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REPORT OF THE SECOND OECD BIOPESTICIDES STEERING GROUP SEMINAR ON THE FATE IN THE ENVIRONMENT OF MICROBIAL CONTROL AGENTS AND THEIR EFFECTS ON NON-TARGET ORGANISMS

Series on Pesticides
No. 64

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Series on Pesticides

No. 64

Report of the Second OECD BioPesticides Steering Group Seminar on the Fate in the Environment of Microbial Control Agents and their Effects on Non-Target Organisms

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
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FOREWORD

This report presents the outcomes of an OECD Seminar on biopesticide issues related to the fate in the environment of microbial control agents and their effects on non-target organisms, which took place on 19 May 2010 at OECD, in Paris, France. This Seminar was held back-to-back with the annual meeting of the BioPesticides Steering Group (BPSG), a sub-group of the OECD Working Group on Pesticides (WGP). The Seminar was the second one of a series of BPSG Seminars that focus on biopesticide-related issues of interest to OECD member countries' governments.

The Seminar was chaired by Jeroen Meeussen (the Netherlands), Chairman of the BPSG. It was jointly organised by the OECD BPSG and the COST 873 initiative (COST is an intergovernmental European framework for international cooperation between nationally-funded research activities. In particular, COST 873 is a large network of leading European and Mediterranean specialists in 22 countries working on bacterial diseases of all species of stone fruits and nuts). Forty-three experts from 12 countries and IBMA (International Biocontrol Manufacturers Association) participated in the Seminar. The list of participants is in Annex 2.

The objectives of the Seminar were to:

(i) identify key issues and challenges in the area of the fate in the environment of microbial control agents and their effect on non-target organisms (NTOs);

(ii) exchange information on national and international activities in the area concerned; and

(iii) make recommendations for further actions and/or possible activities for OECD and COST 873.

The Seminar consisted of presentations addressing the following topics: *Government Experience and Perspectives* and *Stakeholder Experience and Perspectives* (from research institutes), followed by a round-table discussion after each set of presentations. The Seminar participants' conclusions, observations and recommendations are included in the first part of this report. The Seminar Programme is presented in Annex 1. The abstracts of presentations are compiled in Annex 3, while presentations are provided in Annex 4.

The draft Seminar report was approved out-of-session by the Working Group on Pesticides by written procedure finishing on 1st June 2011.

This document is being published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology, which has agreed that it be unclassified and made available to the public.

TABLE OF CONTENTS

INTRODUCTION

1. This report presents the results and recommendations of an OECD Seminar on issues related to the *fate in the environment of microbial control agents and their effect on non-target organisms*. This one-day Seminar, held on 19 May 2010, was chaired by Jeroen Meeussen (the Netherlands), Chairman of the OECD BioPesticides Steering Group (BPSG), and took place at OECD, Paris, France.

2. This Seminar was the second in a series of Seminars on biopesticides organised by the OECD BioPesticides Steering Group (BPSG). This time it was jointly organised by the OECD BPSG and the COST 873 initiative. The BPSG is a sub-group of the OECD Working Group on Pesticides (WGP); COST is an intergovernmental European framework for international cooperation between nationally-funded research activities. In particular, COST 873 is a large network of leading European and Mediterranean specialists in 22 countries working on all bacterial diseases of all species of stone fruits and nuts in the European sphere.

3. BPSG Seminars focus on key issues on biopesticides of interest to OECD governments. "*Fate in the environment of microbial control agents and their effect on non-target organisms*" was selected as the topic of this Seminar considering its significance for the registration of biopesticides. The importance of the fate in the environment of microbial control agents, natural background levels and their effect on non-target organisms will be a topic of a future Working Document to be prepared by the BPSG. These issues were also highlighted in the Workshop on the Regulation of Biopesticides: Registration and Communication issues (OECD Series on Pesticides No. 44, 2009).

PARTICIPANTS

4. People attending the OECD Seminar included:

- members of the OECD Working Group on Pesticides and BioPesticides Steering Group;
- invited experts from key stakeholder groups such as industry (IBMA) and manufacturers of micro-organisms;
- COST 873 experts from research institutes; and
- regulators from governments.

A participant list is provided in Annex 2.

PURPOSE AND SCOPE OF THE SEMINAR

5. The main objectives of the Seminar included:

- to identify key issues and challenges in the area of the fate in the environment of microbial control agents and their effect on non-target organisms (NTOs);
- to provide updates of national and international activities and initiatives in the area of the fate in the environment of microbial control agents and their effect on NTOs;
- to exchange information on OECD countries' current activities in the area of the fate in the environment of microbial control agents and their effect on NTOs;
- to exchange information and needs between scientists and stakeholders in the framework of COST 873.
- to suggest and discuss options of further steps for OECD countries and key stakeholders in OECD and non-OECD countries to address the identified issues; and,
- to recommend possible further steps for OECD and COST 873.

6. In particular the following issues were discussed during the Seminar:

- Natural and released inoculum levels of microbials;
- Stability of microbial strains when released;
- Potential persistence and mobility, in particular in soil;
- Epidemiology of microbial control agents in the environment;
- Systems to control released microbial control agents in the environment; and
- Environmental safety evaluation and risk assessment of microbial control agents.

STRUCTURE OF THE SEMINAR

The Seminar programme is provided in Annex 1. Invited speakers included:

- International experts in this field;
- Government representatives;
- Representatives from industry (IBMA); and
- COST 873 representatives from research institutes.

7. Due to the diversity of issues addressed by the speakers, short discussions were held after each (set of) presentation(s).

SUMMARY OF PRESENTATIONS AND DISCUSSIONS

8. All abstracts and slides of presentations are presented in Annexes 3 and 4.

Introduction by the Seminar Chair, Jeroen Meeussen, The Netherlands

9. The Chair presented the purpose of the Seminar and outlined the structure of the day. He explained that the main focus of the Seminar was to discuss the release of microbes into the environment and issues surrounding persistence/mobility and possible effects on the environment. The Seminar would also consider the relationship with background levels and would include such issues as:

- Natural and release levels
- Persistence and mobility
- Epidemiology
- Systems to control the release
- Environmental safety

10. It was indicated that there would not be a discussion on methodology, invertebrates or secondary metabolites. Metabolites may be the topic of a future Seminar.

11. The Chair then explained the remit and work of the BPSG. The BPSG was established in 1999 and has produced a number of guidance documents mainly on microbials, but also on pheromones. The most recent document has been the Working Document on the Evaluation of Microbials (OECD Series on Pesticides No. 43, 2008). The document outlines working practice in countries but is a not mandatory guidance.

Introduction on the COST 873 initiative

12. Brion Duffy (Agroscope, Wädenswil; Switzerland) gave a presentation to outline the COST 873 initiative (www.cost873.ch) which is an open network framework initiative that anyone can join at anytime. COST 873 considers stone fruit and nut trees which are major and important economic crops across the world. The aim is to stimulate solid research results, coordinate stakeholders and provide training opportunities. This is achieved via courses, publications, disease fact sheets and scientific grants.

COST 973 has four main work areas:

- Diagnostics
- Disease prevention and epidemiology
- Tree host resistance and breeding
- Sustainable control strategies

Government Experience and Perspectives

13. OECD countries presented their views.

Germany

A proposal for an environmental safety evaluation of microbial biocontrol agents - decision tree for the aquatic and terrestrial compartment, by Bilgin Karaoglan (Federal Environment Agency (UBA), Dessau-Rosslau; Germany)

14. Microbials are often considered to be environmentally benign compared to chemicals but this does not mean there are no potential hazards. Examples were given such as effects of *Beauveria bassiana* and *Bacillus subtilis* on bumble bees and effects of *Bacillus thuringiensis kurstaki* and *B. subtilis* on earthworms under laboratory conditions. Therefore this highlights the need for pre-market consideration and regulation.

15. It was stressed that there is currently no internationally accepted guidance. The current proposal is based on the risk assessment scheme developed by Mensink (2005) but has integrated work of others and comments from BPSG member and risk assessors. An overview of the current version of the proposal was given. Background documents used for the German proposal were the EU Directives, US-EPA/OPPTS/Canada-PMRA guidelines, the outcome of the EU review programme, EFSA peer review and other scientific articles and reviews.

16. In response to a question as to whether the proposal is aimed at indigenous or non-indigenous agents, it was indicated that it was mostly aimed at indigenous. Therefore, it was suggested to be very conservative in particular when dealing with a very disturbed environment. This should be taken into account in the decision scheme at the "characterisation box".

17. It was suggested that the characterisation and identity issues can be considered at the pre-submission stage in order to establish the required course of action. It was supported that characterisation was a vital issue for the risk assessment (as are pre-submission meetings).

18. The difficulties of defining what is meant by "indigenous" were highlighted in terms of regions and whether it was at strain or species level. This is further complicated by taxonomy and name changes which make it difficult to establish the true position regarding characterisation.

Netherlands

Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil – non-target effects on soil micro-organisms, by Jacqueline Scheepmaker (National Institute of Public Health and the Environment (RIVM), Bilthoven; The Netherlands)

19. The rationale for undertaking the research was that reference is made in the EU Uniform Principles that approval cannot be given if a microbial is considered to be persistent. Therefore the question was raised as to what is considered to be persistent? How long?

20. The paper is based on entomopathogenic fungal BCAs. It does not address whether persistence is a problem as there is no consideration of effects data on NTOs.

21. Germany highlighted that some of the EU 4th List DARs indicated that there were effects on earthworms (although this may be at high dose). Therefore Germany considered that effects should be considered independently of whether an organism is persistent.

22. Industry suggested that it was no longer economically viable to produce products containing more than 10^6 cfus. Therefore it was unlikely to achieve the high levels.

Environmental risk assessment of microbial pesticides from a regulatory perspective, by Adi Cornelese (Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands)

23. A number of issues and inconsistencies with the EU data requirements and Uniform Principles were highlighted regarding persistence, 'relevant' metabolites and NTOs. These inconsistencies raise a number of questions and also present difficulties for EU Rapporteur Member States (RMS) when attempting to conclude on their assessments. This is further compounded by the lack of guidance in these areas.

24. The conclusion was that data requirements should be reviewed and revised, that suitable guidelines needed to be indicated and that consideration should be given to the need for exposure data.

25. UK highlighted that the difference between the EU and US systems appears to be that, the assumption in the US is that the majority of microbials are of low risk, whereas in the EU it seems to be that there is a need for this assumption to be confirmed before a conclusion can be drawn. Therefore, the UK asked how the EU could learn from this approach. The US indicated that the majority of microbial BCAs originally came from the environment and therefore this forms the initial thinking for this approach.

United States

US experiences and approach to assessing the environmental fate and NTO effects of microbial pest control agents, by William Schneider (BioPesticides Division, EPA, Arlington; USA)

26. Testing is based on four tiers:

Tier I - acute NTO testing
> Tier II – Environmental expression of the microbial
> Tier III – Mesocosm testing of NTO
> Tier IV – simulated or actual field testing with the microbial

27. It was highlighted that the US-EPA does not ask for testing on NT micro-organisms or earthworms. First, microbial communities are extremely variable and usually environmental populations represent the most competitive species that have adapted to their niche. So what would be a significant effect? Testing would warranted be on a case-by-case basis e.g. effect on beneficial root colonizers). Second, earthworms are extremely resistant to pathogens and have very active immune system, and there are no known pathogens of earthworms. This approach is in line with REBECA recommendations.

28. The assessment is based on hazard, exposure and risk. Exposure data is only required if toxicity concerns are identified. Exposure depends on population dynamics, infectivity, use, rates, persistence & mobility, degradation of toxins. Formal exposure analysis is rarely use. US-EPA clarified that effects are based on assuming that the exposure is at the estimated environmental concentration.

29. Denmark supported the view that earthworms are rarely affected by microbes.

30. Industry highlighted that the US system is more of a holistic approach rather than looking at separate compartments. It tends to take a top-down whole system approach rather than working up from the lower organisms. If there are no effects seen at the higher levels then it is assumed that the lower organisms are not being affected.

Italy

Italy's experiences and approach to assessing the environmental fate and NTO effects of microbial pest control agents, by Marco Nuti (University of Pisa; Italy)

31. Italy explained how they used the decision tree developed by Mensink et al (2007, Biocontrol Sci. Technol. 17, 3-20) when carrying out the RMS evaluation of 4 *Bt* strains for the EU review. It was highlighted that the EFSA Qualified Presumption of Safety (QPS) approach was considered not appropriate for PPPs. Italy also highlighted how the exposure was calculated for these BCAs, including the use of the model for predicted environmental density (PED) and summarised their experience regarding impact on NTOs.

32. IBMA suggested that for fate of BCAs it is probably more appropriate to refer to 'impact' rather than 'risk', as the use of the word risk itself implies that there is already risk associated with the BCA. This could create perception issues amongst the general public.

33. US-EPA highlighted that the use of any pesticide is often a trade-off as there will be some kind of effects. Hence, the wording of US requirements is that there are "no adverse effects" rather than "no effects".

Denmark

Fate of microbials in the environment – a structural model for explanation of the fate, by Niels Bohse Hendriksen (Department of Environmental Chemistry and Microbiology, Aarhus University; Denmark)

34. Denmark explained the structural model approach that they are developing for considering the fate of endospore formers BCAs (i.e. *Bt*). Research indicates that a number of factors can be associated with the fate of BCAs such as immigration, number of applications, growth, nutrient availability, rainfall and death (related to factors such as dessication, temperature, sun-light, predation etc).

Stakeholder Experience and Perspectives

35. Research institutes presented their views.

INRA France

Impact of MBCAs on soil micro-organisms and persistence (resilience) in soil, by Claude Alabouvette (Laboratoire de Recherches sur la Flore Pathogène dans le Sol, I.N.R.A., Dijon; France)

36. An explanation was provided on how the fate of a microbial can be followed in the environment using marker strains (e.g. mutants). However, the use of mutants or transformed strains is not permitted in the open environment. Therefore, a way to follow fate in the environment is to design a Specific Characterised Amplified Region (SCAR). It was concluded that that a soil-borne micro-organism re-introduced into a soil will survive but will not proliferate in the absence of any specific selection pressure.

37. The issue of effects on soil microbial communities was also discussed. It is considered that the introduction of a BCA will have minimal effect on the Carbon and Nitrogen cycles. The release of BCAs will have some effects on the soil microflora, but it is considered that these will always be transient. The concern should be no greater than for the use of manure or compost.

University of Innsbruck, Austria

New risk indicator model: an excellent tool to comparatively assess environmental risks posed by microbial and conventional pest control agents, by Hermann Strasser (Institute of Microbiology, University Innsbruck, Innsbruck; Austria)

38. An overview of the recently developed risk indicator model was provided. The model was based on *Metarhizium* and chlorpyrifos, and has five categories: persistence, dispersal potential, non-target range, direct effects and indirect effects. The model allows for comparison between products (conventional and biological).

39. Such an approach may be a useful tool for consideration under the new EU regulation on sustainable use because it has requirements for risk indicators and comparative assessment.

Graz University, Austria

Biological control agents: Interplay with rhizosphere communities and risk assessment, by Henry Müller (Graz University; Austria)

40. The effect on the rhizosphere was explained. Studies demonstrated that effects appear to be negligible and relatively short lived. Some organisms show synergistic effects.

41. It was indicated that there is potential for organisms from the rhizosphere to infect humans. The technique of using *Caenorhabditis elegans* as a possible technique to determine whether a BCA has potential to infect humans was presented. It was suggested that it could be used as a development screen to determine potential toxicity. However, it should not be used as a standalone test and it should rather be used in conjunction with other tests.

Agroscope, Wädenswil; Switzerland

Environmental fate of bacterial biocontrol agents applied to the phylosphere, by Brion Duffy (Agroscope, Wädenswil; Switzerland)

42. An overview of research on the fate of foliar-applied bacterial BCAs to flowering fruit trees was provided. The secondary colonisation tends to be limited and dispersal outside the treated area to be low. In flowering fruit trees it appears that the survival post-bloom on leaves and persistence on fruits is low. Recovery from the soil is limited. There are no observations of impact on target plant or any other plants, microflora, nematodes, honeybees and birds/mammals.

SUMMARY OF THE DISCUSSION, IDEAS FOR FOLLOW-UP, RECOMMENDATIONS FOR POSSIBLE FURTHER OECD WORK AND ACTIONS IN THE FRAMEWORK OF COST 873

43. A number of keys points were summarised as follows:

- The importance of holding pre-submission meetings was stressed.
- For NTOs it was reiterated that the decision scheme could be used.
- It was pointed out that there is a need to consider background levels but there are still issues to resolve around what methods can be used to quantify the levels. Some of the models discussed during the day might be used.
- There is a need to take a broader view when considering BCAs than there is with chemical pesticides, in particular to take into account their mode of action. Use can also be compared to effects from cultural techniques.
- It was noted that comparative assessment is part of the new EU regulation on plant protection products (EC 1107/2009/EC); in addition, establishing harmonised risk indicators is part of the Sustainable Use directive (2009/128/EC). Therefore, it was suggested that some of the risk indicator approaches may be developed for this area.
- It appears that for fate in the environment it is still very much a case-by-case basis when considering microbials and it is still difficult to have general guidance. Therefore there is still a need for a lot of expert judgement during the evaluation.
- It was commented that there are difficulties in being able to develop these work areas further as there are no fora to do so. Therefore, perhaps as suggested at last year's BPSG Seminar, an electronic forum can be used to exchange ideas. There is also a need for consideration of how to co-ordinate model development.
- It was requested whether a faster system could be developed and whether it is possible to achieve a system similar to the US system.

ANNEX 1:

BIOPESTICIDES STEERING GROUP/COST 873 INITIATIVE
SEMINAR ON THE FATE IN THE ENVIRONMENT OF MICROBIAL CONTROL AGENTS AND THEIR EFFECTS ON NON-TARGET ORGANISMS

19 May 2010, OECD, Paris, France

OECD

SEMINAR PROGRAMME

COST 873

Chair: Jeroen Meeussen, The Netherlands

9.00 – 9.45 [PPT 0a] [PPT 0b]	**Introduction** • Purpose and structure of the seminar • Tour de table to introduce participants • Presentation on the OECD and the work of OECD-BPSG *by Jeroen Meeussen, The Netherlands* • Presentation on the COST 873 initiative *by Brion Duffy (*Agroscope, Wädenswil; Switzerland)

9.45 - 10.15	**Government Experience and Perspectives**
	• OECD-countries will present their views:
[PPT 1]	- A proposal for an environmental safety evaluation of microbial biocontrol agents - decision tree for the aquatic and terrestrial compartment *Bilgin Karaoglan* (Federal Environment Agency (UBA), Dessau-Rosslau; Germany)
10.15 – 10.45	***Coffee break***
10.45 – 12.15 [PPT 2]	- Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil – non-target effects on soil micro-organisms *Jacqueline Scheepmaker* (National Institute of Public Health and the Environment (RIVM), Bilthoven; The Netherlands)
[PPT 3]	- US experiences and approach to assessing the environmental fate and NTO effects of microbial pest control agents *William Schneider* (BioPesticides Division, EPA, Arlington; USA)
[PPT 4]	- Environmental risk assessment of microbial pesticides from a regulatory perspective *Adi Cornelese* (Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands)
12.15 - 13.45	***Lunch break***
13.45 – 14.45 [PPT 5]	- Italy's experiences and approach to assessing the environmental fate and NTO effects of microbial pest control agents *Marco Nuti* (University of Pisa; Italy)
[PPT 6]	- Fate of microbials in the environment – a structural model for explanation of the fate *Niels Bohse Hendriksen* (Department of Environmental Chemistry and Microbiology, Aarhus University; Denmark)

14.45 – 15.30 [PPT 7] [PPT 8]	**Stakeholder Experience and Perspectives** • Research Institutes will present their views: - Joint presentation: - Impact of MBCAs on soil micro-organisms and persistence (resilience) in soil *Claude Alabouvette* (Laboratoire de Recherches sur la Flore Pathogène dans le Sol, I.N.R.A., Dijon; France) - New risk indicator model: an excellent tool to comparatively assess environmental risks posed by microbial and conventional pest control agents *Hermann Strasser* (Institute of Microbiology, University Innsbruck, Innsbruck; Austria)
15.30 – 16.00	**Coffee break**
16.00 – 17.15 [PPT 9] [PPT 10]	- Biological control agents: Interplay with rhizosphere communities and risk assessment *Henry Müller* (Graz University; Austria) - Environmental fate of bactarial biocontrol agents applied to the phylosphere *Brion Duffy (*Agroscope, Wädenswil; Switzerland)
17.15 – 17.30	**Summary of the Discussion, Ideas for Follow-up, Recommendations for possible further OECD work and actions in the framework of COST 873** **Discussion** • Natural and released inoculum levels of microbials; • Stability of microbial strains when released; • Potential persistence and mobility, in particular in soil; • Epidemiology of microbial control agents in the environment; • System to control released microbial control agents in the environment; • Environmental safety evaluation and risk assessment of microbial control agents. *Instead of presentations in the morning and a round table discussion in the afternoon it is proposed to have a short discussion after each (set of) presentation(s) due to the diversity of issues.*
17.30	End of the Seminar

ANNEX 2:
LIST OF PARTICIPANTS

BioPesticides Steering Group/COST 873 initiative
Seminar on the fate in the environment of microbial control agents and their effects on non-target organisms

19 May 2010, OECD, Paris, France

Australia/Australie	Dr. Vanessa BURGESS International Coordinator Regulatory Strategy and Compliance Program APVMA
	Mr. Gary FAN Senior Policy Advisor Agricultural and Veterinary Chemicals Section Australian Government Department of Agriculture, Fisheries and Forestry
Austria/Autriche	Mr. Wolfgang KRAEMER Austrian Agency for Health and Food Safety Spargelfeldstraße 191
	Hermann STRASSER Institute of Biotechnology University Innsbruck
	Henry MÜLLER Technical University Graz Institute of Environmental Biotechnolgie
Belgium/Belgique	M. Jérémy DENIS Service Pesticides & fertilizers FPS Public Health
Denmark/Danemark	Dr. Niels Bohse HENDRIKSEN Department of Environmental Chemistry and Microbiology National Environmental Research Institute

	Ms. Birte Fonnesbech VOGEL Ph.D. Chemical Engineer Ministry of the Environment Danish Environmental Protection Agency Pesticides and Genetechnology
France	Claude ALABOUVETTE Président du CES de l'AFSSA Produits phytosanitaires/ microorganismes INRA UMR Microbiologie du sol et de l'environnement
	Mme Anne DUVAL Direction générale de l'alimentation (DGAL) Sous-direction de la qualité et de la protection des végétaux (SDQPV) Ministère de l'alimentation, de l'agriculture et de la pêche Bureau de la réglementation et de la mise sur le m
	Stéphane JACQUES Chargé de mission Pesticides Bureau des Substances et Préparations Chimiques Direction Générale de la Prévention des Risques Service de la Prévention des Nuisances et de la Qualité de l'Environnement
Germany/Allemagne	Mr. Herbert KOEPP Head of unit Unit 204: EC Procedures Federal Office of Consumer Protection and Food Safety Department 2: Plant Protection Products
	Mr. Bilgin KARAOGLAN Environmental Risk Assessment and Management of Plant Protection Products, EU Active Substances Programme Federal Environment Agency (UBA)
	Mr. Johannes JEHLE Institute for Biological Control Julius Kühn-Institute
	Dr. Vera RITZ Chemicals Safety Federal Institute for Risk Assessment (BfR)
Italy/Italie	Mr. Marco NUTI Co-ordinator, Group of Nat. Experts Department of Crop Biology Ministry of Health (ITA) for Biopesticides and Biocides University of Pisa

Netherlands/Pays-Bas	Mr. Jeroen MEEUSSEN EU Co-ordinator Board for the Authorisation of Plant Protection Products and Biocides
	Mrs. Marloes BUSSCHERS toxicologist, human risk assessment Board for Authorization of Plant Protection Products and Biocides
	Ms. Adi CORNELESE Environmental risk assessment Board for the Authorization of Plant Protection Products and Biocides
	Dr. Jacqueline SCHEEPMAKER Risk Assessor Expertise Centre for Substances National Institute for Public Health and the Environment
Sweden/Suède	Ms. Kersti GUSTAFSSON Prinicpal Scientific Adviser Pesticides and Biotechnical Products Swedish Chemicals Agency
Switzerland/Suisse	Mr. Marco D'ALESSANDRO Section Biotechnology Federal Office for the Environemnt
	Dr. Brion DUFFY Plant Protection Division Agroscope Changins-Wädenswil ACW
United Kingdom/Royaume-Uni	Mr. John DALE Project Manager Chemicals Regulation Directorate Health and Safety Executive
	Andrew MASSEY Environmental Specialist Chemicals Regulation Directorate Health and Safety Executive (Operations)
	Steven DOBSON Project Manager Chemicals Regulation Directorate Health and Safety Executive

United States/États-Unis	Dr. William SCHNEIDER Biopesticides and Pollution Prevention Division (7511) US Environmental Protection Agency Office of Pesticide Programs/BPPD (7511C)
	Mr. Charles RANDOLPH Advisor for Education and Environment Permanent Delegation
	Mr. Robert SCHULTZ Information Management Specialist Information Resources and Services Divsion US Environmental Protection Agency
International Biocontrol Manufacturers Association (IBMA)	DE BRUYNE ROLAND Registration Department Koppert Biological Systems
	Mr. Bernard BLUM Head International Affairs International Biocontrol Manufacturers Association (IBMA) Agrometrix Integrated Crop Management
	David CARY Executive Director International Biocontrol Manufacturers' Association
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	Ms. Maria HERRERO Director of Regulatory Affairs Manager Valent BioSciences Corporation/ Sumitomo Chemicals
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	Mr. Peter LUETH Managing Director Prophyta Biologischer Pflanzenschutz GmbH
	Ms. Denise MUNDAY President IBMA SCAE-Valent BioSciences Sarl International Biocontrol Agent Manufacturers' Association
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	Patrick SUTEAU Environment Safety Manager Bayer Crop Science
	Marina NIEMI Verdera Oy
OECD/OCDE	Miss Charis FEENEY-ORCHARD Assistant ENV/EHS OECD

ANNEX 3:
ABSTRACTS OF PRESENTATIONS

Introduction: The OECD and the work of the OECD-BioPesticides Steering Group (BPSG)
By Jeroen Meeussen, Ctgb, Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands

A proposal for an environmental safety evaluation of microbial biocontrol agents - Decision tree for the aquatic and terrestrial compartment
By Bilgin Karaoglan, Federal Environment Agency (UBA), Dessau-Rosslau; Germany

Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil
By Jacqueline W.A. Scheepmaker, RIVM-SEC, National Institute of Public Health and the Environment-Expertise Centre for Substances, Bilthoven, The Netherlands

US Microbial Pesticide Environmental Risk Assessment
By William R. Schneider, Ph.D., Microbiologist, U.S. Environmental Protection Agency, BioPesticides and Pollution Prevention Division, Office of Pesticide Programs, Washington, D.C., USA

Environmental risk assessment of microbial pesticides from a regulatory perspective
By Adi Cornelese, Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands

Italy's experience and approach to the environmental fate and NTO effects of microbial pest control agents
By Marco Nuti, Stefano Cervelli, Elisabetta Rossi & Caterina Cristani, Università di Pisa, Italy

Fate of microbials in the environment - A structural model for explanation of the fate
By Niels Bohse Hendriksen, National Environmental Research Institute, Aarhus University, Denmark

Impact of MBCAs on soil micro-organisms and persistence (resilience) in soil
By Claude Alabouvette, INRA, UMR Microbiologie du Sol et de l'Environnement, Dijon, France

New risk indicator model: an excellent tool to comparatively assess environmental risks posed by microbial and conventional pest control agents
By Hermann Strasser, Institute of Microbiology, University Innsbruck, Innsbruck, Austria

Biological control agents: Interplay with rhizosphere communities and risk assessment
By Gabriele Berg / Henry Müller, Graz University; Austria

Introduction
The OECD and the work of the OECD-BioPesticides Steering Group (BPSG)

By Jeroen Meeussen

(*Ctgb, Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands)*

[PPT 0a]

In 1961 the Organisation for Economic Co-operation and Development (OECD) was established with a trans-Atlantic and then global reach. Today the OECD has 30 member countries. More than 70 developing and transition economies are engaged in working relationships with the OECD.

OECD is a forum in which governments work together to address the economic, social and environmental challenges of interdependence and globalisation. OECD is also a provider of comparative data, analysis and forecasts to underpin multilateral co-operation.

The OECD work on agricultural pesticides (i.e. chemical and biological 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 and reduce risks to human health and the environment resulting from their use. In support of these goals, the Pesticides Programme has undertaken work to:

- (i) identify and overcome obstacles to work-sharing;
- (ii) harmonise data requirements and test guidelines; and
- (iii)harmonise hazard/risk assessment approaches.

The BioPesticides Steering Group (BPSG) was established by the WGP in 1999 to help member countries harmonise the biological pesticides assessment and improve the efficiency of control procedures. Biological pesticides involve: microbials, pheromones and other semiochemicals, plant extracts (botanicals) and invertebrates as biological control agents. The BPSG has been chaired by Canada since its inception and by The Netherlands from mid 2005 onward. The first tasks of the BPSG consisted of:

(i) reviewing regulatory data requirements for three categories of biopesticides (microbials, pheromones and invertebrates); and

(ii) developing formats for dossiers and monographs for microbials, and pheromones and other semio-chemicals.

This was achieved in 2004 and resulted in several OECD-publications in the Series of Pesticides (No. 12, 2001; No. 18, 2003 and No. 21, 2004).

The BPSG then decided to concentrate its efforts on science issues that remain as barriers to harmonisation and work-sharing. This resulted in the preparation of a “working document” which does not provide 'mandatory' guidance but being essentially a set of examples/case studies aimed at helping the regulatory authorities. The document is titled: *“Working Document on the Evaluation of Microbials for Pest Control”* and has been published in OECD Series on Pesticides No. 43, 2008.

The report of the *Workshop on the Regulation of Biopesticides: Registration and Communication issues, 15 – 17 April 2008, EPA, Arlington, USA*, is the most recent publication of the work of the BPSG in the OECD Series on Pesticides (No. 44, 2009).

In 2009 the BPSG organised the first seminar -in a series of seminars- on *Identity and Characterisation of micro-organisms*. Publication of the report of this seminar is in preparation.

The 2nd seminar is titled: *The fate in the environment of microbial control agents and their effects on non-target organisms*. This topic was selected considering its significance for the registration of biopesticides.

The importance of natural and released inoculum levels of microbials; stability of microbial strains when released; potential persistence and mobility; epidemiology of microbial control agents in the environment; system to control released microbial control agents in the environment; environmental safety evaluation and risk assessment of microbial control agents is already stressed in previous mentioned OECD-publications. The objectives, scope and structure of the seminar are described in detail in the 'Seminar outline'.

A proposal for an environmental safety evaluation of microbial biocontrol agents - decision tree for the aquatic and terrestrial compartment

By Bilgin Karaoglan

(Federal Environment Agency (UBA), Dessau-Rosslau; Germany)

[PPT 1]

Microbial biocontrol agents (mBCA) are generally considered to be an environmentally benign control option compared to synthetic pesticides, however this does not mean they are entirely free of hazards to health and the environment. Examples are given such as effects of the genera *Beauveria* and *Bacillus* on bumblebees as well as effects of the genus *Bacillus* on earthworms under laboratory conditions. This demonstrates the need for pre-market safety evaluation. Due to the fact that no internationally accepted guidance is currently available the proposed "decision scheme" developed by J.W.A. Scheepmaker and B. Karaoglan in April 2010 shall aim to provide regulatory guidance on the environmental safety evaluation of mBCAs. The proposal is based on the risk assessment scheme by Mensink (2005) but has integrated work of others and comments from BPSG members and risk assessors. Background documents used for the current proposal are the EU Directives, US-EPA/OPPTS/Canada-PMRA guidelines, the outcome of the EU review programme, EFSA expert meetings and other scientific articles and reviews.

The decision scheme starts with the "characterisation" box, which can be regarded as a fundamental step in the risk assessment determining the choice of test methods, appropriate test species and possible waiver options. Basic information of the specific microorganism requested here should be obtained from data already available (e.g. published literature or non-published studies for registration application) such as taxonomy, natural occurrence, history of its use, host specificity, mode of action, fate and behaviour in the environment and capability to produce metabolites of potential concern. In addition, information on the temperature/growth-relationship of the specific mBCA should already give some indication on the possibility of infectivity or pathogenicity to endothermic vertebrates. In the next step information on the application type and use pattern will be analysed in order to determine the exposure to non-target groups and the extent of exposure. Moreover, the extent and duration of exposure depends on the potential for persistence and multiplication of the microorganism. On a case-by-case approach it should be evaluated whether the mBCA is likely to survive in different environmental compartments, and if so, whether the mBCA will persist in the environment in concentrations considerably higher than the natural background levels. In this respect, the literature review by Scheepmaker and Butt (2010) should be highlighted which provides useful data on natural background levels for three different species of entomopathogenic fungi and the persistence of introduced inocula. It is proposed that this study could be referred to in a waiver/statement to fill the data requirement for EPF for persistence in soil.

In the margin of safety approach worst-case PEC values are compared to effect values in the first tier level if a quantitative risk assessment is considered feasible. An acceptable margin of safety should be determined on a case-by-case basis. However, in cases where a refined risk assessment is needed or dose response relationships are not observed, a qualitative risk assessment approach may be deemed more appropriate.

References:

Mensink B.J.W.G. (2005): How to evaluate the environmental safety of microbial pest control products. Bilthoven, The Netherlands: RIVM. Report no. 10030A00. 62 pp. Confidential: no.

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

Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil

By Jacqueline W.A. Scheepmaker

(RIVM-SEC, National Institute of Public Health and the Environment-Expertise Centre for Substances, Bilthoven, The Netherlands)

[PPT 2]

This presentation is based on highlights from the publication:
Scheepmaker, J.W.A. and Butt, T.M. (2010) Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment in accordance with EU regulations. Biocontrol Science and Technology 20(5): 503-552

The fate of inoculum in the soil is a data requirement under EU regulation. It should be assessed whether the concentrations decline to background levels. As no guidance is present on how to determine background levels this desk study was initiated with focus on entomopathogenic fungi (EPF).

Information on natural background levels of *Metarhizium anisopliae*, *Beauveria bassiana* and *B. brongniartii* was collected. A calculation of the upper natural background level was proposed. This was done by calculation of the geometric mean of each individual study. Then the overall geometric mean with its 95th percentile was determined. This 95th percentile was determined to be the upper background level. Of all three species this was approximately 1000 CFU/g soil.

Information gathered on the decline of applied inoculum showed that the upper background levels are reached within one year by *B. bassiana*, after >4 y by *B. brongniartii* and after >10 y by *M. anisopliae.* Decrease of inoculum can be explained by a wide array of edaphic, biotic, climatic factors and by agricultural practices.

Further it was shown that increase of inoculum only occurs in the presence of high densities of insect hosts or after renewal of the inoculum by repetition of applications. Uncontrolled growth certainly does not occur.

The question whether slow decline to upper background levels is a problem is directly related to the data requirement of effect on non-target organisms in the soil. This topic was not dealt within this publication. However, no long term adverse effects have been shown for earthworms or soil micro-organisms. It is concluded that in case of absence of non-target effects, slow decline of inoculum is not a problem.

US Microbial Pesticide Environmental Risk Assessment

By William R. Schneider, Ph.D., Microbiologist

*(U.S. Environmental Protection Agency,
BioPesticides and Pollution Prevention Division
Office of Pesticide Programs)*

[PPT 3]

The first microbial pesticide, *Bacillus popilliae,* was registered in 1949 by the US Department of Agriculture. The US Food and Drug Administration established the first tolerance (MRL) exemption which was for *Bacillus thuringiensis*. EPA Office of Pesticide Programs, after a lengthy development program involving contractors and researchers, published guidelines for microbial pesticide testing which were published in 1982. These guidelines were revised and republished under new Harmonized Guidelines numbers in 1995. These guidelines were designed to give guidance on how to conduct studies. The data requirements for microbial pesticides were first published in final form in 1984 and recently revised and updated. This revision was published in the Federal Register of October 26, 2007 (79FR61002). The microbial pesticide data requirements are available online at
http://www.gpoaccess.gov/cfr/index.html, in 40 CFR part 158.2100.
The guidelines are available online at
www.epa.gov/ocspp/pubs/frs/home/guidelin.htm in the 885 series.

To encourage the development of biological pesticides, a tier testing system was devised to ensure, to the best extent possible, that only the minimum data sufficient to make scientifically sound regulatory decisions will be required. Accordingly, a risk assessment is prepared using single species tests on a variety of representative test species to produce a worst case analysis. No further ecological testing is required if no non-target species appear to be adversely affected. These tests are performed using a single, maximum hazard dose, although multiple doses are required if signs of toxicity are seen. The test species are selected according to laboratory availability and relationship to target species and are observed for sufficient time to detect pathogenicity. Additional species may need to be tested if warranted by the characteristics of similar microorganisms or, conversely, data requirements may be waived if there is no reason to believe that particular species is at risk. Gross necropsy and culture are done on abnormal tissue if there are signs of infectivity or pathogenicity. If unacceptable non-target toxicity or pathogenicity are seen, environmental expression studies (Tier 2) are required to evaluate exposure. If the exposure studies indicate the possibility of long term exposure to the microbial pesticide, Tier 3 studies (Table 3) have been designed to evaluate the potential for subchronic and/or significant toxic effects. Tier 4 studies allow for simulated or actual field experiments.

Although we have this formal framework, and we feel it serves us well, in actual practice we are more flexible in our data requirements. There are two relevant paragraphs in the regulations, one allows for data waivers. The other allows us to ask for additional data if circumstances warrant. Product Analysis information on the identity of the microorganism allows us to tailor data requirements to each microbial pesticide on a case-by-case basis.

Environmental risk assessment of microbial pesticides from a regulatory perspective

By Adi Cornelese

(Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands)

[PPT 4]

During the EU peer review process of plant protection products containing micro organisms, a meeting among experts involved in risk assessment of plant protection products was organised. During this meeting discussions around interpretation of data requirements and Uniform Principles appeared time consuming and resulted in the setting of data gaps to the applicants aiming at EU registration of products.

In the EU regulation procedure two directives are relevant: Council Directive 91/414/EEC amended by 2001/36/EC Data requirements for micro organisms; and Annex VI to Council Directive 91/414/EEC amended by 2005/25/EC Uniform Principles as regards Plant Protection Products containing micro-organisms.

Fate in the environment

For the assessment of fate and behaviour in the environment a data requirement on viability, competitiveness and population dynamics is set. In the evaluation of data it is to be assessed what the potential for persistence and multiplication is. There needs to be sufficient information on persistence/competitiveness. Persistence in concentrations considerable higher than background is considered unacceptable unless the risk from accumulated plateau is acceptable. It is unclear how this should be interpreted. There is no need for half life studies as was discussed among experts.

A lot of discussion was on metabolites. It appears that throughout the directives different terminology is used. Most requirements consider so called relevant metabolites, these are metabolites that could be of concern for human health and/or the environment. However, to know if a metabolite can be of concern it should be assessed. To be able to assess, the metabolite has to be identified and quantified. It is well known that most micro-organism tend to produce metabolites, sometimes as part of their metabolic system. It is unclear if and how it is possible to characterise all metabolites prior to the assessment of their relevance.

Non-target organisms

In the dossiers and during discussions the usefulness of guideline studies with non-target organisms according to OECD was subject, as OECD guidelines are guidelines for testing of chemicals. Without adaptation these kinds of studies do not provide useful endpoints for micro-organisms. In the data requirements there is no reference to OPPTS microbial test guidelines though these may be more relevant. Another issue is the choice of the test organism. It is important that the most relevant test organism is used for testing. How to choose the most relevant organism? This can be an issue related to mode of action and or exposure.

The data requirements require information on toxicity infectivity and pathogenicity. Toxicity effects are mostly related to toxins or chemicals present in the formulation. Looking at infectivity is just the presence relevant or growth? Should we look at recovery as well in case of pathogenicity. What is a relevant effect and what is the relevant endpoint?

For some organisms it is not easy to determine the required endpoint. For small animals like bees and non-target arthropods testing of infectivity by looking at the m.o. inside the organism may not be feasible. Furthermore, if no authorisation shall be granted if the m.o. has a potential to infect and multiply in non-target arthropods this would mean no m.o. as insecticide can be registered.

The assessment of infectivity and pathogenicity is required unless it can be justified non target organisms will not be exposed. In the assessment of exposure survival, natural background level and information on fate and behaviour should be taken into account. During the EU peer review it appeared that it is considered essential to always quantify exposure. But how? Exposure models exist for chemicals can they be used? The EU review process considered the use of absolute worst case PEC values calculated similar to chemicals acceptable. If a refinement would be required no guidance or ideas on further approaches are defined. It should be questioned if risk mitigation approaches as for chemicals can be relevant. For exposure of NTO no worked out approaches to quantify are available.

Based on the questions posed on the DAR's as presented by member states and on discussions during the expert meeting, it appears there is a large difference in interpretation of the detailed requirements that are necessary to finalise risk assessments of micro-organisms adequately. It is recommended to review and revise the data requirements and Uniform Principles. If we are too strict to the letter of the directives PPP containing m.o. are hampered in their regulation. Suitable test guidelines for micro-organisms need to be indicated. If there is a need to quantify environmental exposure on different levels an agreed methodology should be developed.

Italy's experience and approach to the environmental fate and NTO effects of microbial pest control agents

By Marco Nuti, Stefano Cervelli, Elisabetta Rossi & Caterina Cristani

(Università di Pisa – DBPA & DPDSL - Italy)

[PPT 5]

Among the 40 microbials (active substances or Microbial Pest Control Agents, MPCA) included in Annex I of Dir. 91/414/EEC, of which 10 are still under evaluation, Italy was requested to prepare the draft assessment report (DAR) on four strains of *Bacillus thuringiensis*, i.e. *B.t.* subsp. *aizawai* 1857 and GC91, *B.t.* subsp. *tenebrionis* NB176, *B.t.* subsp. *israelensis* AM65-52. The MPCP (Microbial Pest Control Product) Agree 50WP, based on *Bta* GC-91, is active on the lepidopteran pest species *Lobesia botrana, Eupoecelis ambiguella*; XenTari WG, based on *Bta* ABTS1857, is active against the defoliating caterpillars *Chrysodeixis chalcites* (Northern EU), and *Heliothis plusia* or *Spodoptera* (Southern EU); VectoBac WG, based on *Bti* AM65-52, is active against *Dipteran* insects, particularly the fungus gnats (*Sciaridae*); Novodor SC, based on *Btte* NB176, is active against the foliar feeding Coleopteran beetle larvae *Leptinotarsa decemlineata.* The environmental risk assessment was done essentially in agreement with the decision tree proposed by Mensinck *et al* (Biocontrol Sci.& Tecnol., 17, 3-20, 2007), using as a metric the EED (Estimated Environmental Density) instead of PEC (Predicted Environmental Concentration) which is used for chemical pesticides; PEC can be still used when dealing with the insecticidal protein, although in the latter case the metric ITU (Insecticidal Thuringiensis Units) are more widely used . The above Plant Protection Products (PPPs) were not considered appropriate for the Qualified Presumption of Safety (QPS; EFSA J. 923, 1-48, 2008), and a model had to be developed to assess their environmental fate. The calculation have been done by using the software PEC-TWA_NEW_1_3_1, available on request (stefano.cervelli@ise.cnr.it), and the population densities have been calculated at time zero and following the last application at the highest suggested rate. The following assumptions were made: (a) there is degradation, (b) there is no adsorption, i.e. step-1 worst case scenario), (c) application is made to soil, (d) there is a drift to surface water at the distance of 3 m, in different % according to the crop, (e) there is a variable number of applications (f) there are various intervals between applications. Taking into account the different crops (grape, pepper, ornamental or potatoes), the recommended doses and whether the application is to field or indoor, the various application rates have been defined in terms of Kg/ha, cfu/ha and bio-potency ITU/ha of MPCP. From the very scarce literature data, and extrapolating for the various formulates, the degradation time in soil and surface water (DT_s and DT_{sw}) have been defined, while the number of treatment replications and the time of interval between treatments have been taken from the recommended usage. Then the rates have been re-calculated for each formulated product, in terms of both cfu/ha and ITU/ha, at the minimum and maximum application dosage. The Estimated Environmental Density in soil and water of each MPCP has been finally derived from the above data, taking into consideration the soil bulk density, soil thickness, the water depth, the distance spore drift to water, the drift percentage according to crop, and the application rates, after one single application and at the beginning of the last repeated application. The latter values range 1.05 x E05 – 1.89 x E06 cfu/g and 2.27 x E05 – 8.60 x E06 cfu/liter.

