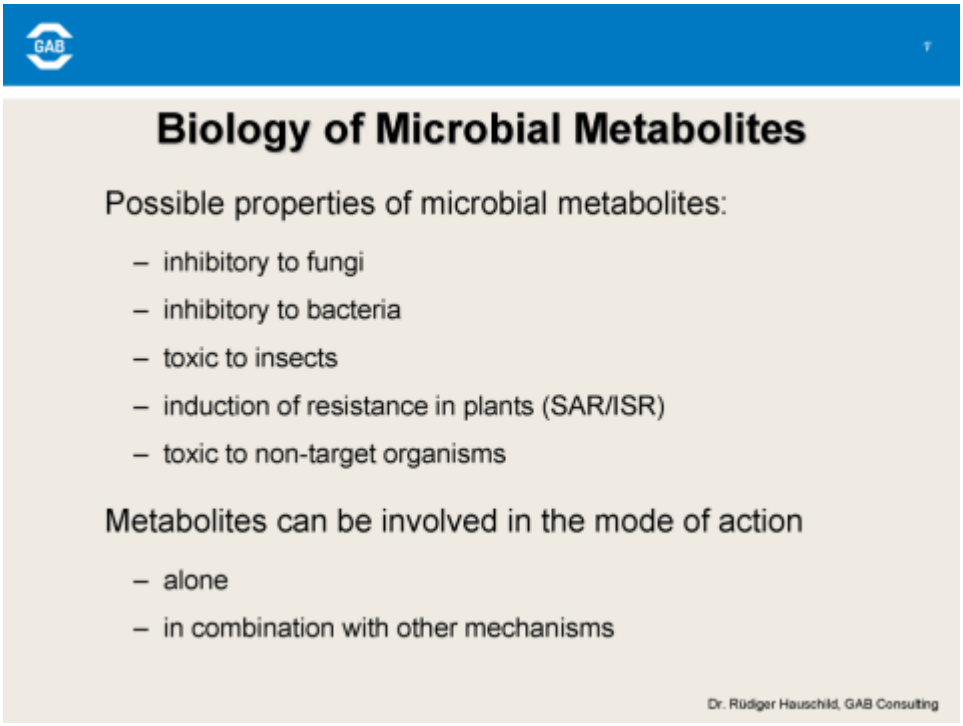
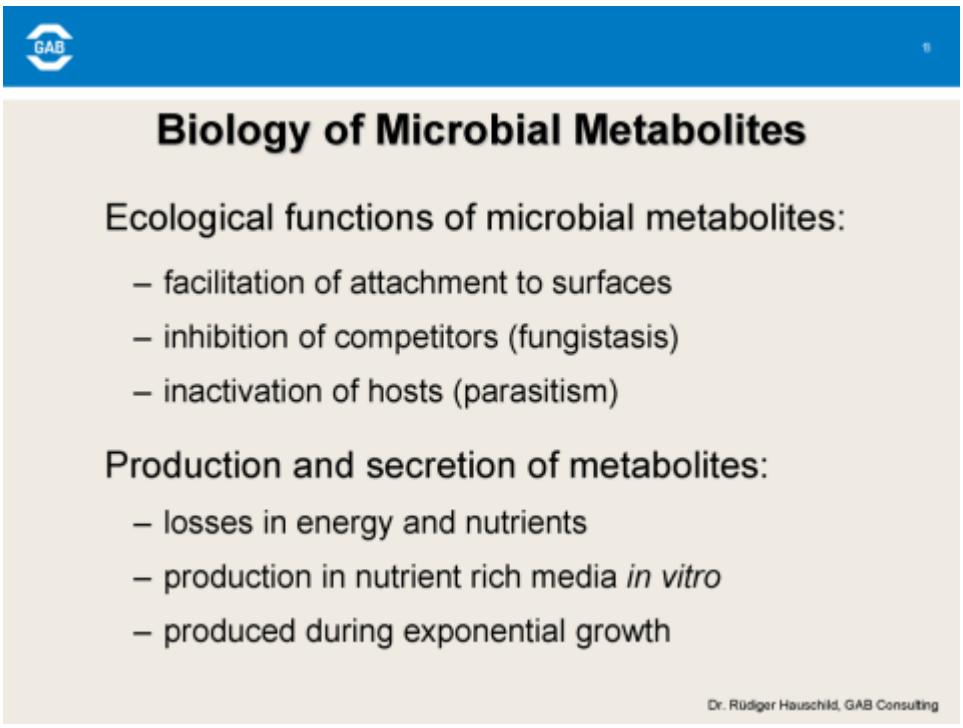
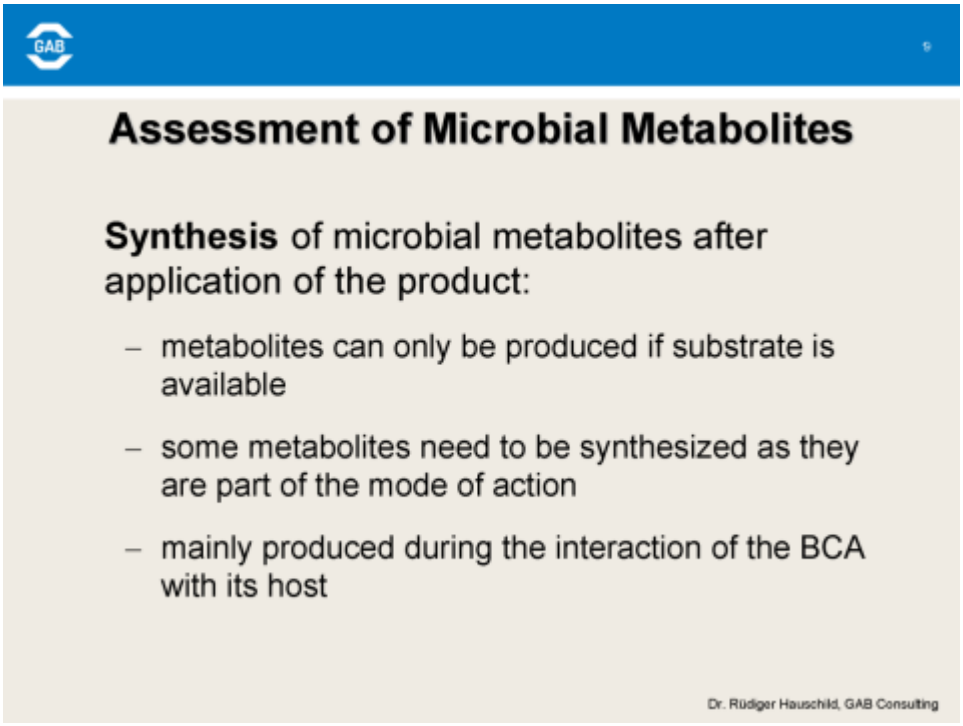
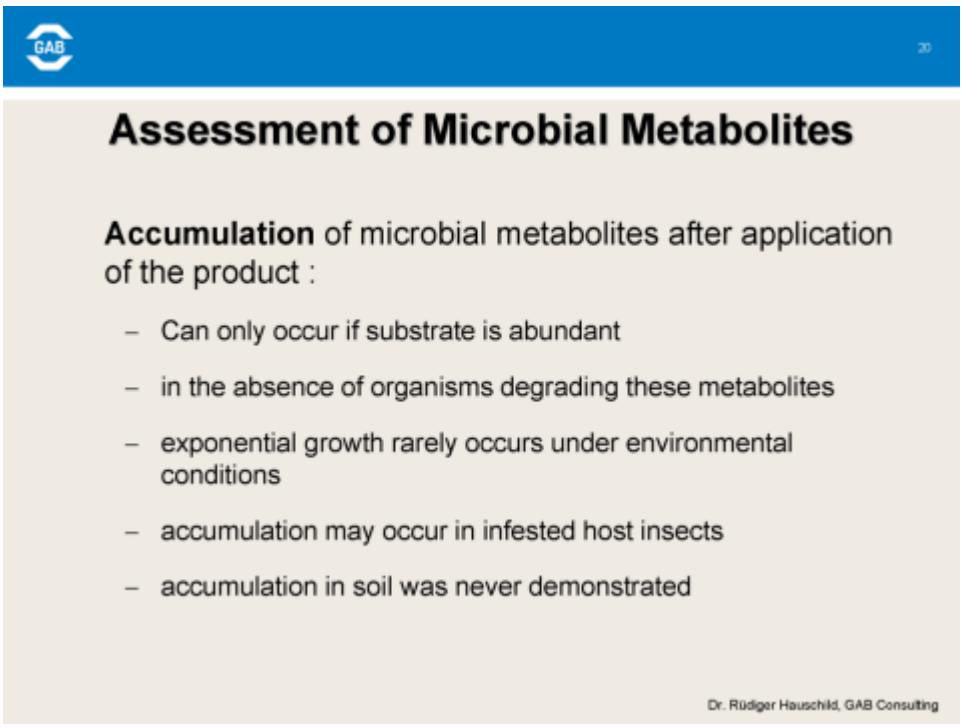

GAB
Biology of Microbial Metabolites
Possible properties of microbial metabolites:
– inhibitory to fungi
– inhibitory to bacteria
– toxic to insects
– induction of resistance in plants (SAR/ISR)
– toxic to non-target organisms
Metabolites can be involved in the mode of action
– alone
– in combination with other mechanisms
Dr. Rüdiger Hauschild, GAB Consulting


GAB
Biology of Microbial Metabolites
Ecological functions of microbial metabolites:
– facilitation of attachment to surfaces
– inhibition of competitors (fungistasis)
– inactivation of hosts (parasitism)
Production and secretion of metabolites:
– losses in energy and nutrients
– production in nutrient rich media *in vitro*
– produced during exponential growth
Dr. Rüdiger Hauschild, GAB Consulting


GAB
9
Assessment of Microbial Metabolites
Synthesis of microbial metabolites after application of the product:
– metabolites can only be produced if substrate is available
– some metabolites need to be synthesized as they are part of the mode of action
– mainly produced during the interaction of the BCA with its host
Dr. Rüdiger Hauschild, GAB Consulting


GAB
20
Assessment of Microbial Metabolites
Accumulation of microbial metabolites after application of the product :
– Can only occur if substrate is abundant
– in the absence of organisms degrading these metabolites
– exponential growth rarely occurs under environmental conditions
– accumulation may occur in infested host insects
– accumulation in soil was never demonstrated
Dr. Rüdiger Hauschild, GAB Consulting

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Assessment of Microbial Metabolites

Accumulation of microbial metabolites independently from Biocontrol

- Through Plant pathogens (*Fusarium*, *Microdochium*)
- Through storage pathogens (*Aspergillus*, *Chaetomium*, *Clostridium*, etc.) under condition where these are favoured
- By entomopathogens (*Lecanicillium*, *Verticillium*, *Isaria*, *Beauveria* etc.)

Dr. Rüdiger Hauschild, GAB Consulting

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Assessment of Microbial Metabolites

Under which conditions do microbial metabolites represent a risk to humans, non-target organisms or the environment?