For the impact the MPCPs on non-target organisms, the following remarks can be made: (a) *Bt* is an "old" product, and there are many scientific papers on its environmental impact; this has caused confusion about the need for specific data at strain level, which are too often lacking; the above data cannot be extrapolated for endpoints to achieve a quantitative risk assessment, (b) in aquatic toxicology tests, turbidity due to high concentration of the product is probably the main cause for some negative effects observed on fish and Daphnia, (c) due to the specificity of Bt, the choice of particular groups of beneficial insects could be better used to describe effects on NTOs; in other words NTO testing is solely based on "surrogate species" (classic ecotoxicological approach), not on "ecologically relevant species" (field testing required), (d) based on literature review on effect assessment, the EFSA PPR Panel concluded that for soft bodied soil organisms (earthworms, enchytraeids, nematodes) and plants in close contact with the soil solution, pore water mediated uptake of pesticides seems mainly responsible for the effects caused, and would therefore be the relevant metric for effects assessment, and consequently also for exposure assessment (EFSA Journal 922, 1-90, 2009): is this true for microbials ? (e) for a number of relevant soil taxa with different life and feeding strategies (e.g. mites and isopods), no information is available; for these organisms, additional routes of uptake (e.g. feed, contact with substrates in soil and litter) may need to be considered for terrestrial risk assessment; (f) soil microorganisms are generally overlooked during environmental risk assessment (SANCO/10329/2002); however, during the last decade consolidated molecular approaches have been developed and used in scientific and technical literature, enabling to monitor the shifts of soil microbiological profiles following the introduction of an exogenous microbial component. Therefore justification for the request of no data requirement, based on the claim that "no changes" have been detected in the past, cannot be accepted because the methods used (almost exclusively vital counts) were inadequate to reveal whether the "changes" occurred or not.

As far as the cumulative risk assessment is concerned, mandatory according to the EU Regulation 396/2005, it could be carried out by using different approaches, i.e. assessing the cumulative risk (more than one substance is present at the same time), or the aggregate risk (all exposure routes are considered), or through the probabilistic evaluation of exposure (use of distributions instead of punctual estimates).

Fate of microbials in the environment
A structural model for explanation of the fate

By Niels Bohse Hendriksen

(National Environmental Research Institute, Aarhus University, Denmark)

[PPT 6]

The structural explanation model focus on explaining the fate of microorganisms used as plant protection agents on plant surfaces, especially vegetables. *Bacillus thuringiensis* is used as an example of microorganisms used as the active ingredient of plant protection products; most of which are used on plants for the control of lepidopteran larvae.

The key factors of the model are that the population size of a certain microorganism on a plant surface is determined by immigration (arrival of viable propagules on the plant surface), growth (an increase in biomass or number of viable propagules through multiplication), emigration (physical loss or removal of viable propagules) and death. The use of microbial pest control agents is a special case of microbial populations on plants, where the immigration at certain time-points, the time of application, is very high (often 100-1000 times) compared to the natural level. *B. thuringiensis* is applied as endospores with a limited germination and accompanied growth on plant surfaces, so the growth is very limited. Emigration seems notably to be dependent on rainfall. Death of spores can, in general terms, be caused by desiccation, temperature, sunlight or predation, for *B. thuringiensis* endospores seems notable UV and some parts of the visible light to be affecting survival. From these considerations it is proposed, that the fate of *B. thuringiensis* applied as a pesticide on plant surfaces mainly are determined by the numbers applied, death caused by sunlight and rain-off. The exposure of the microorganism for sunlight is dependent on whether they are directly or indirectly exposed for the light, which are dependent on the morphology of the plant species, and to some extent on plant age and coverage. Further the light exposure is, in general, dependent on sun-hours, latitude, time of the year, height above water level, shadow and reflections and might also be influenced by the formulation of the specific pesticide. The emigration, notably affected by rain, is dependent on the number of showers, their duration and the amount of rain, and might also be influenced by the formulation of the specific pesticide.

The proposed structural model has importance for 1) the design of experiments on the fate of microorganisms on plants, 2) the understanding and establishment of microbial residues on plants, 3) the understanding the efficacy of microbial pest control agents and 4) risk assessment.

This specific explanation model might give a basis for more general models for the fate of microorganisms used as pest control agents in terrestrial environments.

Impact of MBCAs on soil micro-organisms and persistence (resilience) in soil

By Claude Alabouvette

(INRA, UMR Microbiologie du Sol et de l'Environnement, Dijon, France)

[PPT 7]

Two basic questions have to be addressed before releasing a Microbial Bio-Control Agent: what will be its fate in the environment and what will be its impact on other organisms sharing the same ecological niche.

Behaviour of the MBCA is first studied in laboratory experiments then under conditions of the natural environmental. In the latter case, it is necessary to develop a tool enabling to trace the introduced MBCA among other strains belonging to the same species and naturally occurring in the environment. Indeed, in most cases, the MBCA already exists in soil, but at a density too low to be effective. To trace an introduced strain, the easiest approach is to use a mutant, resistant to an antibiotic or a fungicide. For example, an UV irradiated mutant of Fo47 resistant to Benomyl was used to study the population dynamics of this MBCA in two soils of different physico-chemical properties over one year (Edel-Herman et al., 2009). In the disinfested soils, this strain grew and established itself at a high population density whatever the dose of inoculation and the soil type. On the contrary, in the non-disinfested soil, the MBCA was not able to proliferate. It did not disappear but established at a population density lower than that at which it was introduced. These results illustrate the fact that a naturally occurring micro-organisms re-introduced in the environment from which it has been isolated neither disappear nor proliferate more than the native population (Edel-Herman et al., 2009).

This approach using antibiotic or fungicide resistant mutants can only be used in confined environment since it is not allowed to release mutants in the environment. The most elegant approach consists in designing a SCAR marker that will enable to trace the MBCA among other strains of the same species. This approach was used for the strain T1 of *Trichoderma atroviride*. The results of the population dynamics study in two soils of different physico-chemical properties were analogous to that obtained for Fo47. Indeed the strain T1 did neither disappear nor proliferate in the non disinfested soils (Cordier et al. 2007). Based on these results and on many other from the literature we can conclude that a soil-borne micro-organism re-introduced into a soil will survive but will not proliferate; it will become part of the native populations of the same species.

The impacts of MBCAs on non-target organisms can not be compared to that of chemicals, since most of the non-targets organisms have already been exposed to the natural micro-organisms developed as MBCA. However, it is still required to demonstrate that MBCAs have no deleterious effects on the non-target organisms, especially on the soil microflora. The soil microbial communities play very important roles in the ecosystem, but the soil microbiota are characterized by a redundancy of functions. Thus the functional characteristics of component species are as important as the number of species for maintenance of essential processes, such as nitrogen or carbon cycling. The use of molecular tools enabled to trace the presence of genes encoding for important functions and show that release of a relatively small quantity of a MBCA did not modify the soil functioning (Sessitsch et al., 2002).

Another family of methods enables to globally assess the impact of the introduction of a MBCA on the structure of the microbial communities. Results of such studies showed that even when an impact is detected shortly after introduction of the MBCA the structure of the microbial communities tended to come back quickly to their initial stage. After a few weeks there was no difference in the structures of the microbial communities between the infested soil and the non-infested control (Edel-Herman et al., 2009). Moreover, similar studies have shown that traditional agricultural practices have much more impact on the soil microbiota and the soil functions than release of a MBCA. It is especially the case of manure or compost amendment that releases millions of unknown micro-organisms.

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New risk indicator model: an excellent tool to comparatively assess environmental risks posed by microbial and conventional pest control agents

By Hermann Strasser

(Institute of Microbiology, University Innsbruck, Austria)

[PPT 8]

Several authors have suggested concepts to compare the health or environmental risks or hazards of conventional pesticides, but, unfortunately, the applicability of these tools to biological control agents (BCAs) is limited. The availability of a tool to objectively compare the impact of biological and conventional pesticides is desirable in light for the promotion of biological control options and the measurement of resulting risk reduction in agricultural use.

Based on two insecticides, the microbial biological control agent *Metarhizium anisopliae* and the conventional pesticide chlorpyrifos, the applicability of a newly developed and published risk indicator (RI) system is discussed (Längle & Strasser, 2010). The proposed model is based on five basic components for the calculation of the overall environmental risk score: persistence of the active ingredient, dispersal potential, range of non-target organisms that are affected, and direct and indirect effects on the ecosystem. One further category was implemented also to assess the risks to vertebrate non-target species.

In this presentation the strong points of this “handy tool” for pest control agent evaluation is discussed:

(i) the applicability to biological and conventional pesticides to allow a direct comparison between products,
(ii) the ability to score the risk on an application basis rather than on an active ingredient basis,
(iii) the flexibility of the system that permits the use of regulatory data or published literature, and
(iv) a readily understandable output.

We feel confident, that the proposed RI model can be used to communicate environmental risk and to design lower risk integrated pest management strategies. We recommend that the proposed risk indicator system may serve to define low risk and reduced risk pesticides. We believe that the RI system can help facilitate discussions between stakeholders and regulators regarding the regulatory approaches to microbial and other pest control agents. Yet, it remains debatable whether the RI will be useful in determining acceptability of data waivers for regulatory purposes.

Längle, T. & Strasser, H. (2010) Developing a risk index to comparatively assess environmental risks posed by microbial and conventional pest control agents. Biocont. Sci. Technol. 20 (7), 659-681.

Biological control agents
Interplay with rhizosphere communities and risk assessment

By Gabriele Berg / Henry Müller

(Graz University, Austria)

[PPT 9]

Gabriele Berg
Graz University of Technology, Department of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria, Gabriele.berg@tugraz.at

To use micro-organisms to control plant pathogens, to enhance plant growth and protect them against abiotic stress are environmentally friendly alternatives in agriculture. Although originating from plant-associated microenvironments themselves, beneficial bacteria, if applied to plants in adequate numbers, may perturb indigenous microbial populations and the important ecological functions associated therewith. Therefore, possible non-target effects of the applied antagonists on ecologically important soil-microbes need to be considered. Methods as well as examples from literature and own research are summarized. Regarding the latter a study, which analysed the effect of biological control agents (BCAs) on non-target microbes in the field will be presented. Whereas the bacterial BCAs *Serratia plymuthica* HRO-C48 (RhizoStar®) and *Streptomyces* sp. HRO-71 (RhizoVit®) were applied to control the pathogen *Verticillium dahliae* on strawberry and potato, the bacterial strains *Pseudomonas trivialis* 3Re2-7 (Salavida®), *P. fluorescens* L13-6-12, *S. plymuthica* 3Re4-18 and the fungal antagonists *Trichoderma reesei* G1/8 and *T. viride* G3/2 were introduced to control *Rhizoctonia solani* on lettuce and potato. As the analysed BCAs belong to different microbial groups like grampositive (HRO-71) and gramnegative (HRO-C48, L13-6-12, 3Re2-7, 3Re4-18) bacteria or the ascomycota (G1/8, G3/2) and originated from different micro-habitats like the rhizosphere or the endorhiza, general conclusion could be drawn from our results. After BCA treatment we did not observe any long-term effect on the plant-associated microbes in any tested pathosystem. This was confirmed by results from other studies. Therefore, no sustainable risks could be seen for the indigenous micro-organisms. In addition, it is necessary to assess the potential in human pathogenicity. For this procedure, an assay with the nematode *Caenorhabditis elegans* was developed. Our new findings may help to improve the development as well as the registration procedures of future microbial inoculants.

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ANNEX 4

PRESENTATIONS

Introduction: The OECD and the work of the OECD-BioPesticides Steering Group (BPSG)
By Jeroen Meeussen, Ctgb, Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands

Presentation on the COST 873 initiative
By Brion Duffy, Agroscope, Wädenswil, Switzerland

A proposal for an environmental safety evaluation of microbial biocontrol agents - Decision tree for the aquatic and terrestrial compartment
By Bilgin Karaoglan, Federal Environmental Agency (UBA), Dessau; Germany

Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil
By Jacqueline W.A. Scheepmaker, RIVM-SEC, National Institute of Public Health and the Environment-Expertise Centre for Substances, Bilthoven, The Netherlands

US Microbial Pesticide Environmental Risk Assessment
By William R. Schneider, Ph.D., Microbiologist, U.S. Environmental Protection Agency, BioPesticides and Pollution Prevention Division, Office of Pesticide Programs,Washington, D.C., USA

Environmental risk assessment of microbial pesticides from a regulatory perspective
By Adi Cornelese, Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands

Italy's experience and approach to the environmental fate and NTO effects of microbial pest control agents
By Marco Nuti, Stefano Cervelli, Elisabetta Rossi & Caterina Cristani, Università di Pisa, Italy

Fate of microbials in the environment - A structural model for explanation of the fate
By Niels Bohse Hendriksen, National Environmental Research Institute, Aarhus University, Denmark

Impact of MBCAs on soil micro-organisms and persistence (resilience) in soil
By Claude Alabouvette, INRA, UMR Microbiologie du Sol et de l'Environnement, Dijon, France

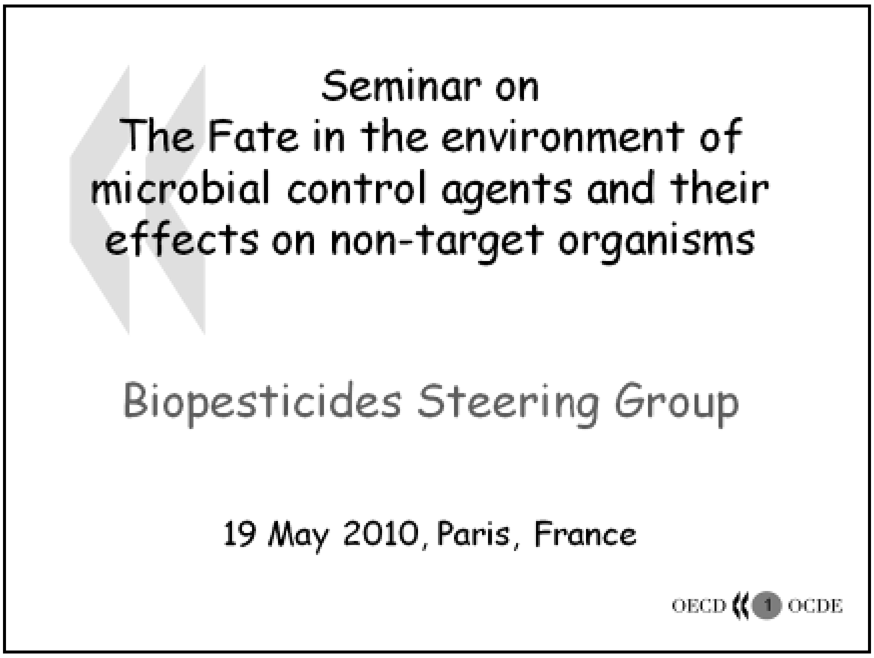
New risk indicator model: an excellent tool to comparatively assess environmental risks posed by microbial and conventional pest control agents
By Hermann Strasser, Institute of Microbiology, University Innsbruck, Innsbruck, Austria

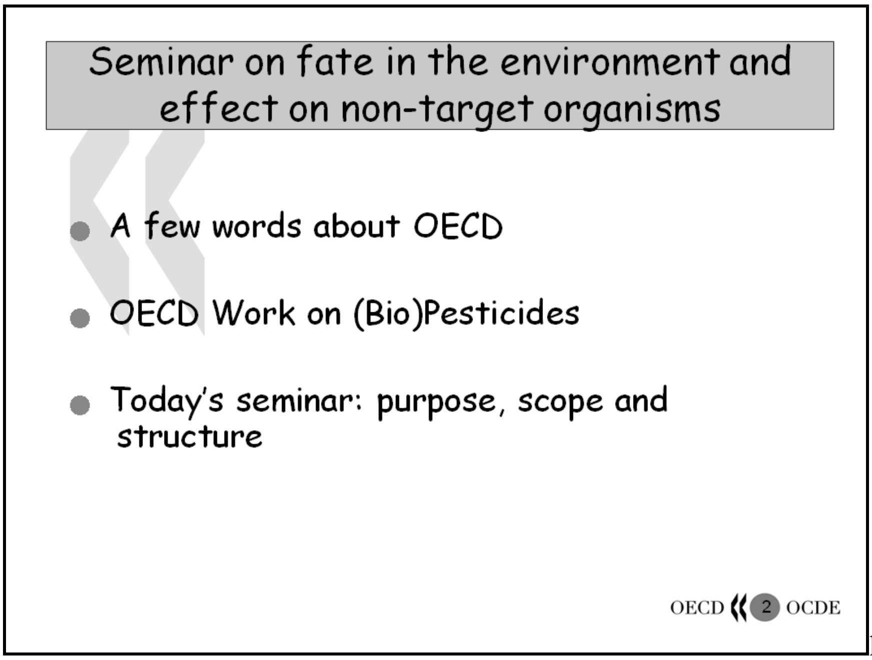
Biological control agents: Interplay with rhizosphere communities and risk assessment
By Gabriele Berg / Henry Müller, Graz University; Austria

Environmental fate of bacterial biocontrol agents applied to the phylosphere
By Brion Duffy, Agroscope, Wädenswil; Switzerland

Presentation on the OECD and the work of OECD-BPSG

By Jeroen Meeussen, The Netherlands





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OECD

- OECD: The Organisation for Economic Co-operation and Development

OECD 3 OCDE

OECD

What is OECD?

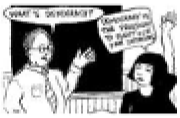
- A forum in which governments work together to address the economic, social and environmental challenges of interdependence and globalisation.
- A provider of comparative data, analysis and forecasts to underpin multilateral co-operation with more than 250 publications per year.

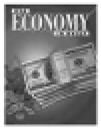
OECD 4 OCDE

OECD's Mission

OECD brings together the governments of countries committed to democracy and the market economy from around the world to:

– Support sustainable economic growth;
– Boost employment;
– Raise living standards;
– Maintain financial stability;
– Assist other countries' economic development;
– Contribute to growth in world trade.





OECD 5 OCDE

A tool for governments

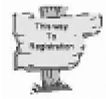
- Started after World War II;
- Transformed in 1961 into the Organisation for Economic Co-operation and Development with trans-Atlantic and then global reach;
- Today the OECD has 30 member countries;
- More than 70 developing and transition economies are engaged in working relationships with the OECD.

OECD 6 OCDE

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.

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OECD - Working Group on Pesticides

- Registration Steering Group
- Risk Reduction Steering Group
- BioPesticides Steering Group

OECD 8 OCDE

OECD-BPSG

The BioPesticides Steering Group (BPSG) was established by the WGP in 1999:

- to help member countries harmonise the methods and approaches used to assess biological pesticides and

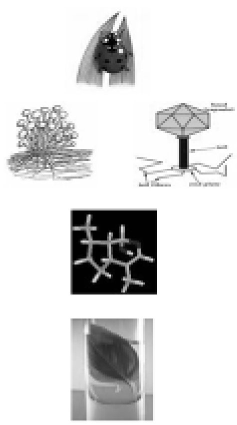
- improve the efficiency of control procedures.

OECD 9 OCDE

OECD-BPSG

Biological Pesticides:

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



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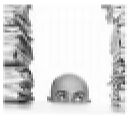
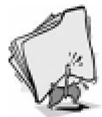
OECD-BPSG

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 11 OCDE

OECD-Publications

Registration requirements:

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

OECD 12 OCDE

OECD-Publications

- OECD Guidance for **Industry Data Submissions** for Microbial Pest Control Products and their Microbial Pest Control Agents (Dossier Guidance for Microbials), August 2006.
- OECD Guidance for **Country Data Review Reports** on Microbial Pest Control Products and their Microbial Pest Control Agents (Monograph Guidance for Microbials), August 2006.

OECD 13 OCDE

OECD-BPSG

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

OECD 14 OCDE

Working Document

"Working Document on the Evaluation of Microbials for Pest Control"

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 15 OCDE

Working Document - chapters

- Taxonomic identification of micro organisms in MPCP
- Genetic toxicity assessment of microbial pesticides
- Exposure (operators, bystanders, consumers)
- Microbial metabolite residues in food
- Efficacy evaluation of microbials

OECD 16 OCDE

Workshop on the Regulation of Biopesticides

- *"Workshop on the Regulation of Biopesticides: registration and Communication issues"* 15-17 April 2008, EPA, Arlington, USA

SuCCeS

The objectives of the workshop were met:

- To collect input to resolve science issues;
- To improve communication and information exchange;
- To take forward some of the conclusions from REBECA.

OECD 17 OCDE

OECD-Publications

- **OECD,** ***Working Document on the Evaluation of Microbials for Pest Control***, OECD Environment, Health and Safety Publications, Series on Pesticides No. 43, 2008.
- **OECD,** ***Report of Workshop on the Regulation of Biopesticides: Registration and Communication issues****, 15 – 17 April 2008, EPA, Arlington, USA*, OECD Environment, Health and Safety Publications, Series on Pesticides No. 44, 2009

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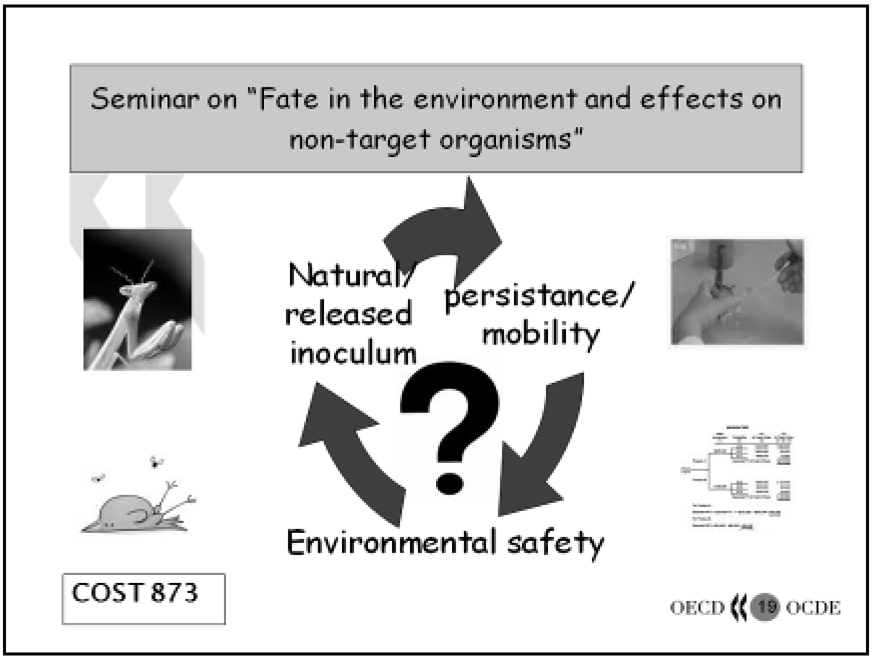
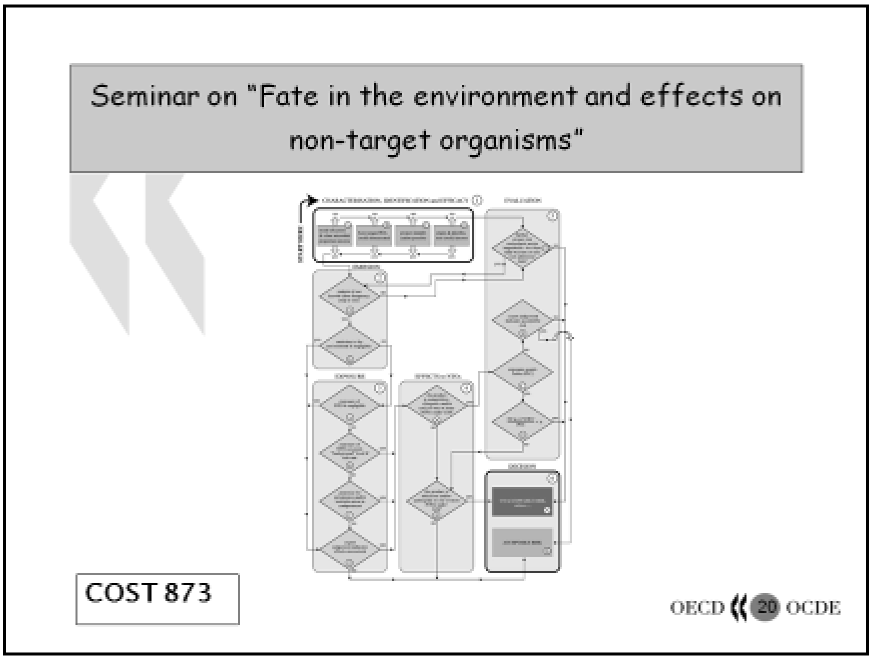

Seminar on "Fate in the environment and effects on non-target organisms"
Natural/ released inoculum
persistance/ mobility
?
Environmental safety
COST 873
OECD 19 OCDE


Seminar on "Fate in the environment and effects on non-target organisms"
COST 873
OECD 20 OCDE

Seminar - Objectives

The objectives of the seminar are:

- identify key issues related in the area of fate in the environment and effect on non-target organisms;
- exchange information on national and international activities in the area concerned; and
- make recommendations for further actions and/or possible activities for OECD and COST 873.

COST 873

OECD 21 OCDE

Seminar - Scope

- Natural and released inoculum levels of microbials;
- Stability of microbial strains when released;
- Potential persistence and mobility, in particular in soil;
- Epidemiology of microbial control agents in the environment;
- System to control released microbial control agents in the environment;
- Environmental safety evaluation and risk assessment of microbial control agents

COST 873

OECD 22 OCDE

Seminar – Scope (cont'd)

- The seminar will focus on risk assessment aspects and not on methodology issues in carrying out (field)experiments.
- The seminar will also not cover invertebrates as biological control agents, nor will it deal with metabolite issues.

COST 873

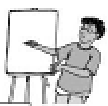
OECD 23 OCDE

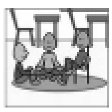
Seminar - Structure

Presentations on:

- government experience and perspectives,
- stakeholder experience and perspectives,

followed by discussion after each set of presentations.





COST 873

OECD 24 OCDE

Seminar - Results

With the focus on the fate in the environment and effects on non-target organisms, the goals of this seminar are

1. for participants to share information and to promote a dialogue on inoculum levels, stability of strains, potential persistence and mobility, epidemiology, control of released MPCA and environmental safety assessment and

2. to suggest future work/issue papers in the field of the fate in the environment and effects on non-target organisms.

COST 873

OECD 25 OCDE

Seminar on "Fate in the environment and effects on non-target organisms"

I wish you an interesting and useful seminar!

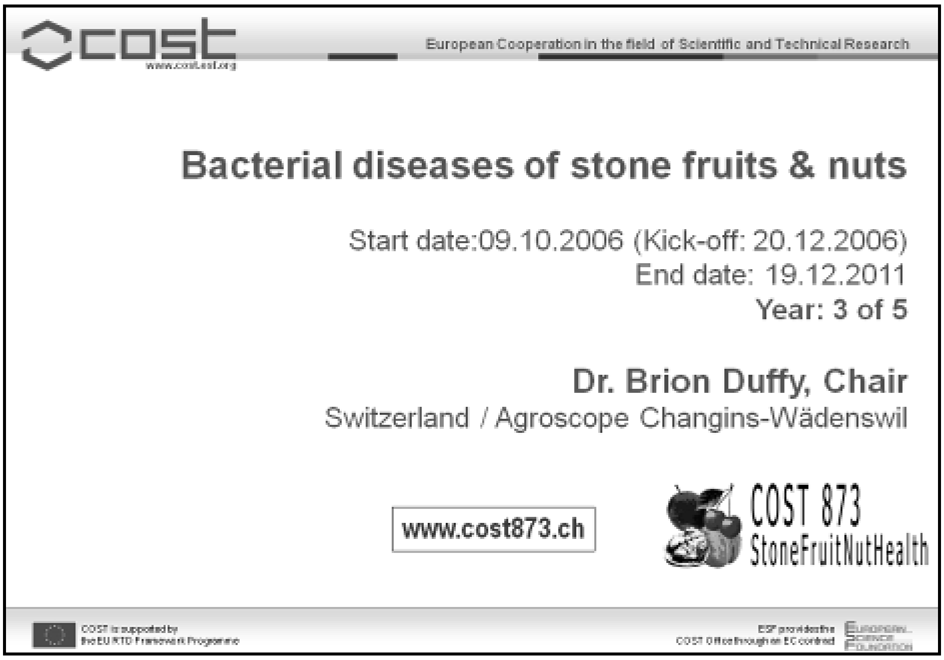
Thank you very much for your attention.

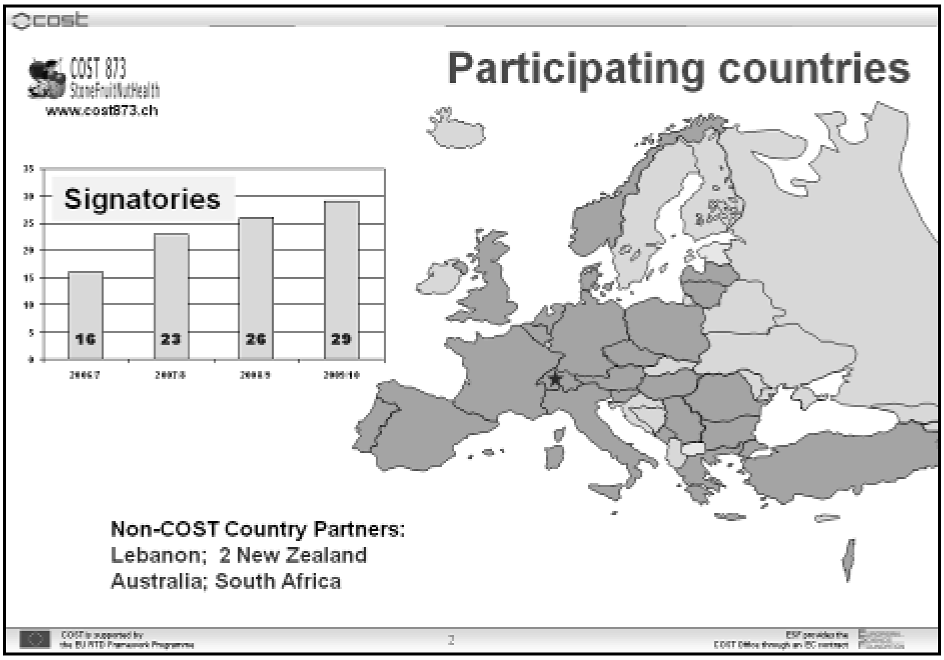
COST 873

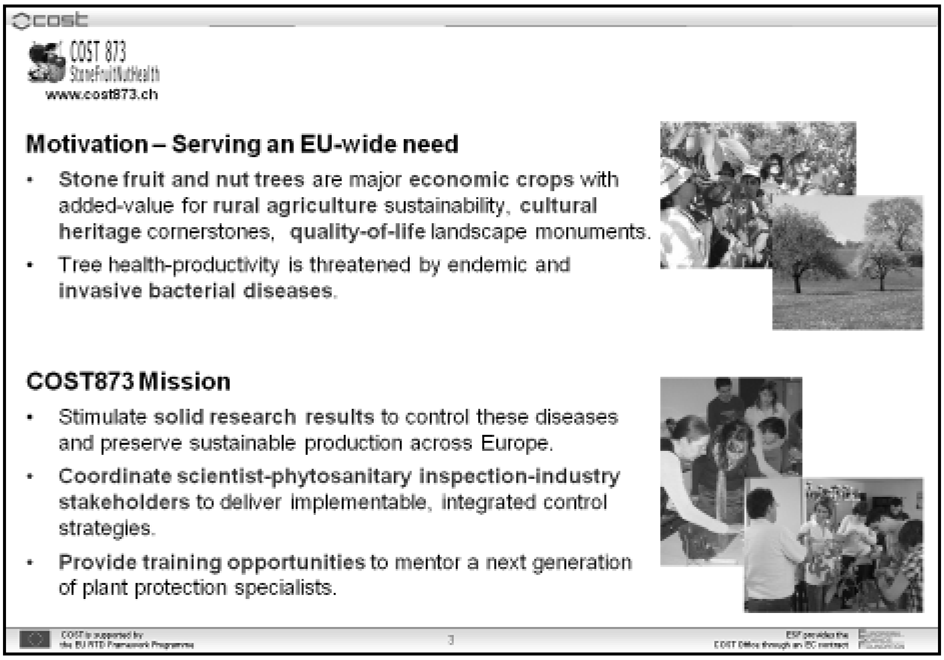
OECD 26 OCDE

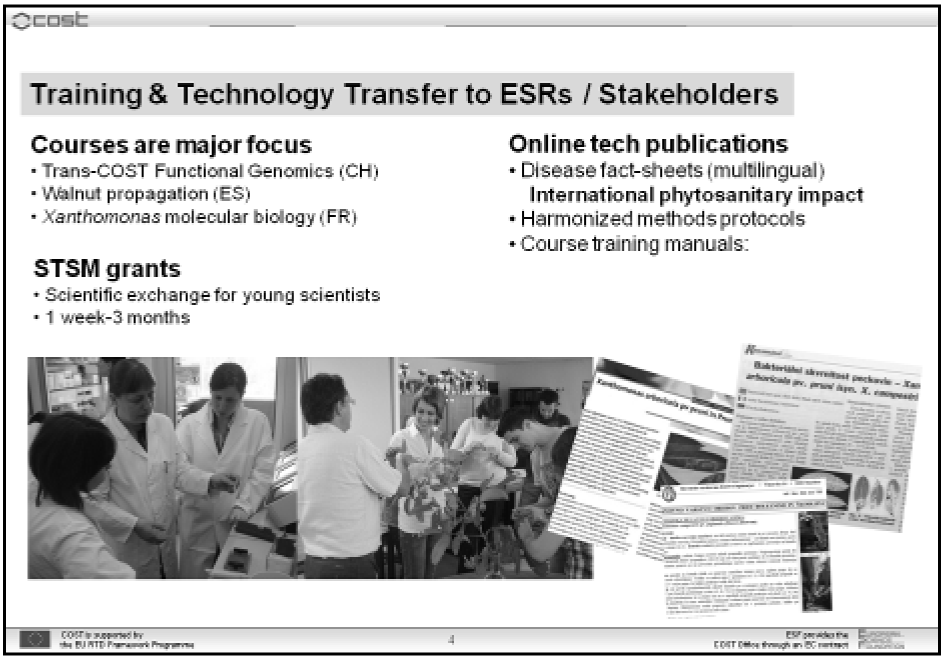
Presentation on the COST 873 initiative

By Bryon Duffy (Agroscope, Wädenswil, Switzerland)






cost
COST 873
StoneFruitNutHealth
www.cost873.ch
Motivation – Serving an EU-wide need
• Stone fruit and nut trees are major economic crops with added-value for rural agriculture sustainability, cultural heritage cornerstones, quality-of-life landscape monuments.
• Tree health-productivity is threatened by endemic and invasive bacterial diseases.
COST873 Mission
• Stimulate solid research results to control these diseases and preserve sustainable production across Europe.
• Coordinate scientist-phytosanitary inspection-industry stakeholders to deliver implementable, integrated control strategies.
• Provide training opportunities to mentor a next generation of plant protection specialists.
COST is supported by the EU RTD Framework Programme
3
ESF provides the COST Office through an EC contract


cost
Training & Technology Transfer to ESRs / Stakeholders
Courses are major focus
• Trans-COST Functional Genomics (CH)
• Walnut propagation (ES)
• Xanthomonas molecular biology (FR)
STSM grants
• Scientific exchange for young scientists
• 1 week-3 months
Online tech publications
• Disease fact-sheets (multilingual)
International phytosanitary impact
• Harmonized methods protocols
• Course training manuals:
COST is supported by the EU RTD Framework Programme
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COST 873 StoneFruitNutHealth www.cost873.ch

Research objectives – WG mission

WG1 - Diagnostics & Early Detection

- **Diagnostic methods** – develop, optimise, validate reliable, sensitive, simple tools
- **Pathogen genetics/biology/ecology** – elucidate prey to design better control strategies (i.e., identify pathogen Achilles heel!)

WG2 – Disease Prevention & Epidemiology

- **Phytosanitary technology transfer** – new diseases, new disease forecasting models, streamline plant inspection/monitoring to prevent and contain disease threats

WG3 – Tree Host Resistance & Breeding

- **Plant resistance resources** – identify new sources by mining germplasm collections
- **SMART breeding** – apply genetics/genomics to develop molecular markers

WG4 – Sustainable Control Strategies

- **Biocontrol / safe chemicals** – discover, develop, integrate disease management tools
- **Facilitate market entry** – resistant varieties, evaluate efficacy/biosafey for registration

COST is supported by the EU RTD Framework Programme 5 ESF provides the COST Office through an EC contract

COST 873 StoneFruitNutHealth www.cost873.ch

Systems approach integrating science, regulators, growers, industry stakeholders

Committee of Practioners

Facilitates dialogue between scientists and stakeholders :

- effective transfer of research advances to end-users
- offer platform for end-user input to focus research efforts that meet real needs

oepp eppo — ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT OECD — IBMA International Biocontrol Manufacturers Association — Euphresco Phytosanitary ERA-NET — IOBC-WPRS OILB-SROP — ISHS

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A proposal for an environmental safety evaluation of microbial biocontrol agents - Decision tree for the aquatic and terrestrial compartment

By Bilgin Karaoglan (Federal Environmental Agency (UBA), Dessau; Germany)

Introduction

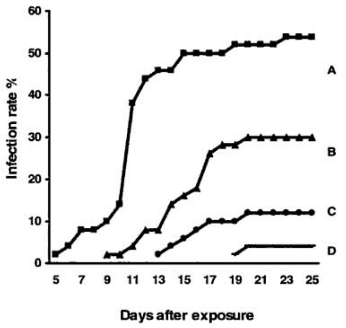
- microbial biocontrol agents (mBCA) are generally considered to be an environmentally benign control option compared to synthetic pesticides
- does that mean they are entirely free of hazards to health and the environment?

Introduction

Major concerns:

- introduction of living organisms is perhaps an irreversible step
- potential for toxicity and pathogenicity

Non-target effects: Examples



Mortality due to infection by Beauveria bassiana *in the bumblebee* Bombus terrestris *in laboratory cage experiments mimicking conditions in a bumblebee hive, means of 5 replicates of 10 bumblebees each.*
A = direct exposure of individual bumblebees to sprays of 10^8 cfu/ml;
B = exposure to treated flowers only, 2 h after spraying at 10^8 cfu/ml;
C = exposure to treated flowers only, 48 h after spraying at 10^8 cfu/ml;
D = exposure to treated flowers only, 2 h after spraying at 5×10^6 cfu/ml.

- *Beauveria bassiana* infects bumblebees. Maximum Infection rate was 54% under laboratory conditions (Hokkanen et al. 2003; In: Environmental Impacts of Microbial Insecticides: Need and Methods for Risk Assessment, Progress in Biological Control)
- field tests did not indicate high risk to bumblebees.
- Authors noted that the laboratory study offered more conductive temp/humidity conditions for the fungi (similar to the ground hives)

Non-target effects: Examples

Effects on pollinators:

- In a laboratory study by Mommaerts et al. (2009) *B. terrestris* was exposed to mBCA at its maximum field recommended concentration (MFRC): Two products containing *B. bassiana* and *B. subtilis* were classified as harmful to bumblebees.

Mommaerts et al. (2009). Pest Managment Science. 65(9):949-955

Effects on earthworms:

- Few strains of *B. thuringiensis kurstaki* and *B. subtilis* revealed pathogenic effects in earthworms (see also DAR).

→ pre-market safety evaluation (regulation) is needed to protect the environment

Key background documents

legal framewok

EU:

- Comm. Dir. 2001/36/EC
- Council Dir. 2005/25/EC

guidelines

(USA / CAN):

- OPPTS Guidelines
- PMRA Guidelines

EU review programme

- DARs of the for the „4th list microorganisms" (MPCAs on the EU market before 25 July 1993)
- EFSA PRAPeR expert meeting reports (M1-M3)

scientific articles, reviews

- Review article on „natural inoculum levels of entomopathogenic fungal BCAs in soil in relation to RA and in acordance with EU Regulations" (Scheepmaker and Butt, 2010)

Abbreviations:
DAR = Draft Assessment Report
EFSA = European Food Safety Authority
PMRA = Pest Management Regulatory Agency
OPPTS = Office of Prevention, Pesticides and Toxic Substances
PRAPeR = Pesticide Risk Assessment Peer Review

Meta analysis of DARs (4th list)

Desk study by Mensink JWG (2006) for 11 DARs

	MPCA	RMS
1.	*Lecanicillium muscarium*	The Netherlands
2.	*Metarhizium anisopliae* var. anisopliae	The Netherlands
3.	*Adoxophyes orana* Granulovirus	Germany
4.	*Beauveria bassiana* ATCC 74040	Germany
5.	*Beauveria bassiana* GHA	Germany
6.	*Cydia pomonella* Granulovirus	Germany
7.	*Phlebiopsis gigantea*	Estonia
8.	*Pythium oligandrum*	Sweden
9.	*Streptomyces griseoviridis* K61	Estonia
10.	*Trichoderma harzianum* Rifai	Sweden
11.	*Verticillium albo-atrum*	The Netherlands
	Bacillus thuringiensis aizawai ABTS 1857	Italy
	Bacillus thuringiensis aizawai GC-91	Italy
	Bacillus thuringiensis tenebrionis NB176	Italy
	Bacillus thuringiensis israeliensis AM 6552	Italy
	Bacillus thuringiensis kurstaki SA 11, SA 12	Denmark
	Bacillus thuringiensis kurstaki PB 54	Denmark
	Bacillus thuringiensis kurstaki ABTS-351	Denmark

Meta analysis of DARs (4th list)

Dossier inspection (for DARs available at Dec 06) revealed

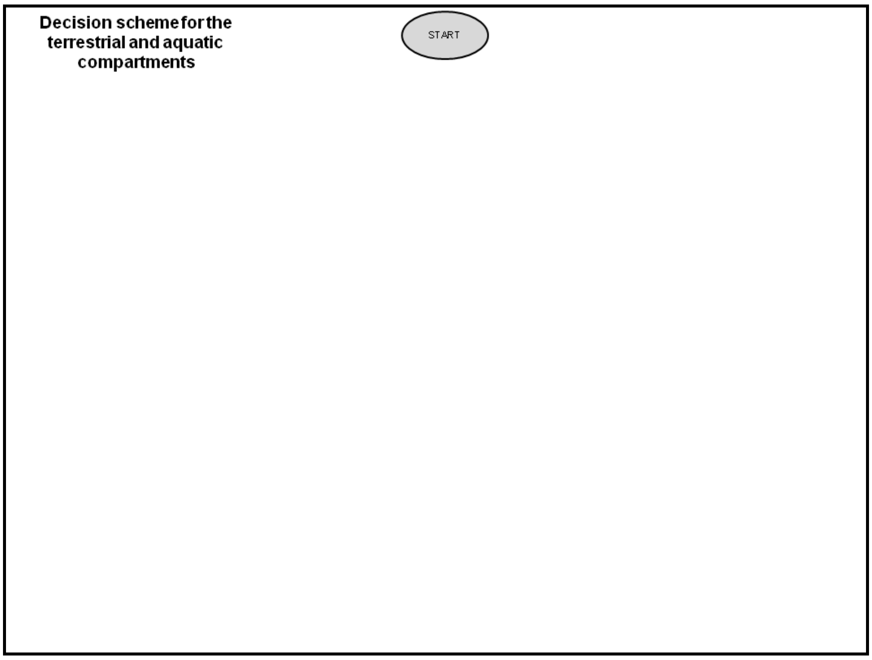
- **relatively small contribution by confidential study reports for registration purposes**
- **Contribution by scientific literature and waivers (whether phrased by the notifiers or the RMS) was relatively equal**
- **the outcome of the "mini-desk study" also holds true for the remaining submitted DARs of the 4th list**

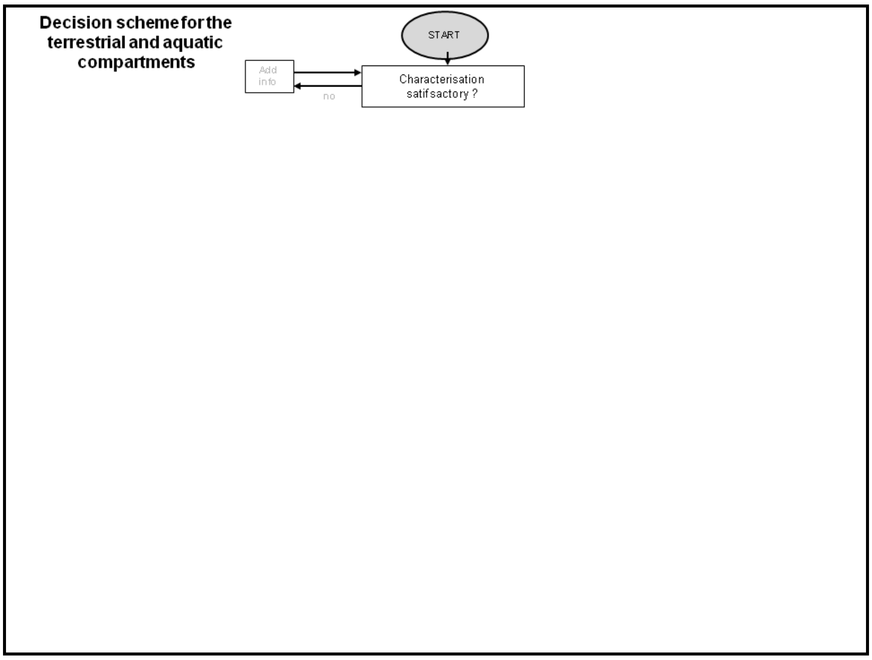
Decision scheme

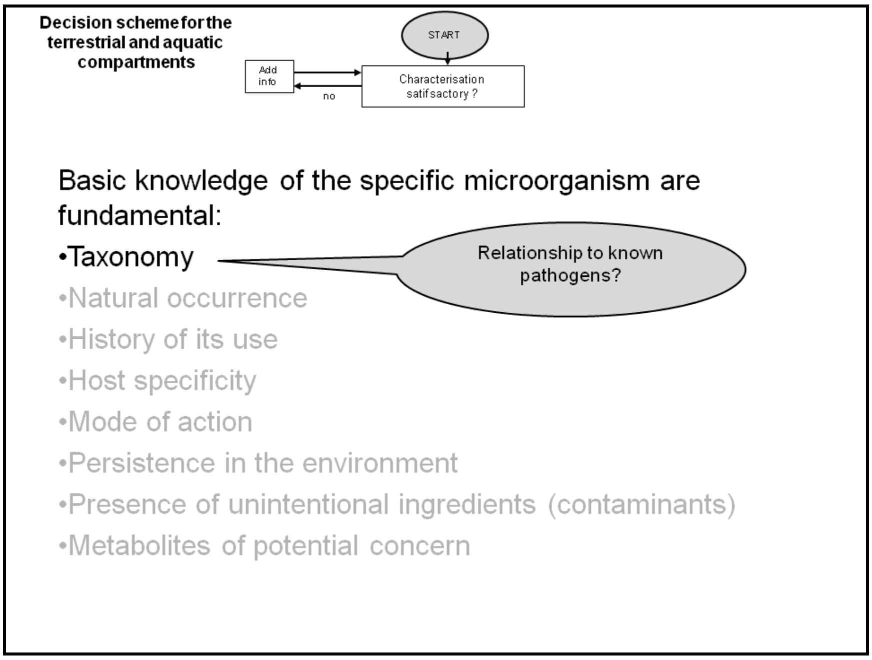
- Currently no internationally accepted harmonized guidance documents available on environmental safety evaluation for regulatory purposes
- Former decision scheme was developed in 2007
  Mensink BJWG and Scheepmaker JWA (2007). Biocontrol Science and Technology, 2007; 17(1): 3-20
- The present proposal „decision scheme" developed by Scheepmaker and Karaoglan (2010) shall aim to provide regulatory guidance on the environmental safety evaluation of mBCA

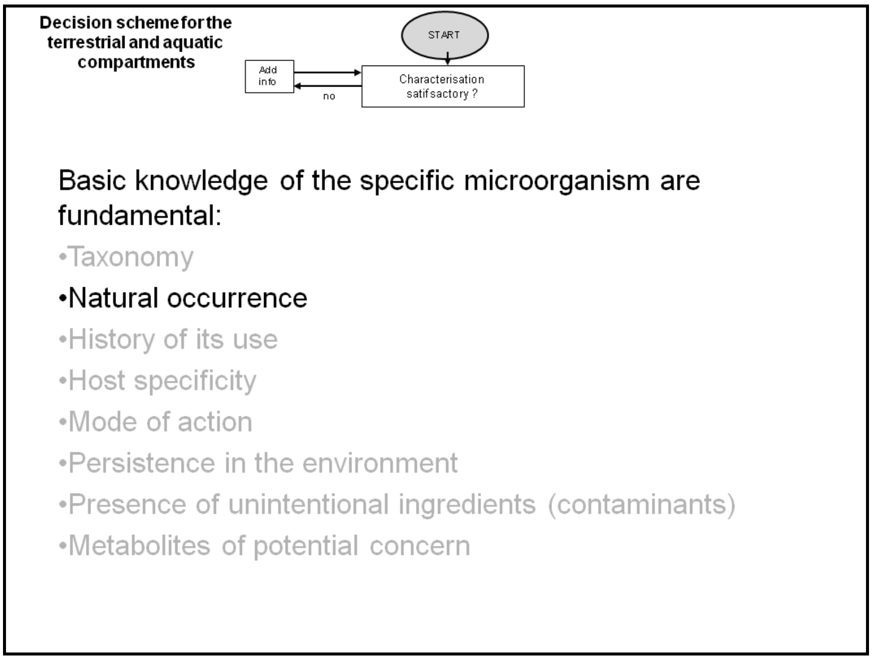
Decision scheme

- "Decision scheme for the terrestrial compartments" was distributed in November 27, 2010 among BPSG-participants
- Comments received from AUS, AUT, CAN, CH, DE, DK, NL,UK, USA
- Revised "Decision scheme" for both aquatic and terrestrial compartments was submitted to the OECD in April 29, 2010


Decision scheme for the terrestrial and aquatic compartments
START


Decision scheme for the terrestrial and aquatic compartments
START
Add info
no
Characterisation satifsactory ?


Decision scheme for the terrestrial and aquatic compartments
START
Add info
Characterisation satifsactory ?
no
Basic knowledge of the specific microorganism are fundamental:
•Taxonomy
Relationship to known pathogens?
•Natural occurrence
•History of its use
•Host specificity
•Mode of action
•Persistence in the environment
•Presence of unintentional ingredients (contaminants)
•Metabolites of potential concern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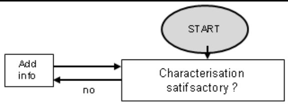
Decision scheme for the terrestrial and aquatic compartments



Basic knowledge of the specific microorganism are fundamental:

- Taxonomy
- Natural occurrence
- History of its use
- Host specificity
- Mode of action
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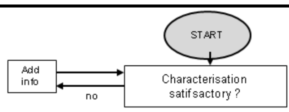
- Metabolites of potential concern

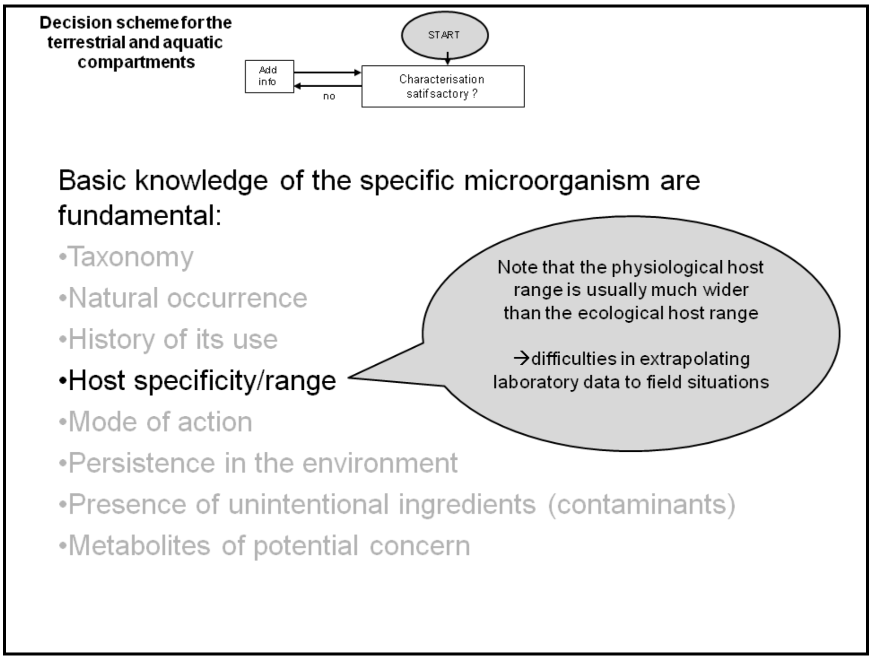
Decision scheme for the terrestrial and aquatic compartments



Basic knowledge of the specific microorganism are fundamental:

- Taxonomy
- Natural occurrence
- History of its use
- Host specificity/range
- Mode of action
- Persistence in the environment
- Presence of unintentional ingredients (contaminants)
- Metabolites of potential concern

The basic information on the host range should already give some indication on the possibility of infectivity or pathogenicity to other species than the target organism →radial taxonomical testing approach


Decision scheme for the terrestrial and aquatic compartments
START
Add info
no
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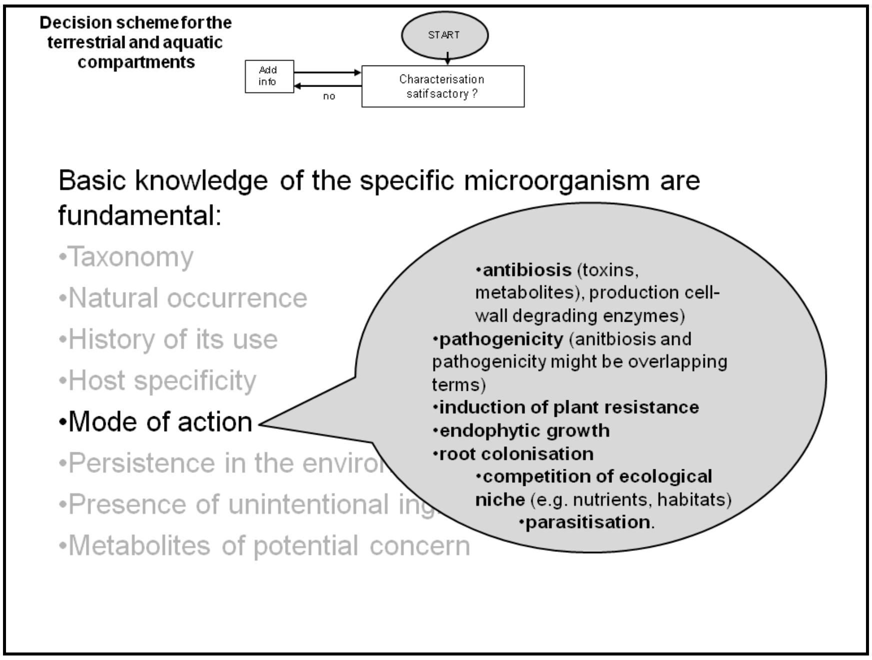
Basic knowledge of the specific microorganism are fundamental:
•Taxonomy
•Natural occurrence
•History of its use
•Host specificity/range
•Mode of action
•Persistence in the environment
•Presence of unintentional ingredients (contaminants)
•Metabolites of potential concern
Note that the physiological host range is usually much wider than the ecological host range
→difficulties in extrapolating laboratory data to field situations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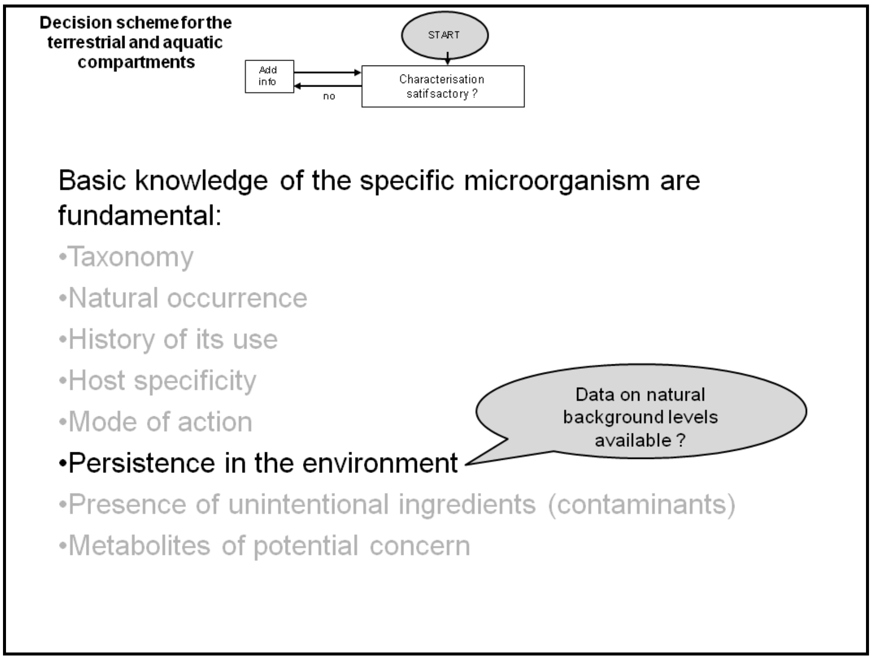
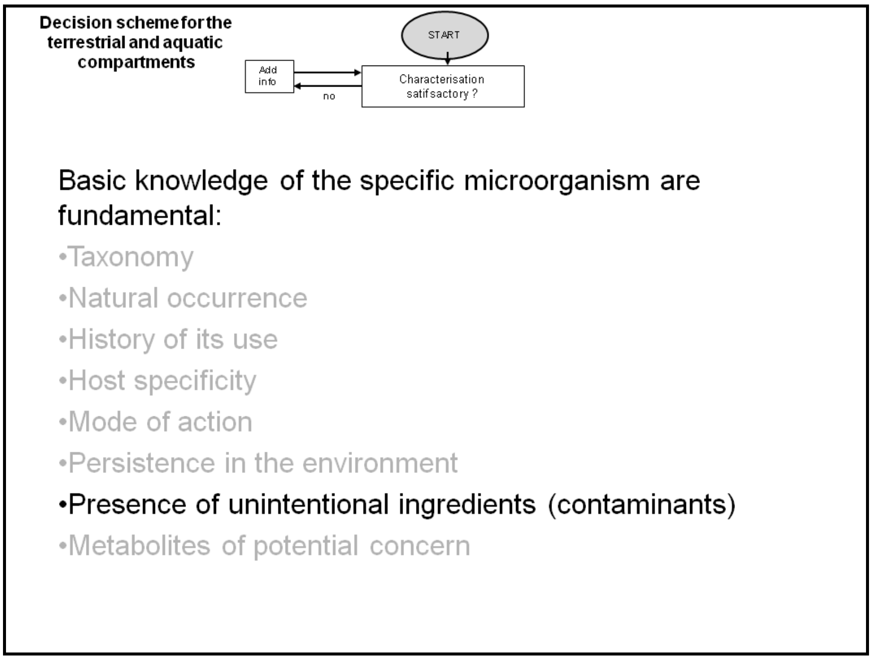
•Natural occurrence
•History of its use
•Host specificity
•Mode of action
•Persistence in the enviro
•Presence of unintentional ing
•Metabolites of potential concern
•antibiosis (toxins, metabolites), production cell-wall degrading enzymes)
•pathogenicity (anitbiosis and pathogenicity might be overlapping terms)
•induction of plant resistance
•endophytic growth
•root colonisation
•competition of ecological niche (e.g. nutrients, habitats)
•parasitisation.


Decision scheme for the terrestrial and aquatic compartments
START
Add info
no
Characterisation satifsactory ?
Basic knowledge of the specific microorganism are fundamental:
•Taxonomy
•Natural occurrence
•History of its use
•Host specificity
•Mode of action
•Persistence in the environment
Data on natural background levels available ?
•Presence of unintentional ingredients (contaminants)
•Metabolites of potential concern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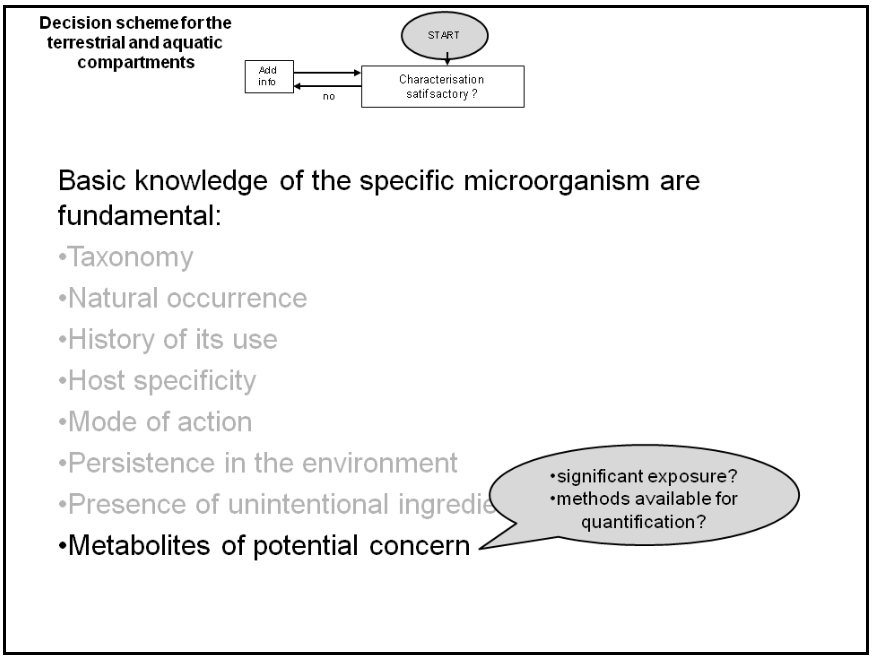
•Persistence in the environment
•Presence of unintentional ingredients (contaminants)
•Metabolites of potential concern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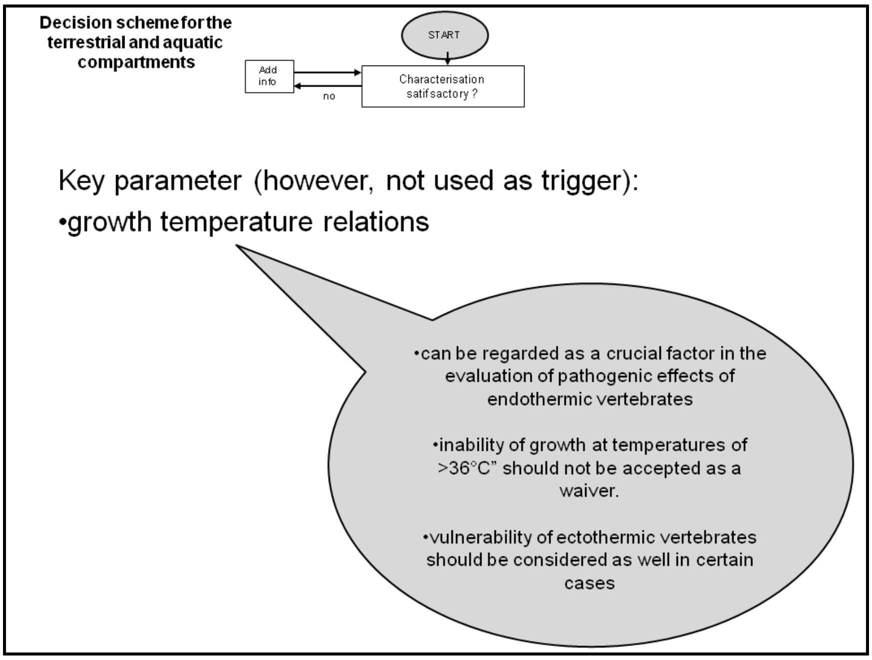
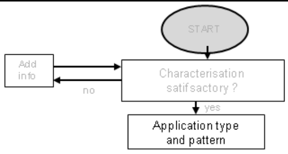
•Natural occurrence
•History of its use
•Host specificity
•Mode of action
•Persistence in the environment
•Presence of unintentional ingredie
•Metabolites of potential concern
•significant exposure?
•methods available for quantification?


Decision scheme for the terrestrial and aquatic compartments
START
Add info
Characterisation satifsactory ?
no
Key parameter (however, not used as trigger):
•growth temperature relations
•can be regarded as a crucial factor in the evaluation of pathogenic effects of endothermic vertebrates
•inability of growth at temperatures of >36°C" should not be accepted as a waiver.
•vulnerability of ectothermic vertebrates should be considered as well in certain cases

Decision scheme for the terrestrial and aquatic compartments



Pattern of use determines

•Exposure to NTOs

•The extent of exposure

Important information:

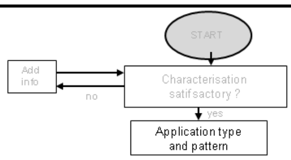
•Dosage

•Frequency

•The site (e.g. crop, bare soil)

•Type of application (e.g. spraying, seed treatment)

Decision scheme for the terrestrial and aquatic compartments



Pattern of use determines:

•Exposure to NTOs

•The extent of exposure

Important information

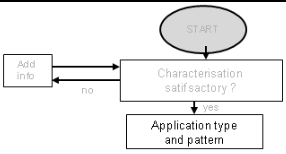
•Dosage (maximum application rate)

•Frequency

•The site (e.g. crop, bare soil)

•Type of application (e.g. spraying, seed treatment)

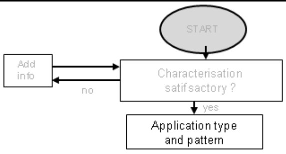
Decision scheme for the terrestrial and aquatic compartments



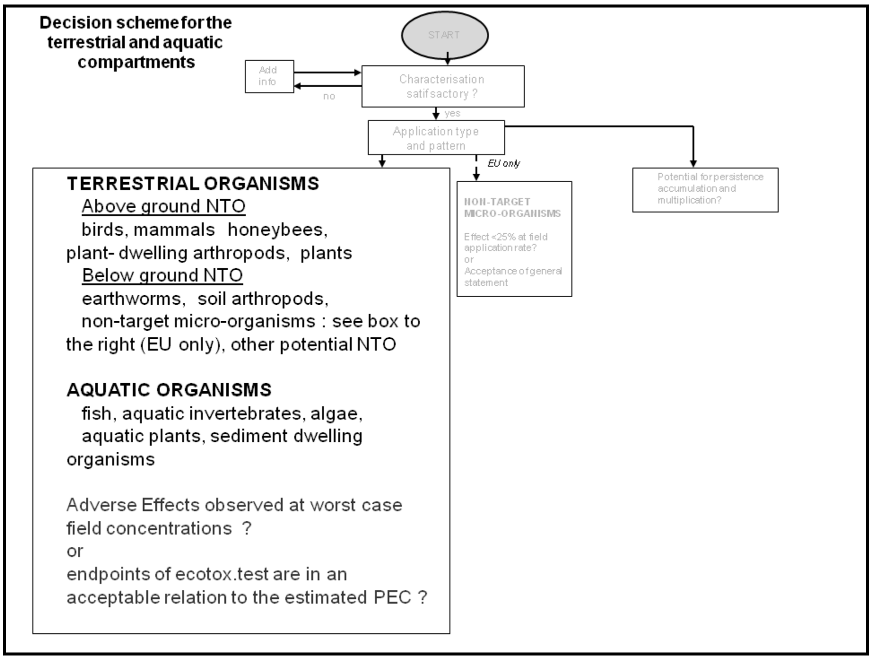
A first distinction can be made between indoor and outdoor applications

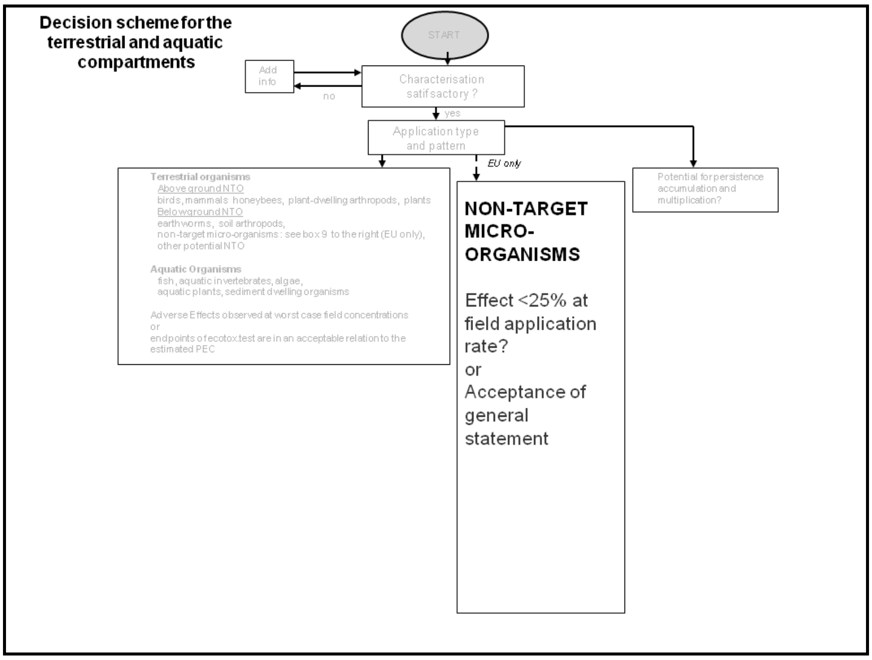
However, definitions of individual protected/covered crop systems are lacking (→Guidance Document in near future)

Decision scheme for the terrestrial and aquatic compartments

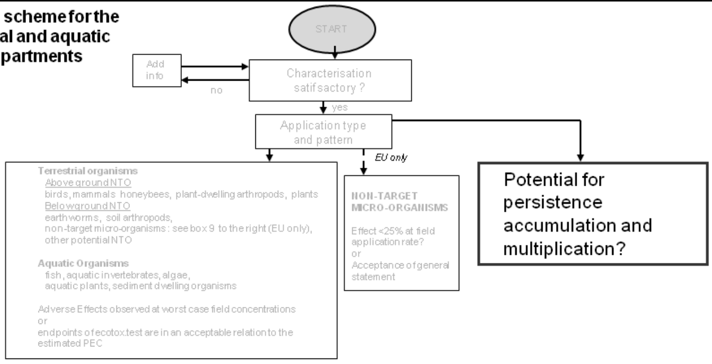


Application type	NTOs of concern
Indoor (no/negligible exposure)	Beneficial insects (IPM), Pollinators in flowering crops Discharge to surface water or STP?
outdoor – spray to bare soil	Birds, mammals, soil dwelling arthropods, earthworms and micro-organisms, emerging plants Fish, daphnia, algae, benthic invertebrates, aquat. macrophytes
outdoor – spray to crop	Birds, mammals, Bees (only in flowering crops), Non-target arthropods (soil and plant-dwelling) Earthworms and micro-organisms, plants Fish, daphnia, algae, benthic invertebrates, aquat. macrophytes
outdoor – seed treatments	Granivorous birds & mammals Soil dwelling arthropods Earthworms and micro-organisms
outdoor – aerial spraying	Requires testing of all relevant NTOs Risk mitigation options?

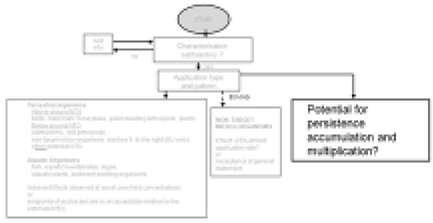

Decision scheme for the terrestrial and aquatic compartments
START
Add info
no
Characterisation satifsactory ?
yes
Application type and pattern
EU only
Potential for persistence accumulation and multiplication?
NON-TARGET MICRO-ORGANISMS
Effect <25% at field application rate?
or
Acceptance of general statement
TERRESTRIAL ORGANISMS
Above ground NTO
birds, mammals honeybees, plant- dwelling arthropods, plants
Below ground NTO
earthworms, soil arthropods,
non-target micro-organisms : see box to the right (EU only), other potential NTO
AQUATIC ORGANISMS
fish, aquatic invertebrates, algae, aquatic plants, sediment dwelling organisms
Adverse Effects observed at worst case field concentrations ?
or
endpoints of ecotox.test are in an acceptable relation to the estimated PEC ?


Decision scheme for the terrestrial and aquatic compartments
START
Add info
no
Characterisation satifsactory ?
yes
Application type and pattern
EU only
Potential for persistence accumulation and multiplication?
Terrestrial organisms
Above ground NTO
birds, mammals honeybees, plant-dwelling arthropods, plants
Below ground NTO
earthworms, soil arthropods,
non-target micro-organisms : see box 9 to the right (EU only),
other potential NTO
Aquatic Organisms
fish, aquatic invertebrates, algae,
aquatic plants, sediment dwelling organisms
Adverse Effects observed at worst case field concentrations
or
endpoints of ecotox.test are in an acceptable relation to the estimated PEC
NON-TARGET MICRO-ORGANISMS
Effect <25% at field application rate?
or
Acceptance of general statement

Decision scheme for the terrestrial and aquatic compartments



Decision scheme for the terrestrial and aquatic compartments



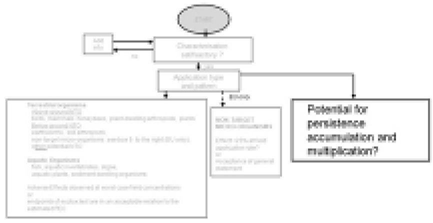
On a case-by-case approach it should be evaluated whether the mBCA, based on its identity and characterization, is likely to survive in the soil.

→particularly important for alien mBCAs

Properties affecting persistence negatively:

- The microorganism does not germinate and/or proliferate/or multiply in the soil.
- It cannot readily gain energy from hardly degradable substances of limited biodegradability like lignin.

Decision scheme for the terrestrial and aquatic compartments



Data on natural concentrations in the soil and persistence examined in a literature review by Scheepmaker and Butt (2010).

Approx. 1000 CFU/g soil
upper natural background level for the entomopathogenic fungi

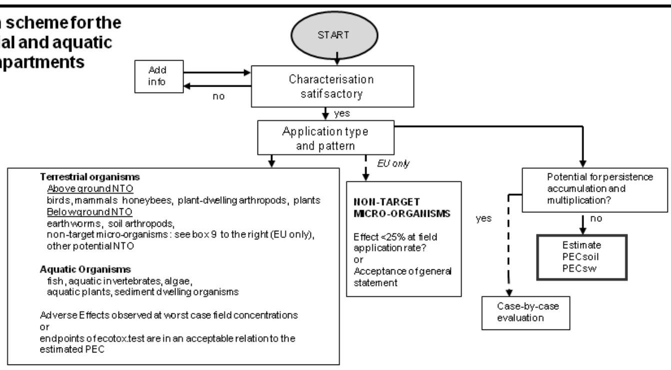
Beauveria bassiana
Beauveria brongniartii
Metarhizium anisopliae

→could be referred to in a waiver/statement to fill the data requirement for EPF for persistence in soil.

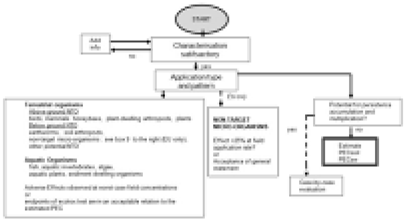
→extrapolation to other strains / MPCAs possible?

→need of further reviews, for instance for the genera *Bacillus* and *Trichoderma*.

Decision scheme for the terrestrial and aquatic compartments



Decision scheme for the terrestrial and aquatic compartments



Estimate PECsoil

$$PEC_{ini} = \frac{A \cdot (1 - f_{int})}{100 \cdot d \cdot bd}$$

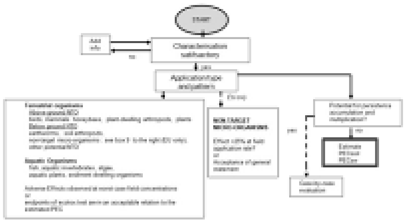
where A = application rate [g/ha]
f_{int} = fraction intercepted by plant cover
d = depth of the soil layer [cm] = 5 cm
bd = bulk soil density [g/cm³] = 1.5 g/cm³

Differences compared to chemical pesticides:

- Total application rate will be considered
- Wording "PIEC" (*Predicted Initial Environmental Concentration*) was suggested, alternatively population density
- Use of interception values derived from chemical pesticides is questionable (factors affecting interception: e.g. formulation type, similarity to chemical PPPs?)

→approach is considered conservative for the 1st tier assessment

Decision scheme for the terrestrial and aquatic compartments



Estimate PECsw

$$PEC_{ini} = \frac{a \cdot dr}{V_{sw} \cdot 100}$$

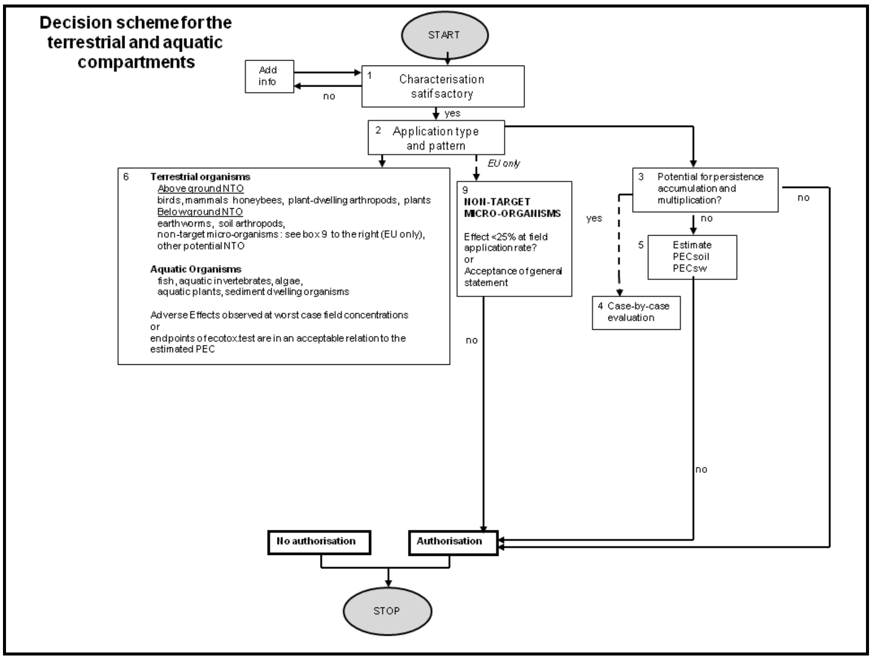
where a = application rate [µg/m²]
dr = % spray drift
V_{sw} = water volume per m² [300 l/m²]

- Total application rates should be considered in the first tier assessment
- %drift based on drift tables by Ganzelmeier et al. (1995), revised by Rautmann et al. (2001)
- Other option: AgDrift (an Internet accessible model developed by the Spray Drift Task Force for the US pesticide registration) can be regarded as a counterpart to the EU method.

→ Validity for mBCAs needs to be checked

→ Entry paths such as runoff, drainage and aerial deposition are not considered here and require different approaches

→ A rough estimation of the initial concentrations seems appropriate in the first tier level

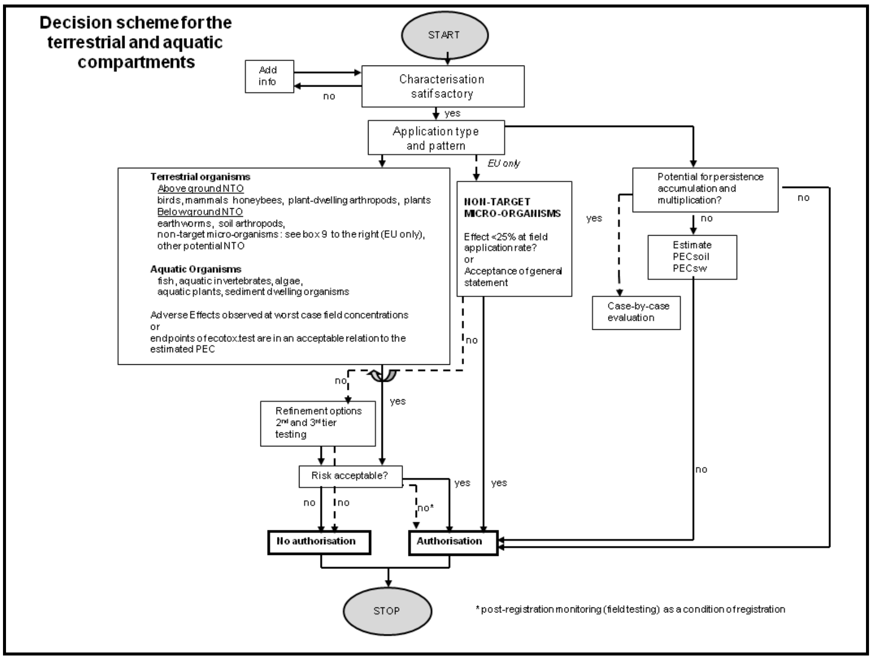


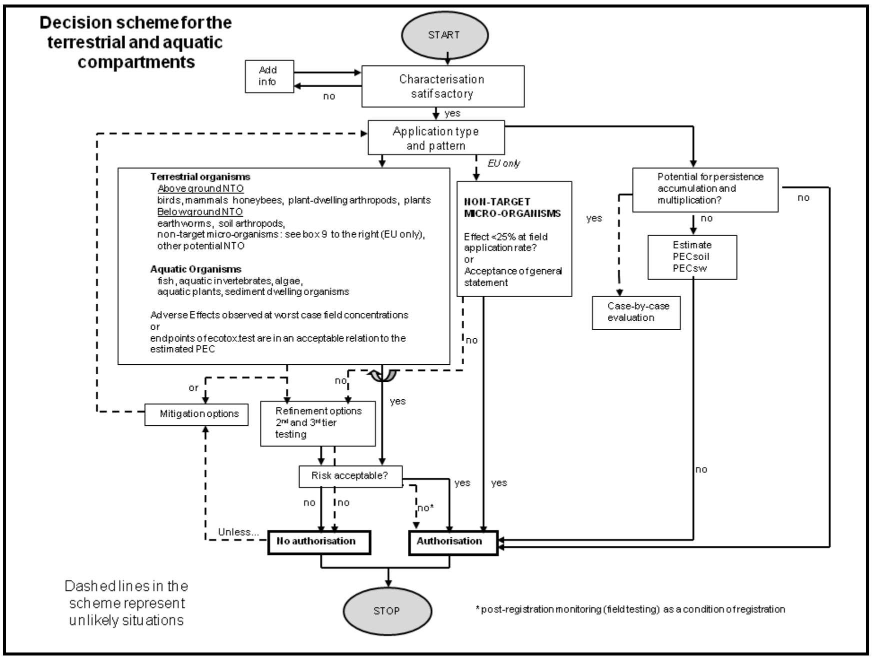
Risk to non target organisms (NTOs)
According to Annex VI (2005/25/EC) - no authorisation if:

Note that TER (Toxicity Exposure Ratio) was replaced by the more general wording MOS (Margin of Safety) because effects of mBCA are not necessarily attributed to toxicity

MOS = ecotoxicological endpoint*/PEC * may be a NOEC, LC50 or EC50

- Birds and terrestrial vertebrates: MOS_a <10, MOS_{lt}<5
- Fish and Daphnids: MOS_a <100, MOS_{lt}<10
- Algae: <10
- Bees: HQ > 50
- Other arthropods: effects >30%
- Earthworms: MOS_a <10, MOS_{lt}<5
- Soil micro-organisms: nitrogen or carbon mineralization processes in laboratory studies are affected by more than 25 % after 100 days


Decision scheme for the terrestrial and aquatic compartments
START
Add info
Characterisation satisfactory
no
yes
Application type and pattern
EU only
Terrestrial organisms
Above ground NTO
birds, mammals honeybees, plant-dwelling arthropods, plants
Belowground NTO
earthworms, soil arthropods,
non-target micro-organisms: see box 9 to the right (EU only),
other potential NTO
Aquatic Organisms
fish, aquatic invertebrates, algae,
aquatic plants, sediment dwelling organisms
Adverse Effects observed at worst case field concentrations
or
endpoints of ecotox test are in an acceptable relation to the estimated PEC
NON-TARGET MICRO-ORGANISMS
Effect <25% at field application rate?
or
Acceptance of general statement
Potential for persistence accumulation and multiplication?
yes
no
Estimate PECsoil PECsw
Case-by-case evaluation
no
yes
Refinement options 2nd and 3rd tier testing
Risk acceptable?
no
no
yes
no*
yes
No authorisation
Authorisation
STOP
* post-registration monitoring (field testing) as a condition of registration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or
no
yes
Mitigation options
Refinement options 2nd and 3rd tier testing
Risk acceptable?
no
no
yes
no*
yes
Unless...
No authorisation
Authorisation
Dashed lines in the scheme represent unlikely situations
STOP
* post-registration monitoring (field testing) as a condition of registration

POINTS of DISCUSSION

- Box 9: Until now adverse effects of mBCA to soil-microorganisms have not been reported.
  Is there a need for testing to address potential risk on soil micro-organisms?
  Or can a statement be accepted for soil micro-organisms?
- Annex VI (European Commission, 2005) does not give criteria for the length of period that the applied concentration is allowed to be higher than the background concentrations. This should be discussed.
- It should also be discussed whether the concentrations of EPF after application can be allowed to be a factor 10, 100 or 1000 higher than the background concentrations for the following reason: mBCAs are only effective at high concentrations. There is a dose/effect relation. Below a certain threshold level the effect sharply decreases although the concentration may still be higher than the background level. The question is whether the decrease of effects caused in non-target organisms is similar to the decrease observed in target organisms? This question is not easy to answer and needs an in-depth literature research.
- Safety factors are not considered applicable to pathogenic mBCAs.
  However, should a safety factor be determined for non-target organisms in case of toxins/metabolites of toxicological concern and relevant exposure? Can the same safety factors as used for chemical PPPs be applied?