1. metabolites are toxic
2. metabolites are contained in the product
3. metabolites are produced and accumulated after product application
4. accumulation occurs at sites that are exposed to non-target organisms

Only in case points 1 and (2 or 3+4) are fulfilled, further analysis is required

Dr. Rüdiger Hauschild, GAB Consulting

Experiences with data requirements Summary

Assessment of Microbial Metabolites

Most microorganisms used in biocontrol are well known

Some metabolites are synthesized as part of the mode of action

Metabolites can be contained in the product

Synthesis occurs often during interaction with the host

Accumulation of microbial metabolites with harmful effects on non-target organisms in the environment is unlikely to occur and was never observed

Evaluation can be based on product data and published literature

Dr. Rüdiger Hauschild, GAB Consulting

Presentation 8

Experiences from industry in the USA in the risk assessment of secondary metabolites produced by microbial pesticides

Keith Pitts (Marrone Bio Innovations, Inc., Davis; USA) and Alison Hamer (TSGE Consulting Ltd. UK, representing Marrone Bio Innovations)

Marrone Bio Innovations

OECD: The 6th Biopesticides Steering Group

Seminar on "Hazard and Risk Assessment of Secondary Metabolites"

Experiences from industry in the USA in the risk assessment of secondary metabolites produced by microbial pesticides

May 18, 2015
Paris, France

Safe Harbor Statement

Forward-Looking Statements

This presentation may include forward-looking statements. These statements reflect the current views of the Company's senior management with respect to future events and financial performance. These statements include forward-looking statements with respect to the Company's business and industry in general, including statements regarding potential market size of Company products, anticipated product launches, target geographic markets, factors for the barriers to entry into the market, and strategies for growth. Statements that include the words "expect," "intend," "plan," "believe," "project," "forecast," "estimate," "may," "should," "anticipate" and similar statements of a future or forward-looking nature identify forward-looking statements for purposes of the federal securities laws or otherwise. Forward-looking statements address matters that involve risks and uncertainties such as the timing of and costs associated with the launch of products, the difficulty in predicting the timing or outcome of product research and development efforts and regulatory approvals. Accordingly, there are or will be important factors that could cause the Company's actual results to differ materially from those indicated in these statements. The statements made herein speak only as of the date of this presentation.

Page 2

Marrone Bio Innovations

Marrone Bio Innovations Overview

Company Highlights

- Incorporated in June 2006
- **4** commercially available products, **2** additonal approved & **1** submitted for EPA registration
- Library of 18,000+ proprietary microorganisms screened against multiple targets
- Wholly-owned, operational fermentation facility in Bangor, MI
- Commercial sales in North America, LATAM, parts of ME&A; early in our long term growth curve
- Listed on NASDAQ as MBII August 2, 2013; Follow-on June 6, 2014

Commercial Products Today

REGALIA GRANDEVO VENERATE ZEQUANOX

Marquee Partners / Distributors

syngenta FMC Scotts DSM Valagro IAP evogene Crop Production Services ENGAGE AGRO CNI

Robust Pipeline

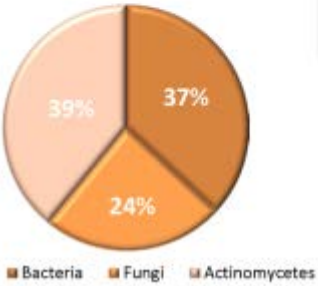
- Pipeline products: nematicides, herbicides, fungicide and biostimulants
- Many more earlier stage candidates across all categories
- 40 allowed patents globally, 15 U.S. issued patents; 232 pending patent applications globally, 32 are pending in the U.S
- USDA NOP-OMRI compliant formulations

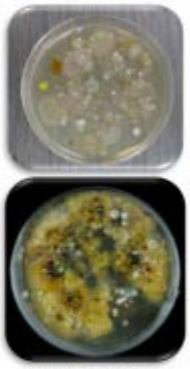
Marrone Bio Innovations

Page 3

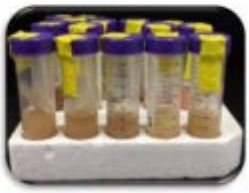
Discovery: Sourcing and Isolation of Microorganisms

Soil and other types of samples collected from unique habitats and niches





Individual fungal, bacterial, and actinomycete colonies picked from primary plate



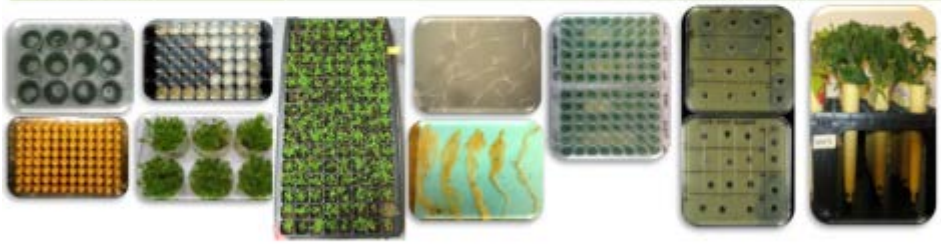
Water extracts of fermentation broths are used for bioassays

Marrone Bio Innovations

Page 4

Primary Screen Testing

Insecticide	Fungicide	Herbicide	Nematicide	Algaecide	Bactericide	Biostimulants
Lygus *Beet armyworm* *Corn rootworm*	*Botrytis cinerea* *Phytophthora capsici*	Crabgrass Lettuce	*Meloidogyne spp.*	*Chlamydomonas reinhardtii*	*Xanthomonas campestris* *Pseudomonas syringae*	*Tomatoes, Corn, Radish, Soy & Others*



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Discovery and Characterization

Isolation

Samples from around the world from areas of high biodiversity are collected and cultured.

Fermentation

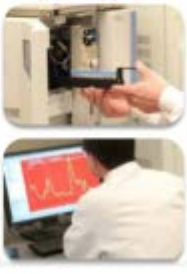
Purity is confirmed and water extracts of fermentation broths are prepared for bioassays.

Biological Testing

Biological testing against weeds, insects, plant pathogens, nematodes, algae, and for growth promotion.

Natural Product & Analytical Chemistry

Identify pesticidal compounds; Eliminate harmful species-strains/metabolites; Develop analytical assays for mfg QC.



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MBI Commercial Products-USA

Product	Active	Uses	Registration Date	Enforcement Method	Notes
Grandevo	*Chromobacterium subtsugae*	F, O, H&G	August 26, 2011 & May 1, 2012	Cabbage Looper Bioassay	Pending in EU, Canada, Mexico and Brazil
Zequanox	*Pseudomonas fluorescens (protegens)*	Water	March 9, 2012	Zebra/Quagga Mussel Bioassay	Registered in Canada; Pending in EU
Opportune	*Streptomyces acidiscabies*	F, O, H&G	April 26, 2012	Thaxtomin A HPLC	Registered in Canada (non-food)
Venerate	*Burkholderia rinojensis*	F, O, H&G	February 28, 2014	Beet Armyworm Bioassay	Pending in Canada, Mexico and Brazil

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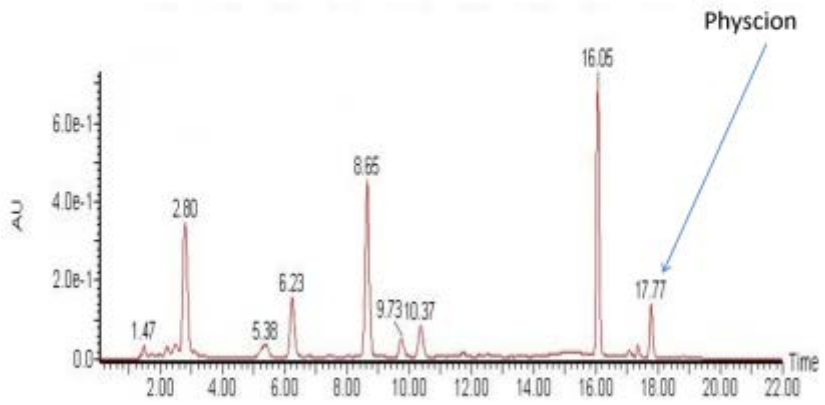
R. sachalinensis extract



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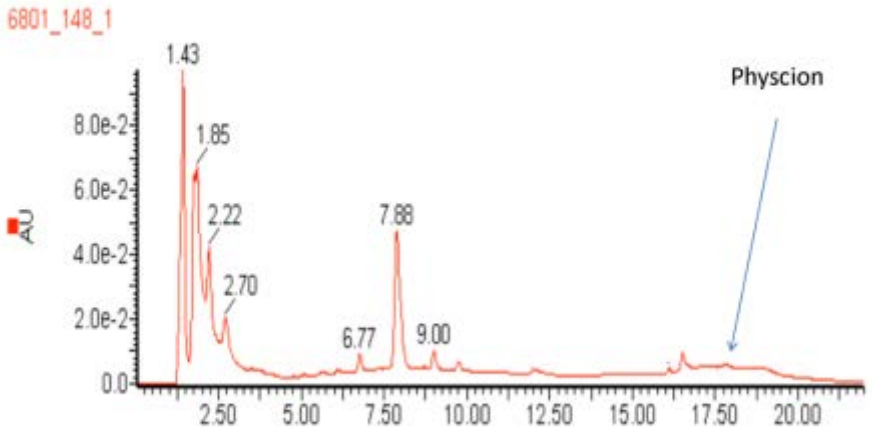
Rheum rhabarbarum (Rhubarb) extract



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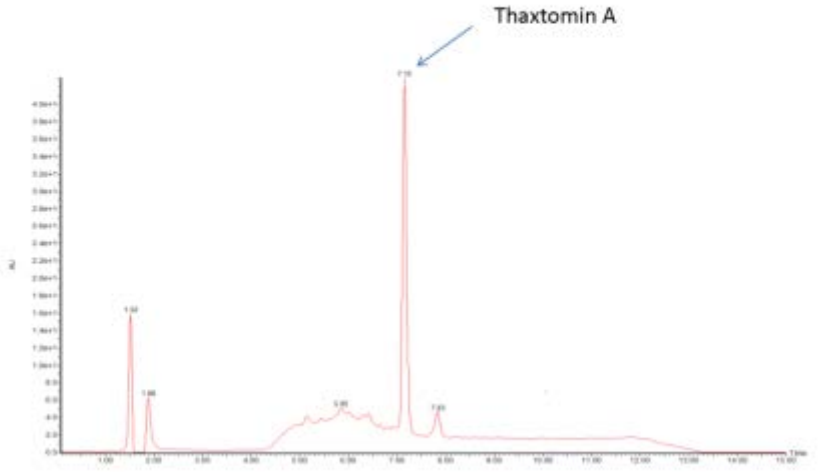
Streptomyces acidiscabies