  (In the current scheme safety factors have not been addressed)

Conclusion

- Quantitative RA in the first tier sufficient (worst case)
- Qualitative RA approach may be more appropriate for specific mode of actions and test organisms (e.g. birds&mammals)
- Conclusion of the former decision scheme by Mensink & Scheepmaker (2007) is still valid:

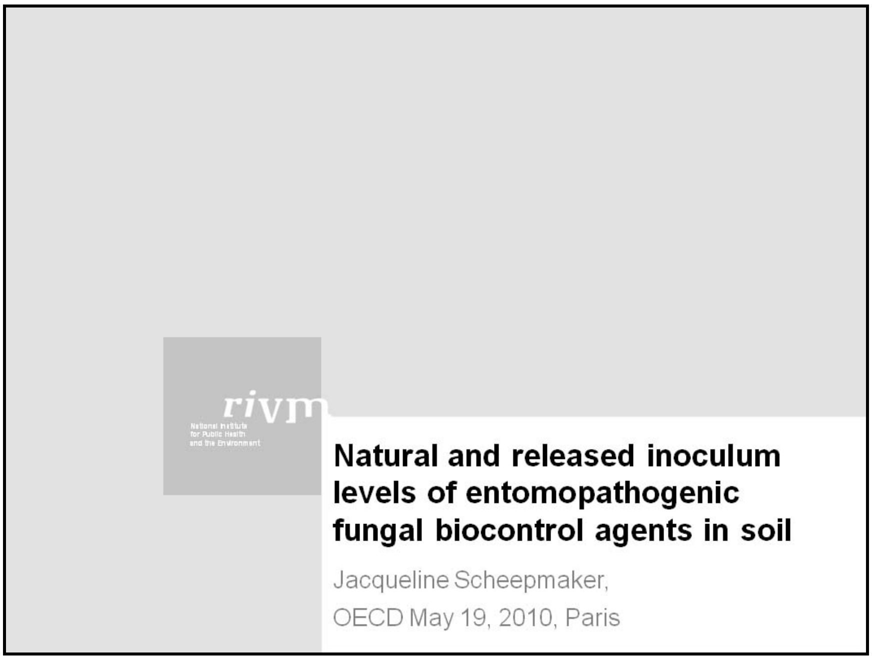
"Case by case expert judgement remains necessary in view of limited knowledge of microbial ecology, limited experience with regulatory test protocols and taxonomic difficulties in relation to the indigenousness of active micro-organisms"

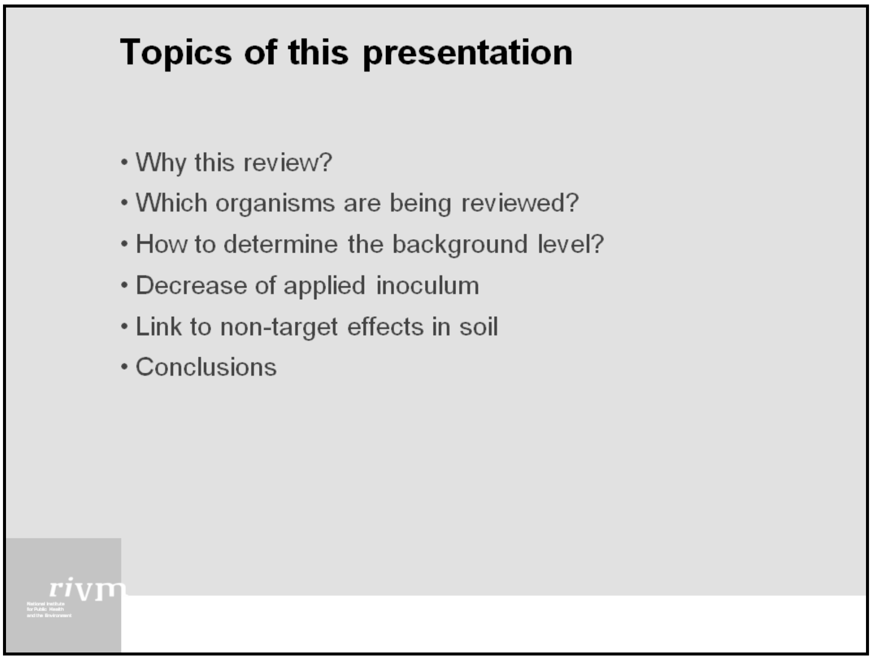
Mensink & Scheepmaker (2007): Biocontrol Science and Technology, 2007; 17(1): 3-20

Thanks for your attention

Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil – non-target effects on soil micro-organisms

By Jacqueline Scheepmaker (National Institute of Public Health and the Environment (RIVM), Bilthoven; The Netherlands)





Why this review?

Persistence in soil is data requirement in the EU

Why this review?

Persistence in soil is data requirement

Uniform principles Annex VIB of 91/414/EC (new regulation 1107/2009) for micro-organisms are unclear about criteria

Uniform principles of Annex VIB of 91/414/EC (the new regulation 1107/2009)

state that:

"authorisation shall not be granted if it can be expected that the micro-organism will persist in the environment in concentrations considerably higher than the natural background levels."

10x, 100x, 1000x ?

Is that bad?

For how long?

How high?

How to determine?

Why this review?

Persistence in soil is data requirement

Uniform principles are unclear about criteria

No guidance on persistence of inoculum

Why this review?

Persistence in soil is data requirement

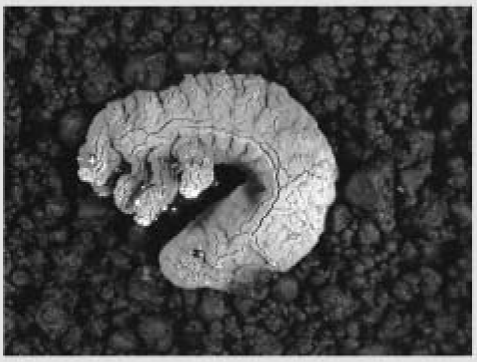
Uniform principles are unclear about criteria

No guidance on persistence of inoculum

In-depth desk study a solid base for statements/OECD monograph

rivm

Which organisms?



Focus on entomopathogenic fungi (EPF) only

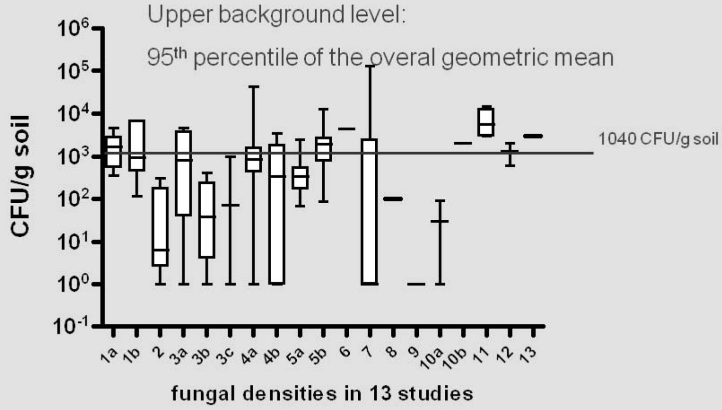
Metarhizium anisopliae, Beauveria bassiana and B. brongniartii

rivm

How to determine the background level?

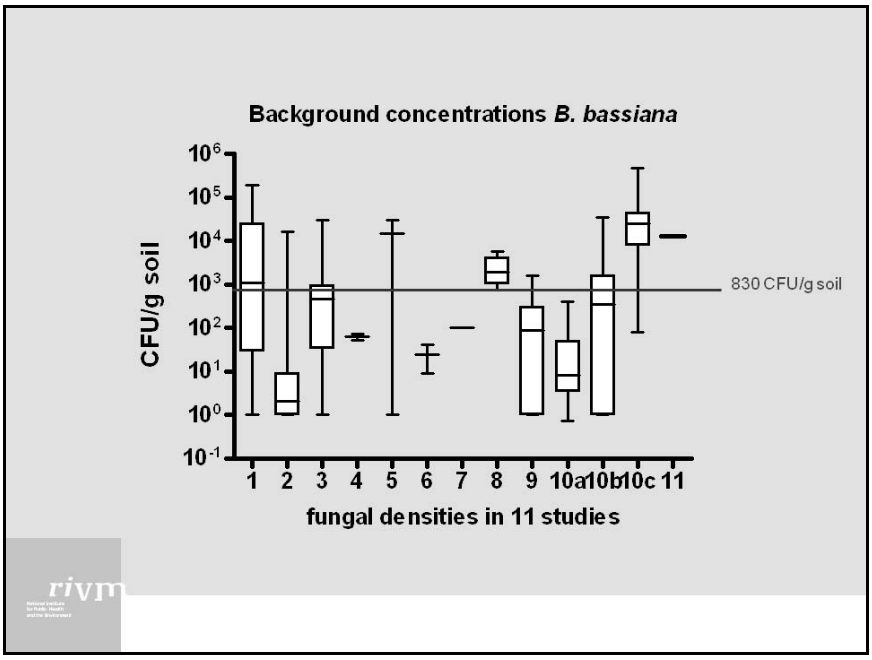
rivm

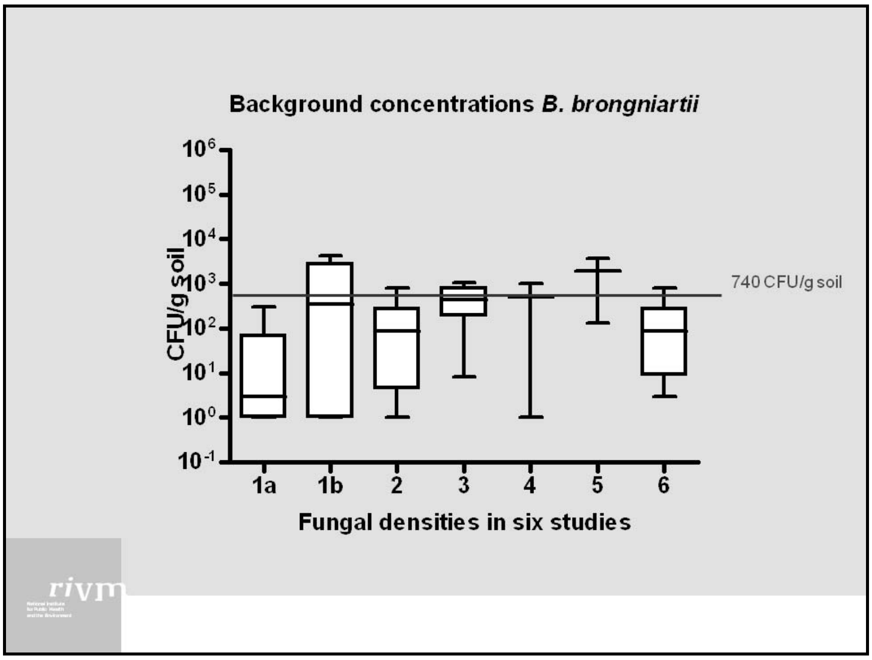
Background concentrations of *M. anisopliae*



1 Switzerland	4, 5	Brazil	9	Tasmania	13 New Zealand	
2 Denmark	6, 7	Tasmania	10, 11	Australia		
3 Finland	8	Macquarie island	12	Austria		

rivm

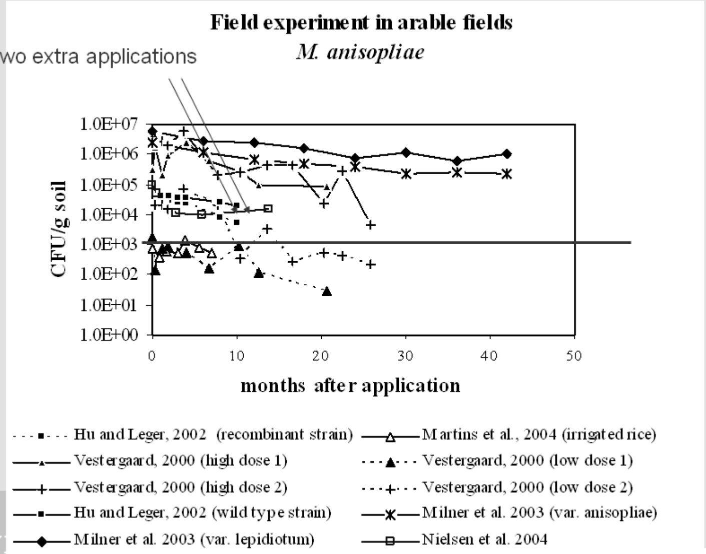

Background concentrations B. bassiana
CFU/g soil
830 CFU/g soil
1 2 3 4 5 6 7 8 9 10a 10b 10c 11
fungal densities in 11 studies
rivm

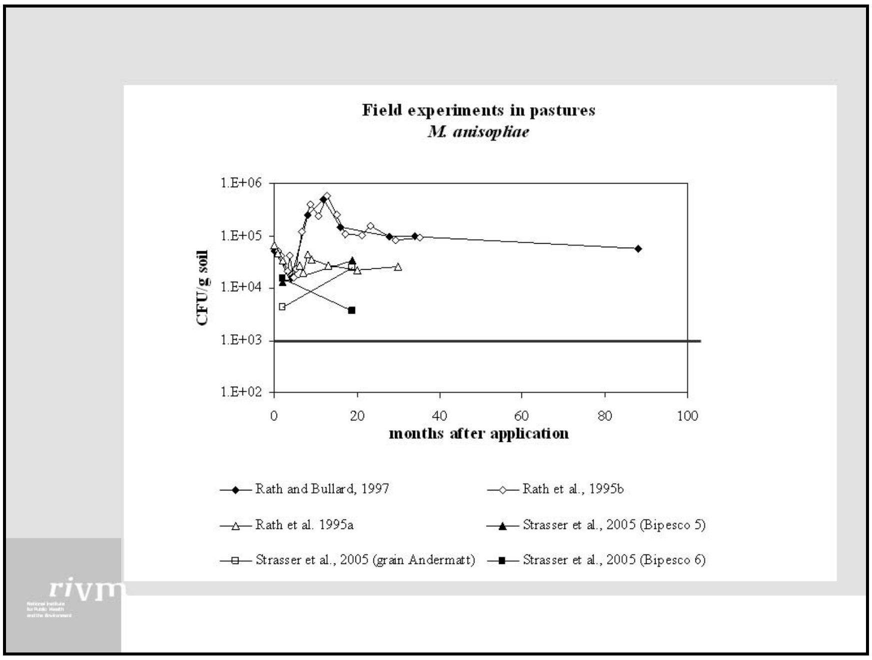

Background concentrations B. brongniartii
CFU/g soil
740 CFU/g soil
1a 1b 2 3 4 5 6
Fungal densities in six studies
rivm

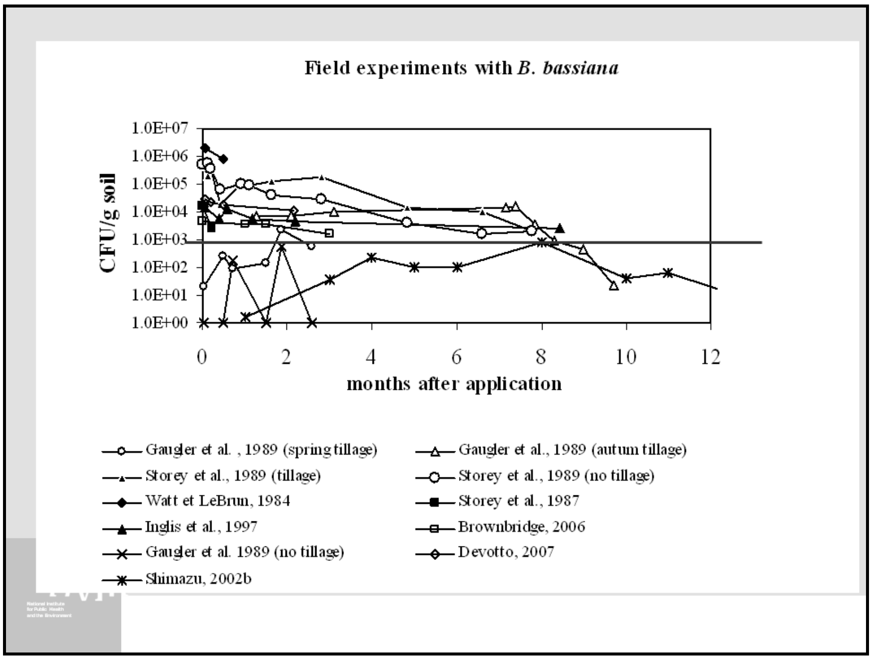
Four examples of decrease of applied inoculum

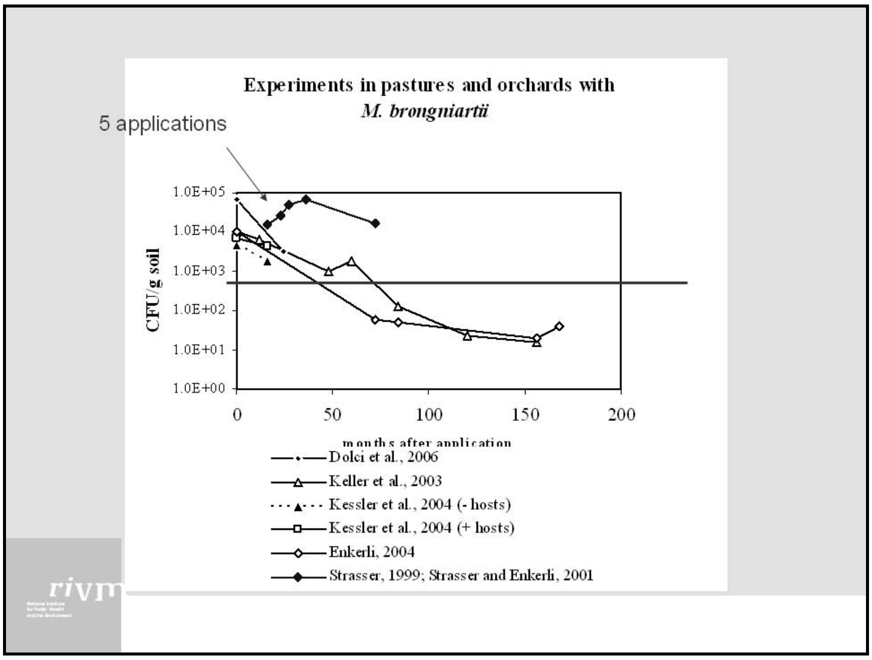
Will the applied inoculum decline to the upper background levels?

Two extra applications




Field experiments in pastures
M. anisopliae
CFU/g soil
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
0
20
40
60
80
100
months after application
Rath and Bullard, 1997
Rath et al., 1995b
Rath et al. 1995a
Strasser et al., 2005 (Bipesco 5)
Strasser et al., 2005 (grain Andermatt)
Strasser et al., 2005 (Bipesco 6)


Field experiments with B. bassiana
CFU/g soil
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
2
4
6
8
10
12
months after application
Gaugler et al. , 1989 (spring tillage)
Gaugler et al., 1989 (autum tillage)
Storey et al., 1989 (tillage)
Storey et al., 1989 (no tillage)
Watt et LeBrun, 1984
Storey et al., 1987
Inglis et al., 1997
Brownbridge, 2006
Gaugler et al. 1989 (no tillage)
Devotto, 2007
Shimazu, 2002b



Factors explaining decline in inoculum levels

- Edaphic
  - Soil texture, moisture, pH
- Biotic
  - Parasitism, predation and antagonism by a wide array of soil organisms
- Agricultural practices
  - Tillage, crop, fertilizer and pesticide use
- Climatic factors
  - Temperature, moisture and UV-radiation

Relation with non-target effects in soil

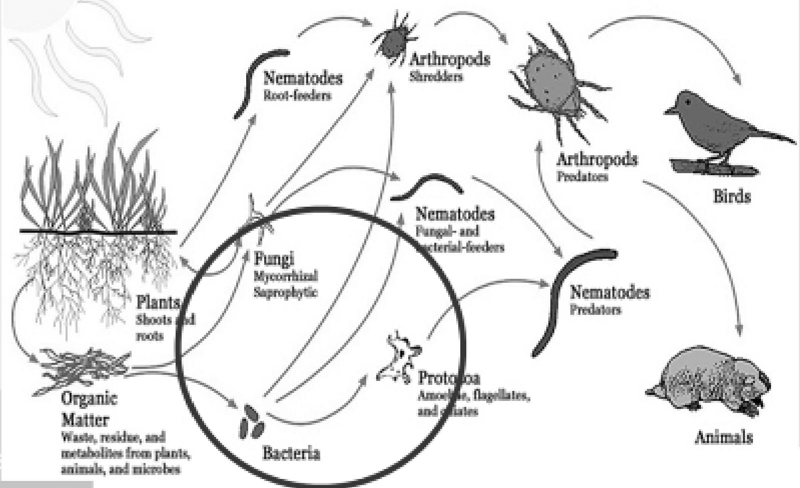
Is persistence of inoculum a problem?
The review cannot give the answer.

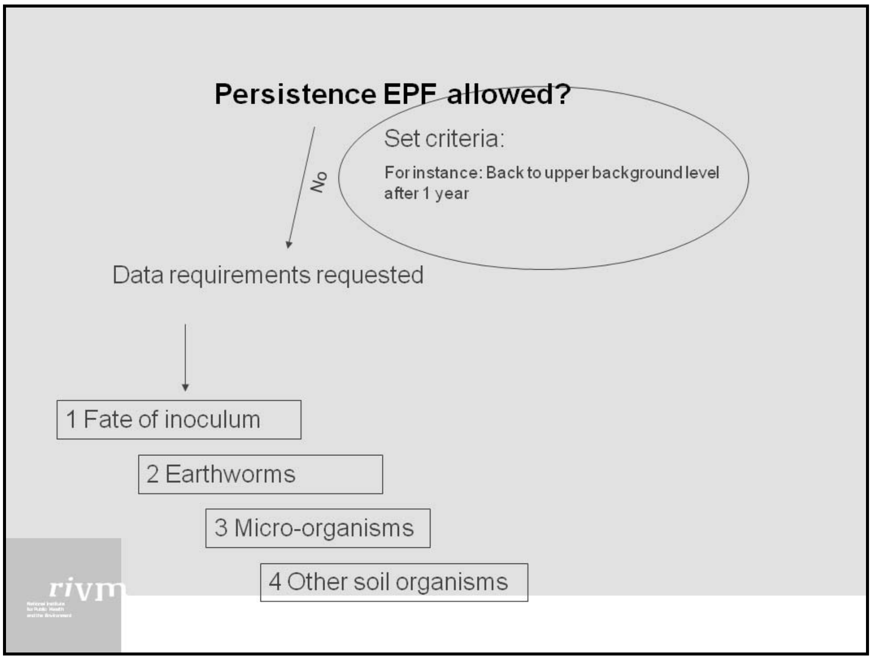
Data requirements:

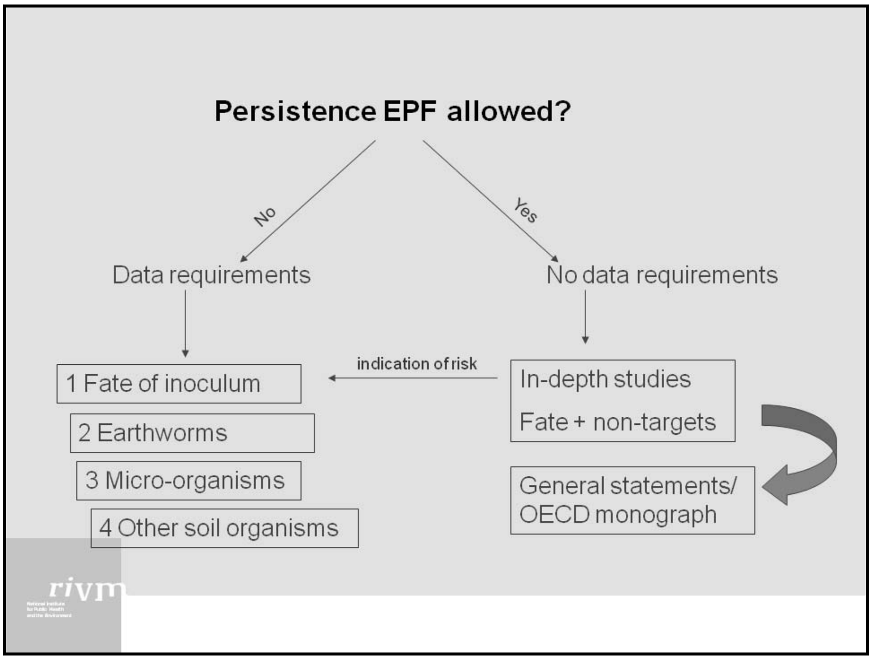
- Earthworms
- Micro-organisms
  - standard nitrification and respiration experiments

rivm

Effects of biologicals to non-target micro-organisms in the soil




Persistence EPF allowed?
No
Set criteria:
For instance: Back to upper background level after 1 year
Data requirements requested
1 Fate of inoculum
2 Earthworms
3 Micro-organisms
4 Other soil organisms
rivm


Persistence EPF allowed?
No
Yes
Data requirements
No data requirements
1 Fate of inoculum
2 Earthworms
3 Micro-organisms
4 Other soil organisms
indication of risk
In-depth studies
Fate + non-targets
General statements/
OECD monograph
rivm

Conclusions

No fear for uncontrolled growth is necessary for EPF

Increases due to reproduction in host insects or renewal of inoculum

Steady decline for all species

Upper background levels of approximately 1000 CFU/ g soil reached after
1 year by *Beauveria bassiana*
> 4 years *Beauveria brongniartii*
>10 years *Metarhizium anisopliae*

In-depth studies on non-target effects are necessary

Can be used as the base for statements/OECD monographs

rivm

Review

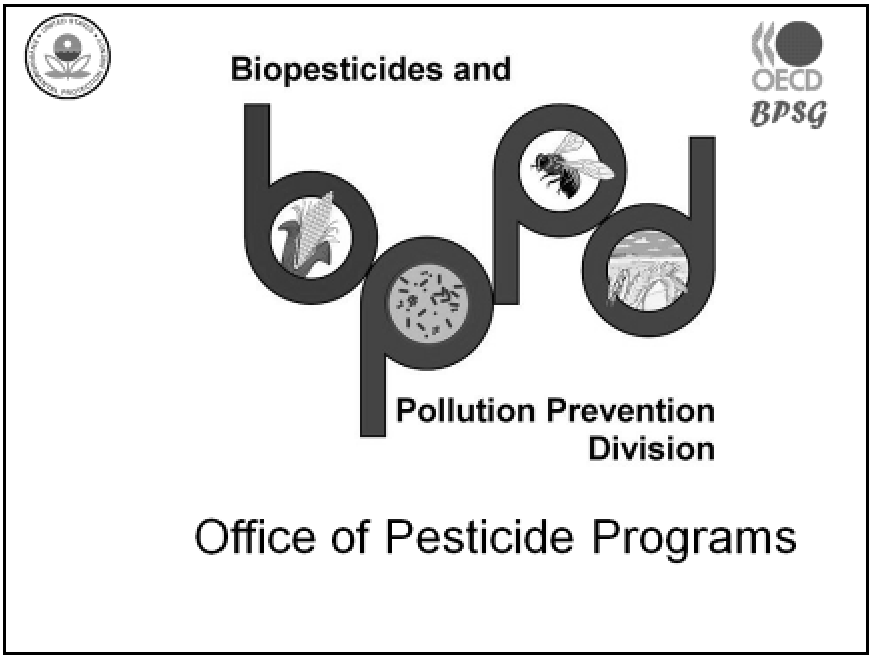
Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment in accordance with EU regulations

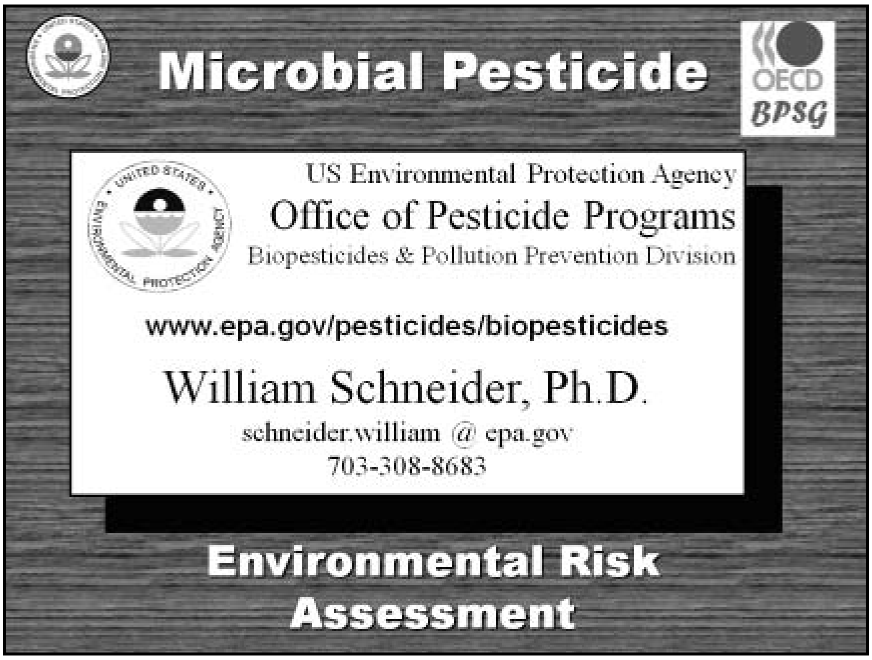
J.W.A. Scheepmaker and T.M. Butt (2010)
Biocontrol Science and Technology 20(5): 503-552

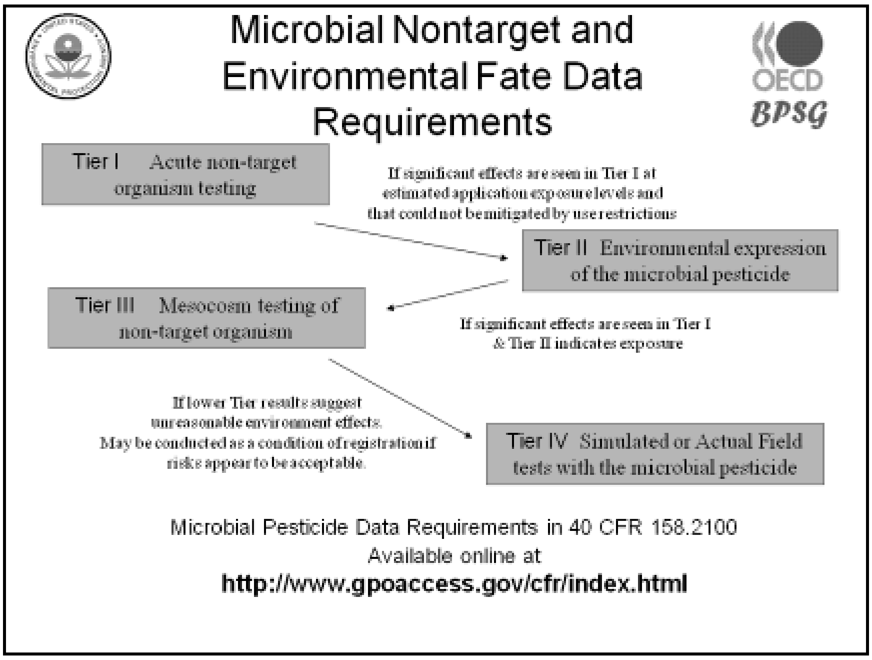
rivm

US experiences and approach to assessing the environmental fate and NTO effects of microbial pest control agents

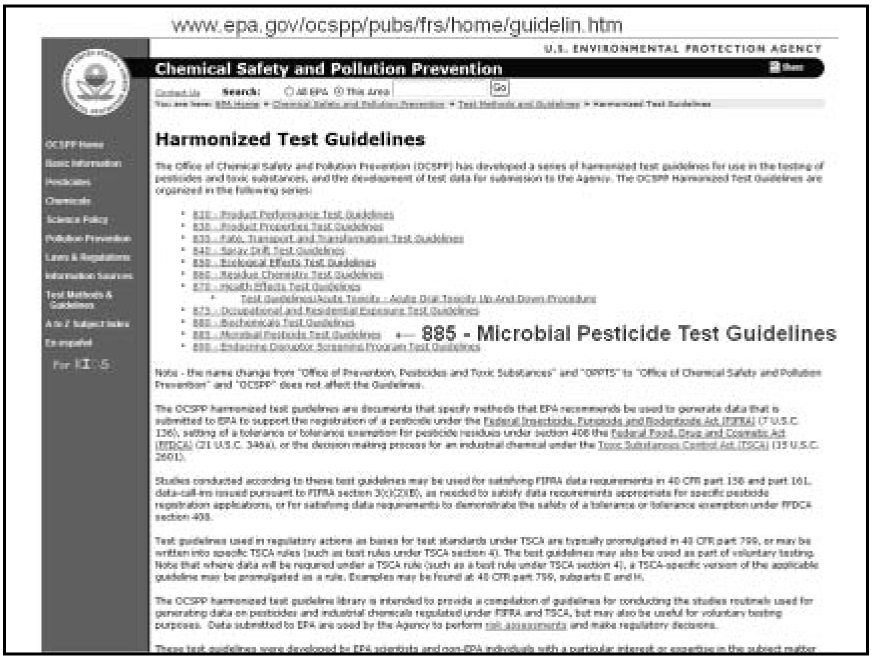
By William Schneider, U.S. Environmental Protection Agency, BioPesticides and Pollution Prevention Division, Office of Pesticide Programs, USA



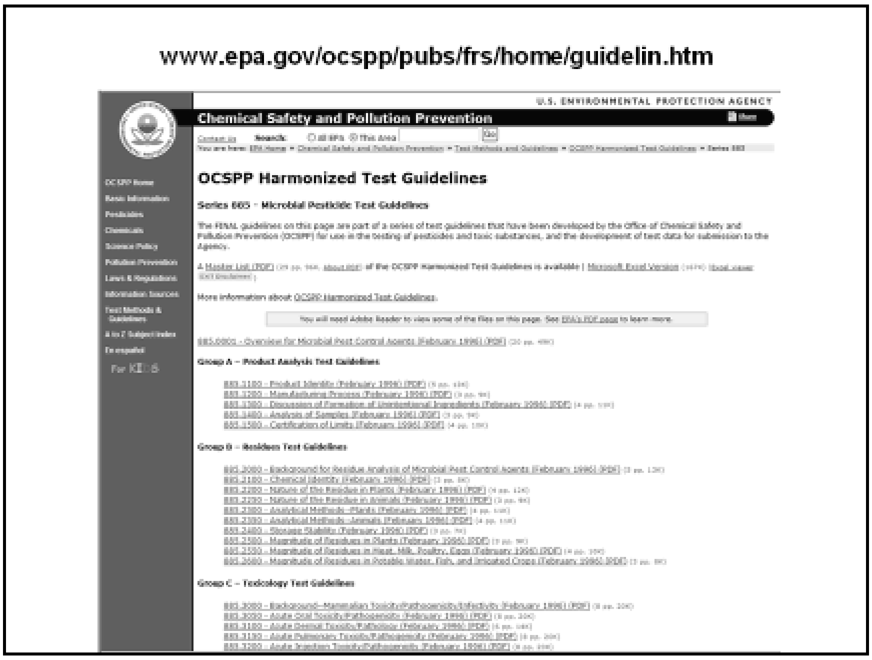




Guideline	Data Requirement	When Required	Recommended Test Species
Tier I			
885.4050	Avian oral toxicity	For all uses except indoor/ greenhouse uses with no exposure	Upland game, e.g. Quail or Waterfowl, e.g. Mallard Duck
885.4100	Avian inhalation toxicity/pathogenicity	When the nature of the microbial pesticide and/or its toxins indicates potential pathogenicity to birds.	Upland game, e.g. Quail
885.4150	Wild mammal toxicity/pathogenicity	Only if human health toxicity tests are not sufficient for assessment	Representative of a potentially exposed species
885.4200	Freshwater fish toxicity/ pathogenicity	Only if significant exposure to aquatic organisms	Coldwater fish -nonaquatic use + Warmwater fish -aquatic use
885.4240	Freshwater invertebrate toxicity/pathogenicity	Only if significant exposure to aquatic organisms	Generally Daphnia
885.4280	Estuarine/Marine fish testing Estuarine and marine invertebrate	Only if significant exposure to estuarine or marine environment (especially if directly applied)	A fish species likely to ingest target pests. Invertebrate species: shrimp
885.4300	Nontarget plant testing	Only if the microbial pesticide is taxonomically related to a known plant pathogen	Variable depending on microbial pesticide properties and uses – details in 885.4300
885.4340	Nontarget insect testing	Only if directly infective to a target insect, i.e. may cause epizootic	Usually: Ladybird beetle, Green lacewing, & Parasitic wasp
885.4380	Honey bee testing	For all uses except indoor/ greenhouse uses with no exposure	Larvae or adults



www.epa.gov/ocspp/pubs/frs/home/guidelin.htm
U.S. ENVIRONMENTAL PROTECTION AGENCY
Chemical Safety and Pollution Prevention
Harmonized Test Guidelines
885 - Microbial Pesticide Test Guidelines



www.epa.gov/ocspp/pubs/frs/home/guidelin.htm
U.S. ENVIRONMENTAL PROTECTION AGENCY
Chemical Safety and Pollution Prevention
OCSPP Harmonized Test Guidelines

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885.4380	Honey bee testing	For all uses except indoor/ greenhouse uses with no exposure	Larvae or adults

Additional Nontarget Testing

OECD BPSG

- Nontarget microorganisms
  - Microbial communities are extremely variable and difficult to evaluate
  - Environmental populations generally represent the most competitive species that have adapted to that particular environmental niche.
  - What would be a significant effect?
  - Testing on a specific microorganism might be warranted on a case-by-case basis, e.g. if the MPCA is likely to kill beneficial root colonizers, e.g. Mycorrhizal fungi
- Earthworms
  - Earthworms are extremely resistant to pathogens and have very active immune systems
  - No known pathogens of earthworms
- REBECA
  - Recommended not testing microorganisms and earthworms

Adverse Effects Analysis

OECD BPSG

- If adverse effects are observed at maximum doses, then sequentially lower doses should be tested to determine an LD50, LC50 or ID50 as described in the Tier 1 testing guidelines
- The adverse effects will also require Tier II environmental expression testing in a contained environment, e.g. greenhouse or aquarium
  - We recognize the difficulty in obtaining valid environmental expression data
  - Tier II data has rarely been required for a microbial pesticide registration

OECD
BPSG

Tier II	Exposure studies (almost never needed for US products so far)	
885.5200	Terrestrial environmental expression tests in a greenhouse environment, or a full laboratory growth requirements analysis indicating no survival	Required if toxic or pathogenic effects are observed in Tier I tests on terrestrial species Representative field studies may be acceptable
885.5300	Freshwater environmental expression tests in an aquarium, or full laboratory growth requirements analysis indicating no survival	Required if toxic or pathogenic effects are observed in Tier I tests on aquatic species
885.5400	Marine or estuarine environmental expression tests in an aquarium, or full laboratory growth requirements analysis indicating no survival	Required if toxic or pathogenic effects are observed in Tier I tests on marine or estuarine species that might be exposed as indicated by Tier II studies

EPA

Microbial Pesticides Test Guidelines
OPPTS 885.5000
Background for Microbial Pesticides Testing

OPPTS 885.5000 Background for microbial pesticides testing.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is OPP guideline 155A–1.

(b) **Purpose.** Tier II tests are intended to demonstrate whether a microbial pest control agent (MPCA) is able to survive or replicate in the environment, thereby indicating which nontarget organism(s) may be exposed to the microbial agent, as well as provide an indication of the level of exposure. A determination of the environmental expression of an MPCA includes an evaluation of the growth of the agent when introduced into a new environment—the way the MPCA may alter its growth habits, take advantage of new environmental conditions or take advantage of changes in the existing equilibrium among microbial species (e.g. in a commensal association, an association in which one species benefits and the other is unaffected). The expression of a microorganism's presence may result in its insertion into a new habitat and continued propagation in the new habitat. Terrestrial and aquatic (freshwater and marine) environments are to be considered, as indicated by results from Tier I, the product type and the product usage. Advance consultation with Agency personnel is recommended since requirements may be modified on a case-by-case basis. The Tier II guidelines consist of descriptions of tests to determine the environmental expression of an MPCA in a terrestrial environment (OPPTS 885.5200), in a freshwater environment (OPPTS 885.5300), and in a marine or estuarine environment (OPPTS 885.5400). If Tier II data is negative (no adverse effects), no further testing is needed. Tier progression is fully explained in OPPTS 885.0001.

(c) **Definitions.** The following definitions apply to OPPTS 885.5200, 885.5300, and 885.5400.

Contained environment. Refers to the use of natural materials from the proposed use-environment (sediment, soil, plants, and marine/estuarine liquids) arranged as naturally as possible, and held within a plastic, glass, or other container to prevent the escape of the microbial agent.

Substance to be tested. A typical end-use product (EP) or the technical grade of the active ingredient (TGAI) shall be tested.

Observation period. This should be of sufficient duration to determine survival, growth, or decline (to the limit of detection) of the MPCA in the appropriate environment unequivocally. Decline of the MPCA should be followed for a sufficient period of time to detect regrowth of the agent after a prolonged adaptation period.

1

Tier III	Triggered by toxic effects in Tier I studies and exposure indicated by Tier II studies	
885.4600	Avian chronic pathogenicity and reproduction test	If pathogenic effects seen in Tier 1 avian studies If Tier II studies indicate likely long-term exposure
885.4650	Aquatic invertebrate range testing	If intended for use in water or expected to be transported to water and pathogenicity or infectivity seen in Tier I studies
885.4700	Fish life cycle studies	If intended for use in water or expected to be transported to water and pathogenicity or infectivity seen in Tier I studies
885.4750	Aquatic ecosystem test	If the assessment based on the potential exposure and Tier II, II, & III testing indicate adverse effects on aquatic nontarget organisms

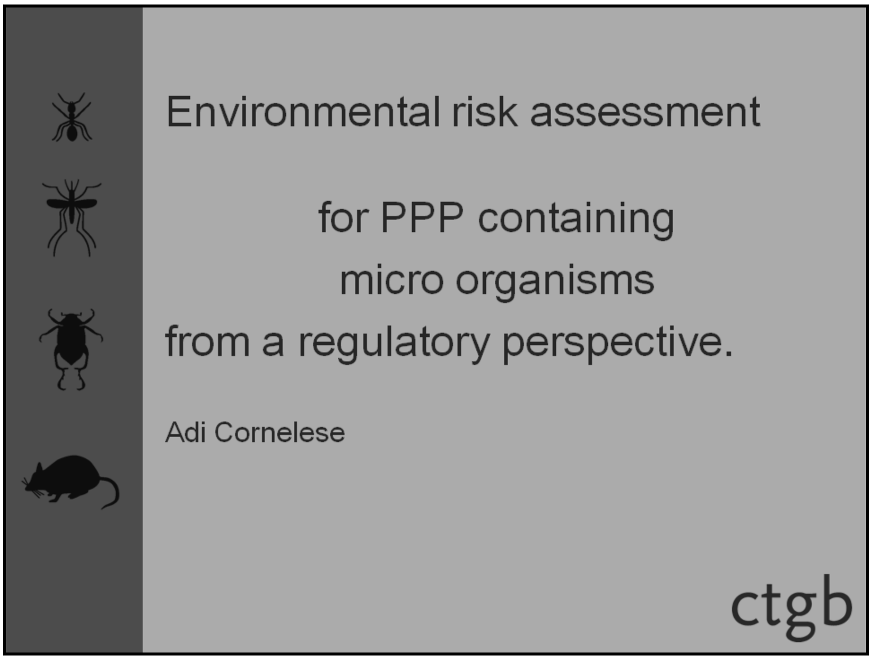
Tier IV	Field testing, may be conducted as a condition of registration if mitigation appears to be adequate	
850.2500 850.1950	Field testing for terrestrial wildlife and Field testing for aquatic organisms	If toxic or pathogenic effects are observed in Tier I tests on marine or estuarine species that might be exposed by the use pattern. If toxicity or pathogenicity is seen in aquatic studies (including plants)
850.2500	Simulated or actual field tests (birds, mammals)	If pathogenic effects at field exposure levels are seen at Tier III and test containment would not prevent escape
850.1950	Simulated or actual field test (aquatic organisms)	Short term studies required If lab data, use patterns, and exposure rate indicate that adverse short-term or acute effects are likely to occur - and - Long term studies required if laboratory data indicate that adverse long term, cumulative, or life-cycle effects may result from intended use.
850.2500	Simulated or actual field tests (insect predators, parasites)	
850.3040	Simulated or actual field tests (insect pollinators)	
850.4300	Simulated or actual field tests (plants)	

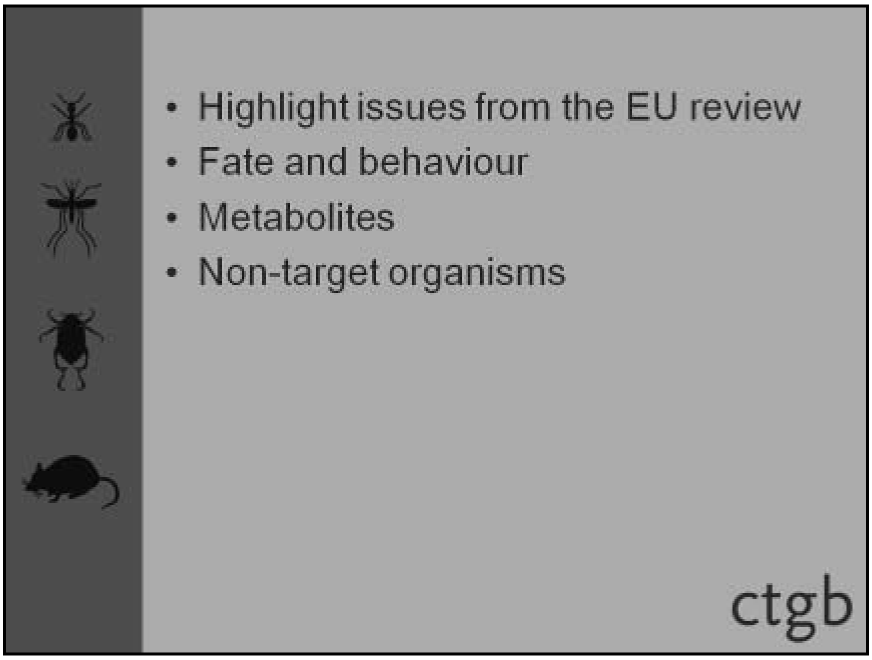
Risk Assessment

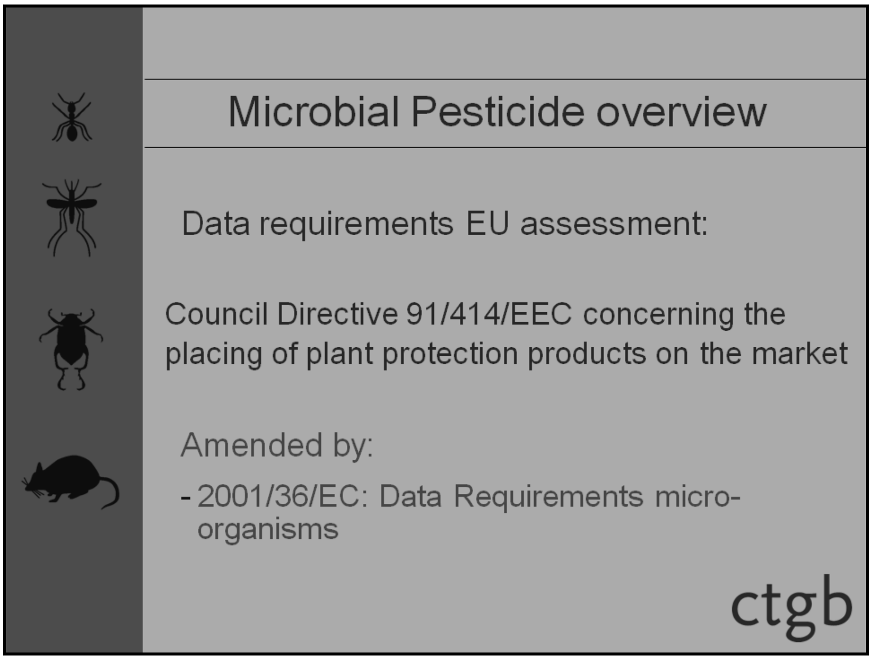
- Hazard
  - Toxicity, Pathogenicity to representative nontarget species
- Exposure
  - Exposure data required only if toxicity concerns are identified
  - Aspects of environmental microbial exposure
    - Population dynamics, infectivity
    - Scale of use, use patterns, application rates
    - Persistence, degradation of toxins, mobility
  - Formal Exposure Analysis seldom used for microbial pesticides
    - Difficult analysis for microbials that multiply in the environment
      - Extremely variable test results
      - Environmental populations are very competitive
      - Generally drop to background levels
  - May use application rates as a worst case
- Risk
  - Non-targets, humans, domestic animals, endangered species

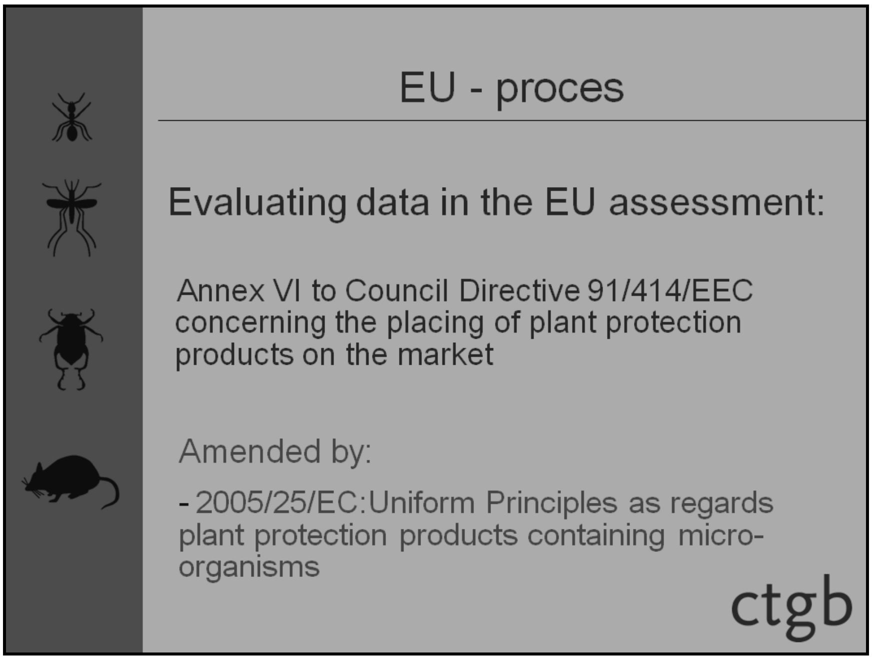
Environmental risk assessment of microbial pesticides from a regulatory perspective

By Adi Cornelese (Board for the Authorisation of Plant Protection Products and Biocides, Wageningen, The Netherlands)






Microbial Pesticide overview
Data requirements EU assessment:
Council Directive 91/414/EEC concerning the placing of plant protection products on the market
Amended by:
- 2001/36/EC: Data Requirements micro-organisms
ctgb


EU - proces
Evaluating data in the EU assessment:
Annex VI to Council Directive 91/414/EEC concerning the placing of plant protection products on the market
Amended by:
- 2005/25/EC:Uniform Principles as regards plant protection products containing micro-organisms
ctgb

Fate and behaviour

- 2001/36: Data requirement on viability, competitiveness and population dynamics in several soils typical of the various Community regions, and surface water.
- UP evaluation:
  - The potential for persistence and multiplication has to be assessed
- UP decision making:
  - unacceptable adverse effects due to fate and behaviour based on origin, properties and survival.
  - Sufficient information on persistence/competitiveness.
  - Persistence in concentrations considerable higher than background unless the risk from accumulated plateau is acceptable

ctgb

Exposure to air

- Data requirement on concentration in air
- Criterion in UP is to evaluate long range transport.

But no RA criteria. How to interpret?

ctgb

Metabolites

- Unclear terminology

  Metabolites of concern --- relevant metabolites --- residual metabolites

- Data requirements

  - relevant metabolites (i.e. of concern for human health and/or the environment), conditions should be characterised.

- UP evaluation part

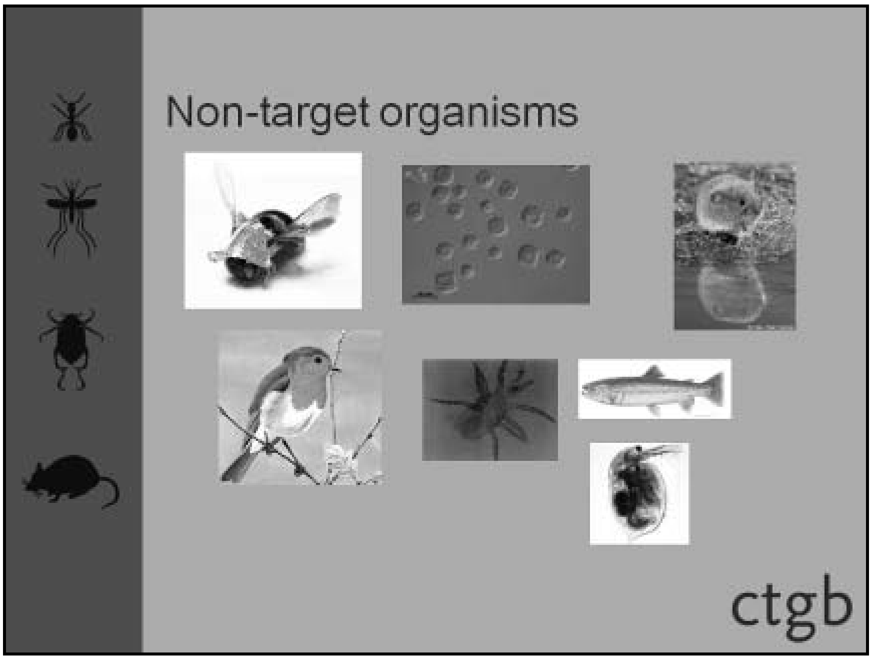
  - Assessment for any known relevant metabolite for each environmental compartment as for chemical metabolites is required.

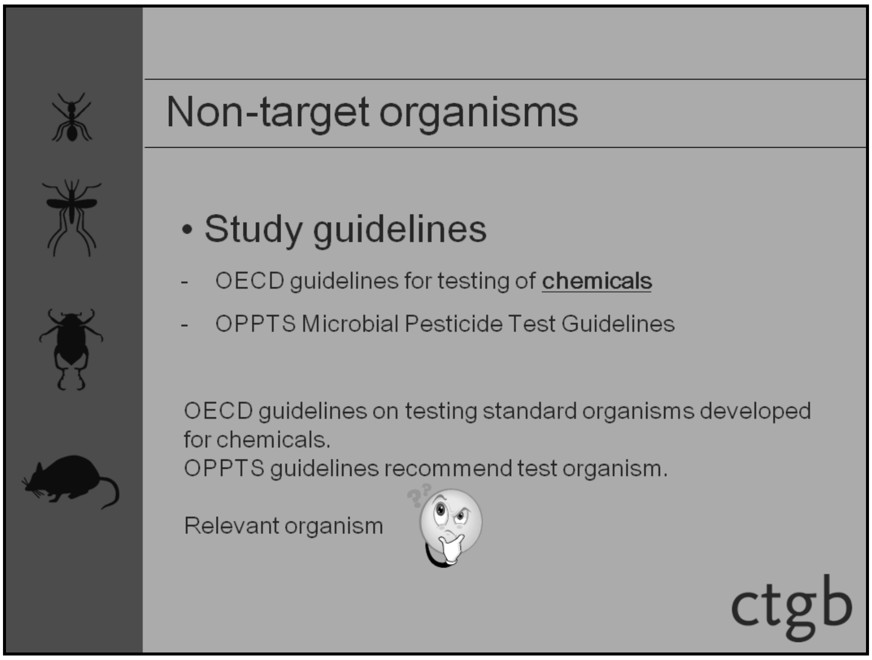
ctgb

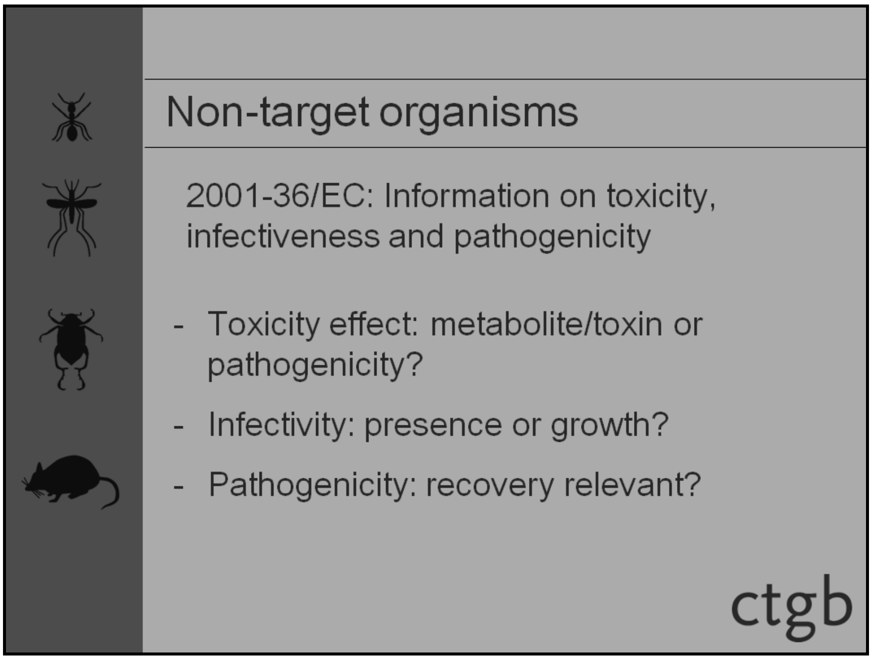
Metabolites

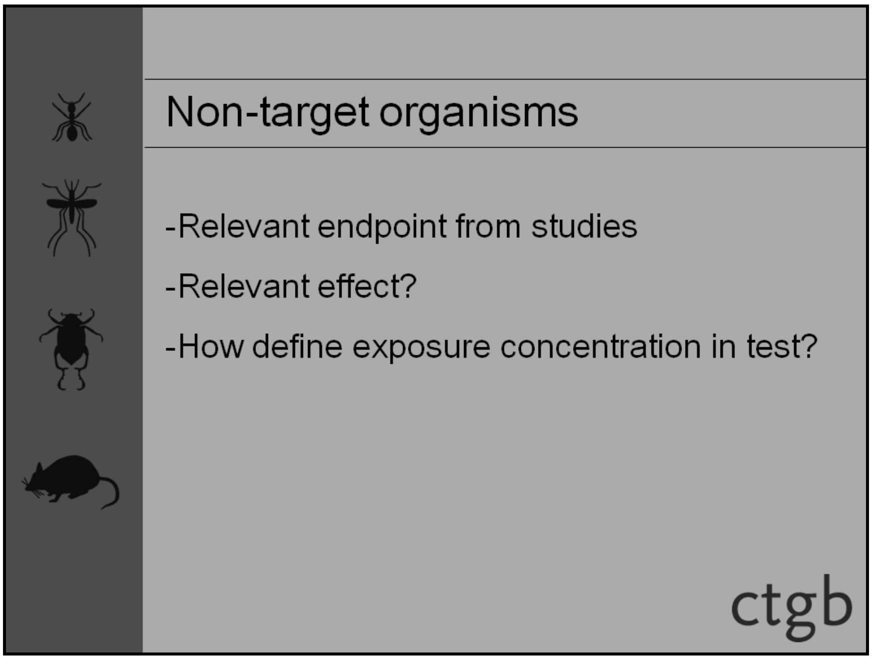
- Question – What means relevant?
- How should this information be derived?
- Should we characterise all? How?

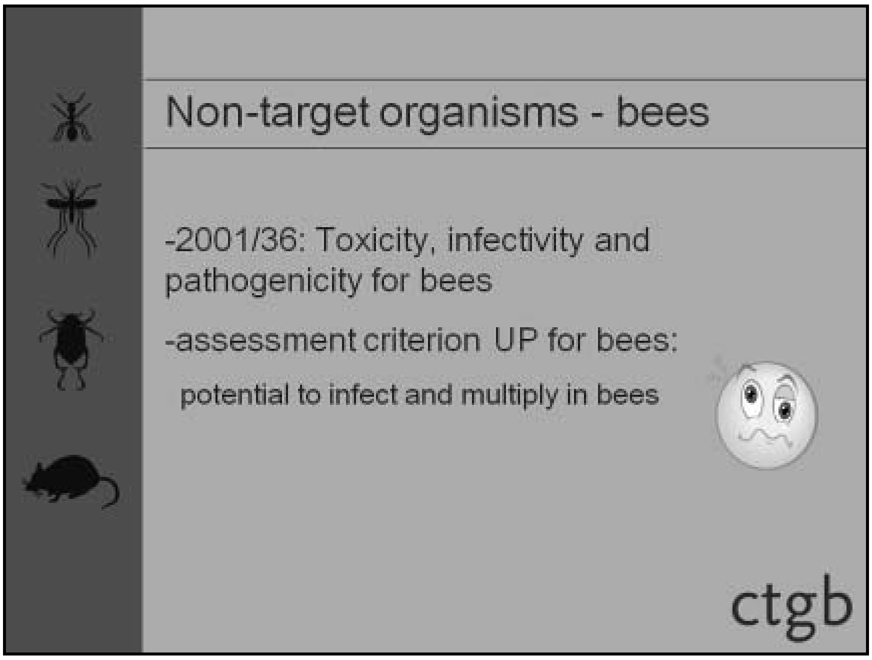
ctgb

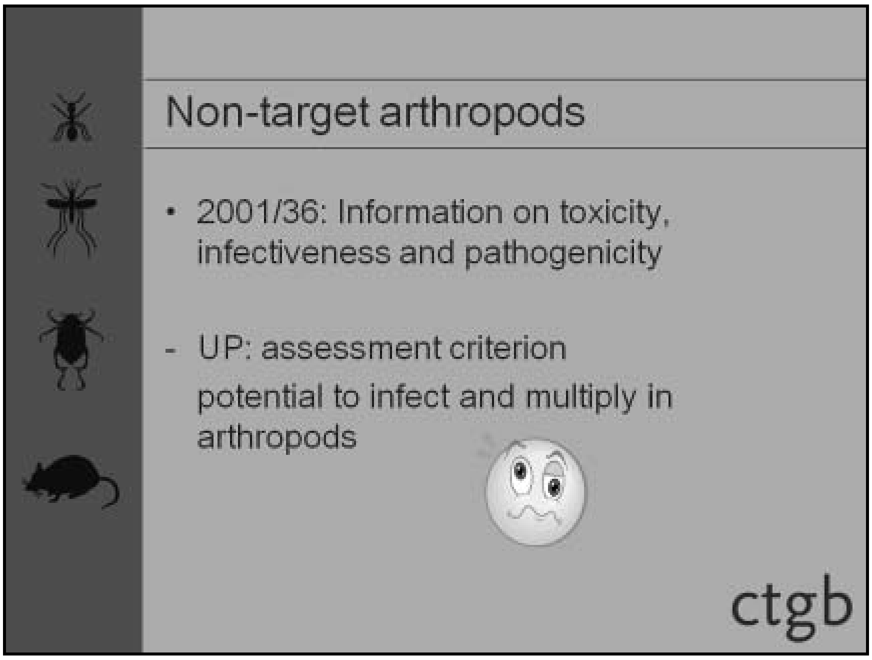

Non-target organisms
ctgb

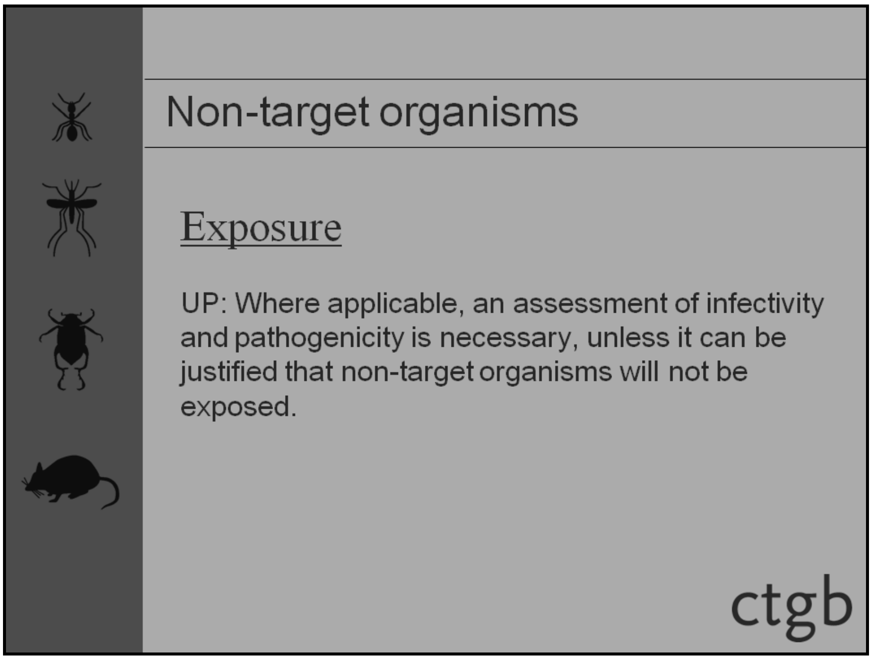

Non-target organisms
• Study guidelines
- OECD guidelines for testing of chemicals
- OPPTS Microbial Pesticide Test Guidelines
OECD guidelines on testing standard organisms developed for chemicals.
OPPTS guidelines recommend test organism.
Relevant organism
ctgb

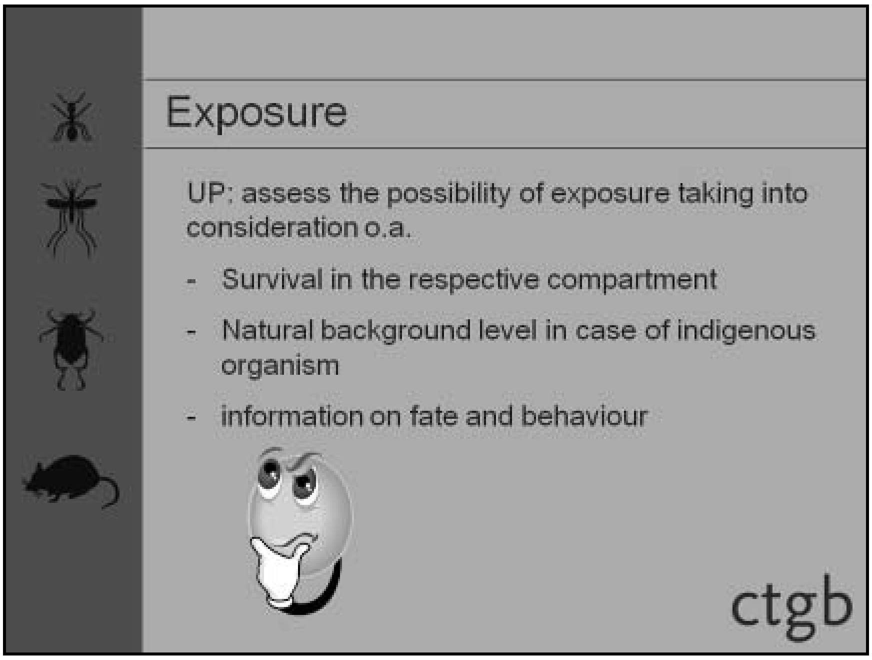

Non-target organisms
2001-36/EC: Information on toxicity, infectiveness and pathogenicity
- Toxicity effect: metabolite/toxin or pathogenicity?
- Infectivity: presence or growth?
- Pathogenicity: recovery relevant?
ctgb


Non-target organisms
-Relevant endpoint from studies
-Relevant effect?
-How define exposure concentration in test?
ctgb


Non-target organisms - bees
-2001/36: Toxicity, infectivity and pathogenicity for bees
-assessment criterion UP for bees:
potential to infect and multiply in bees
ctgb


Non-target arthropods
• 2001/36: Information on toxicity, infectiveness and pathogenicity
- UP: assessment criterion
potential to infect and multiply in arthropods
ctgb


Non-target organisms
Exposure
UP: Where applicable, an assessment of infectivity and pathogenicity is necessary, unless it can be justified that non-target organisms will not be exposed.
ctgb


Exposure
UP: assess the possibility of exposure taking into consideration o.a.
- Survival in the respective compartment
- Natural background level in case of indigenous organism
- information on fate and behaviour
ctgb

Exposure assessment

Exposure assessment only seems relevant if the m.o. can survive

EU review process; considered essential to quantify exposure

How to quantify?

ctgb

Exposure assessment

- Exposure models for chemicals?
- Absolute worst case acceptable for soil, water, air?
- Exposure of NTO?

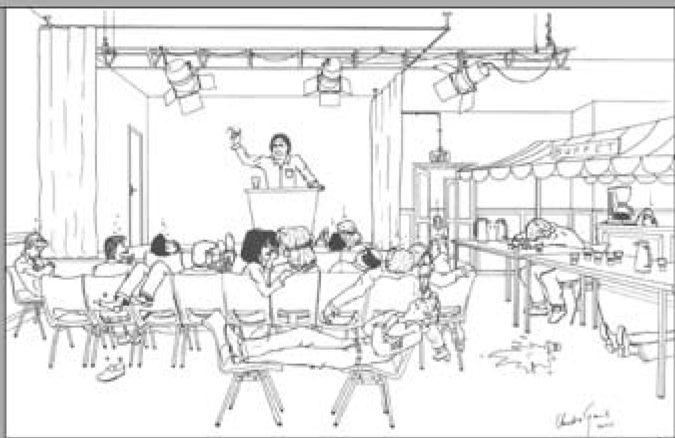
ctgb

Conclusive remarks

- Data requirements and Uniform Principles need to be reviewed and revised
- Suitable test guidelines need to be indicated
- Need to quantify exposure?

ctgb

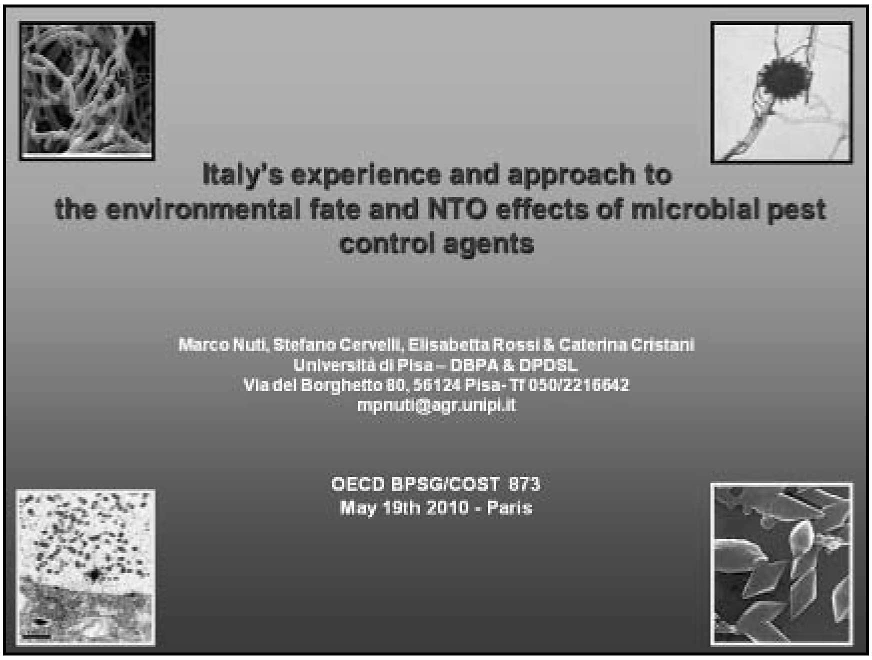
Thank you for your attention

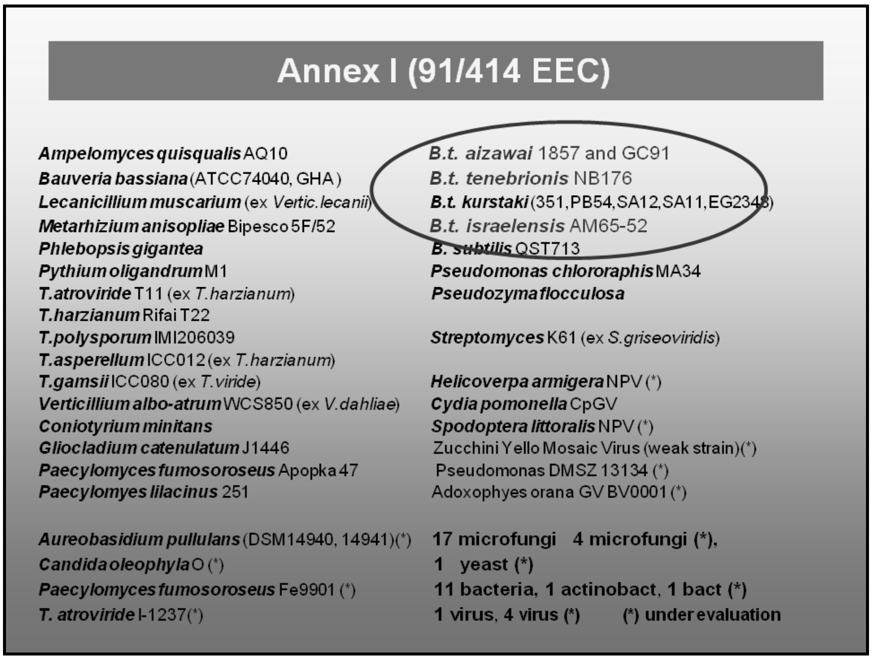


ctgb

Italy's experiences and approach to assessing the environmental fate and NTO effects of microbial pest control agents

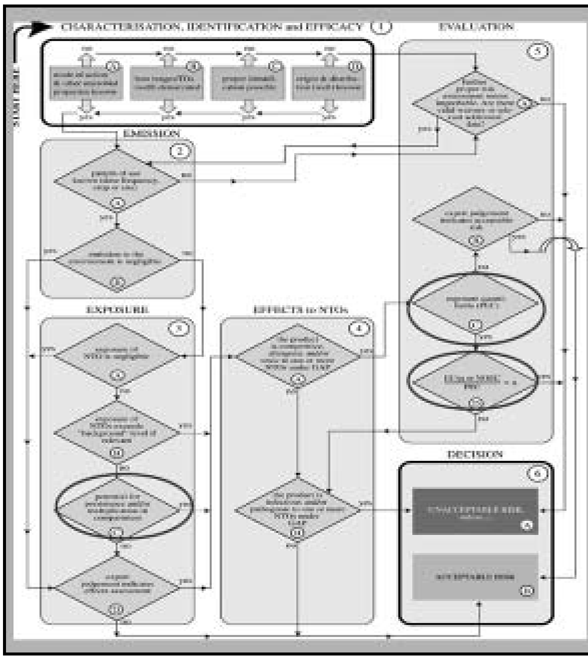
By Marco Nuti, Stefano Cervelli, Elisabetta Rossi & Caterina Cristani (University of Pisa; Italy)





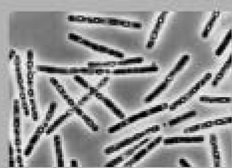
Strains and targets

Product	Strain	Insecticidal activity
Agree 50 WP	*Bacillus thuringiensis* ssp.*aizawai* CG-91	Wide range of lepidopteran pest species *Lobesia botrana, Eupoecelis ambiguella*
XenTari WG	*Bacillus thuringiensis* ssp. *aizawai* ABTS1857	Defoliating caterpillars *Chrysodeixis chalcites* (Northern EU), *Heliothis plusia* or *Spodoptera* (Southern EU)
VectoBac WG	*Bacillus thuringiensis* ssp. *israelensis* AM65-52	*Dipteran* insects Fungus gnats (*Diptera Sciaridae*)
Novodor SC	*Bacillus thuringiensis* ssp. *tenebrionis* NB176	Foliar feeding Coleopteran beetle larvae *Leptinotarsa decemlineata*



ERA DECISION TREE

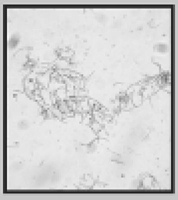
For m.o. : "concentration" cannot be used, we used "density" instead (i.e. PED instead of PEC, PNED instead of PNEC etc)



Mensinck BJ *et al* (2007) Biocontrol Sci.Technol.17, 3-20

Scientific problems emerged during the assessment of *Bt*s as PPPs for inclusion in Annex I (2007-2010)

- identity at strain level
- biological cycle
- contaminants
- mutagenesis & cancerogenesis
- exposure assessment
- environmental risk assessment (fate & NTOs)
- cumulative risks
- scientific soundness of data



Plant protection products considered not appropriate for QPS (Qualified Presumption of Safety)

Bacillus thuringiensis aizawai ABTS 1857

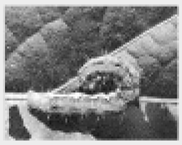
Bacillus thuringiensis aizawai GC 91

Bacillus thuringiensis israeliensis AM 65-52

Bacillus thuringiensis kurstaki PB 54

Bacillus thuringiensis kurstaki ABTS 351

Bacillus thuringiensis kurstaki SA11



The EFSA Journal (2008) 923, 1-48

A new approach for assessing fate and persistence: assumptions and methods

Assumptions

- YES degradation
- NO adsorption (step-1 worst case scenario)
- Application to soil
- YES drift to surface water at the distance of 3 m, different % according to the crop
- Various numbers of applications
- Various intervals between applications
- Results obtained at time zero and following the last application at the highest suggested rate

Methods

- The population densities have been calculated at time zero and following the last application at the highest suggested rate
- The calculation have been done by using the software PEC-TWA_NEW_1_3_1 (developed by Dr. S. Cervelli stefano.cervelli@ise.cnr.it)

ACTIVE INGRED	PRODUCT	CROP	APPLICATION TO	*Conc TP* ----------- *(CFU/g)* *(Biopotency* ITU/g)	Application Rate		
					(kg/ha)	(CFU/ha)	(biopotency ITU/ha)
Bta GC-91	Agree 50 WP (50%)	Grape	Field	$6x10^{10}$ $5x10^{7}$	0.4-0.8	$2.4\text{-}4.8x10^{13}$	$2\text{-}4x10^{10}$
Bta ABTS-1857	XenTari WG (54%)	Pepper h<50 cm	Field – Indoor	$4.7x10^{10}$ $2.8x10^{7}$	0.27-0.54	$1.3\text{-}2.5x10^{13}$	$0.75\text{-}1.5x10^{10}$
Bti AM65-52	VectoBac WG (37.4%)	Ornamental	Indoor	$4.8x10^{10}$ $9.1x10^{6}$	30	$1.4x10^{15}$	$2.7x10^{11}$
Btte *NB-176*	Novodor SC (10%)	Potatoes h>50 cm	Field	$8.3x10^{9}$ $1.5x10^{8}$	0.33-0.77	$2.7\text{-}6.4x10^{12}$	$0.5\text{-}1.2x10^{11}$

Active Ingredient	Formulate	$DT50_s$ (day)	$DT50_{sw}$ (day)	Replications	Interval (days)
Bta GC-91	Agree 50 WP	120	50	4	7
Bta ABTS-1857	XenTari WG	120	50	6	7
Bti AM65-52	VectoBac WG	120	50	3	8
Btte NB-176	Novodor SC	120	50	3	5

MPCP*	Rate MPCP (kg/ha)	MPCA**	Rate MPCA (kg TP/ha)	Rate CFU (CFU/ha)	Density CFU (CFU/g MPCA)	Concentr Toxin (ITU/mg MPCP)	Rate Toxin (ITU/ha)	Concentr Toxin ITU/g MPCA
Agree 50 WP 50%	0.80	Bta	0.40	2.40E+13	6.00E+10	2.5E+04	2.0E+10	5.0E+07
	1.60	GC-91	0.80	4.80E+13	6.00E+10	2.5E+04	4.0E+10	5.0E+07
XenTari WG 54%	0.50	Bta	0.27	1.27E+13	4.70E+10	1.5E+04	7.5E+09	2.8E+07
	1.00	ABTS-1857	0.54	2.54E+13	4.70E+10	1.5E+04	1.5E+10	2.8E+07
VectoBac WG 37.4%	80.21	Bti AM65-52	30.00	1.44E+15	4.80E+10	3.4E+03	2.7E+11	9.1E+06
Novodor SC 10%	3.30	Btte	0.33	2.74E+12	8.30E+09	1.5E+04	5.0E+10	1.5E+08
	7.70	NB-176	0.77	6.39E+12	8.30E+09	1.5E+04	1.2E+11	1.5E+08

Chemical - physical characteristics of the model

Soil Bulk Density	= 1.5	g/ml
Soil Thickness	= 5	cm
Water Depth	= 30	cm
Rate AM65-52 TP	= 1.8×10^{13}	CFU/ha
Bt spore DT_{50} in soil	= 120	day
Bt spore DT_{50} in water	= 50	day
Distance spore drift to water	= 3	m
Drift percentage	= according to the crop	
Rate Agree 50 WP	= 0.40-0.80	kg/ha
Max spore rate CG-91	= 4.8×10^{13}	CFU/ha
Rate XenTari WG	= 0.27-0.54	kg/ha
Max spore rate ABTS-1857	= 2.5×10^{13}	CFU/ha
Rate VectoBac WG	= 30	kg/ha
Max spore rate AM65-52	= 1.4×10^{15}	CFU/ha
Rate Novodor SC	= 0.33-0.77	kg/ha
Max spore rate NB-176	= 6.4×10^{12}	CFU/ha

Bacillus thuringiensis aizawai

Agree 50 WP strain GC-91

Applications	CFU g^{-1}	CFU L^{-1}
1	5.01×10^4	1.09×10^6
4	1.38×10^5	3.76×10^6

XenTari WG strain ABTS-1857

Applications	CFU g^{-1}	CFU L^{-1}
1	3.23×10^4	4.96×10^4
6	1.05×10^5	2.27×10^5

Bacillus thuringiensis israeliensis

VectoBac WP strain AM65-92

Applications	CFU g^{-1}	CFU L^{-1}
1	8.25×10^5	3.09×10^6
3	1.89×10^6	8.60×10^6

Bacillus thuringiensis tenebrionis

Novodor SC strain NB-176

Applications	CFU g^{-1}	CFU L^{-1}
1	6.07×10^3	1.50×10^5
3	1.41×10^4	4.15×10^5

Predicted environmental density (PED) following repeated applications

E R A : impact on NTOs

- *Bt* is an "old" product, and there are many scientific papers on its environmental impact; this has caused confusion about the need for specific data at strain level, which are too often lacking. The above data cannot extrapolated for endpoints to achieve quantitative RA

- In aquatic toxicology tests, turbidity due to high concentration of the product is probably the main cause to some negative effects on fish and Daphnia

- The ecotoxicological approach of chemical products is probably not the best suited for microbials

- Due to the specificity of Bt, the choice of particular groups of beneficial insects could be better used to describe effects on NTOs. Indeed NTO testing is based on "surrogate species" (classic ecotoxicological approach) and not on "ecologically relevant species" (field testing required)

Examples of functional groups (exposure through trophic interactions)

Herbivores

sucking: **aphids (Hemiptera: Aphididae), spider mites (Acarina), leafhoppers (e.g. Hemiptera: Cicadellidae), thrips (Thysanoptera:Thripidae), certain Heteroptera and Nematoda**
chewing: **leaf beetles (Coleoptera: Chrysomelidae), Lepidoptera larvae, Diptera larvae, grasshoppers (Orthoptera Ensifera), gastropods (Mollusca Gastropoda)**

Predators

beetles: **Coleoptera (e.g. Coccinellidae, Carabidae, Staphilinidae);** predatory bugs: **Heteroptera (e.g. Nabidae, Anthocoridae);** predatory flies: **Diptera (e.g. Syrphidae)**
lacewings: **Neuroptera (e.g. Chrysopidae, Hemerobidae);** thrips: **Thysanoptera (e.g. Aeolothrips);** spiders & harvestmen: **Araneae and Opiliones**
mites: **Acarina (e.g. Phytoseiidae);**nematodes **(e.g. Heterorhabditidae, Steinernematidae)**

Parasitoids **Hymenoptera (e.g. Ichneumonidae, Braconidae, Aphelinidae)**
Pollinators **solitary and social bees, bumble bees (Hymenoptera: Apidae), hover flies (Diptera: Syrphidae)**
Decomposers **Diptera larvae (e.g. Phoridae), Nematoda, springtails (Collembola), mites (Acarina), earthworms (Haplotaxida: Lumbricidae), Isopoda**
Plant symbionts **rhizobacteria, mycorrhiza**
In the categorisation of relevant NTO species, additional species of economic, aesthetic or cultural relevance or species of conservational importance considered as threatened or endangered may also need to be included.

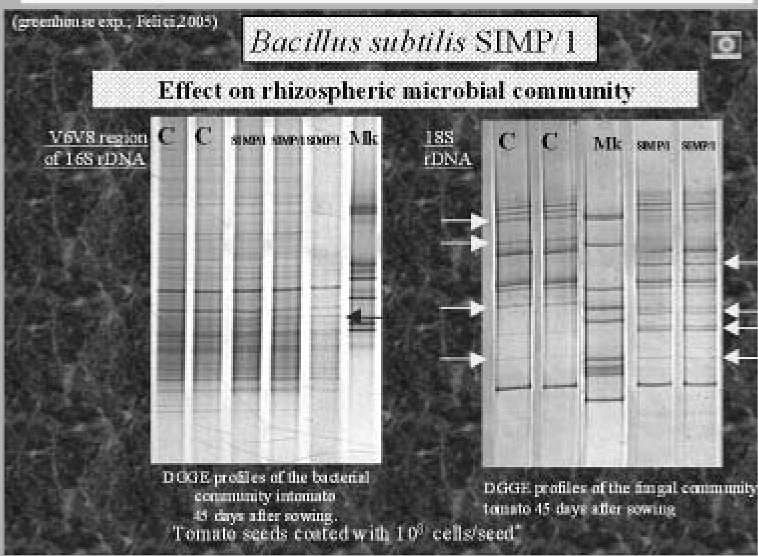
E R A : impact on NTOs

- Based on literature review on effect assessment, the EFSA PPR Panel concluded that for soft bodied soil organisms (earthworms, enchytraeids, nematodes) and plants in close contact with the soil solution, **pore water mediated uptake of pesticides** seems mainly responsible for the effects caused, and would therefore be **the relevant metric** for effects assessment, and consequently also for exposure assessment (EFSA Journal 922, 1-90, 2009). Is this true for microbials ?

- For a number of relevant soil taxa with different life and feeding strategies (e.g. mites and isopods), no information is available. For these organisms, additional routes of uptake (e.g. feed, contact with substrates in soil and litter) may need to be considered for terrestrial risk assessment.

- Soil microorganisms are generally overlooked (SANCO/10329/2002)

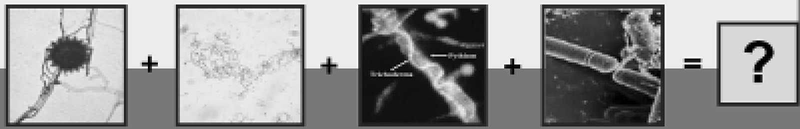
monitoring the effects of a BCA on soil m.o. with DGGE



cumulative risk assessment
(Reg. 396/2005)

1. it is not carried out at the moment
2. various approaches:

- cumulative risk (more than one substance is present at the same time)
- aggregate risk (all exposure routes are considered)
- probabilistic evaluation of exposure (use of distributions instead of punctual estimates)



Risk index for Microbial Control Agents

Five basic components have been proposed for the calculation of the overall environmental risk score:

1) Persistence of the substance
2) Dispersal potential
3) Range of (non)-target organisms that are affected
4) Direct and indirect effects on the ecosystem
5) Risks regarding vertebrate toxicity

Laengle,T. & H.Strasser, (2009) Developing a risk index to comparatively assess environmental risks posed by microbial and conventional pest control agents. Biocontrol Sci. Technol.

research areas (see also Chandler et al. 2008)

- Better knowledge of natural MCA phylogeny
- Understanding of MCA biogeography, including the factors determining the distribution of species and strains or affecting gene fluxes
- Better knowledge of factors affecting persistence and spread of MCA, including the interactions with biotic/abiotic environment and communities fluctuation
- Identification of background levels of MCA in agricultural and natural ecosystems, including prevalence and diversity at gene-community level, biomass and ecosystem functions
- Better knowledge of population dynamic, including host-pathogen, and of indirect effetct on NTOs

Fate of microbials in the environment - a structural model for explanation of the fate

By Niels Bohse Hendriksen (Department of Environmental Chemistry and Microbiology, Aarhus University; Denmark)

Fate of microbials in the environment – a structural model for explantion of the fate

Niels Bohse Hendriksen,
National Environmental Research Institute,
Aarhus University, Denmark

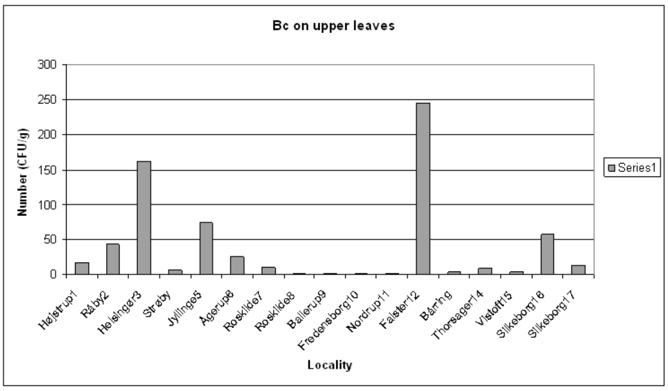
The structural model

- Endospore formers (*Bacillus thuringiensis*)
- On plants

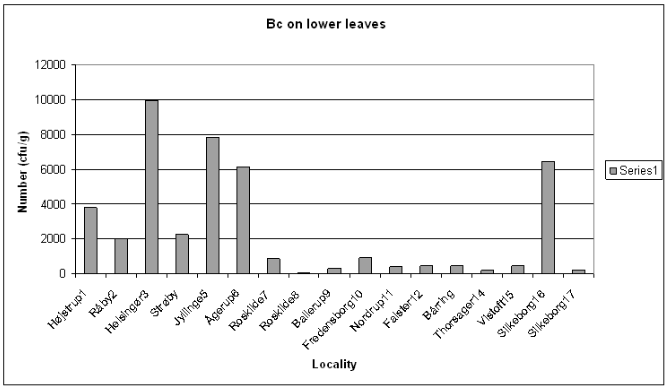
Why ?