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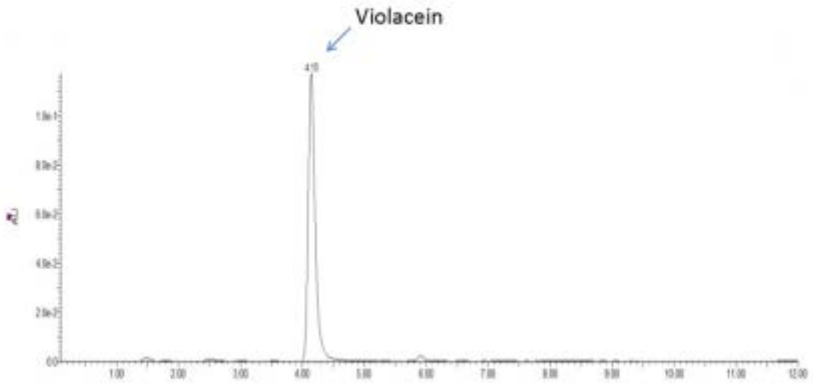
Chromobacterium subtsugae



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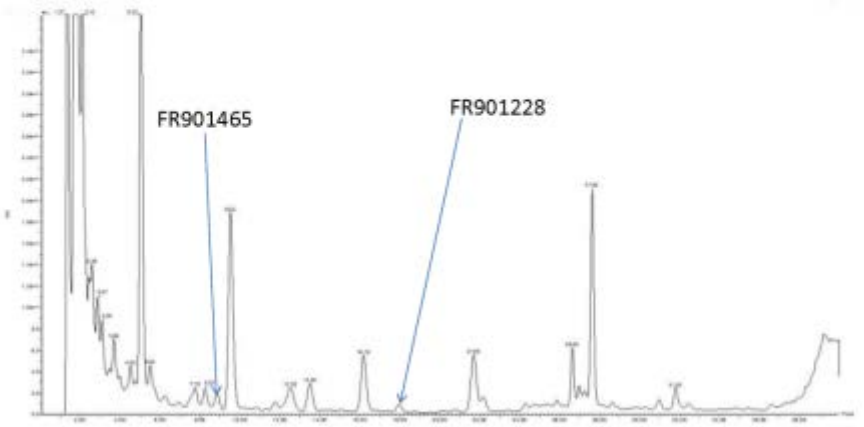
Burkholderia sp. Strain A396



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MBI-005 (Opportune)-Path to Registration in North America

MBI-005 TGAI non-viable Streptomyces acidiscabies strain RL-110[T]	
870.1100	Acute Oral
870.1200	Acute Dermal
870.1300	Acute Inhalation
870. 2400	Primary Eye
870.2500	Dermal Irritation
870.2600	Skin Sensitization (Buehler)
885.3200	Acute Intravenous Toxicity and Pathogenicity
885.3050	Acute Oral Toxicity and Pathogenicity
885.3150	Acute Pulmonary Toxicity and Pathogenicity
885.3400	Hypersensitivity Incidents
885.3500	Cell Culture
885.4100	Avian Toxicity and Pathogenicity
885.4150	Wild Mammal Toxicity and Pathogenicity
885.4280	Estuarine/Marine Organism Toxicity and Pathogenicity
885.4050	Avian Oral Toxicity, Tier 1
885.4200	Freshwater Fish, Tier 1
885.4240	Freshwater Aquatic Invertebrate (Daphnia), Tier 1
885.4240	Freshwater Aquatic Invertebrate (Hyalella), Tier 1
885.4340	Ladybird Beetle
885.4340	Green Lacewing
885.4340	Parasitic Wasp
885.4380	Honeybee, Tier 1
885.4300	Non-target Terrestrial Plant Studies, Tier 1
885.4300	Non-target Aquatic Plant Studies, Tier 1
850.4550	Cyanobacteria, 96-hour

- EPA Registered April 26, 2012, Unconditional
- Non-viable Streptomyces acidiscabies strain RL-110T cells and spent fermentation media, 17% TGAI
- EPA Reg. No. 84059-11 TGAI; 84059-12 EP
- Enforcement method: HPLC Thaxtomin A
- Exempt from Requirement of a Tolerance
- Agricultural, Ornamental and Home Uses

- EPA Guidance on killed microbial viability

- *PMRA Registered February 7, 2014*
- *PRMA-Ames study; 90-day study*
- *Non-food Ornamental and Home Uses Only*
- *PMRA Reg. No. 31165 (TGAI) and 31505 (EP)*

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MBI-203: Path to Registration in USA-Round One, 2011

MBI-203 TGAI and EP Chromobacterium subtsugae strain PRAA4-1[T]			
870.1100	Acute Oral	885.4240	Freshwater Aquatic Invertebrate (Daphnia), Tier 1
870.1200	Acute Dermal	885.4240	Freshwater Aquatic Invertebrate (Hyalella), Tier 1
870.1300	Acute Inhalation	885.4340	Ladybird Beetle
870. 2400	Primary Eye	885.4340	Green Lacewing
870.2500	Dermal Irritation	885.4340	Parasitic Wasp
870.2600	Skin Sensitization (Buehler)	885.4380	Honeybee, Tier 1
885.3200	Acute Intravenous Toxicity and Pathogenicity	885.4340	Honeybee Larval Study
885.3050	Acute Oral Toxicity and Pathogenicity	885.4300	Non-target Terrestrial Plant Studies, Vegetative Vigor, Tier 1
885.3150	Acute Pulmonary Toxicity and Pathogenicity	885.4300	Non-target Terrestrial Plant Studies, Seedling Emergence, Tier 1
885.3400	Hypersensitivity Incidents	MBI-203 Registration Information	
885.3500	Cell Culture	EPA Registered August 26, 2011 Unconditional	
885.4100	Avian Toxicity and Pathogenicity	Chromobacterium subtsugae strain PRAA4-1[T] cells and spent fermentation media, 30% TGAI	
885.4150	Wild Mammal Toxicity and Pathogenicity	EPA Reg. No. 84059-9 (TGAI)	
		EPA Reg. No. 84059-10 (EP)	
885.4280	Estuarine/Marine Organism Toxicity and Pathogenicity	Enforcement-Efficacy method: Cabbage Looper Killing Units	
		Exempt from Requirement of a Tolerance	
885.4050	Avian Oral Toxicity, Tier 1	Agricultural, Ornamental and Home Uses	
885.4200	Freshwater Fish, Tier 1		

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MBI-203: Path to Registration in USA-Round Two, 2012+

MBI-203 DF (GRANDEVO)-30% Chromobacterium subtsugae strain PRAA4-1[T]			
870.1100	Acute Oral	OECD 471	Reverse mutation in five histidine-requiring strains of Salmonella typhimurium using a treat and plate methodology
870.1200	Acute Dermal	OECD 473	Induction of chromosomal aberrations in cultured human peripheral blood lymphocytes
870.1300	Acute Inhalation	OECD 476	Mutation at the hprt locus of mouse lymphoma L5178Y cells (MLA) using the Microrite fluctuation technique
870. 2400	Primary Eye	OPPTS 835.3160	Biodegradability- Sealed vessel CO2 evolution test
870.2500	Dermal Irritation	OEPP/EPPO 170 (4), 2010 OPPTS 850.3040	Determination of side effects after application of *Chromobacterium subtsugae* strain PRAA4-1[T] (Grandevo[TM]/MBI-203 DF2) on honeybees (*Apis mellifera* L.) in buckwheat sp. in a semi-field study in North Carolina, USA 2012
OECD 213 & 214	Honeybee Acute Oral and Contact Study	Other	
OECD 222	Effects on Reproduction and Growth on Earthworms	PMRA	90-day Oral and Prenatal Development Studies
OPPTS 885.4300	A 96-hour Toxicity Test with Cyanobacteria	EU	Modeling the dynamics of secondary metabolite formation in the environment; background levels
OECD 201	Algal Growth Inhibition Assay		
OECD 202	Acute toxicity to Daphnia	EPA Registered May 1, 2012 Unconditional *Chromobacterium subtsugae* strain PRAA4-1[T] cells and spent fermentation media, 30% TGAI EPA Reg. No. 84059-17 *Spray dried-inactivated Chromobacterium subtsugae* Enforcement method: Cabbage Looper Killing Units Exempt from Requirement of a Tolerance Agricultural, Ornamental and Home Uses *Currently pending: Mexico, Canada, Brazil and EU*	
OECD 203	Acute Toxicity to Rainbow trout		
OPPTS 885.4340	Acute Toxicity to beneficial insects: *Typhlodromus pyri*		
OPPTS 885.4340	Acute Toxicity to beneficial insects: *Aphidius rhopalosiphi*		

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Development of dossier for the EU – MBI-203

Literature search and review

- Literature search conducted to support EPA submission in 2009
- For EU submission we updated the search to EFSA Guidance – extensive further work in 2014
- Valuable review papers had been published (2010, 2011) regarding the secondary metabolite 'marker compound' violacein
- We could use a significant published data set on bacterial species producing the substance to satisfy the data requirements
- How useful was new search to US EPA process?
  - Literature review alone not required unless tied to a discussion about a certain data requirement
  - Specific guideline-by-guideline rationales that discuss relevant published literature references are needed

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Dossier features for the EU – MBI-203

- Proposal for active substance definition
  - The active substance is comprised of bacterial cells and naturally occurring substances of biological origin that form a characteristic chromatographic profile when analysed by HPLC
- Extensive metabolite research and characterisation work conducted
- Detailed fractionation/bioassay work was done to elucidate mode of action of fermentate components
- Marker compound violacein occurs at very low levels <0.1% in the active substance

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Dossier features for the EU – MBI-203

- Relevance of test material
  - We demonstrate commercially relevant test material in each phase of GLP toxicology and ecotoxicology studies
  - Transparent presentation of meticulous QA data on batches
  - Studies exposed test system to secondary metabolite at relevant levels
- Robust genotoxicity package
  - Suite of three negative studies
  - Data point 5.2.3
    "Genotoxicity of cellular micro-organisms will be studied after breaking of the cells, wherever possible. Justification should be provided on the method of sample preparation used".
    Various methods for cell breaking were tested to meet this requirement
- Storage stability study includes analysis for secondary metabolite in parallel with confirmation of retention of activity by bioassay

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Lessons Learned to Date

MY LESSONS LEARNED TO DATE	
Opportunities	**Challenges**
Regulatory agencies have been collaborative in adapting regulatory paradigm to accommodate novel products;	While exciting, innovation can be challenging and frustrating; *metabolites have always been present in biopesticides*; over-regulation can (1) stifle move to lower risk tools, (2) stifle innovation, (3) push industry to look for loopholes
Microbial-botanical hybrid appears to be emerging framework—identity, MOA and pathogenicity addressed with microbial underpinning; botanical guidance for mammalian and NTO testing;	Shortage of clear guidance; still a tendency to apply synthetic chemical criteria/protocols in many instances, though not always applicable or appropriate;
Killed microbe allows access to markets where non-native microbes have been of concern, e.g. Brazil, South Africa, Australia, New Zealand;	Mode of action and product identification still remain significant challenges, e.g. lack of CFUs;
Review and approval timelines shorten with each review; both MBI and regulatory agencies are learning and implementing based on growing experience.	New area of innovation; expertise still developing—patience, reason and flexibility is essential for all parties. We'll only learn by doing.