- Residues
- Efficacy
- Risk assesment
- Scientific

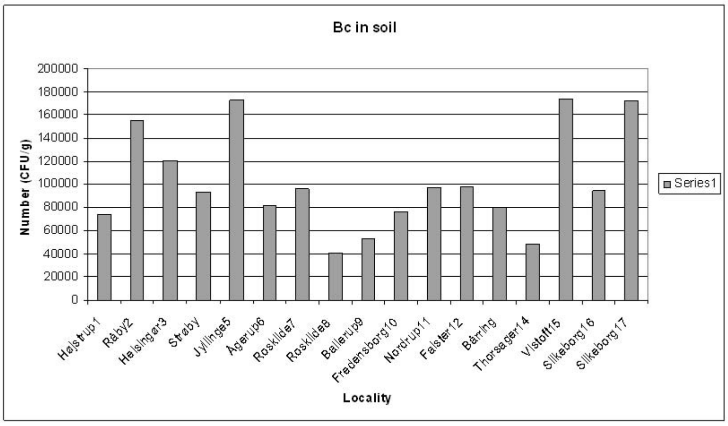
B. cereus like bacteria on leaves in top of plants



B. cereus like bacteria on leaves close to soil

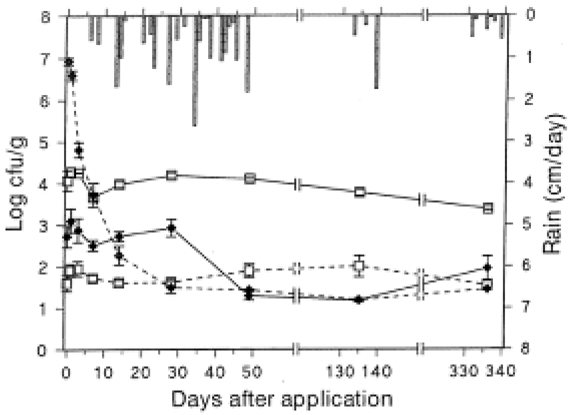


B. cereus like bacteria in soil



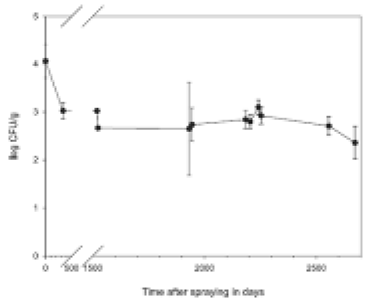
Fate of *B. thuringiensis kurstaki* on white cabbage

Fig. 1. Numbers of *B. thuringiensis* DMU67R detected (without heat treatment) on cabbage leaves (◆) and in topsoil (□) in squares with addition of *P. brassicae* larvae and treated with DMU67R either on soil (solid lines) or on leaves (broken lines). Error bars represent SEM ($n = 3$). Hanging columns show daily rainfall.

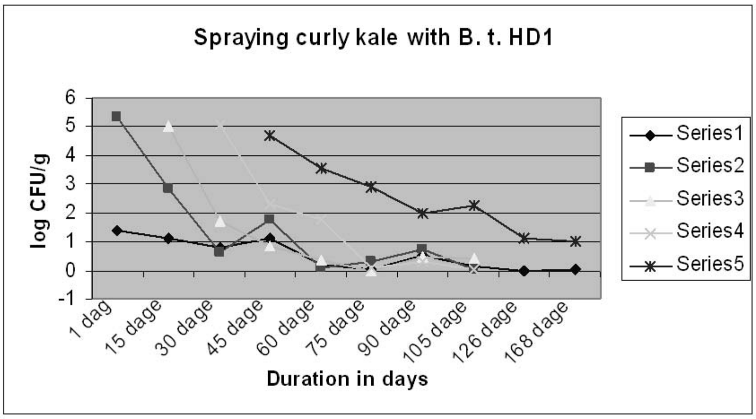


Long-time survival of *B. thuringiensis* in soil

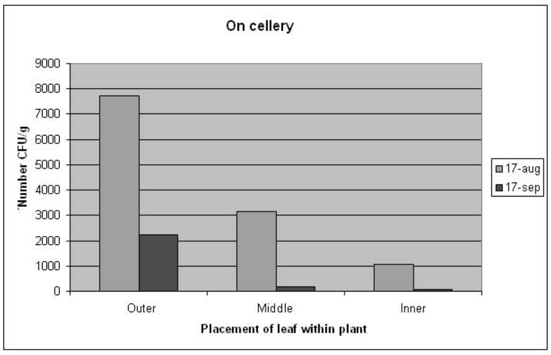
Fig. 1. Numbers of *Bacillus thuringiensis* DMU67R detected in bulk soil samples from the treated area sprayed with *B. thuringiensis* DMU67R in 1993. The results shown are from 1993 to 2000. Error bars represent SEM.



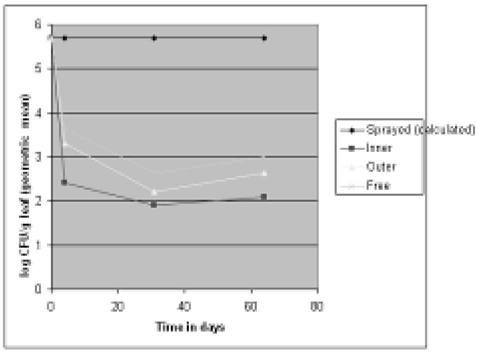
Fate of *Bt* on curly kale



Fate BtHD1 on celery



Fate of Bt HD1 on white cabbage



Population size = I + G – E - D

- I = Immigration (arrival of viable propagules on leaf)
- G = Growth (an increase in biomass or number of viable propagules through multiplication)
- E = Emigration (physical loss or removal of viable propagules)
- D = Death

Immigration

- The use of *B. thuringiensis*
- A special case where immigration at certain time points is very high
- Number of applications not very important

Growth

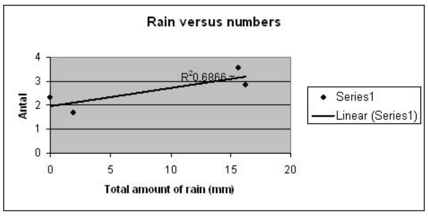
- Applied as spores
- No germination on leaf
- No growth on leaf surfaces and at nutrient concentrations at leaf surfaces
- However, Bt isolated as vegetative cells from leaves

- Growth very limited

Emigration

- Rain-off – reported to have an insignificant effects or to cause a sharp decline in activity
- Simulated rainfall caused 50% to 95% loss of spores depending on amount
- Dependent on numbers, amount and duration ?

Rain versus number on leaves

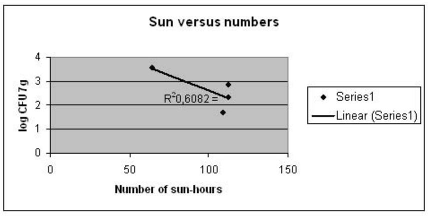


Death

- Dessication
- Temperature
- Sun-light
- Predation

- UV and some parts of the visible light effects survival
- Spores much lesser effected than vegetative cells
- Initial half-life 10 – 24 hours

Hours with sun versus number



Population size= I + $_G$– D - ε

- I = Numbers ”applied”
- G= Growth (insignificant)
- D= Death (sunlight dependent)
- E= Emmigration (rain-off – unpredictable)

PS= I – k_1Exp(I_1) – k_2Exp(I_2) – E(n,a,d)

- I = Numbers applied
- I_1= Numbers directly exposed for sun
- I_2= Numbers indirectly exposed for sun

(Dependent on plant species, plant age, coverage)

- k_1= Decay constant for direct exposed

(Dependent on sun-hours, latitude, time of the year, height above water level, shadow, reflections, formulation, glasshouse)

- k_2= Decay constant for indirectly exposed

(Dependent on plant species, plant age, coverage)

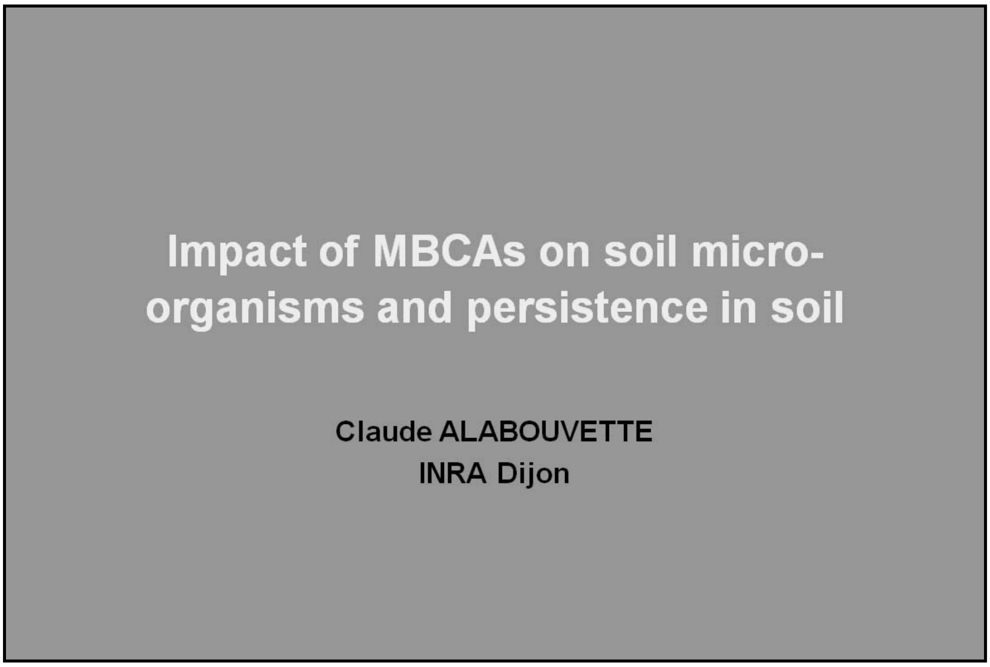
- E = Stochastic function dependent on number, amount and duration of rain and formulation

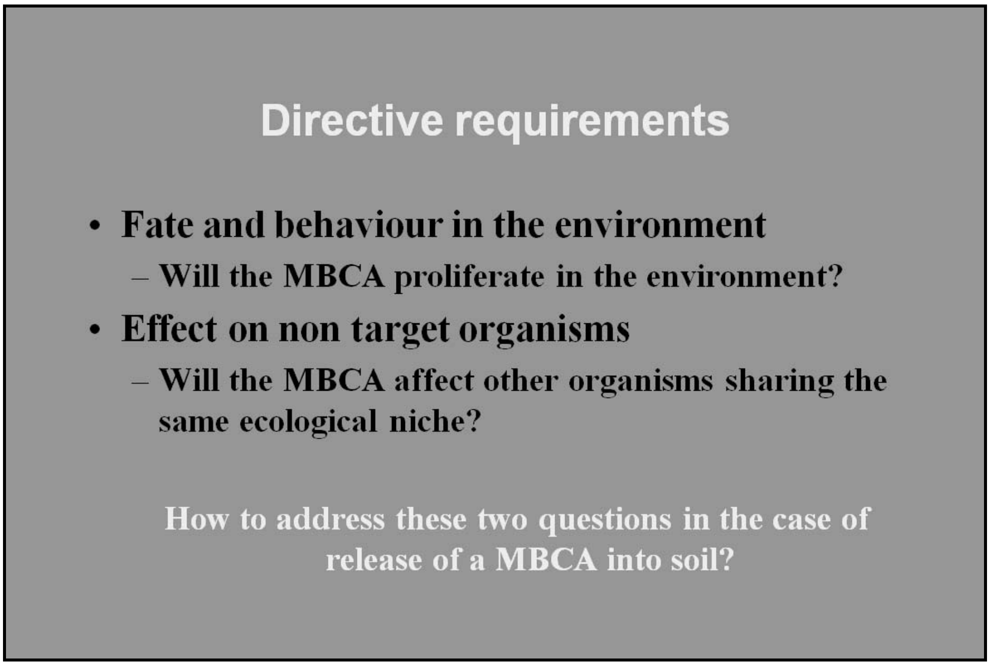
The explanation model

- General for use of microbial pesticides on vegetables ?
- Factors affecting survival dependent on microorganism ?
- Coefficients dependent of use ?

Impact of MBCAs on soil micro-organisms and persistance (resilience) in soil

Claude Alabouvette (Laboratoire de Recherches sur la Flore Pathogène dans le Sol, I.N.R.A., Dijon; France)



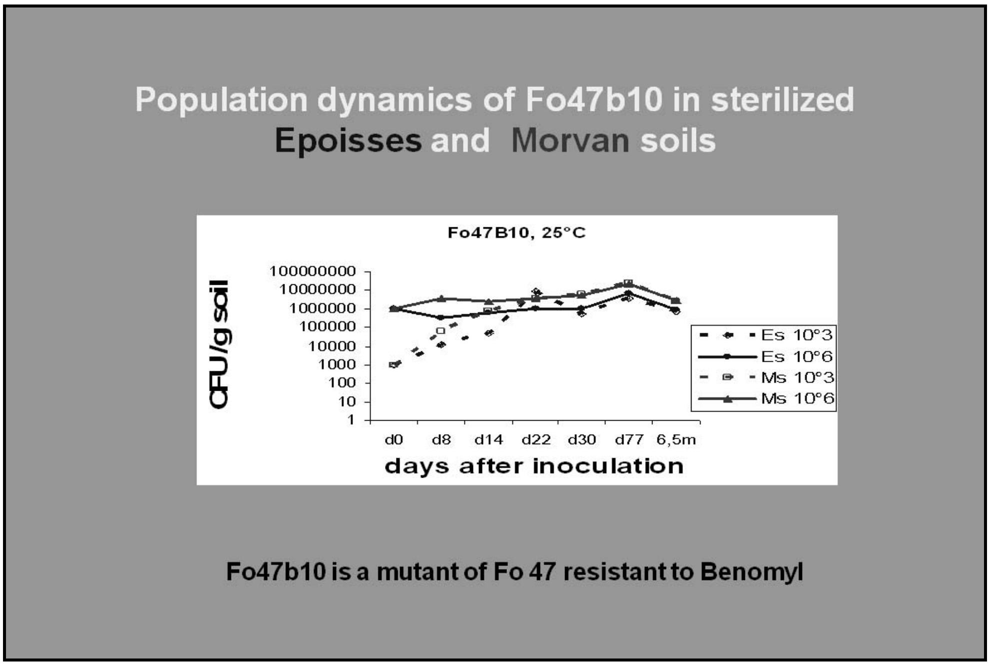


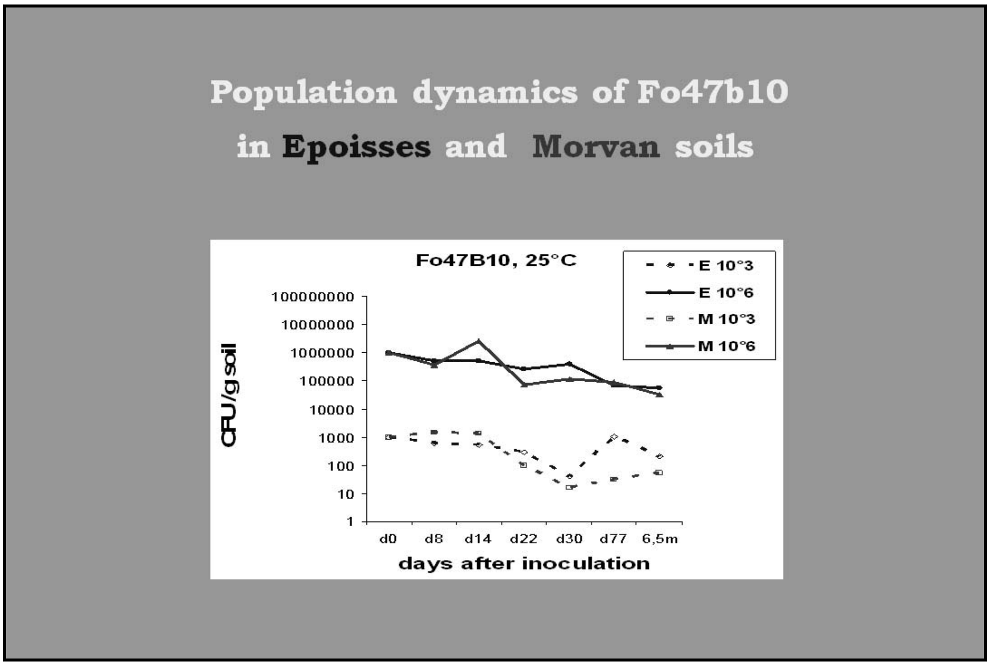
Population dynamics in the soil

- How to follow the strain introduced in the environment ?
  - Use of a marked strain in microcosm studies
    - A mutant resistant to an antibiotic or a fungicide
    - A transformed strain expressing a reporter gene: GFP, DsRed2
  - Identification of a SCAR marker to follow the native organism in the environment

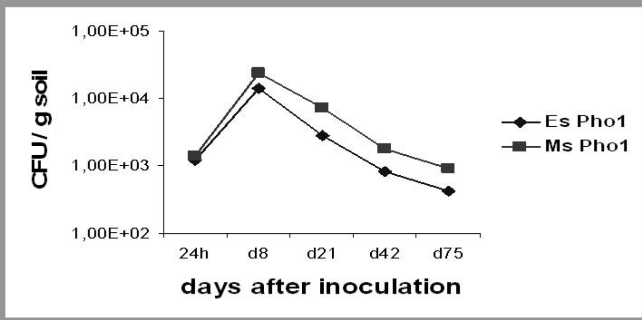
Use of a mutant resistant to Benomyl

- The use of a mutant resistant to a fungicide is the easiest method to study the fate in soil
- But this method can not be used in the fields


Population dynamics of Fo47b10 in sterilized
Epoisses and Morvan soils
Fo47B10, 25°C
100000000
10000000
1000000
100000
10000
1000
100
10
1
CFU/g soil
d0
d8
d14
d22
d30
d77
6,5m
days after inoculation
Es 10°3
Es 10°6
Ms 10°3
Ms 10°6
Fo47b10 is a mutant of Fo 47 resistant to Benomyl

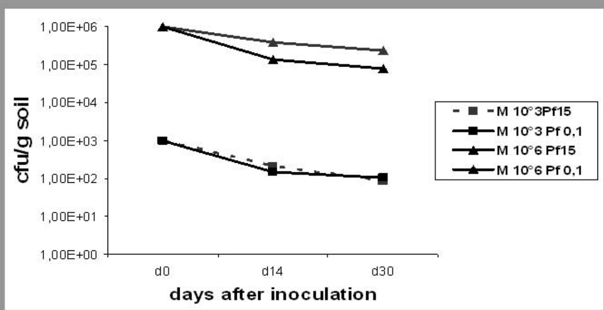

Population dynamics of Fo47b10
in Epoisses and Morvan soils
Fo47B10, 25°C
E 10°3
E 10°6
M 10°3
M 10°6
100000000
10000000
1000000
100000
10000
1000
100
10
1
CFU/g soil
d0
d8
d14
d22
d30
d77
6,5m
days after inoculation

Population dynamics of *Phomopsis circii* in sterilised Epoisses and Morvan soils



Phomopsis circii **is an aerial pathogen of *Circium***

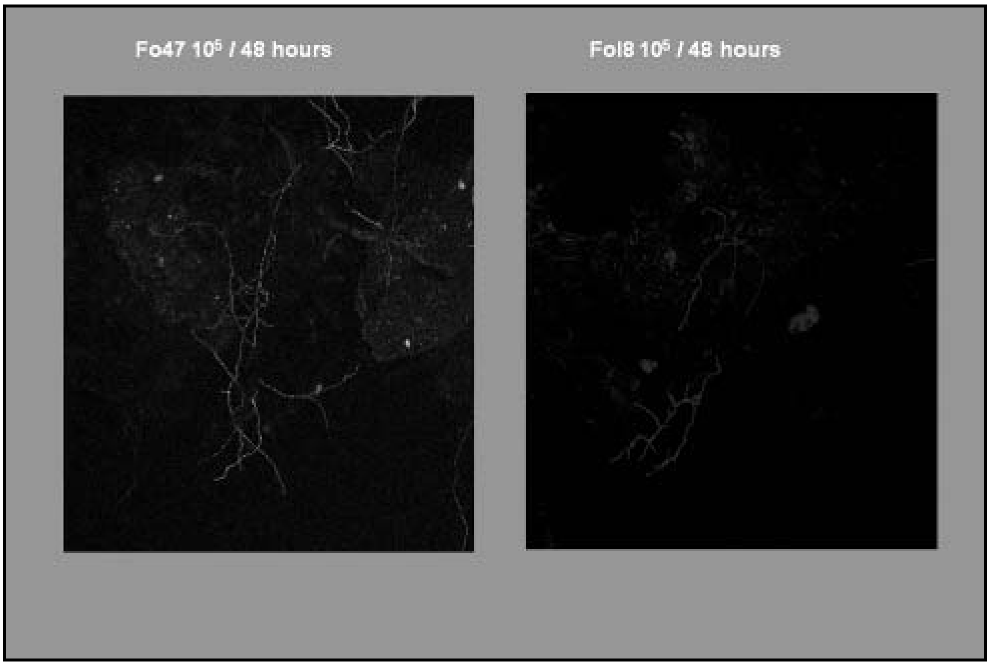
Effect of water potential on population dynamics of Fo47b10 in Morvan soil

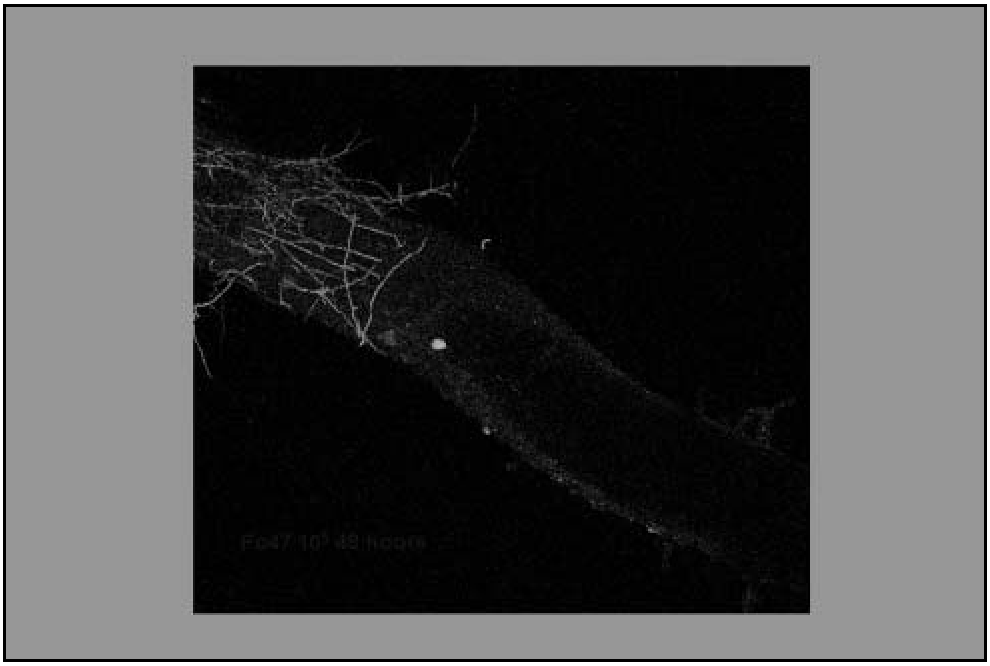


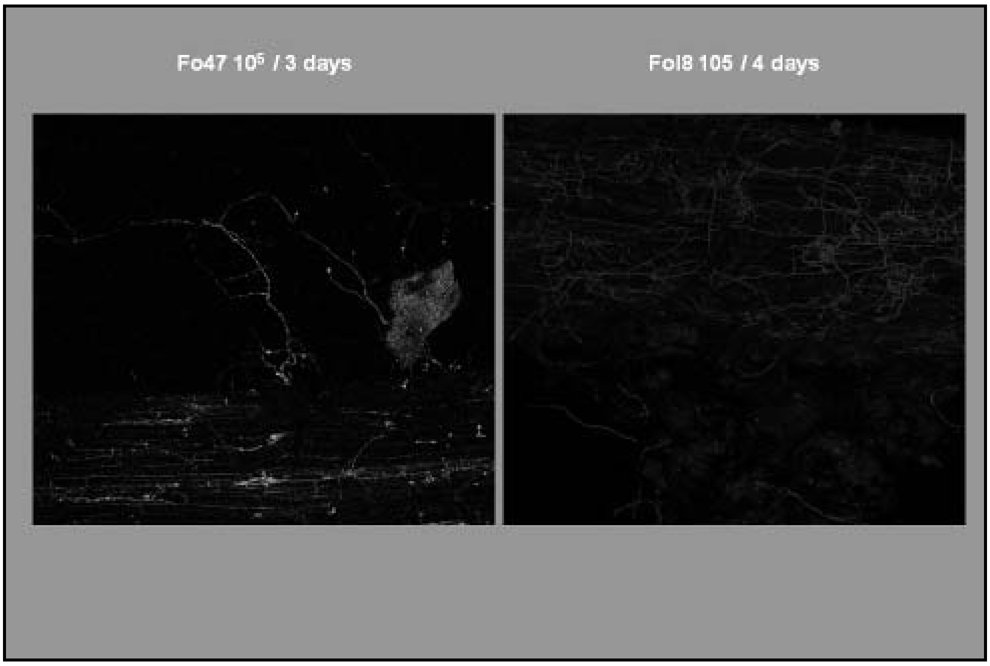
Pf 15 and Pf 0.1

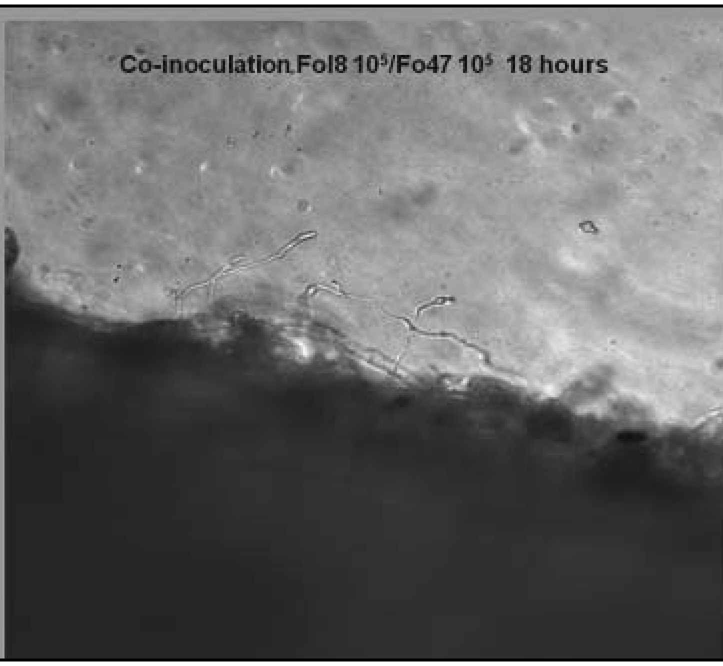
Use of transformed strains expressing fluorescent markers

- This method is useful to visualize the introduced MBCA in soil
- We used it to study interactions between a protective and a pathogenic strain of *Fusarium oxysporum* in the rhizosphere of tomato.




Fo47 10^5 48 hours

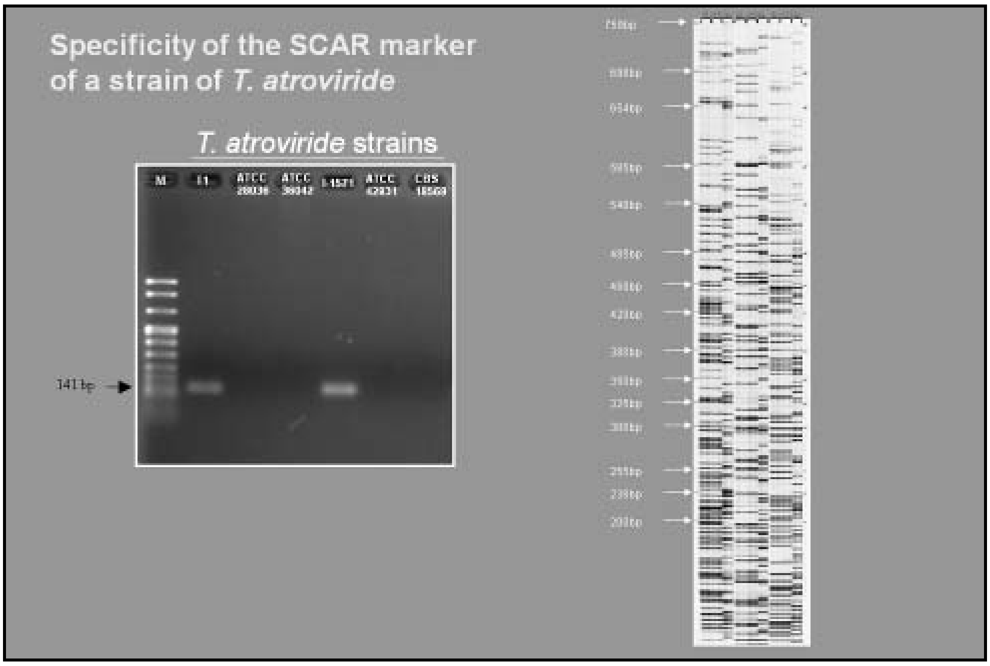

Fo47 10^5 / 3 days
Fol8 105 / 4 days

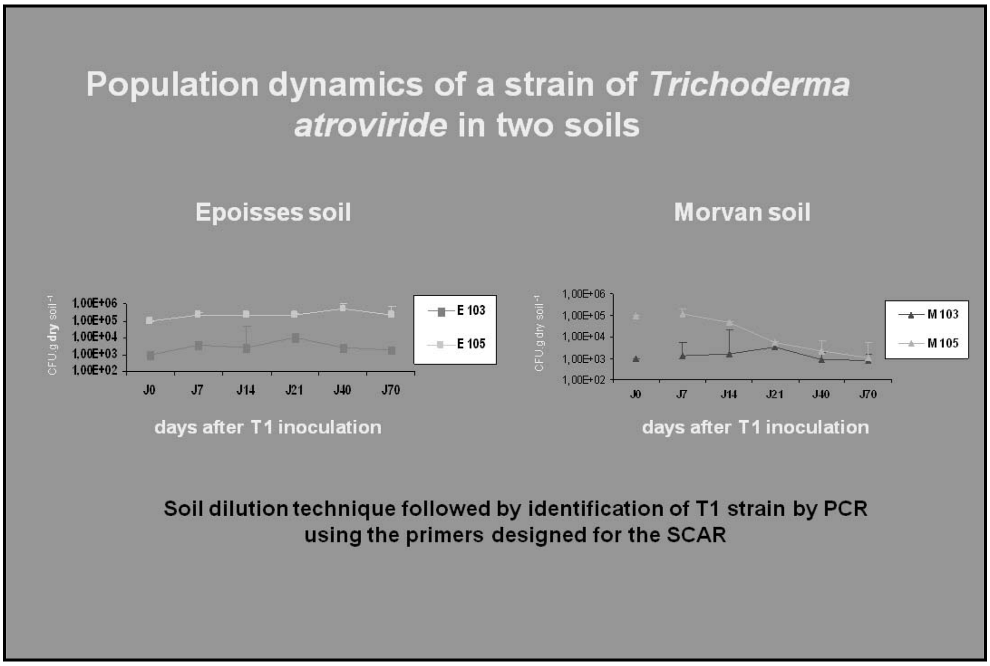


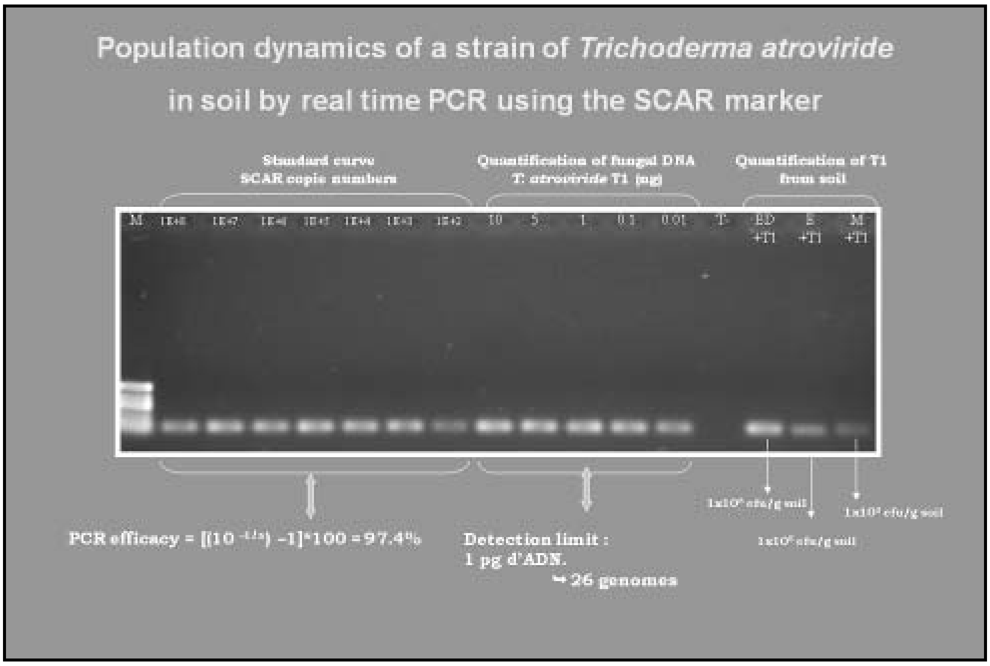
Identification of a SCAR marker

Since the used of mutants or transformed strains is not allowed in the environment, the only method to follow the population dynamics in the open environment is to design a

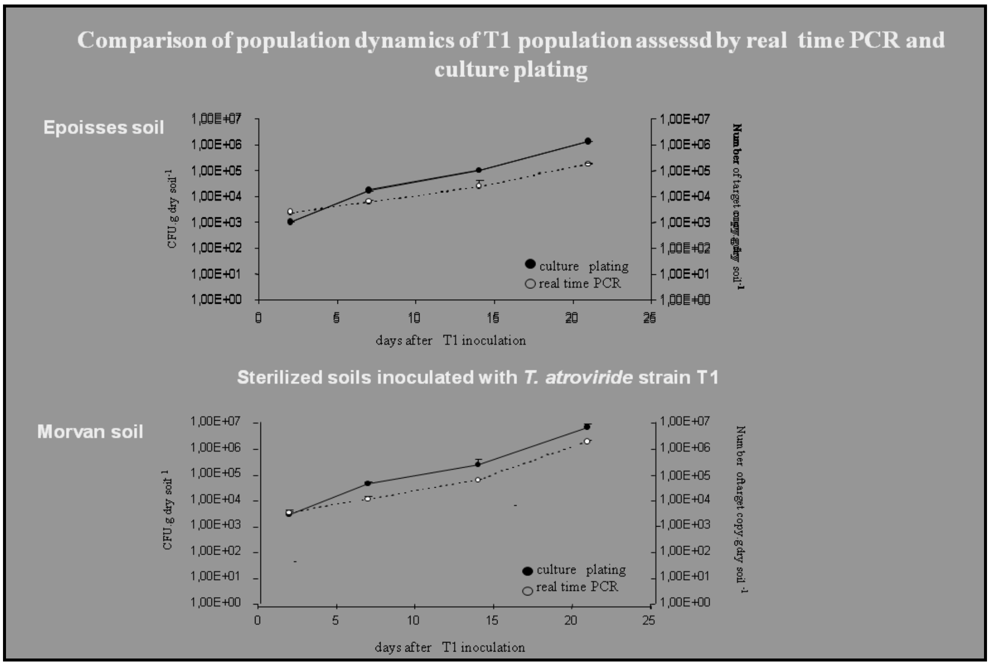
Specific **C**haracterized **A**mplified **R**egion


Specificity of the SCAR marker
of a strain of *T. atroviride*
T. atroviride strains
M
T1
ATCC 28038
ATCC 36042
I-1571
ATCC 42831
CBS 16563
341 bp

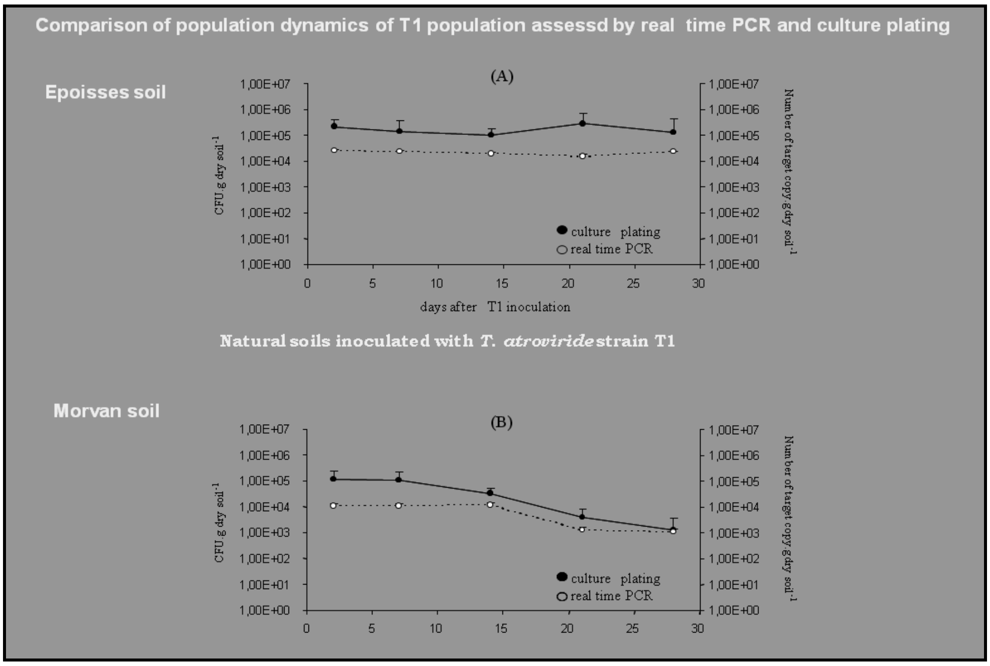

Population dynamics of a strain of *Trichoderma atroviride* in two soils
Epoisses soil
Morvan soil
CFU.g dry soil⁻¹
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
J0
J7
J14
J21
J40
J70
E 103
E 105
M 103
M 105
days after T1 inoculation
days after T1 inoculation
Soil dilution technique followed by identification of T1 strain by PCR using the primers designed for the SCAR



Population dynamics of a strain of *Trichoderma atroviride*
in soil by real time PCR using the SCAR marker
Standard curve
SCAR copie numbers
Quantification of fungal DNA
T. atroviride T1 (ng)
Quantification of T1
from soil
M
10
5
1
0.1
0.01
T-
ED +T1
E +T1
M +T1
PCR efficacy = [(10 -1/a) -1]*100 = 97.4%
Detection limit :
1 pg d'ADN.
→ 26 genomes
cfu/g soil



Comparison of population dynamics of T1 population assessd by real time PCR and culture plating
Epoisses soil
Sterilized soils inoculated with *T. atroviride* strain T1
Morvan soil
CFU g dry soil -1
Number of target copy g dry soil -1
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
0
5
10
15
20
25
days after T1 inoculation
culture plating
real time PCR



Effects on non target organisms

- Aquatic organisms
- Bees
- Arthropods other than bees
- Earth worms
- Soil microorganisms

Effects on soil micro-organisms

- How to address effects on soil microbial communities?
- Why to assess non target effects on soil micro-organisms?
- What is important for the soil functioning?
  - Population density
  - Microbial functions involved in soil fertility, soil health and ecosystemic services

Effects on soil micro-organisms

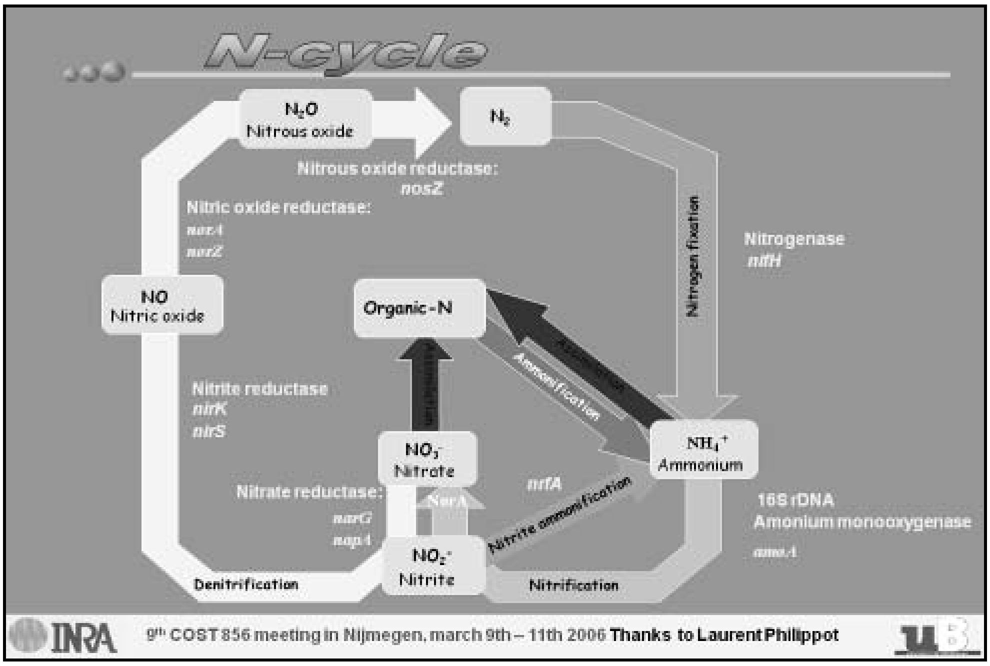
- It is important to preserve the soil functions (soil fertility, soil health)
- Functional redundancy is a characteristics of the soil microflora
- It would be interesting to assess the effects of microbial inoculation on soil functions for example on the C and N cycles

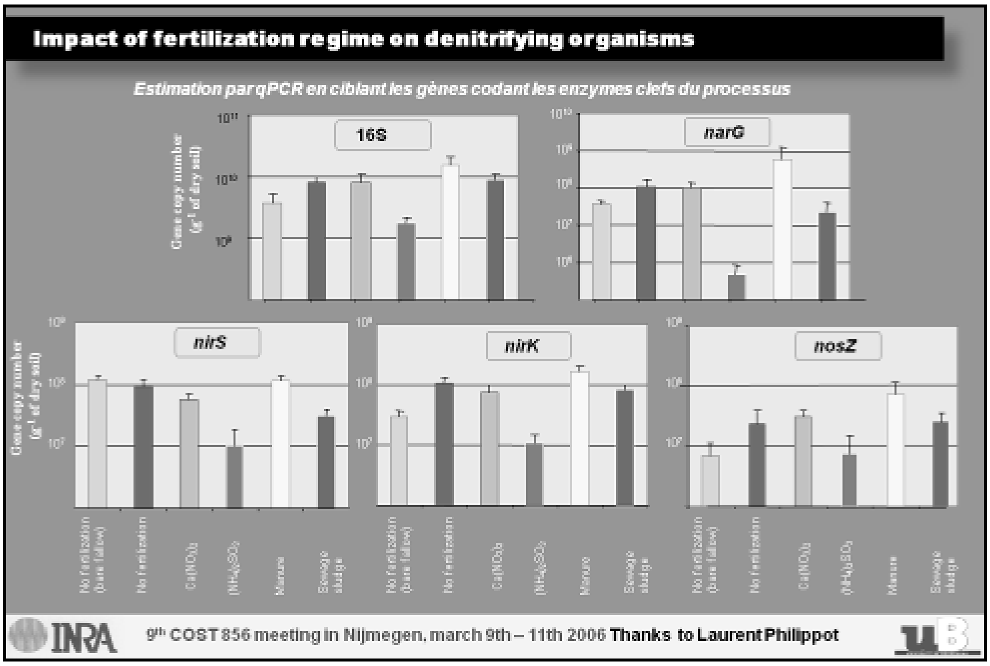
C Cycle

- The main enzymes (Cellulases , Cellobiases, Xylanases, Arabinases, ß-1-4 glucosidase, Exo and endo ß-1-4 glucanases) responsible for mineralization of organic carbon are produced by many different organisms
- Release of a MBCA has no impact on C mineralization

N Cycle

- The sequences of genes coding for the different steps of the N cycle are known,
- It is therefore possible to address the impact of MBCA release on the N cycle by QPCR taging the genes


N-cycle
N2O
Nitrous oxide
N2
Nitrous oxide reductase:
nosZ
Nitric oxide reductase:
norA
norZ
Nitrogenase
nifH
Nitrogen fixation
NO
Nitric oxide
Organic-N
Assimilation
Ammonification
Nitrite reductase
nirK
nirS
NO3-
Nitrate
NH4+
Ammonium
Nitrate reductase:
narG
napA
NasA
nrfA
Nitrite ammonification
16S rDNA
Amonium monooxygenase
amoA
NO2-
Nitrite
Denitrification
Nitrification
INRA
9th COST 856 meeting in Nijmegen, march 9th – 11th 2006 Thanks to Laurent Philippot

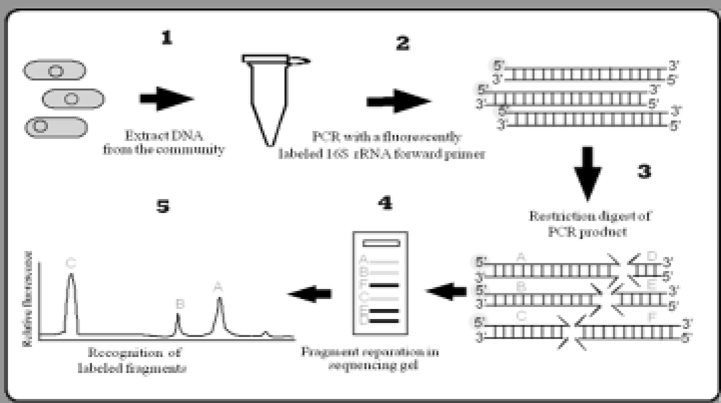

Impact of fertilization regime on denitrifying organisms
Estimation par qPCR en ciblant les gènes codant les enzymes clefs du processus
16S
narG
nirS
nirK
nosZ
Gene copy number (g-1 of dry soil)
No fertilization (bare fallow)
No fertilization
Ca(NO3)2
(NH4)2SO4
Manure
Sewage sludge
INRA
9th COST 856 meeting in Nijmegen, march 9th – 11th 2006 Thanks to Laurent Philippot

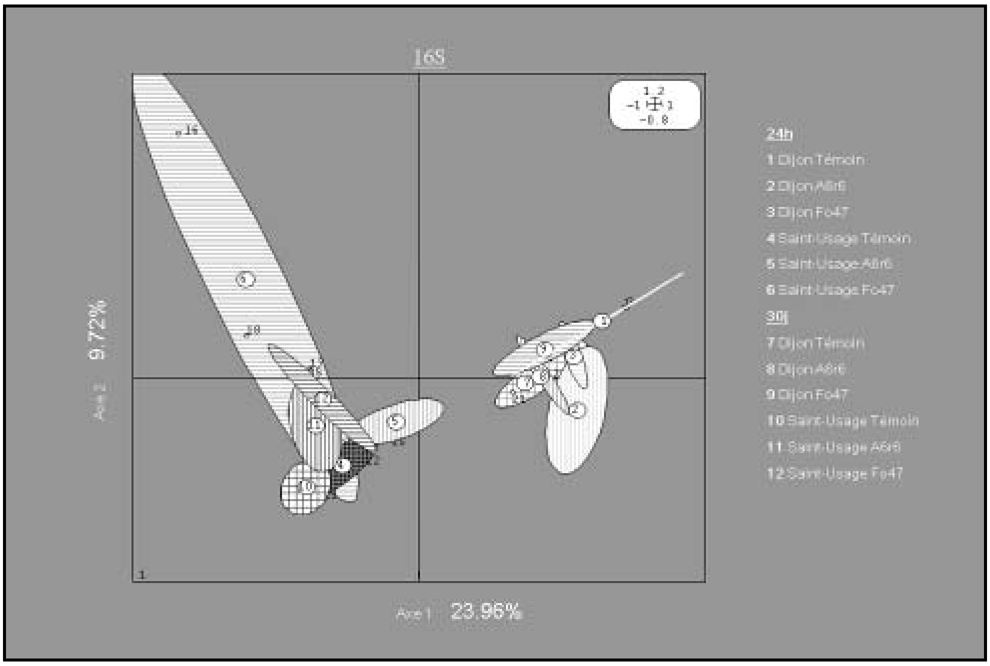
Other approaches

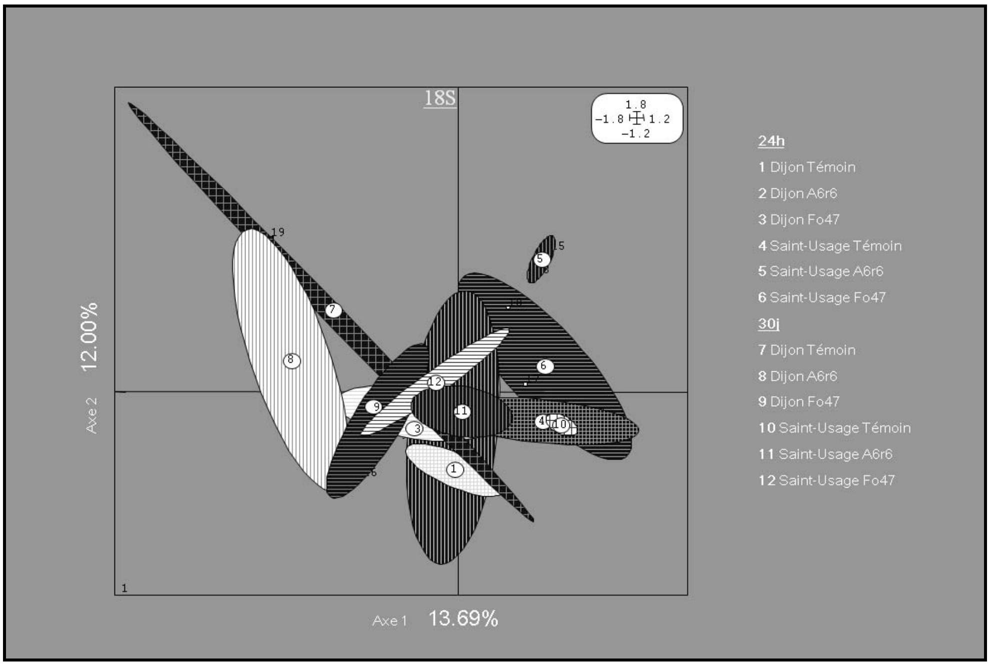
- Other methods such as RISA, DGGE or T-RFLP enable to detect the impact of MBCA introduction on the community structures of soil organisms

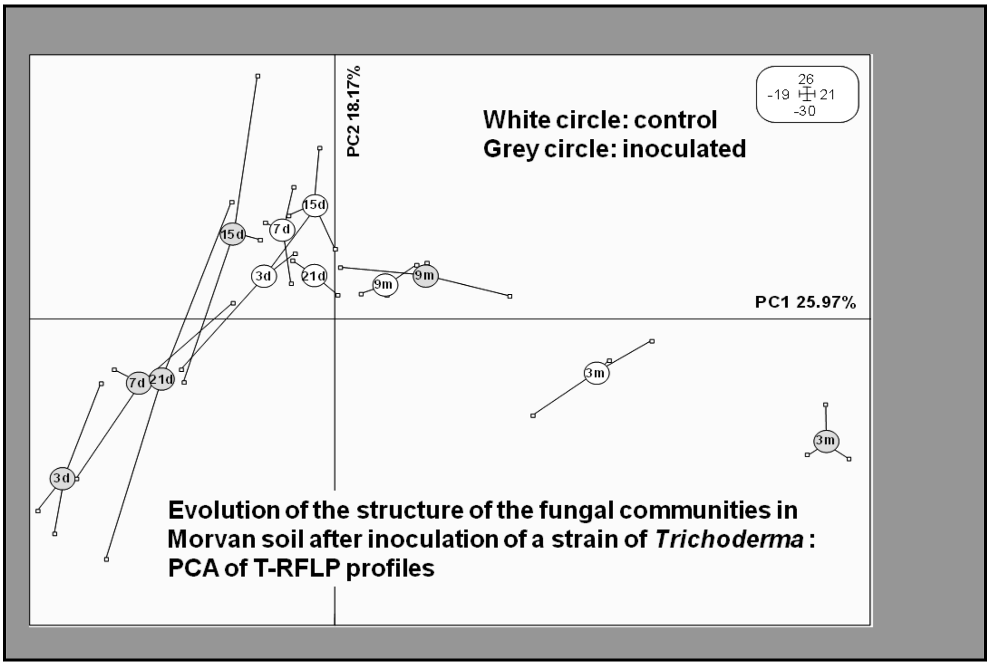
STRUCTURE of the bacterial and fungal communities

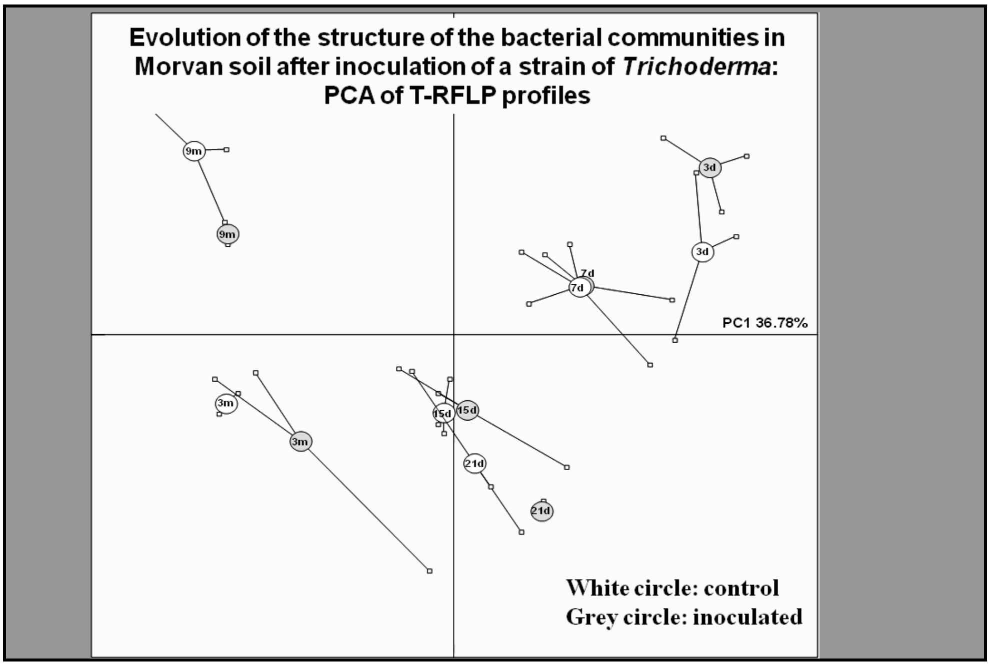
- Terminal Restriction Fragment Length Polymorphism (T-RFLP)

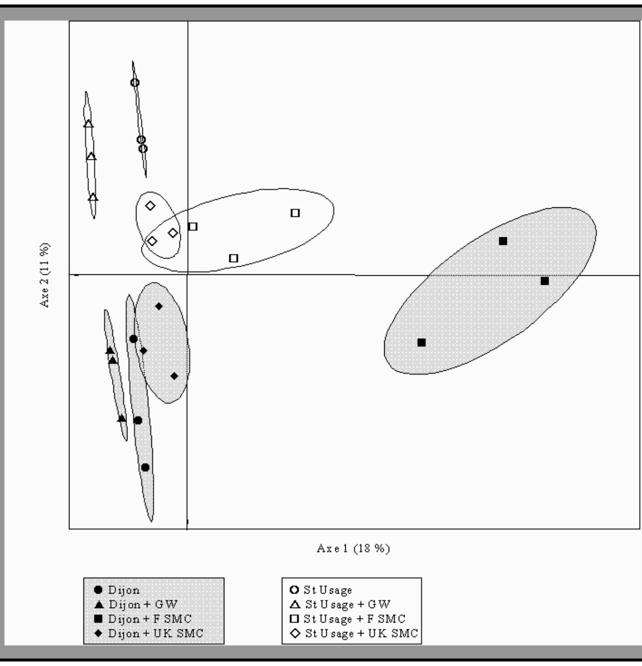



16S
1.2
-1
1
-0.8
9.72%
Axe 2
Axe 1
23.96%
24h
1 Dijon Témoin
2 Dijon A6r6
3 Dijon Fo47
4 Saint-Usage Témoin
5 Saint-Usage A6r6
6 Saint-Usage Fo47
30j
7 Dijon Témoin
8 Dijon A6r6
9 Dijon Fo47
10 Saint-Usage Témoin
11 Saint-Usage A6r6
12 Saint-Usage Fo47


18S
1.8
-1.8
1.2
-1.2
12.00%
Axe 2
Axe 1
13.69%
24h
1 Dijon Témoin
2 Dijon A6r6
3 Dijon Fo47
4 Saint-Usage Témoin
5 Saint-Usage A6r6
6 Saint-Usage Fo47
30j
7 Dijon Témoin
8 Dijon A6r6
9 Dijon Fo47
10 Saint-Usage Témoin
11 Saint-Usage A6r6
12 Saint-Usage Fo47


PC2 18.17%
26
-19
21
-30
White circle: control
Grey circle: inoculated
PC1 25.97%
Evolution of the structure of the fungal communities in Morvan soil after inoculation of a strain of *Trichoderma* : PCA of T-RFLP profiles


Evolution of the structure of the bacterial communities in Morvan soil after inoculation of a strain of *Trichoderma*: PCA of T-RFLP profiles
PC1 36.78%
White circle: control
Grey circle: inoculated



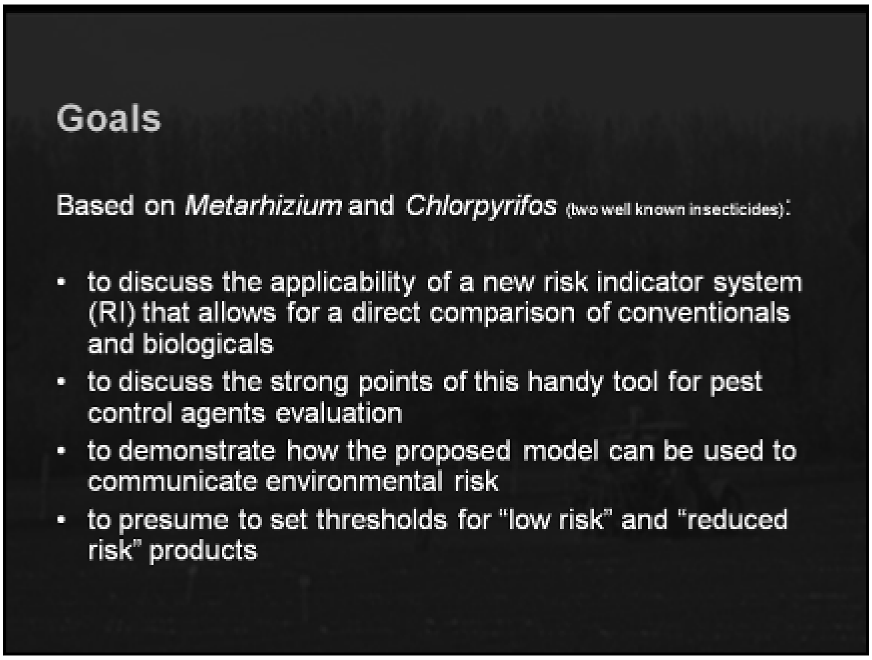
Effect of compost amendment on the structure of the bacterial communities

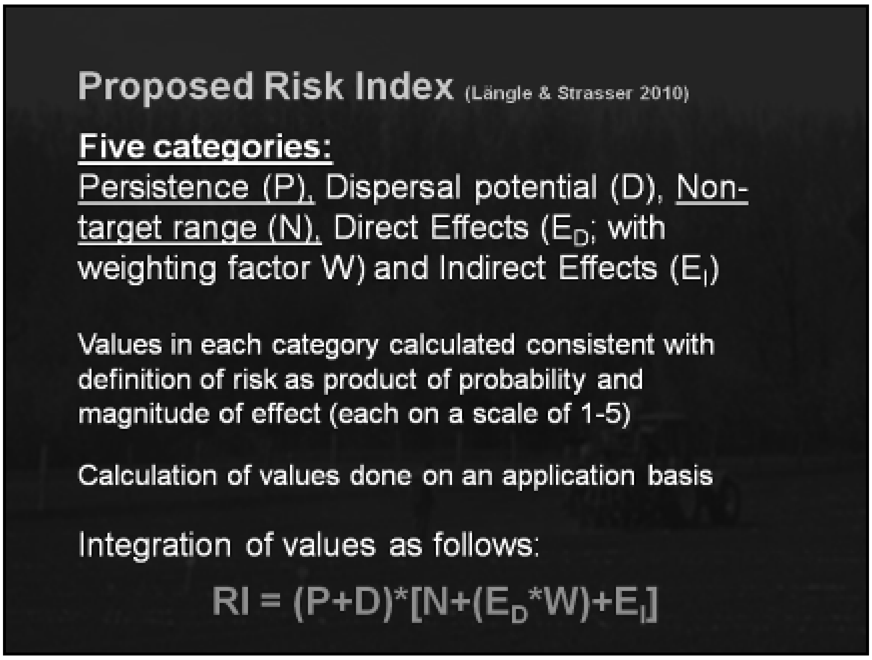
Conclusion

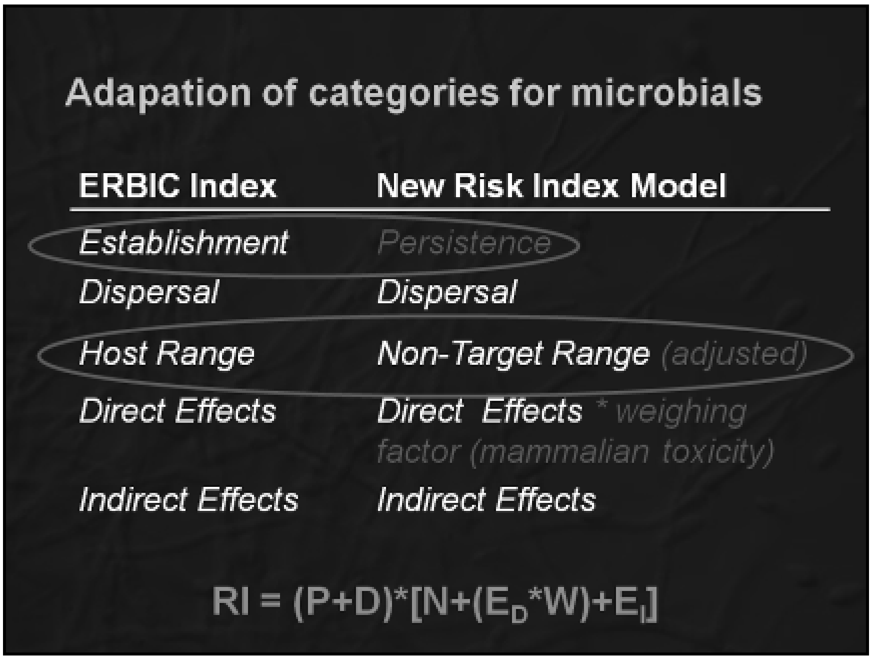
- A soil microorganism re-introduced into soil will establish, but never become predominant in absence on any specific selection pressure
- As any other agricultural practice, MBCAs release has some effects on the soil microflora but these effects are always transients
- There should be no more concern for MBCA release than for manure or compost use

New risk indicator model: an excellent tool to comparatively assess environmental risks posed by microbial and conventional pest control agents

By Hermann Strasser (Institute of Microbiology, University Innsbruck, Innsbruck; Austria)




Proposed Risk Index (Längle & Strasser 2010)
Five categories:
Persistence (P), Dispersal potential (D), Non-target range (N), Direct Effects (E_D; with weighting factor W) and Indirect Effects (E_I)
Values in each category calculated consistent with definition of risk as product of probability and magnitude of effect (each on a scale of 1-5)
Calculation of values done on an application basis
Integration of values as follows:
$RI = (P+D)*[N+(E_D*W)+E_I]$


Adapation of categories for microbials
ERBIC Index
New Risk Index Model
Establishment
Persistence
Dispersal
Dispersal
Host Range
Non-Target Range (adjusted)
Direct Effects
Direct Effects * weighing factor (mammalian toxicity)
Indirect Effects
Indirect Effects
$RI = (P+D)*[N+(E_D*W)+E_I]$

Scoring and rationales

Persistence factor (P)

Score	Persistence in target absence (use columns 2 or 3, depending on available information)	
1^2(=1)	No BCA detectable in soil 1 yr after use (or at levels found naturally for indigenous species)	$T_{0.5}$ < 30 d
2^2(=4)	>0 % - 16 % of original CFUs 1 yr after use (for indigenous species: at natural levels after 2 yrs)	$T_{0.5}$ = 0.1 a - 0.25 a (36 d - 91 d)
3^2(=9)	16 % - 40 % of original CFUs 1 yr after use	$T_{0.5}$ = 0.25 a - 0.75 a (91 d - 274 d)
4^2(=16)	40 % -62 % of original CFUs 1 yr after use	$T_{0.5}$ = 0.75 a – 1.5 a
5^2(=25)	No significant reduction of CFUs 1 yr after use	$T_{0.5}$ > 1.5 a

$T_{0.5}$ stands for half life in the absence of the target.

$$RI = (P+D)*[N+(E_D*W)+E_I]$$

Persistence (after foliar spray)

M. anisopliae (RI Score 1)

M. anisopliae were found at levels naturally for indigenous species

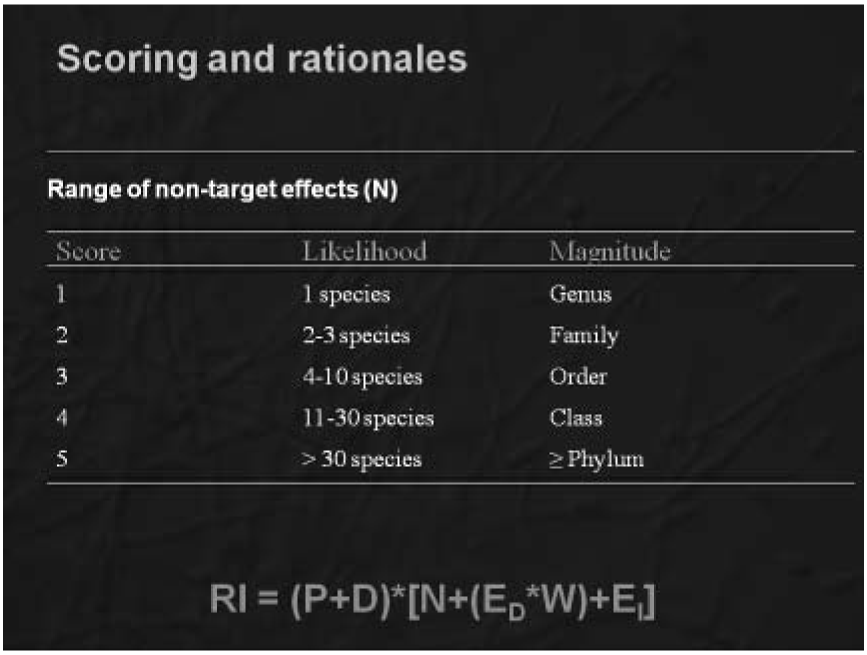
The application of *M. anisopliae* lead to an decrease of total CFU counts but did not influence significantly the shares of different ecological groups (Kirchmair et al. 2009)

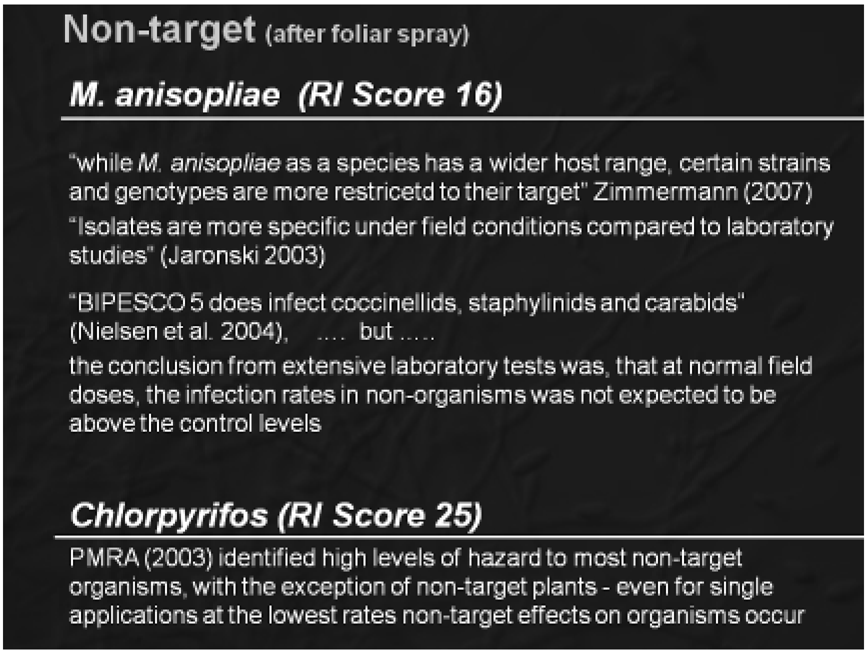
The data presented by Scheepmaker & Butt (2010) all show a decline of *Metarhizium* persistence, ..., even when the upper background level was achieved within a range of a few weeks and 10 years

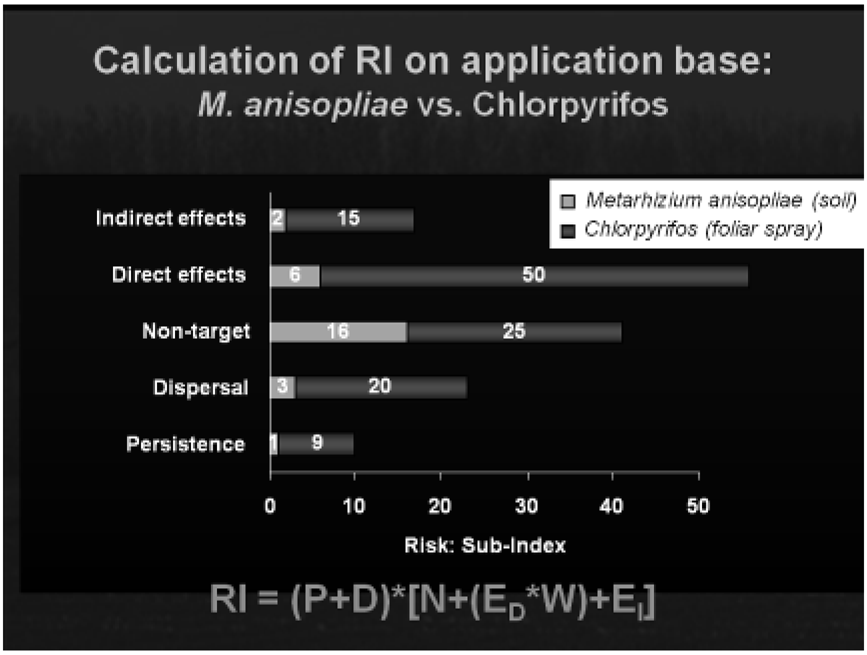
"MO´s will survive but will not proliferate - it will come part of the native populations of the same species" (Alabouvette & Cordies, in press)

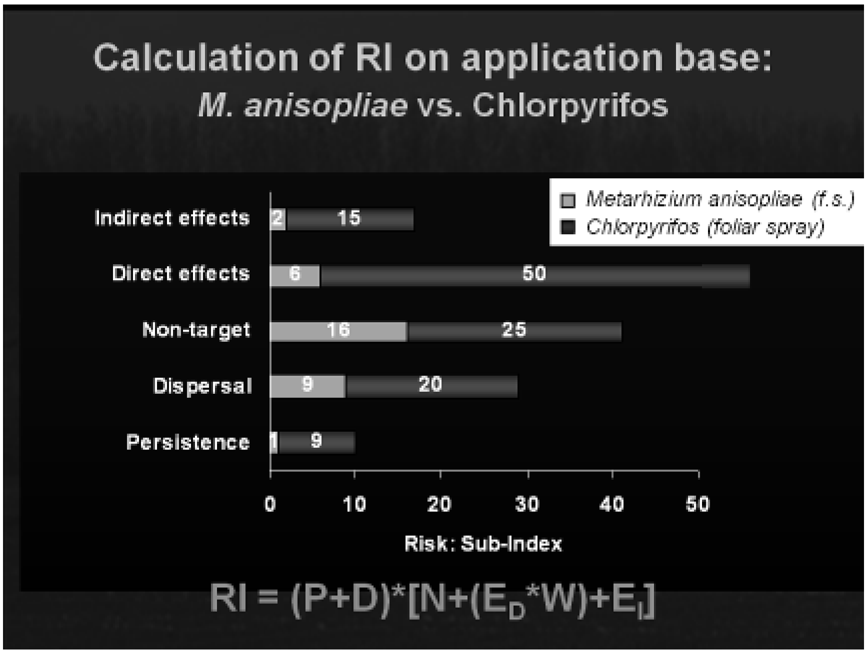
Chlorpyrifos (RI Score 9)

It shows moderate persistence; half life is up to 200 days (PMRA 2003)


Scoring and rationales
Range of non-target effects (N)
Score Likelihood Magnitude
1 1 species Genus
2 2-3 species Family
3 4-10 species Order
4 11-30 species Class
5 > 30 species ≥ Phylum
RI = (P+D)*[N+(E_D*W)+E_I]


Non-target (after foliar spray)
M. anisopliae (RI Score 16)
"while M. anisopliae as a species has a wider host range, certain strains and genotypes are more restricetd to their target" Zimmermann (2007)
"Isolates are more specific under field conditions compared to laboratory studies" (Jaronski 2003)
"BIPESCO 5 does infect coccinellids, staphylinids and carabids" (Nielsen et al. 2004), ... but .....
the conclusion from extensive laboratory tests was, that at normal field doses, the infection rates in non-organisms was not expected to be above the control levels
Chlorpyrifos (RI Score 25)
PMRA (2003) identified high levels of hazard to most non-target organisms, with the exception of non-target plants - even for single applications at the lowest rates non-target effects on organisms occur


Calculation of RI on application base:
M. anisopliae vs. Chlorpyrifos
Metarhizium anisopliae (soil)
Chlorpyrifos (foliar spray)
Indirect effects
Direct effects
Non-target
Dispersal
Persistence
2
15
6
50
16
25
3
20
1
9
0
10
20
30
40
50
Risk: Sub-Index
RI = (P+D)*[N+(E_D*W)+E_I]

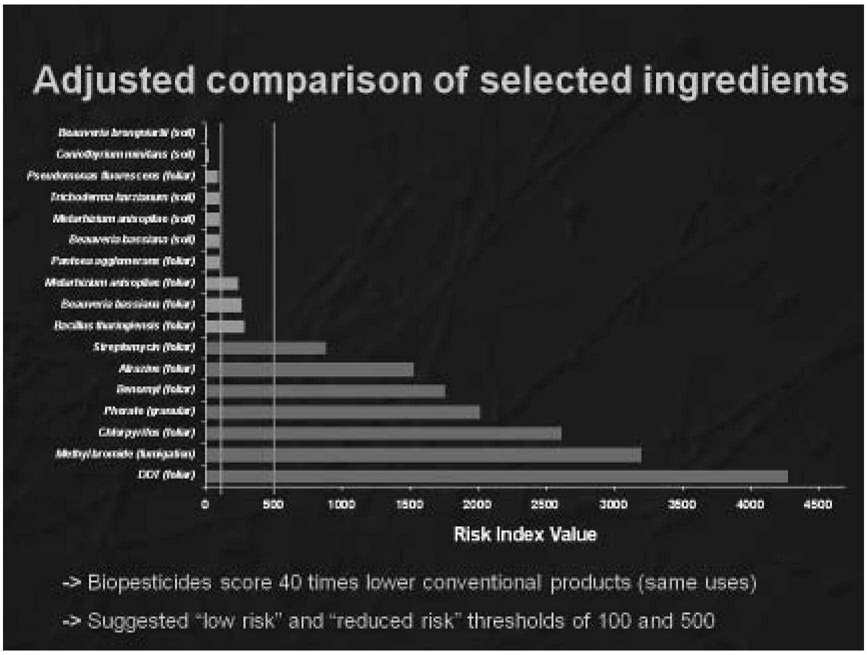

Calculation of RI on application base:
M. anisopliae vs. Chlorpyrifos
Metarhizium anisopliae (f.s.)
Chlorpyrifos (foliar spray)
Indirect effects
Direct effects
Non-target
Dispersal
Persistence
2
15
6
50
16
25
9
20
1
9
0
10
20
30
40
50
Risk: Sub-Index
RI = (P+D)*[N+(E_D*W)+E_I]

Risk scores and calculated RI for selected PPP

Active ingredient	Persistence factor	Dispersal factor		Host Range		Direct effect		Indirect Effects		Vertebrate effects	Risk score
		Distance	Quantity	Species	Taxonomic level	Likelihood	Magnitude	Likelihood	Magnitude		
Metarhizium anisopliae (soil)	1	3	1	4	4	3	2	1	2	1	96
Metarhizium anisopliae (foliar spray)	1	3	3	4	4	3	2	1	2	1	240
Pantoea agglomerans (foliar spray)	1	3	2	3	3	2	1	1	1	2	98
Pseudomonas fluorescens (foliar spray)	1	2	3	3	3	2	1	1	1	1.5	91
Chlorpyrifos (foliar spray)	9	5	4	5	5	5	5	5	3	2	2610
DDT (foliar spray)	25	5	4	5	5	5	5	5	4	2	4275
Methyl bromide (fumigation)	25	5	5	5	5	5	3	3	3	2	3200

Strong points of this RI tool

- the applicability to biologicals and conventionals to allow a direct comparison between products
- the ability to score the risk on an application basis rather than on an active ingredient basis
- the flexibility of the system that permits the use of regulatory data or published literature
- a readily understandable output


Adjusted comparison of selected ingredients
Trichoderma harzianum (soil)
Beauveria bassiana (foliar)
Bacillus thuringiensis (foliar)
Streptomycin (foliar)
Atrazine (foliar)
Benomyl (foliar)
Phorate (granular)
Chlorpyrifos (foliar)
Methyl bromide (fumigation)
DDT (foliar)
0 500 1000 1500 2000 2500 3000 3500 4000 4500
Risk Index Value
-> Biopesticides score 40 times lower conventional products (same uses)
-> Suggested "low risk" and "reduced risk" thresholds of 100 and 500


Benefits in Registration & Development
• Summary parameter based on studies, literature or expert judgement
• Comparative risk assessment in registration
• Selection of safest control option in IPM
• Low risk definition: threshold?
• Tool for industry in product development

Conclusion

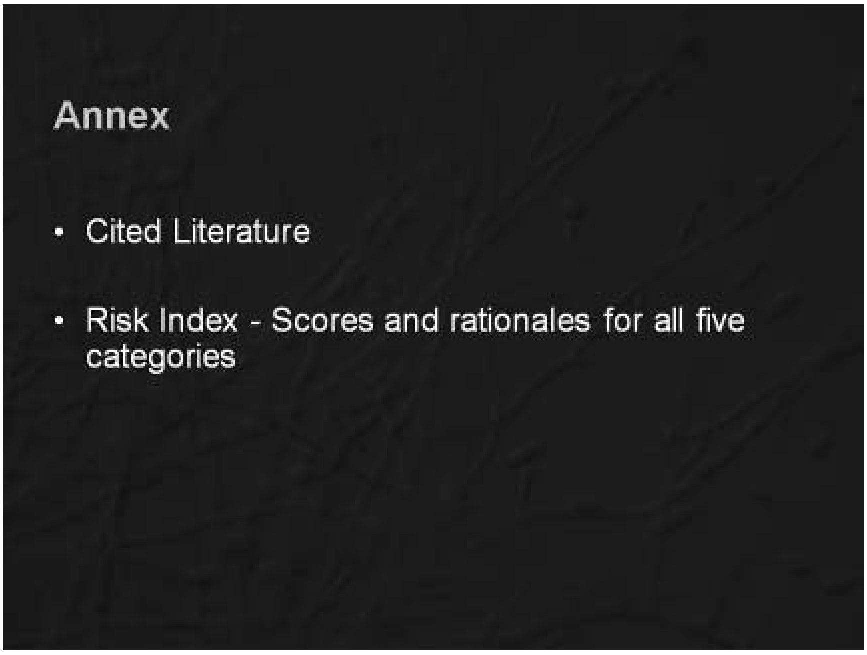
It is believed that the proposed RI system can help facilitate discussions between stakeholders and regulators regarding the regulatory approaches to microbial and other pest control substances

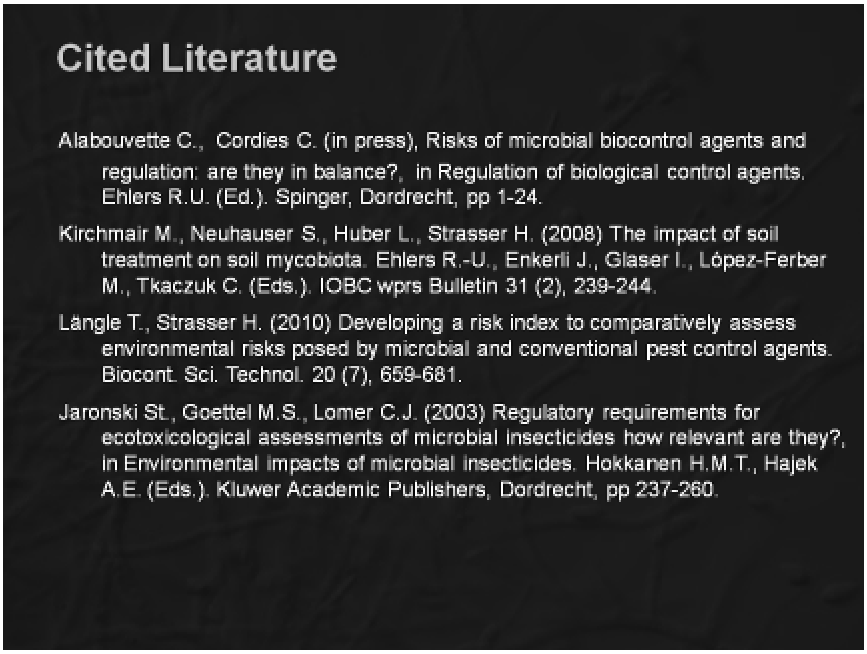
It is debatable whether the RI system will be useful in determining acceptability of data waivers for regulatory purposes

THANK YOU

This work was supported by the European Commission:
RAFBCA (QLK1-CT2001-01391)
and
REBECA (SSPE-022709).

I also wish to thank:
Brian Belliveau (Pest Management Regulatory Agency of Canada), Leslie Cass (Agriculture and Agri-Food Canada), Anita Fjelsted (Danish Ministry of the Environment), Stefan Jaronski (USDA ARS Northern Plains Agricultural Research Laboratory), Mark Goettel (Lethbridge Research Centre), Olaf Strauch (University of Kiel), Stefan Hutwimmer (University Innsbruck) and three anonymous reviewers, for their helpful discussion and kindly reviewing the manuscript.


Annex
• Cited Literature
• Risk Index - Scores and rationales for all five categories


Cited Literature
Alabouvette C., Cordies C. (in press), Risks of microbial biocontrol agents and regulation: are they in balance?, in Regulation of biological control agents. Ehlers R.U. (Ed.). Spinger, Dordrecht, pp 1-24.
Kirchmair M., Neuhauser S., Huber L., Strasser H. (2008) The impact of soil treatment on soil mycobiota. Ehlers R.-U., Enkerli J., Glaser I., López-Ferber M., Tkaczuk C. (Eds.). IOBC wprs Bulletin 31 (2), 239-244.
Längle T., Strasser H. (2010) Developing a risk index to comparatively assess environmental risks posed by microbial and conventional pest control agents. Biocont. Sci. Technol. 20 (7), 659-681.
Jaronski St., Goettel M.S., Lomer C.J. (2003) Regulatory requirements for ecotoxicological assessments of microbial insecticides how relevant are they?, in Environmental impacts of microbial insecticides. Hokkanen H.M.T., Hajek A.E. (Eds.). Kluwer Academic Publishers, Dordrecht, pp 237-260.

Cited Literature

Nielsen C., Eilenberg J., Harding S., Vestergaard S. (2004) Biological control of weevils (*Strophosoma melanogrammum* and *S. capitatum*) in greenery plantations in Denmark. Pesitcide Res. 91, pp. 84.

PMRA (2003) Re-evaluation Document REV99-01, Re-evaluation of Organophosphate pesticides - Proposed acceptability for continuing registration - PACR2003-03, pp. 1-56.

Scheepmaker J.W.A., Butt T.M. (2010) Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment and in accordance with EU regulations. Biocont. Sci. Technol. 20 (5), 503-552.

Zimmermann G. (2007) Review on saftey of the entomopathogenic fungus *Metarhizium anisopliae*. Biocont. Sci. Technol. 17, 879-92.