Marrone Bio Innovations

Thank You

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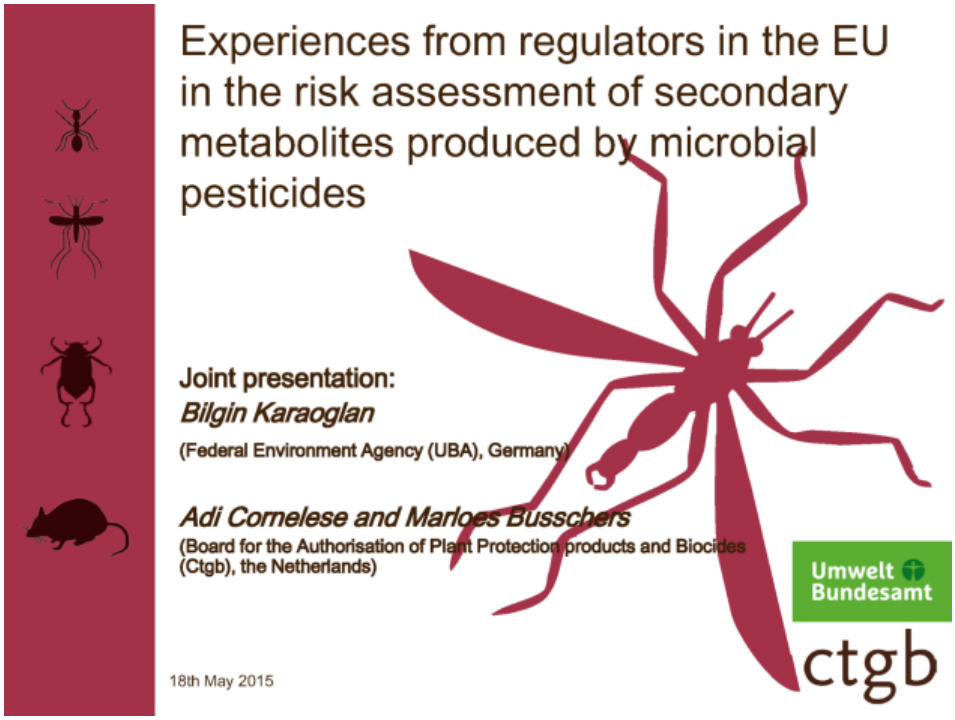
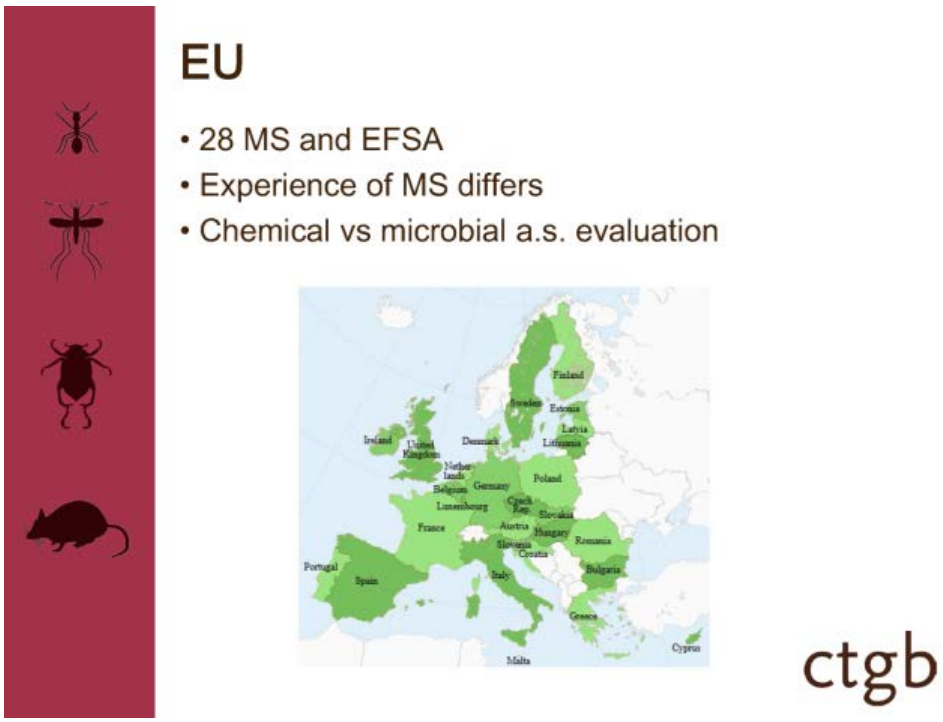
Presentation 9

Experiences from regulators in the EU in the risk assessment of secondary metabolites produced by microbial pesticides

Bilgin Karaoglan (Federal Environment Agency (UBA), Dessau-Rosslau; Germany) and Adi Cornelese and Marloes Busschers (Board for the Authorisation of Plant Protection products and Biocides (Ctgb), Wageningen; The Netherlands)





Reservations or doubts in EU MS and EFSA

- M.o. are living organisms
- M.o. can produce many metabolites
- Metabolites can be relevant toxins
- Dependent on strain, (environmental) conditions and target organism
- Present in formulation or involved in MoA/produces upon infection
- Only minimal amount of study data
- Strict interpretation of data requirements and decision making criteria

ctgb

Problems in the discussion

- Impossible to address all possible metabolites:
  - Differ from lab – field
  - Differ from field to field (environmental condition, target organism)
  - How to identify/quantify?
  - How to prove non-existence?
- What level of evidence is needed?
- Needle in hay stack

ctgb

Definition

Relevant metabolite

if expected to be of concern to human health and/or the environment

- If plant protection action is known to be due to the **residual effect** of toxin/metabolite, or
- if **significant residues** of toxins/metabolites are to be expected not related to the effect of the active substance

> Chemical dossier for toxin/metabolite must be submitted

ctgb

Pragmatic interpretation

Level of evidence needed
- Biology and MoA
- Information literature on (related) species/strain
- (Eco)tox study results
- External, not e.g. endotoxin of Bt

- Relevant metabolites?
- Exposure/risk

ctgb

546/2011 Uniform principles

B. Evaluation

Micro-organisms may produce a range of different metabolites (e.g. bacterial toxins or mycotoxins) many of which may have toxicological significance, and one or more of which may be involved in the mode of action of the plant protection product. The characterisation and identification of relevant metabolites must be assessed and the toxicity of these metabolites must be addressed.

Information on production and/or relevance of metabolites may be deduced from:

(a) toxicity studies;

(b) biological properties of the micro-organism;

(c) relationship to known plant, animal or human pathogens;

(d) mode of action;

(e) analytical methods.

On the basis of this information, metabolites may be considered as possibly being relevant. Therefore potential exposure to these metabolites must be assessed, in order to decide on their relevance.

ctgb

Secondary metabolites

- Production process:
  - ›If present in the product than only in liquid fermentation.
  - ›Range and quantities depend on culture medium
- Leading in mode of action and produced upon infection

✓ If present in fermentation it may be identified and quantified, however range and quantities depend on culture medium

✓ Secondary metabolites involved in MoA cannot be easily identified nor quantified

ctgb

Environmental risk assessment

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

- Relevant metabolite stable outside m.o.
- Toxic effect of metabolite is independent of presence of m.o.
- Relevant metabolite is expected to occur in environment in concentrations considerably higher than under natural conditions

ctgb

Environmental assessment

- Secondary metabolites that are part of natural metabolic systems are expected to degrade rapidly
- all conditions, as described in data requirements, are not likely to occur at the same time

ctgb

Examples of EU review

- *Bacillus pumilus* QST 2808
- *Streptomyces lydicus* strain WYEC 108
- *Metarhizium anisopliae* var. *anisopliae*

✓ dossier content on metabolites
✓ result EU review

ctgb

Bacillus pumilus QST 2808

- HPLC analysis of the fermentation broth was performed to prioritise potential active ingredients by comparing against a list of known compounds (various metabolites, potential toxins).
- The whole fermentation product plus any substances produced by the strain have been evaluated in the technical and end-use product toxicity testing schemes.

ctgb

Datagaps

- data to address the possible production of toxins/secondary metabolites (other than those produced by *Bacillus Cereus*); sections identity, (eco)tox and residues
- demonstrate that, under the conditions of use, any toxins/secondary metabolites produced by *Bacillus pumilus* QST 2808 will not occur in the environment in concentrations higher than under natural conditions

ctgb

Streptomyces lydicus strain WYEC 108

- Chemical components measured in fermentation broth.
- Known metabolites addressed in the dossier for human exposure. Extensive data search was done
- Research showed no mammalian toxins or active metabolites or degradation products of toxicological significance are known to be produced by *S. lydicus* strain WYEC 108.
- hydroxamate siderophores (plant growth enhancing), chitinase (plant enzyme) and some other enzymes.

ctgb

Datagaps

- strain specific evidence to prove the inability of the microorganism to produce toxins/secondary metabolites of concern.
- data on toxin/secondary metabolites production for the sections tox and residues
- Satisfactory information to demonstrate that, under the conditions of use, any toxins/secondary metabolites produced by *Streptomyces lydicus* WYEC108 will not occur in the environmental compartments in concentrations considerably higher than under natural conditions.

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Metharhizium anisopliae var. *anisopliae*

- EPF, subject of the RAFBCA project. Large dataset available. One workpackage focussed on metabolites.
- Identification and quantification in cultures by using external standards of cytochalasin (A, B, C, D, E, H, and J) and destruxins (A, B, CHL, and E diol).
- Information on potential swainsonine production
- Crude extracts and several purified toxins of different strains of *M. anisopliae* var. *anisopliae* were tested in two Ames tests and a Vitotox assay and gave negative results .

ctgb

- Metabolites destruxins A, B and E were analysed in plant material after soil/foliar application, in soil including drainage water, and in insect cadavers.
- In insects only destruxin A,B and E
- stability and persistence of destruxins A, B and E was assessed under various environmental conditions.

ctgb

Datagaps

- the production of toxins cannot be excluded and therefore the risk assessment cannot be finalised for humans.
- As the issue of toxins is not fully addressed the consumer risk assessment remains open.
- To assess the production and the fate and behaviour of any relevant metabolite in the environment. Information on the fate of destruxins is in the dossier however, a datagap is set for groundwater.

ctgb

RMS approach

- adequate literature research
- Biology and MoA
- Tested in tox batches?
- Metabolites involved in MoA?
- Potential for toxicological relevance?
- Measurement of potentially relevant metabolites in fermentation product ?
- Significant amount in product and/or environment?

If addressed properly in dossier, risk assessment can be completed

ctgb

DE RMS for several MPCAs with secondary metabolite production ability:

MPCA	Product / Type	old (List 4) / new (NAS) Substance	EFSA Conclusion
Bacillus subtilis QST 713	Serenade WP	NAS (Annex I Renewal)	Review Report (2006); (EFSA Conclusion not available)
Bacillus amyloliquefaciens D747 *	CX 9030 (WG)	NAS	EFSA Journal 2014;12(4):3624
Beauveria bassiana ATTC 74040	Naturalis-L (OD)	List 4	EFSA Journal 2013;11(1):3031
Beauveria bassiana GHA	BotaniGard 22 WP	List 4	EFSA Journal 2013;11(1):3031

* strain D747 formerly assigned to the species *B. subtilis*

Abbreviations: MPCA= Microbial Pest Control Agent; NAS = New Active Substance

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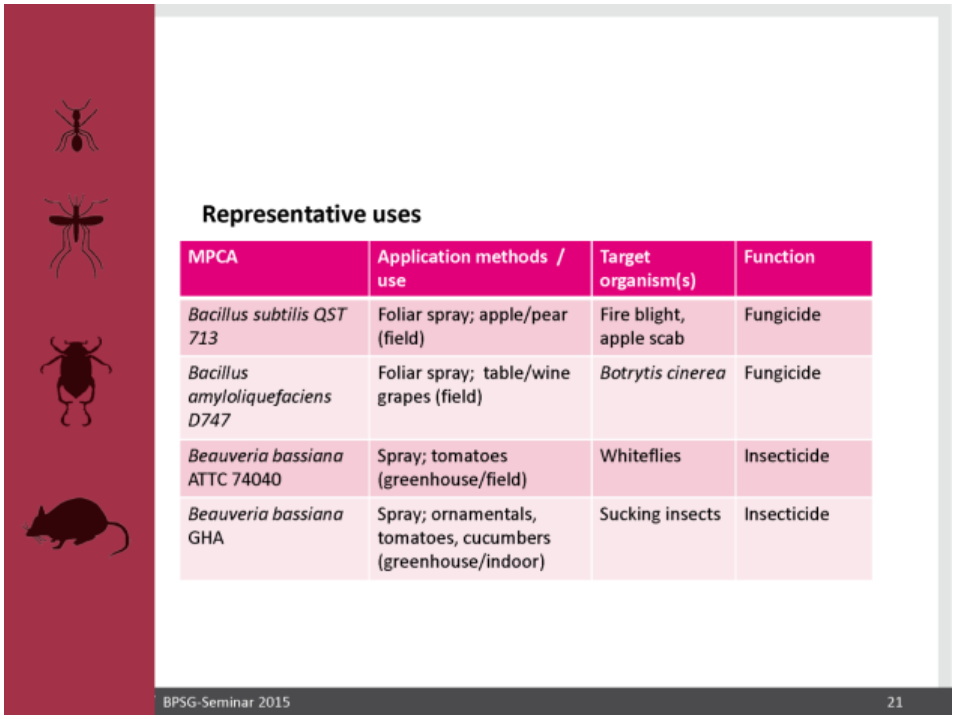

Representative uses

MPCA	Application methods / use	Target organism(s)	Function
Bacillus subtilis QST 713	Foliar spray; apple/pear (field)	Fire blight, apple scab	Fungicide
Bacillus amyloliquefaciens D747	Foliar spray; table/wine grapes (field)	*Botrytis cinerea*	Fungicide
Beauveria bassiana ATTC 74040	Spray; tomatoes (greenhouse/field)	Whiteflies	Insecticide
Beauveria bassiana GHA	Spray; ornamentals, tomatoes, cucumbers (greenhouse/indoor)	Sucking insects	Insecticide

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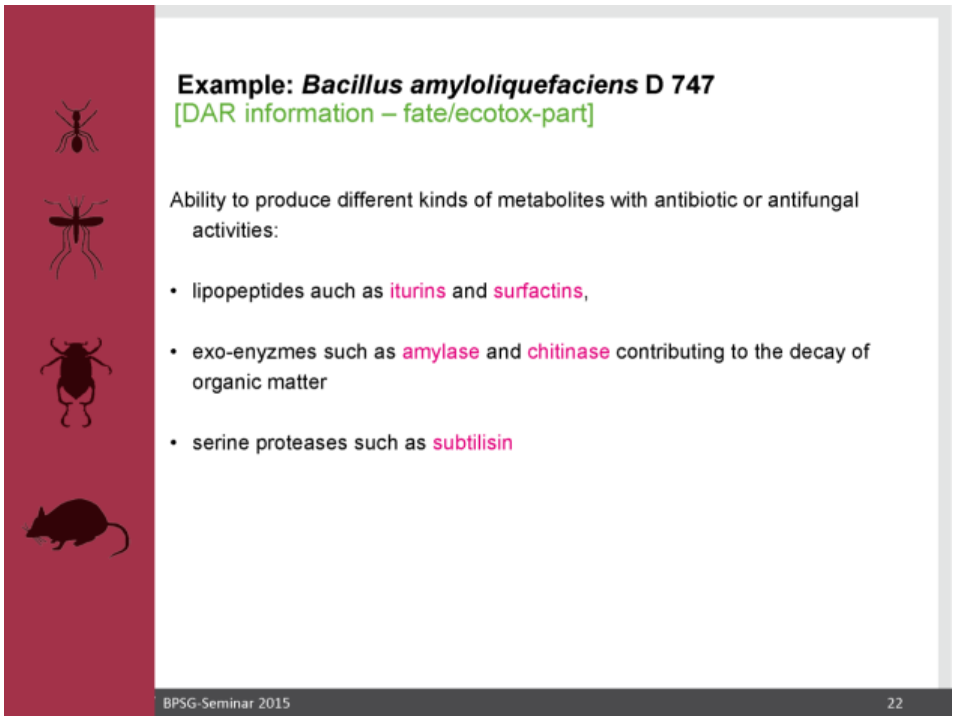

Example: *Bacillus amyloliquefaciens* D 747

[DAR information – fate/ecotox-part]

Ability to produce different kinds of metabolites with antibiotic or antifungal activities:

- lipopeptides auch as iturins and surfactins,
- exo-enyzmes such as amylase and chitinase contributing to the decay of organic matter
- serine proteases such as subtilisin

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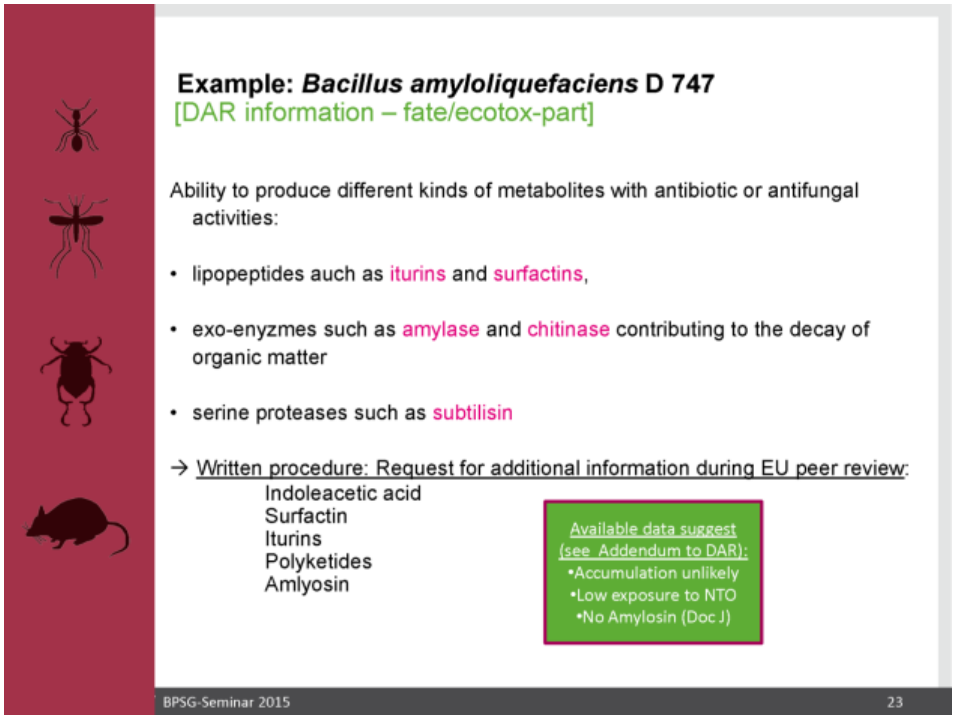
Example: Bacillus amyloliquefaciens D 747
[DAR information – fate/ecotox-part]
Ability to produce different kinds of metabolites with antibiotic or antifungal activities:
• lipopeptides auch as iturins and surfactins,
• exo-enyzmes such as amylase and chitinase contributing to the decay of organic matter
• serine proteases such as subtilisin
→ Written procedure: Request for additional information during EU peer review:
Indoleacetic acid
Surfactin
Iturins
Polyketides
Amlyosin
Available data suggest (see Addendum to DAR):
•Accumulation unlikely
•Low exposure to NTO
•No Amylosin (Doc J)
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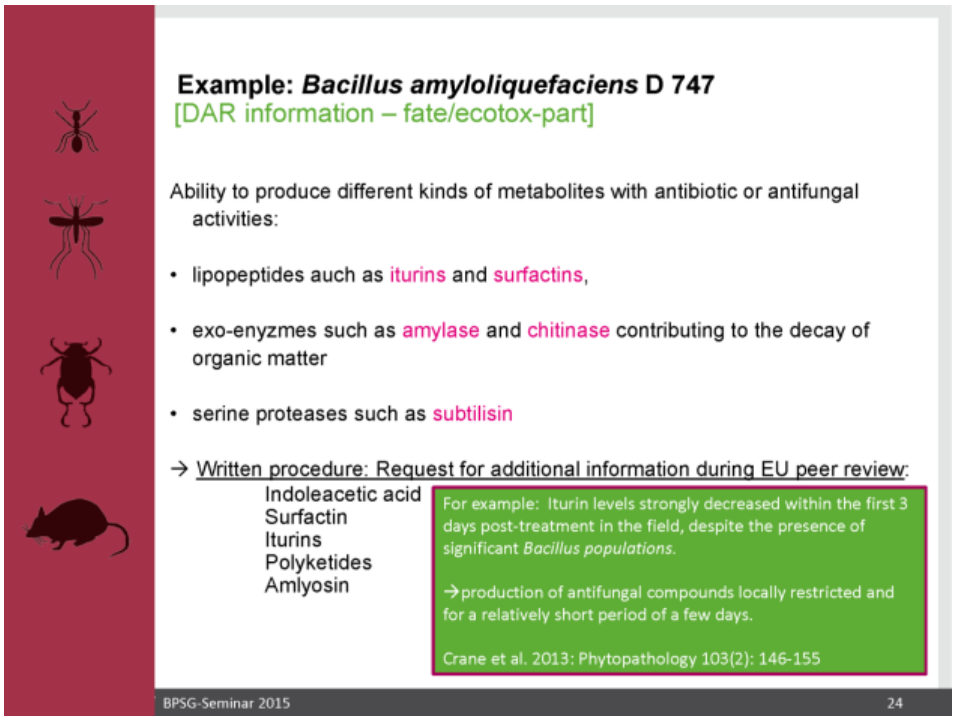
Example: Bacillus amyloliquefaciens D 747
[DAR information – fate/ecotox-part]
Ability to produce different kinds of metabolites with antibiotic or antifungal activities:
• lipopeptides auch as iturins and surfactins,
• exo-enyzmes such as amylase and chitinase contributing to the decay of organic matter
• serine proteases such as subtilisin
→ Written procedure: Request for additional information during EU peer review:
Indoleacetic acid
Surfactin
Iturins
Polyketides
Amlyosin
For example: Iturin levels strongly decreased within the first 3 days post-treatment in the field, despite the presence of significant Bacillus populations.
→production of antifungal compounds locally restricted and for a relatively short period of a few days.
Crane et al. 2013: Phytopathology 103(2): 146-155
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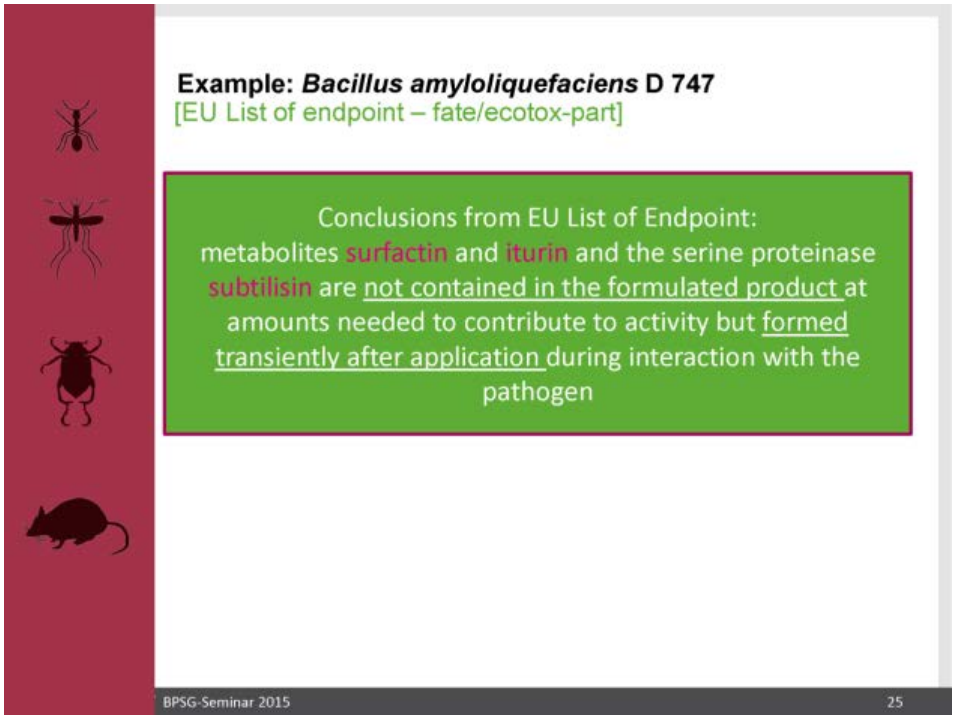
Example: Bacillus amyloliquefaciens D 747
[EU List of endpoint – fate/ecotox-part]
Conclusions from EU List of Endpoint:
metabolites surfactin and iturin and the serine proteinase subtilisin are not contained in the formulated product at amounts needed to contribute to activity but formed transiently after application during interaction with the pathogen
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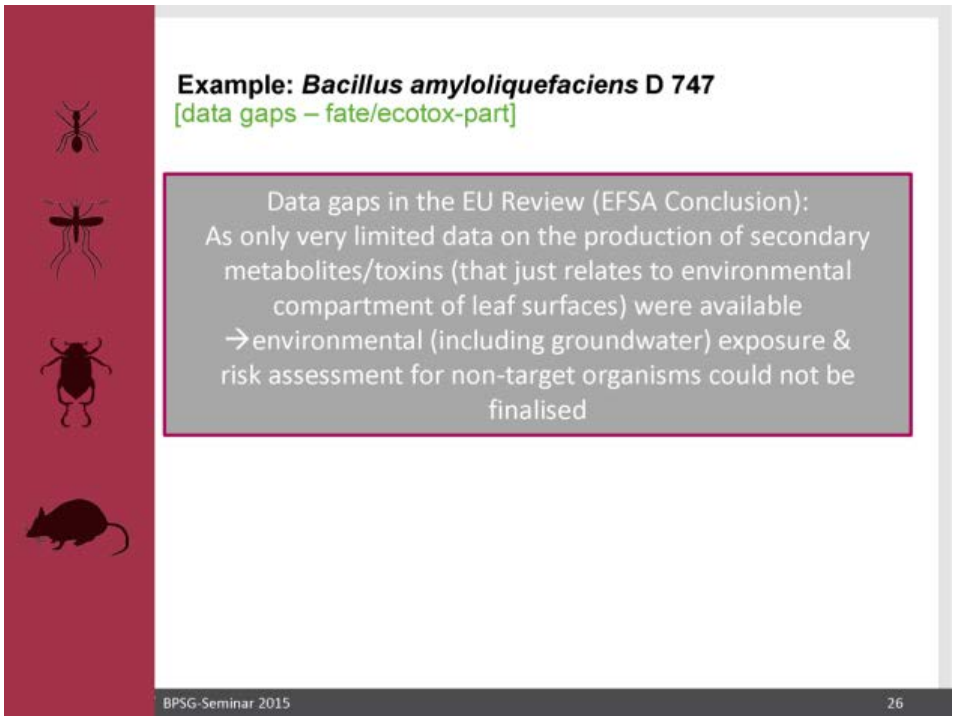
Example: Bacillus amyloliquefaciens D 747
[data gaps – fate/ecotox-part]
Data gaps in the EU Review (EFSA Conclusion):
As only very limited data on the production of secondary metabolites/toxins (that just relates to environmental compartment of leaf surfaces) were available
→environmental (including groundwater) exposure & risk assessment for non-target organisms could not be finalised
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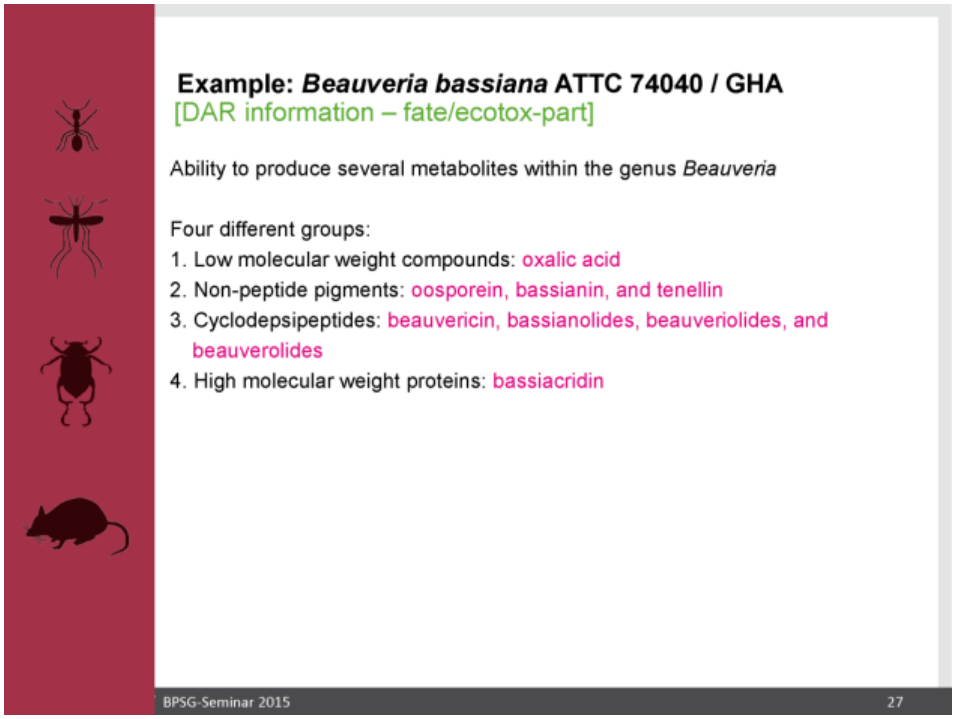
Example: Beauveria bassiana ATTC 74040 / GHA
[DAR information – fate/ecotox-part]
Ability to produce several metabolites within the genus Beauveria
Four different groups:
1. Low molecular weight compounds: oxalic acid
2. Non-peptide pigments: oosporein, bassianin, and tenellin
3. Cyclodepsipeptides: beauvericin, bassianolides, beauveriolides, and beauverolides
4. High molecular weight proteins: bassiacridin
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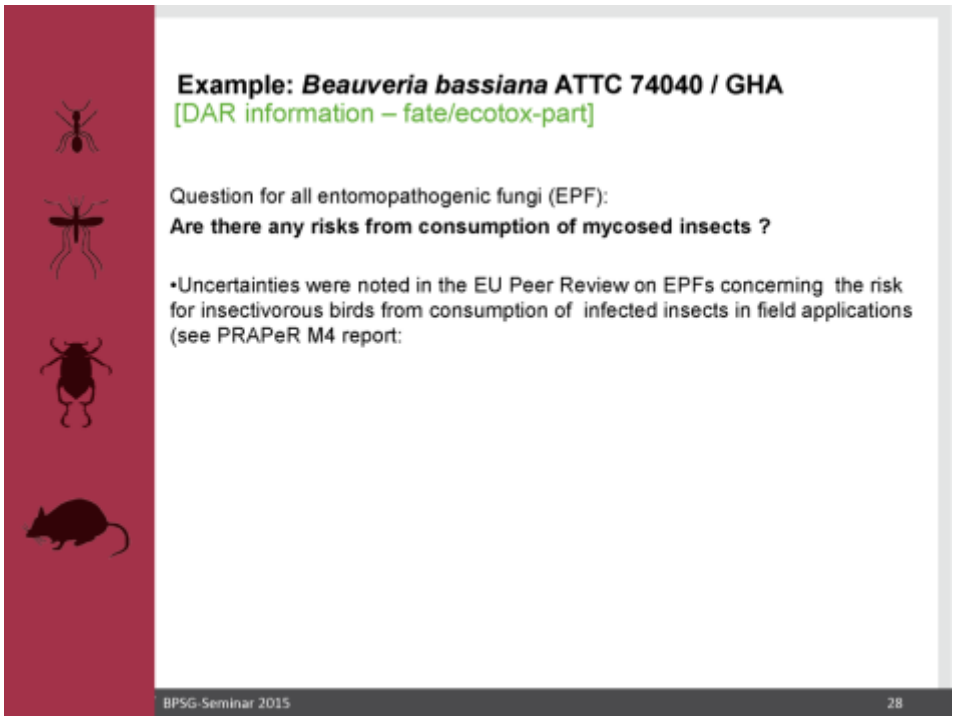
Example: Beauveria bassiana ATTC 74040 / GHA
[DAR information – fate/ecotox-part]
Question for all entomopathogenic fungi (EPF):
Are there any risks from consumption of mycosed insects ?
•Uncertainties were noted in the EU Peer Review on EPFs concerning the risk for insectivorous birds from consumption of infected insects in field applications (see PRAPeR M4 report:
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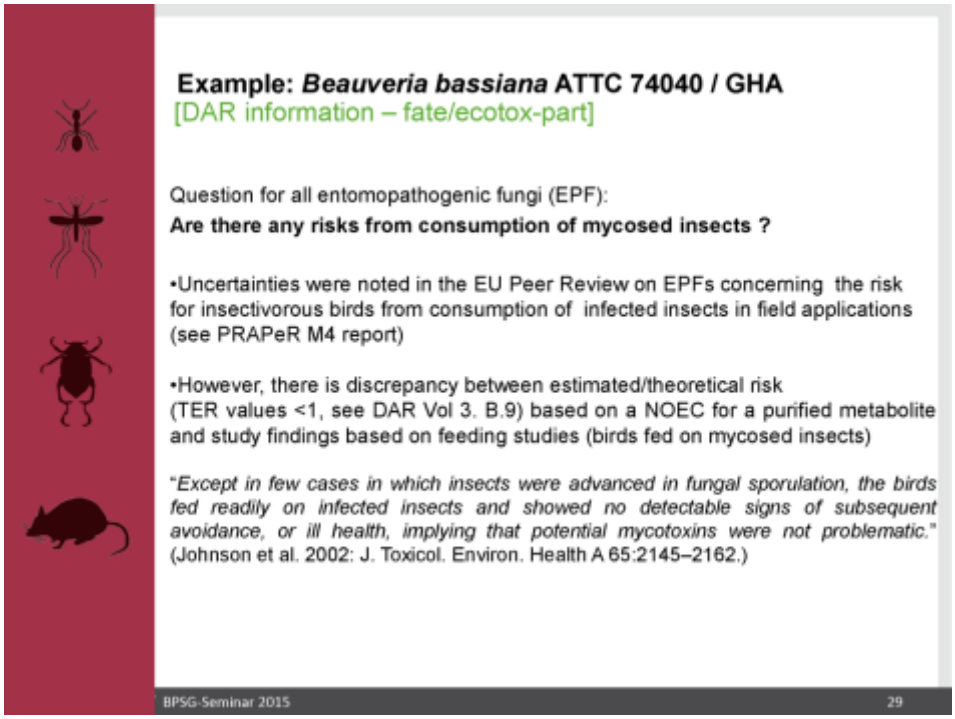
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•However, there is discrepancy between estimated/theoretical risk (TER values <1, see DAR Vol 3. B.9) based on a NOEC for a purified metabolite and study findings based on feeding studies (birds fed on mycosed insects)
"Except in few cases in which insects were advanced in fungal sporulation, the birds fed readily on infected insects and showed no detectable signs of subsequent avoidance, or ill health, implying that potential mycotoxins were not problematic." (Johnson et al. 2002: J. Toxicol. Environ. Health A 65:2145–2162.)
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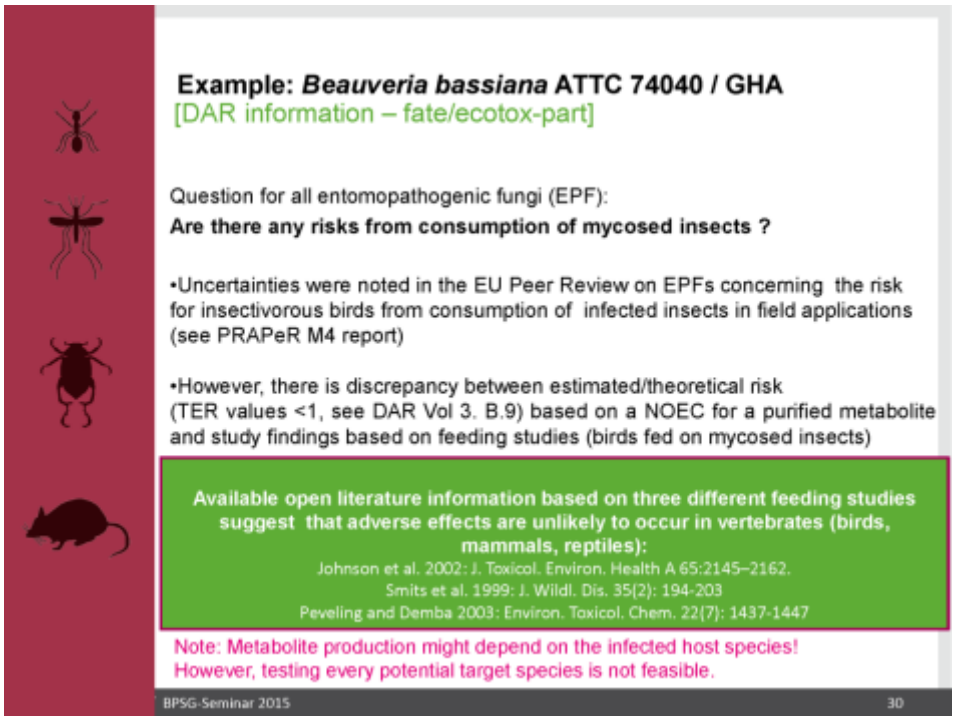
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Available open literature information based on three different feeding studies suggest that adverse effects are unlikely to occur in vertebrates (birds, mammals, reptiles):
Johnson et al. 2002: J. Toxicol. Environ. Health A 65:2145–2162.
Smits et al. 1999: J. Wildl. Dis. 35(2): 194-203
Peveling and Demba 2003: Environ. Toxicol. Chem. 22(7): 1437-1447
Note: Metabolite production might depend on the infected host species!
However, testing every potential target species is not feasible.
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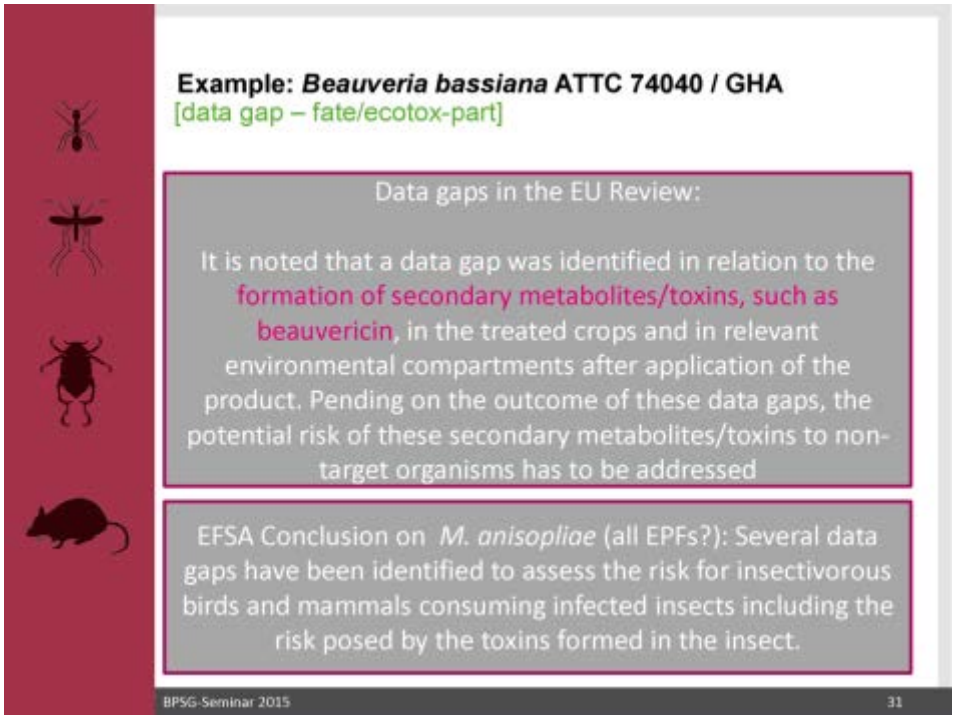
Example: Beauveria bassiana ATTC 74040 / GHA
[data gap – fate/ecotox-part]
Data gaps in the EU Review:
It is noted that a data gap was identified in relation to the formation of secondary metabolites/toxins, such as beauvericin, in the treated crops and in relevant environmental compartments after application of the product. Pending on the outcome of these data gaps, the potential risk of these secondary metabolites/toxins to non-target organisms has to be addressed
EFSA Conclusion on M. anisopliae (all EPFs?): Several data gaps have been identified to assess the risk for insectivorous birds and mammals consuming infected insects including the risk posed by the toxins formed in the insect.
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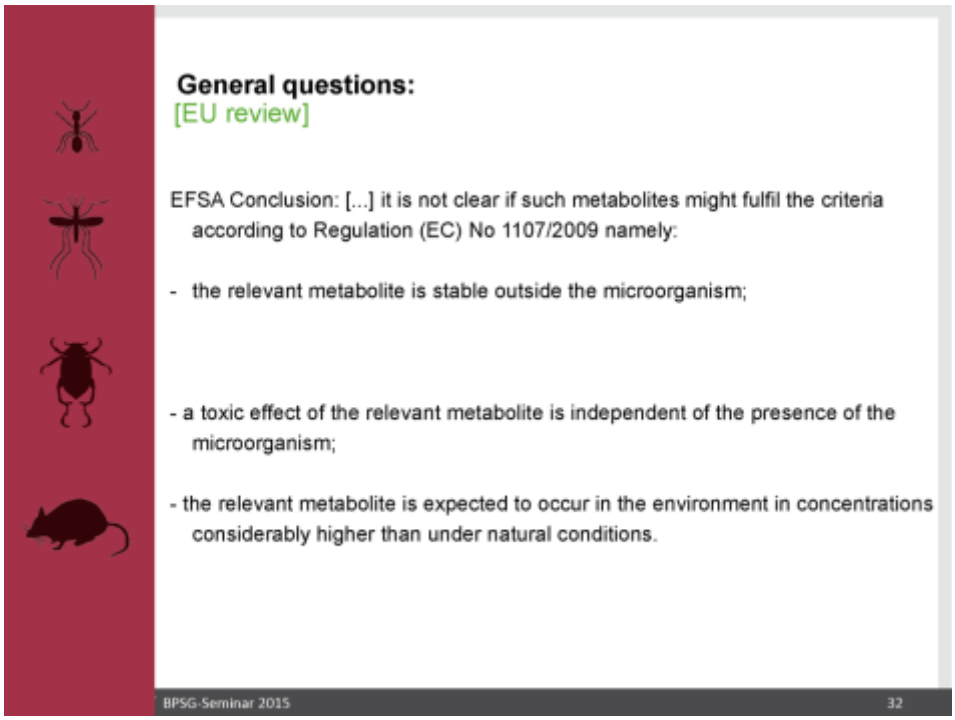
General questions:
[EU review]
EFSA Conclusion: [...] it is not clear if such metabolites might fulfil the criteria according to Regulation (EC) No 1107/2009 namely:
- the relevant metabolite is stable outside the microorganism;
- a toxic effect of the relevant metabolite is independent of the presence of the microorganism;
- the relevant metabolite is expected to occur in the environment in concentrations considerably higher than under natural conditions.
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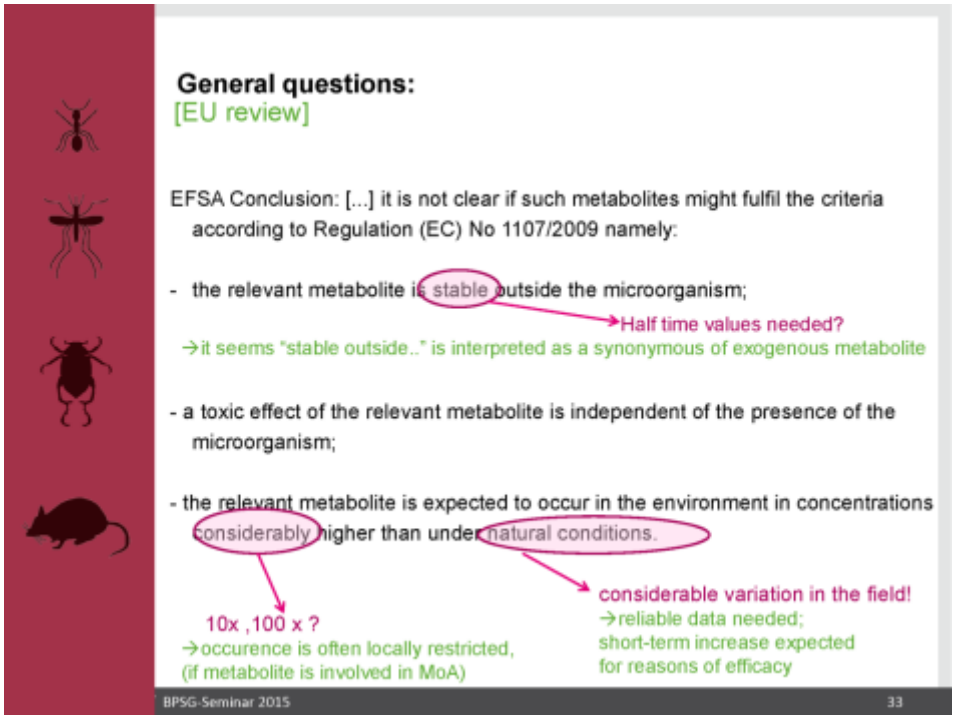
General questions:
[EU review]
EFSA Conclusion: [...] it is not clear if such metabolites might fulfil the criteria according to Regulation (EC) No 1107/2009 namely:
- the relevant metabolite is stable outside the microorganism;
Half time values needed?
→it seems "stable outside.." is interpreted as a synonymous of exogenous metabolite
- a toxic effect of the relevant metabolite is independent of the presence of the microorganism;
- the relevant metabolite is expected to occur in the environment in concentrations considerably higher than under natural conditions.
10x ,100 x ?
→occurence is often locally restricted, (if metabolite is involved in MoA)
considerable variation in the field!
→reliable data needed;
short-term increase expected for reasons of efficacy
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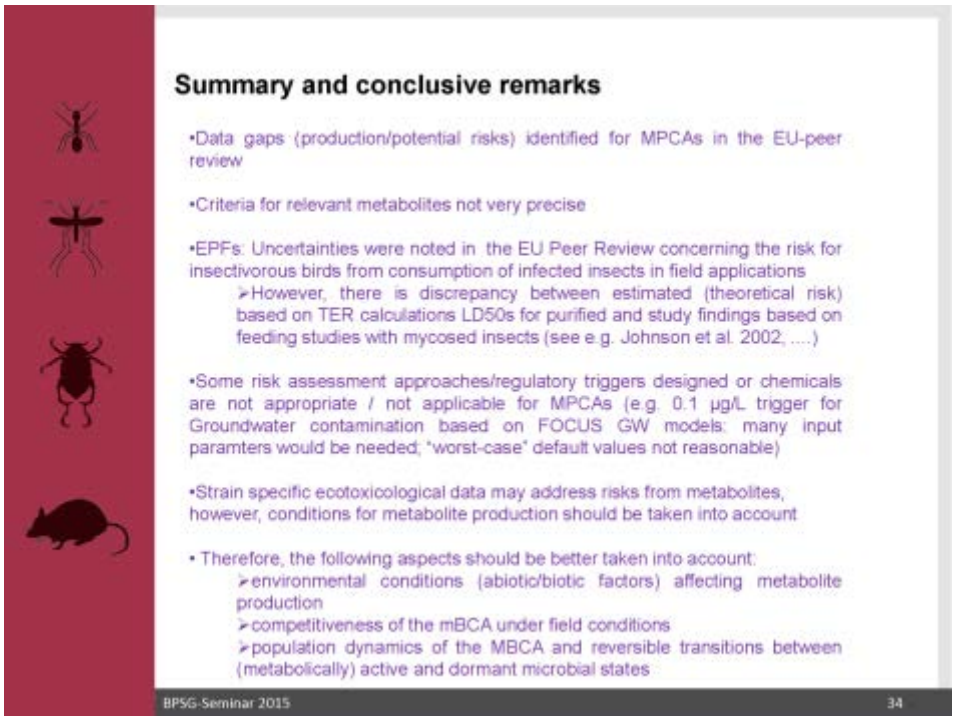
Summary and conclusive remarks
•Data gaps (production/potential risks) identified for MPCAs in the EU-peer review
•Criteria for relevant metabolites not very precise
•EPFs: Uncertainties were noted in the EU Peer Review concerning the risk for insectivorous birds from consumption of infected insects in field applications
➢However, there is discrepancy between estimated (theoretical risk) based on TER calculations LD50s for purified and study findings based on feeding studies with mycosed insects (see e.g. Johnson et al. 2002, ...)
•Some risk assessment approaches/regulatory triggers designed or chemicals are not appropriate / not applicable for MPCAs (e.g. 0.1 µg/L trigger for Groundwater contamination based on FOCUS GW models: many input paramters would be needed; "worst-case" default values not reasonable)
•Strain specific ecotoxicological data may address risks from metabolites, however, conditions for metabolite production should be taken into account
• Therefore, the following aspects should be better taken into account:
➢environmental conditions (abiotic/biotic factors) affecting metabolite production
➢competitiveness of the mBCA under field conditions
➢population dynamics of the MBCA and reversible transitions between (metabolically) active and dormant microbial states
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Presentation 10

Experiences from regulators in the USA in the risk assessment of secondary metabolites produced by microbial pesticides

Shannon Borges (Environmental Protection Agency, Washington, DC; United States)

Experience from Regulators in the USA in the Risk Assessment of Secondary Metabolites Produced by Microbial Pesticides: U.S. EPA Regulatory Perspective

May 18, 2015
6th Biopesticides Steering Group Seminar
Paris, France

Shannon Borges
Lead Biologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs

Secondary Metabolites of Microbial Pesticides

- Microbial pesticide defined in U.S. Code of Federal Regulations (CFR) at 40 CFR § 158.2100
- Secondary metabolite not officially defined
- Concern for risk assessment is on those compounds that might be toxic or hazardous; may be more appropriate to think in terms of toxin or hazardous compound produced by the microbe (see OCSPP guideline 885.0001)

Secondary Metabolites and Risk Assessment

- Hazard primarily considered in risk assessment
- Identification and description of known toxic compounds is required; emphasis is on testing to identify effects
- Data to indicate potential problems related to toxic compounds included in:
  - Product Analysis
  - Toxicology testing (mammals)
  - Nontarget organism testing

Product Analysis

- Data requirements in 40 CFR § 158.2120
- Product Identity (885.1300) – Description of microbial pesticide.
  - Must include description of properties with known or potential hazard
  - Describe mode of action if known
- Discussion of Formation of Unintentional Ingredients (885.1300)
  - Must describe formation of substances other than the microbial agent and intentionally added ingredients
  - Also, toxic or sensitizing compounds known or suspected to be present

Hazard Testing

- Emphasis is on effects testing for the risk assessment
- Tiered testing system
- Tier I toxicity/pathogenicity testing believed to be sufficiently designed to detect acute toxicity of toxic components of microbial pest control agents
- If effects are observed in Tier I tests, then testing is done at higher tiers or using guidelines developed for chemical pesticides to refine hazard

Hazard Testing – Mammalian Toxicology

Tier I Toxicology Testing (40 CFR § 158.2140)

- Studies to evaluate toxicity and pathogenicity of microbial pesticide:
  - Acute oral toxicity/pathogenicity (885.3050)
  - Acute pulmonary toxicity/pathogenicity (885.3150)
  - Acute injection toxicity/pathogenicity (885.3200)
- Studies to evaluate toxicity of chemical components of microbial pesticide:
  - Acute oral toxicity (870.1100)
  - Acute dermal toxicity (870.1200)
  - Acute inhalation toxicity (870.1300)
  - Primary eye irritation (870.2400)
  - Primary dermal irritation (870.2500)
  - Reporting of hypersensitivity incidents (885.3400)

Hazard Testing – Mammalian Toxicology

- Toxic effects observed (in the absence of pathogenicity and infectivity) in Tier I trigger the need for Tier II acute oral testing with the toxic component
- If a full evaluation is needed, additional testing can be done employing other guidelines more specific to toxicity testing with chemical compounds (OCSPP series 870 guidelines)
- Unusual that this level of testing occurs

Testing to Determine Exposure - Mammals

- Residue testing according to 40 CFR § 158.2130 is required when:
  - The results of testing indicate the potential to cause adverse human health effects or the product characterization indicates that the microbial pesticide has a significant potential to produce a mammalian toxin, and
  - The use pattern is such that residues may be present in or on food or feed crops

Hazard Testing – Nontarget Organisms

Tier I toxicity/pathogenicity with nontargets (40 CFR § 158.2150)

- Avian oral toxicity (885.4050)
- Avian inhalation toxicity/pathogenicity (885.4100)
- Wild mammal toxicity/pathogenicity (885.4150)
- Freshwater fish toxicity/pathogenicity (885.4200)
- Freshwater invertebrate toxicity/pathogenicity (885.4240)
- Estuarine/Marine fish and invertebrate testing (885.4280)
- Nontarget plant testing (885.4300)
- Nontarget insect testing (885.4340)
- Honeybee testing (885.4380)

Hazard Testing – Nontarget Organisms

- If toxic effects are observed at Tier I, testing may advance to Tier II to characterize exposure
- Subchronic testing may be appropriate, which may alleviate the need for Tier II testing
- Higher tiers (Tiers III and IV) are more appropriate for investigating pathogenic effects. If toxicity is still a concern with testing at lower tiers, testing with other guidelines is more appropriate (OCSPP series 850 guidelines)
- Unusual that this level of testing occurs

Toxicity Concerns – How Are They Usually Handled?

- Limit exposure
  - Testing may indicate safe levels; batch level testing may be required
  - Testing on indicator organisms (e.g., *Bt* tolerance exemption)
  - Limit application methods or timing (e.g., *Myrothecium verrucaria*)
  - Limit uses (e.g., non-food only)
- May determine that the pesticide is more appropriately registered as a conventional pesticide

Emerging Regulatory Issues

- Role of manufacturing process
  - Importance of establishing equivalence
  - May require bridging studies
- Microbial pesticides not identified to species level
- "Killed" microbials and appropriate testing
- At what point are microbial toxins better handled as conventional pesticides

Links

- U.S. Code of Regulations
  www.ecfr.gov
- OCSPP Microbial Testing Guidelines
  www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series885.htm
- OCSPP Health Effects Test Guidelines (conventionals)
  www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series870.htm
- OCSPP Ecological Effects Test Guidelines (conventionals)
  www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series850.htm
- EPA Label Review Manual
  www2.epa.gov/pesticide-registration/label-review-manual

Questions?