Scoring and rationales

Persistence factor (P)

Score	Persistence in target absence (use columns 2 or 3, depending on available information)	
1^2 (=1)	No BCA detectable in soil 1 yr after use (or at levels found naturally for indigenous species)	$T_{0.5} < 30$ d
2^2 (=4)	> 0 % - 16 % of original CFUs 1 yr after use (for indigenous species: at natural levels after 2 yrs)	$T_{0.5} = 0.1$ a - 0.25 a (36 d - 91 d)
3^2 (=9)	16 % - 40 % of original CFUs 1 yr after use	$T_{0.5} = 0.25$ a - 0.75 a (91 d - 274 d)
4^2 (=16)	40 % -62 % of original CFUs 1 yr after use	$T_{0.5} = 0.75$ a – 1.5 a
5^2 (=25)	No significant reduction of CFUs 1 yr after use	$T_{0.5} > 1.5$ a

$T_{0.5}$ stands for half life in the absence of the target.

$$RI = (P+D)*[N+(E_D*W)+E_I]$$

Scoring and rationales

Dispersal factor (D)

Score	Distance	Quantity
1	< 10 m	< 1 %
2	< 100 m	< 5 %
3	< 1 000 m	< 10 %
4	< 10 000 m	< 25 %
5	> 10 000 m	> 25 %

Factors: spray drift, bioaccumulation, leaching or run off, infected MO´s

$$RI = (P+D)*[N+(E_D*W)+E_I]$$

Scoring and rationales

Range of non-target effects (N)

Score	Likelihood	Magnitude
1	1 species	Genus
2	2-3 species	Family
3	4-10 species	Order
4	11-30 species	Class
5	> 30 species	≥ Phylum

$$RI = (P+D)*[N+(E_D*W)+E_I]$$

Scoring and rationales

Direct effects (ED, Method 1)

Score	Likelihood	Magnitude
1	Very unlikely (< 1 %)	< 5 % mortality
2	Unlikely (1 %-10 %)	< 50 % mortality
3	Possible (10 %-50 %)	> 50 % mortality or > 10 % short term suppression
4	Likely (50 %-80 %)	> 50 % mortality ore > 10 % permanent suppression
5	Very likely (> 80 %)	> 10 % long term suppression or local extinction

$$RI = (P+D)*[N+(E_D*W)+E_I]$$

Scoring and rationales

Direct effects (ED, Method 2)

Score	Highest risk quotient
$1^2 (=1)$	< 0.1
$2^2 (=4)$	< 1
$3^2 (=9)$	< 10
$4^2 (=16)$	< 100
$5^2 (=25)$	> 100

Square of the score assigned for the highest risk quotient as described by the PMRA

$$RI = (P+D)*[N+(E_D*W)+E_I]$$

Scoring and rationales

Indirect effects (EI)

Score	Likelihood	Magnitude
1	Very unlikely	no significant impact on whole ecosystem
2	Unlikely	minor and short-term impact on parts of ecosystem
3	Possible	significant short-term impact on parts of ecosystem
4	Likely	significant long-term impact on parts of ecosystem
5	Very likely	significant long-term impact on whole ecosystem

$$RI = (P+D)*[N+(E_D*W)+E_I]$$

Scoring and rationales

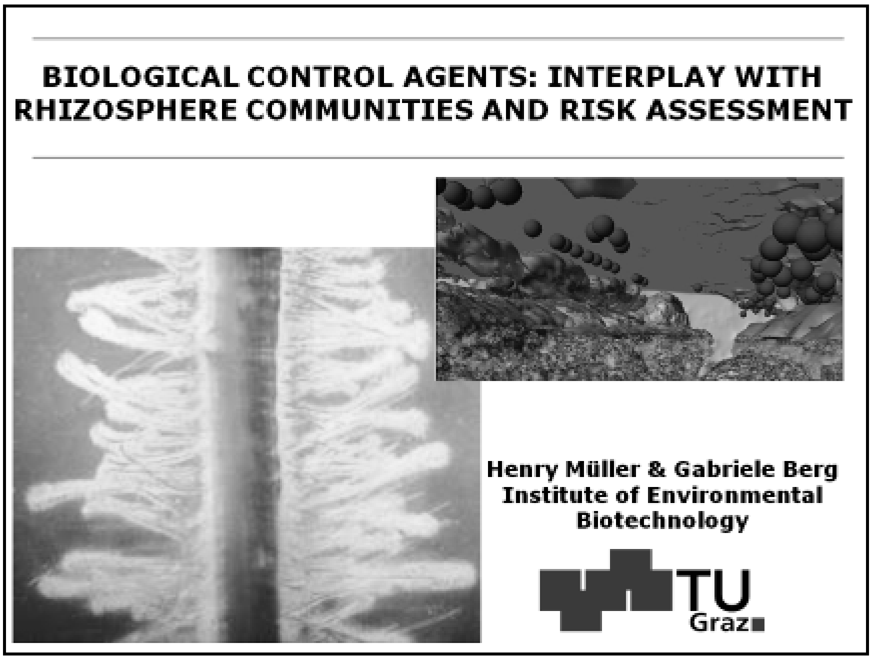
Vertebrate toxicity (W)

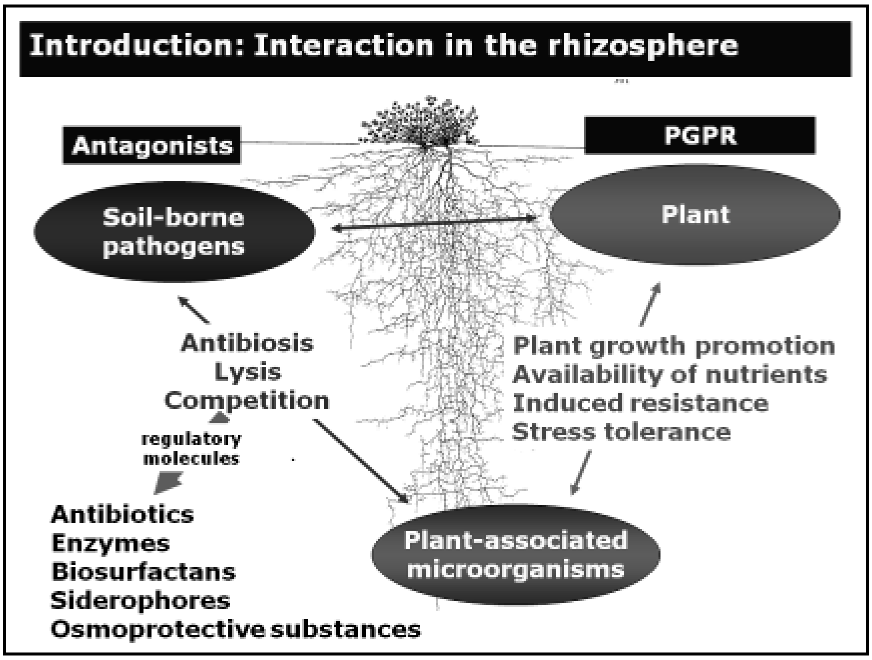
Factor	Magnitude
1	No vertebrates are affected
1.5	Poikilothermic vertebrates are affected by non-target effects
2	Homeothermic vertebrates are affected

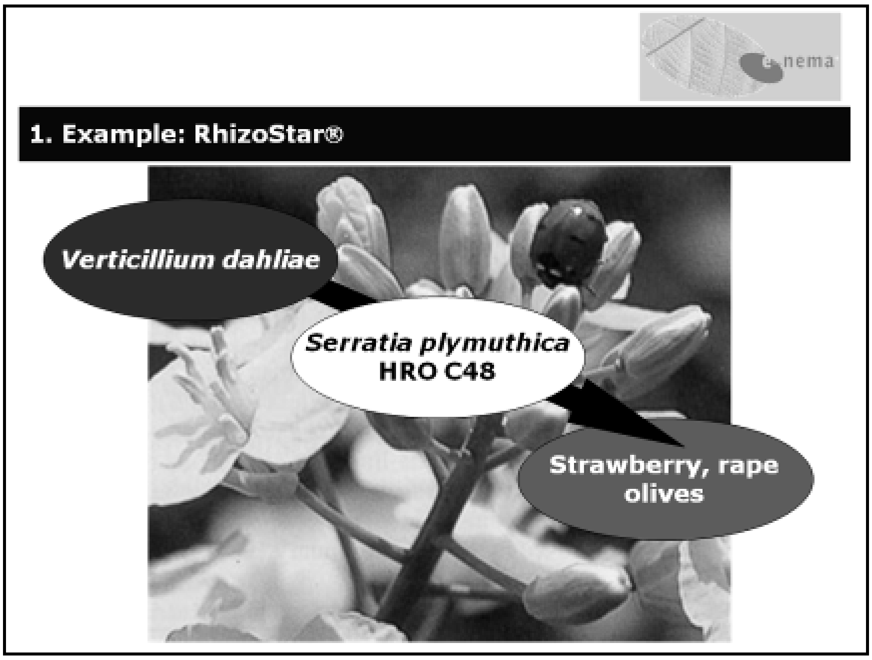
$$RI = (P+D)*[N+(E_D*W)+E_I]$$

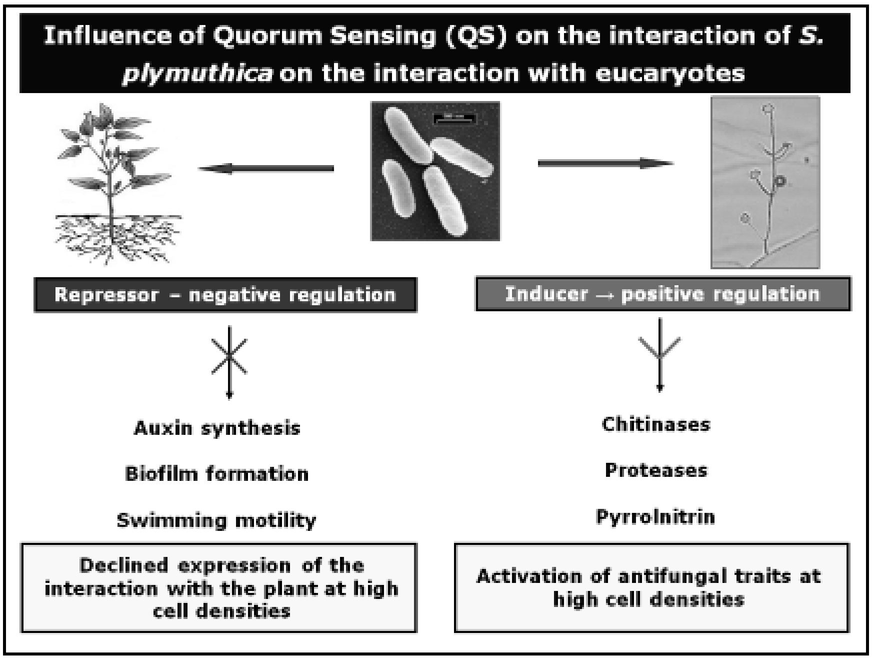
Biological control agents: Interplay with rhizosphere communities and risk assessment

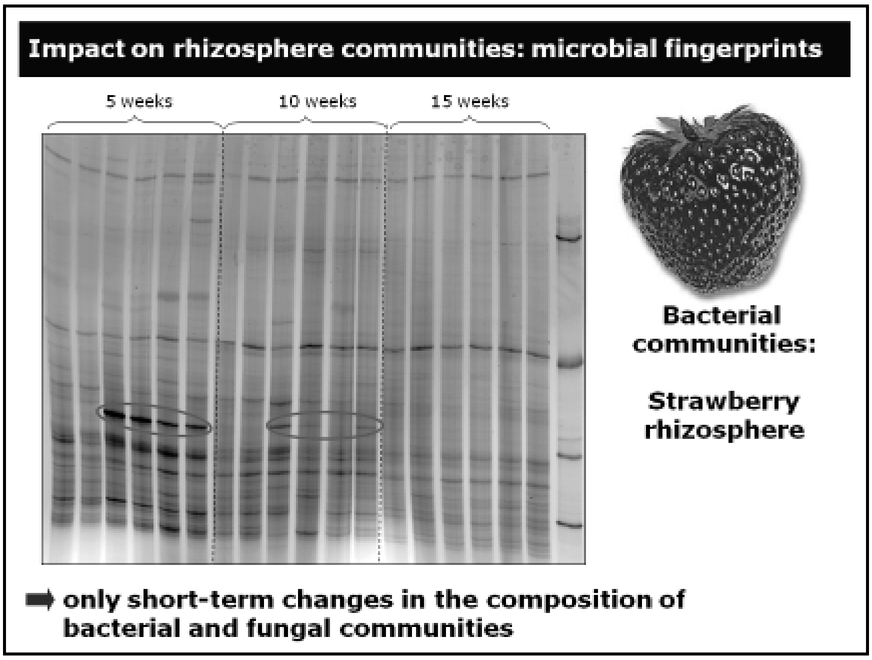
By Henry Müller and Gabriele Berg (Graz University; Austria)

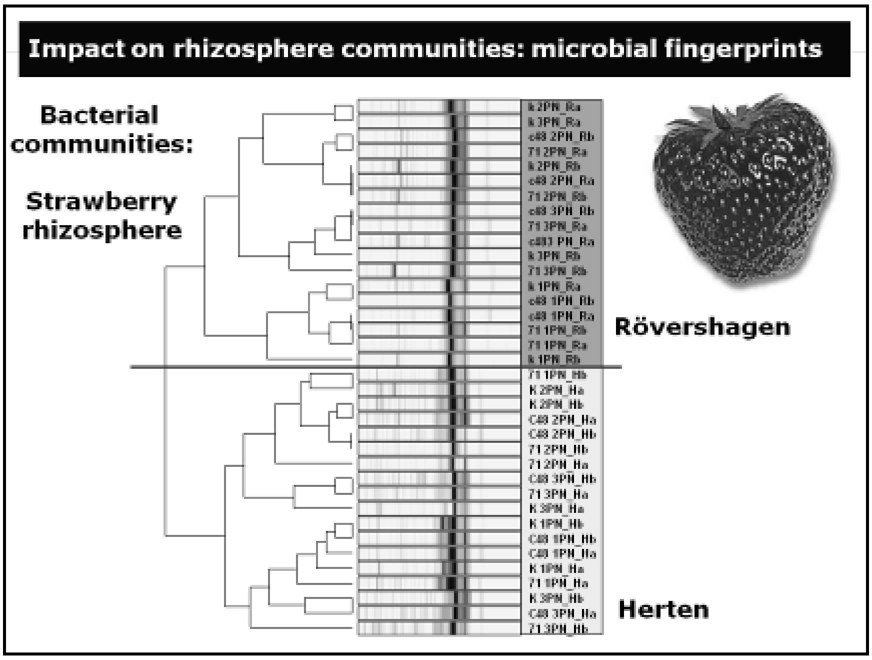


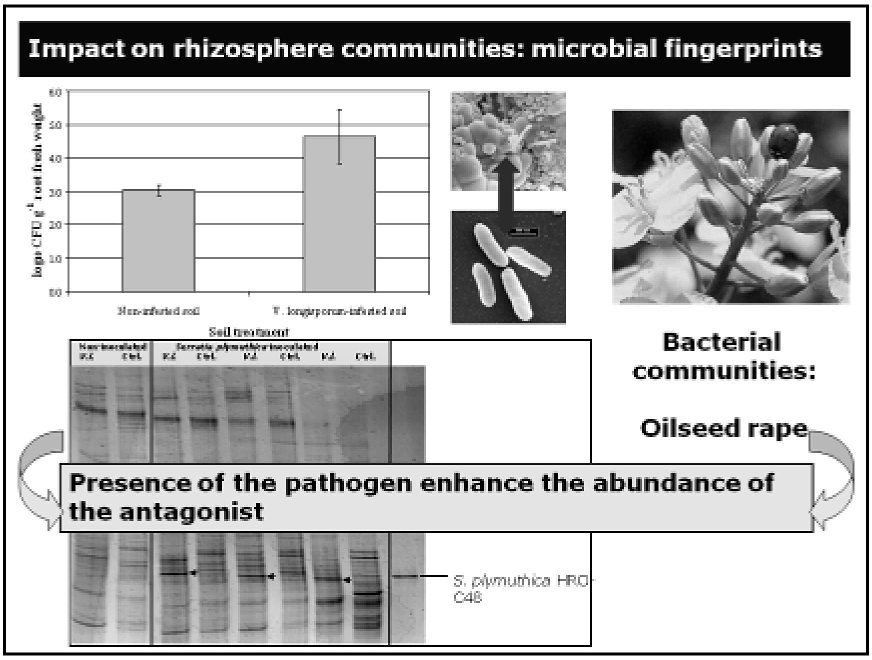


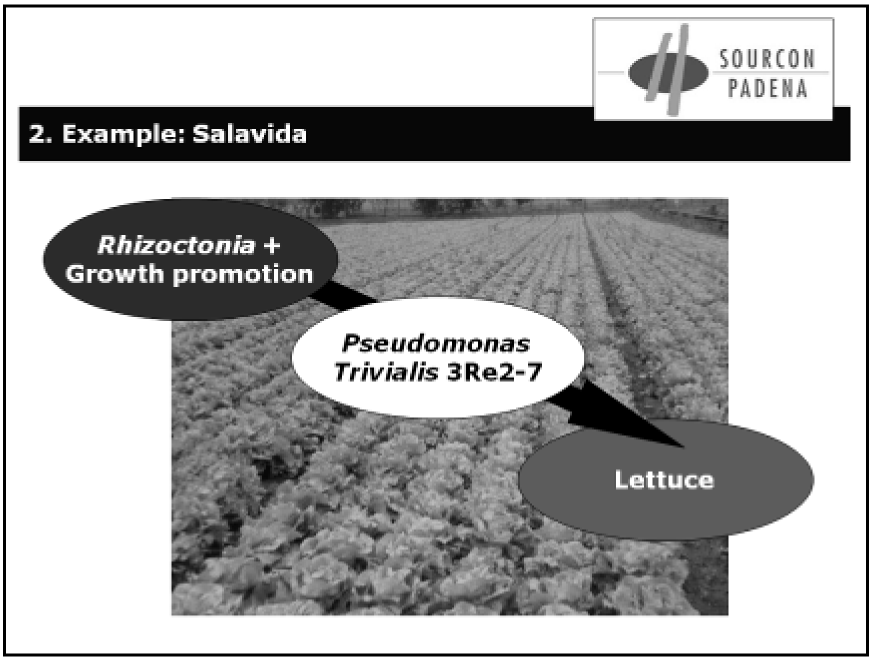

1. Example: RhizoStar®
Verticillium dahliae
Serratia plymuthica
HRO C48
Strawberry, rape
olives

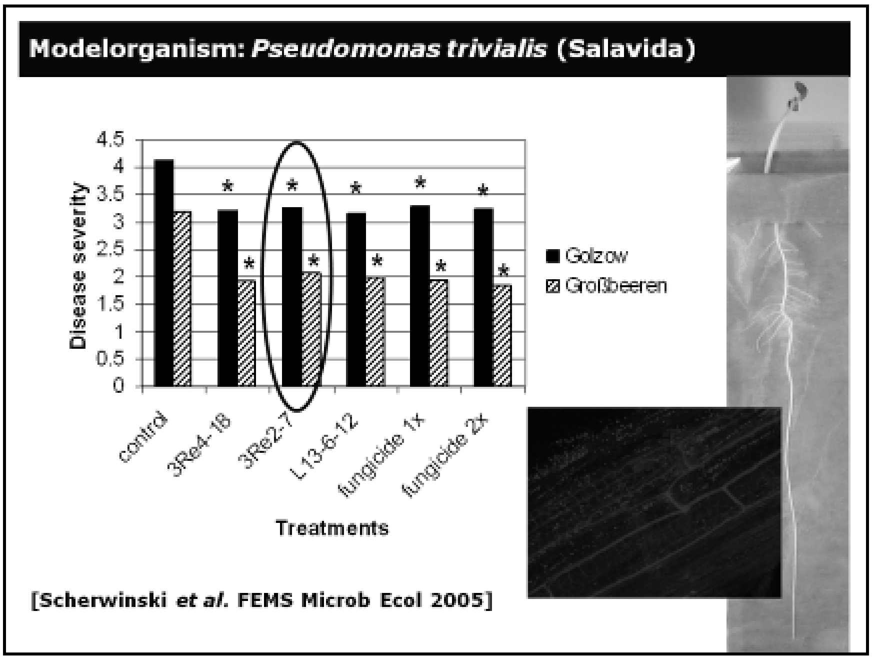

Influence of Quorum Sensing (QS) on the interaction of S. plymuthica on the interaction with eucaryotes
Repressor – negative regulation
Inducer → positive regulation
Auxin synthesis
Biofilm formation
Swimming motility
Chitinases
Proteases
Pyrrolnitrin
Declined expression of the interaction with the plant at high cell densities
Activation of antifungal traits at high cell densities


Impact on rhizosphere communities: microbial fingerprints
5 weeks
10 weeks
15 weeks
Bacterial communities:
Strawberry rhizosphere
only short-term changes in the composition of bacterial and fungal communities

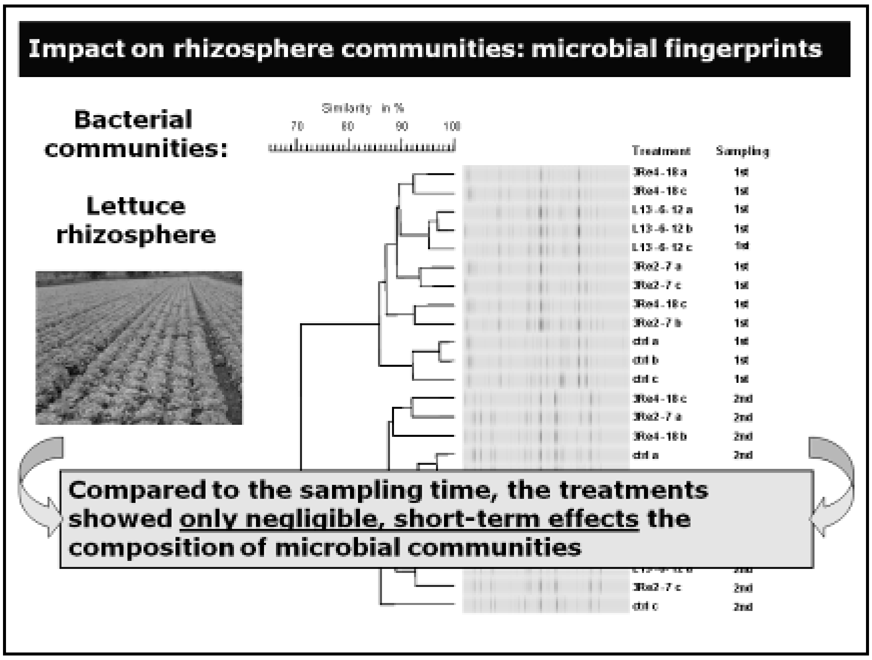

Impact on rhizosphere communities: microbial fingerprints
Bacterial communities:
Strawberry rhizosphere
k 2PN_Ra
k 3PN_Ra
c48 2PN_Rb
71 2PN_Ra
k 2PN_Rb
c48 2PN_Ra
71 2PN_Rb
c48 3PN_Rb
71 3PN_Ra
c48 3PN_Ra
k 3PN_Rb
71 3PN_Rb
k 1PN_Ra
c48 1PN_Rb
c48 1PN_Ra
71 1PN_Rb
71 1PN_Ra
k 1PN_Rb
71 1PN_Hb
K 2PN_Ha
K 2PN_Hb
C48 2PN_Ha
C48 2PN_Hb
71 2PN_Hb
71 2PN_Ha
C48 3PN_Hb
71 3PN_Ha
K 3PN_Ha
K 1PN_Hb
C48 1PN_Hb
C48 1PN_Ha
K 1PN_Ha
71 1PN_Ha
K 3PN_Hb
C48 3PN_Ha
71 3PN_Hb
Rövershagen
Herten


Impact on rhizosphere communities: microbial fingerprints
Soil treatment
Bacterial communities:
Oilseed rape
Presence of the pathogen enhance the abundance of the antagonist
S. plymuthica HRO-C48


SOURCON PADENA
2. Example: Salavida
Rhizoctonia + Growth promotion
Pseudomonas Trivialis 3Re2-7
Lettuce



Modelorganism: Pseudomonas trivialis (Salavida)
Disease severity
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
control
3Re4-18
3Re2-7
L13-6-12
fungicide 1x
fungicide 2x
Treatments
Golzow
Großbeeren
[Scherwinski et al. FEMS Microb Ecol 2005]



Impact on rhizosphere communities: microbial fingerprints
Bacterial communities:
Lettuce rhizosphere
Similarity in %
70
80
90
100
Treatment
Sampling
3Re4-18 a 1st
3Re4-18 c 1st
L13-6-12 a 1st
L13-6-12 b 1st
L13-6-12 c 1st
3Re2-7 a 1st
3Re2-7 c 1st
3Re4-18 c 1st
3Re2-7 b 1st
ctrl a 1st
ctrl b 1st
ctrl c 1st
3Re4-18 c 2nd
3Re2-7 a 2nd
3Re4-18 b 2nd
ctrl a 2nd
3Re2-7 c 2nd
ctrl c 2nd
Compared to the sampling time, the treatments showed only negligible, short-term effects the composition of microbial communities

Impact on rhizosphere communities: microbial fingerprints

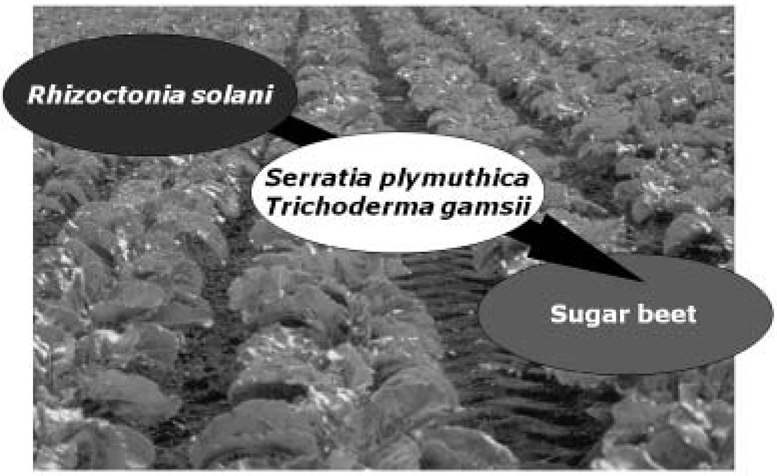
Similarity of microbial communities

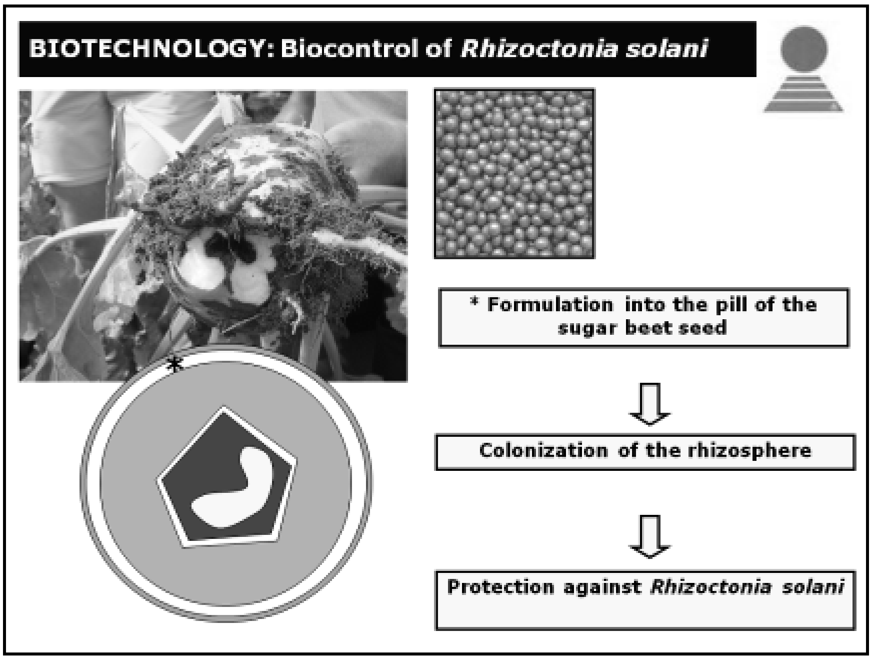
community	field site	growth stage	treatment
bacteria	60	66-75	78-100
pseudomonads	62	70-78	82-100
fungi	59	70-72	69-79

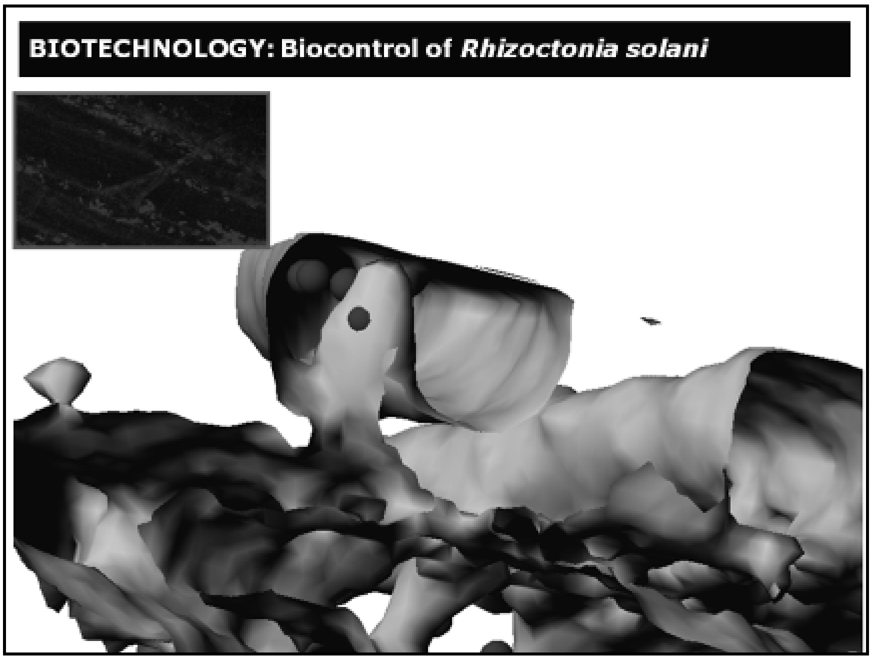
Compared to the growth stage and the field site, the treatments showed <u>only negligible, short-term effects</u> the composition of microbial communities

[Scherwinski *et al.* Biocontrol 2005]

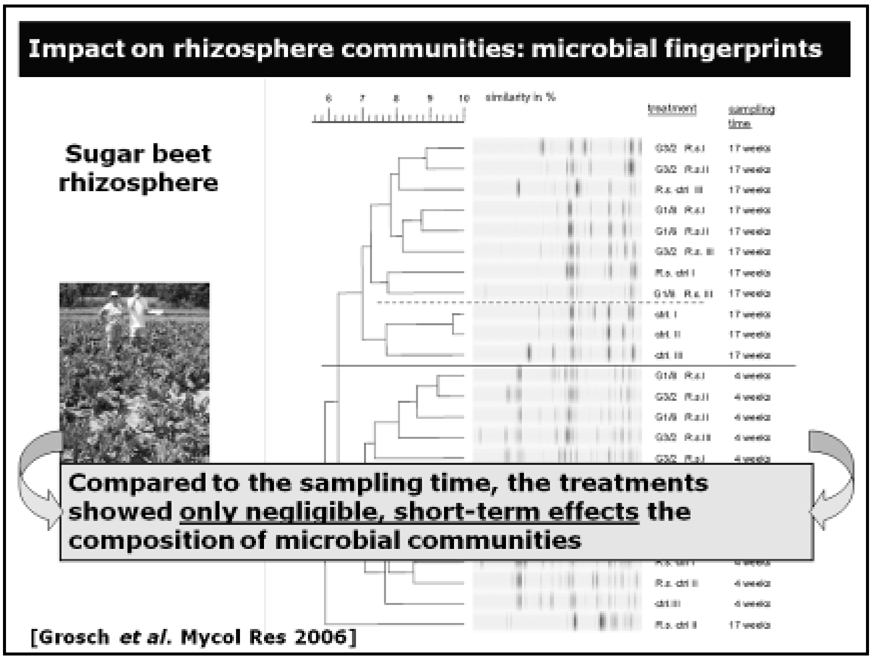
3. Example




BIOTECHNOLOGY: Biocontrol of *Rhizoctonia solani*
* Formulation into the pill of the sugar beet seed
Colonization of the rhizosphere
Protection against *Rhizoctonia solani*


BIOTECHNOLOGY: Biocontrol of *Rhizoctonia solani*

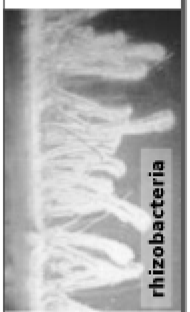

Serratia plymuthica and Trichoderma gamsii
in the sugar beet rhizosphere
80 µm
128.48 µm


Impact on rhizosphere communities: microbial fingerprints
Sugar beet
rhizosphere
similarity in %
treatment
sampling time
Compared to the sampling time, the treatments
showed only negligible, short-term effects the
composition of microbial communities
[Grosch et al. Mycol Res 2006]

Human pathogenicity?

Biosafety: Pathogens from the rhizosphere?

Plant

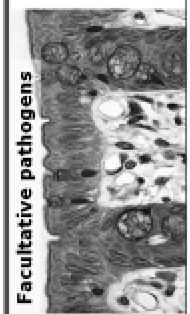


Pathogenicity:
- **Recognition**
- **Adherence**
- **Antibiosis/Toxicity**
- **Resistance** against antibiotics
- **Biofilm** formation

Fitness:
- **Competition** for nutrients and minerals (siderophores)
- **Production of hydrolytic enzymes**
- **Osmotolerance**
- **Versatility**

Induced resistance in plants
Production of phytohormons Growth at 37°C

Humans



[Berg *et al.* Environ Microb 2005]

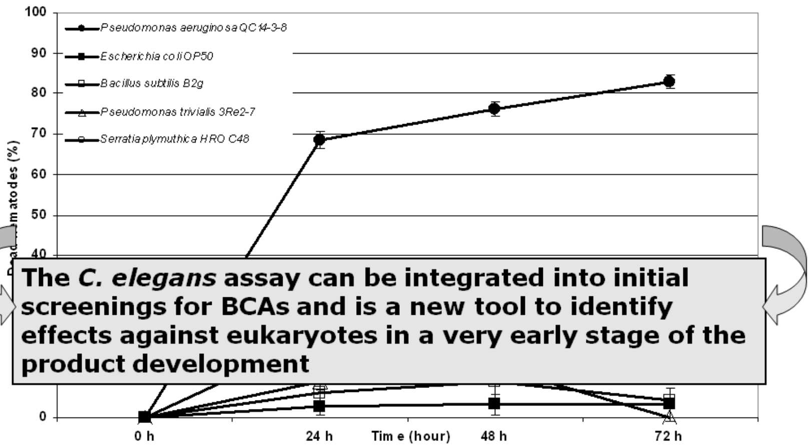
Why is *Caenorhabditis elegans* so useful?

- Can be safely used
- Easy observation (transparent)
- Easily and cheaply housed and cultivated
- Short life cycle reduces experimental cycle

- Genome is similar to that of humans (40% homologous)
- Attractive organism in the study of human diseases

- Slow killing assay – NGMII medium
  - Egg preparation - synchronization of worms
  - Six-well-plates with bacteria
  - Positive control (*Pseudomonas aeruginosa*)
  - Negative control (*Escherichia coli* OP50)
  - Test – strains
  - [Köthe et al. Cell. Microbiol. 2003]

C. elegans assay: BCAs



The *C. elegans* assay can be integrated into initial screenings for BCAs and is a new tool to identify effects against eukaryotes in a very early stage of the product development

Zachow et al. Europ. J. Plant Pathol. 2009

Conclusion: Interplay in the rhizosphere

Fate of microbial inoculants in the rhizosphere

- Manyfold interactions/interplays take place in this highly dynamic habitat.
- The balance of structure and functions is important for plant growth and health.
- Microbial fingerprinting is an appropiate way to analyse the impact on ubiquitous microflora
- BCAs *Serratia*, *Pseudomonas* and *Trichoderma* have only minor, short-time effects.
- Presence of target pathogens stimulates the population of BCAs

COOPERATIONS AND THANKS

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Prof. Ben Lugtenberg (Uni Leiden)
Dr. Dilfuza Egamberdiyeva (Uni Taschkent)
Prof. Martin Grube (Uni Graz)
Prof. Erich Leitner (TU Graz)

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Erdbeerhof Rövershagen
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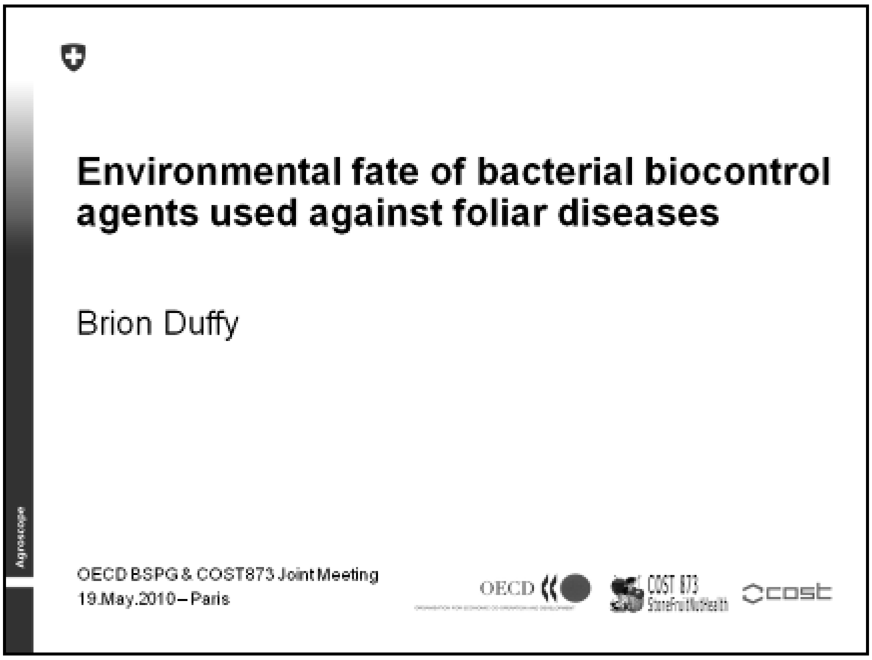
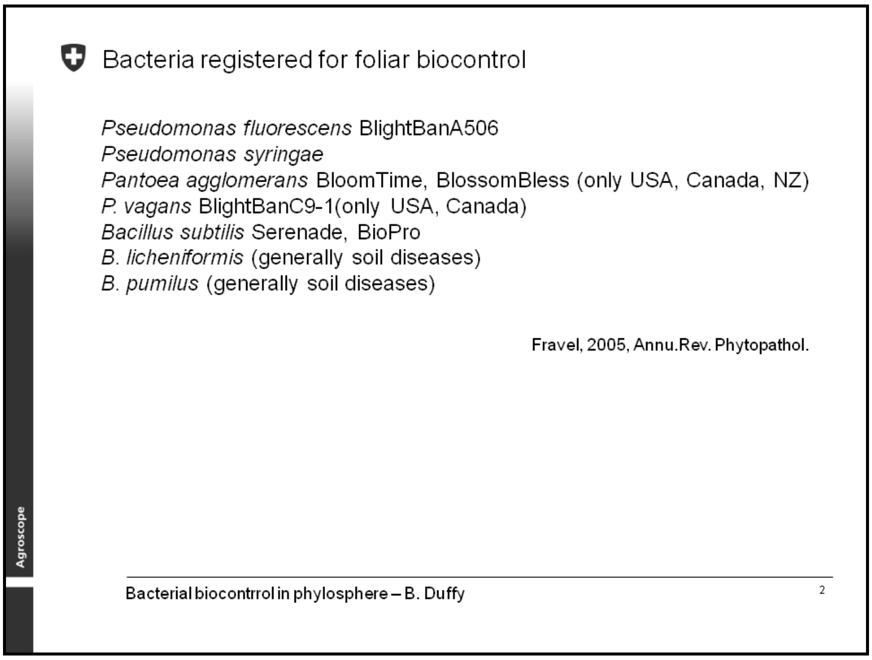
KWS Saat AG
Dr. Wolfgang Vogt
Sourcon-Padena AG

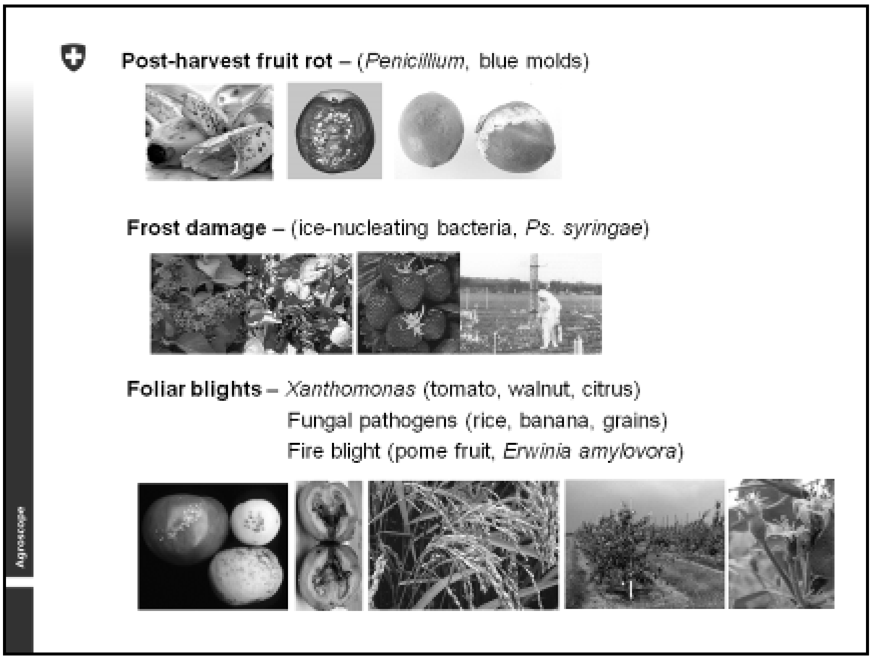
Prof. Gabriele Berg

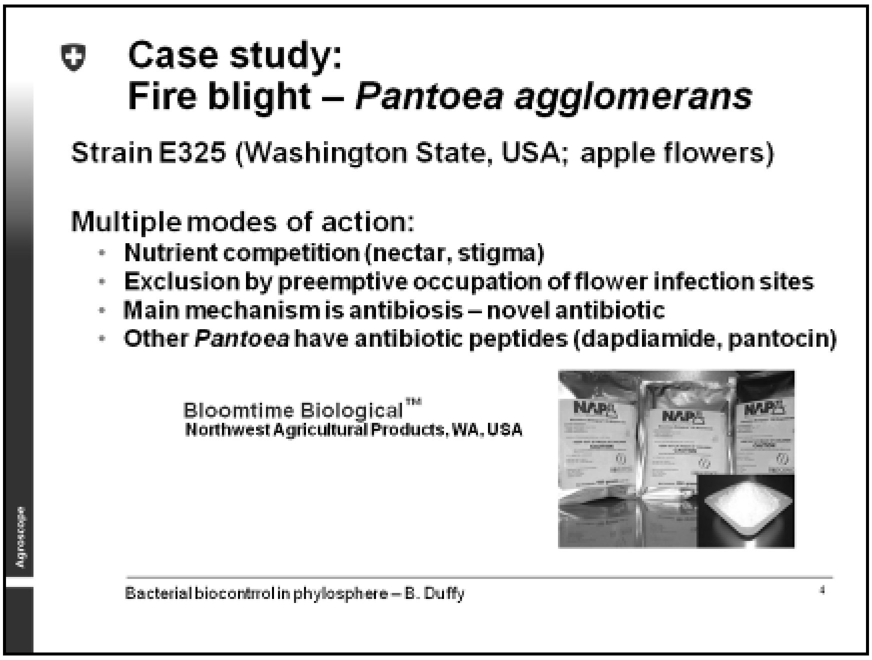
Environmental fate of bactarial biocontrol agents applied to the phylosphere

By Brion Duffy (Agroscope, Wädenswil; Switzerland)






Post-harvest fruit rot – (Penicillium, blue molds)
Frost damage – (ice-nucleating bacteria, Ps. syringae)
Foliar blights – Xanthomonas (tomato, walnut, citrus)
Fungal pathogens (rice, banana, grains)
Fire blight (pome fruit, Erwinia amylovora)
Agroscope


Case study:
Fire blight – Pantoea agglomerans
Strain E325 (Washington State, USA; apple flowers)
Multiple modes of action:
• Nutrient competition (nectar, stigma)
• Exclusion by preemptive occupation of flower infection sites
• Main mechanism is antibiosis – novel antibiotic
• Other Pantoea have antibiotic peptides (dapdiamide, pantocin)
Bloomtime Biological™
Northwest Agricultural Products, WA, USA
Bacterial biocontrol in phylosphere – B. Duffy
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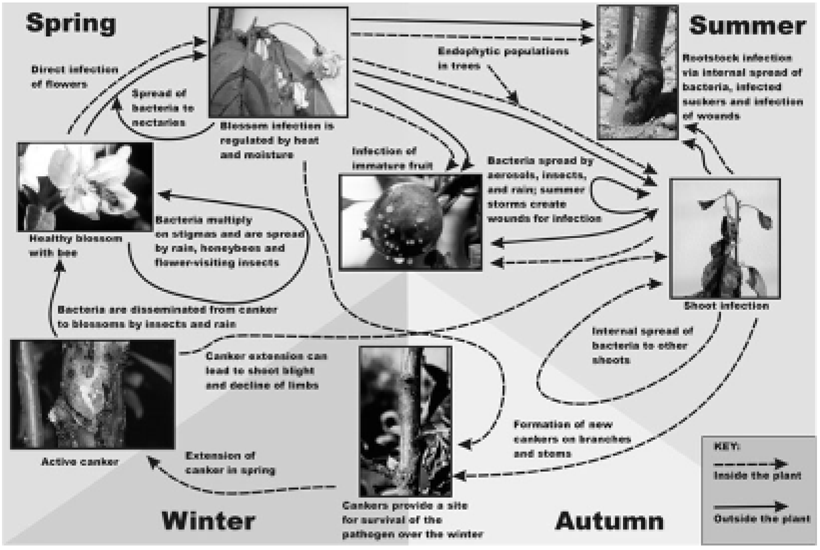
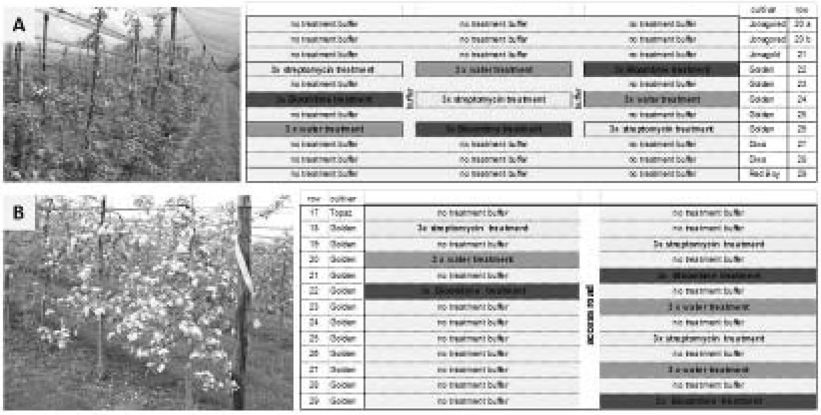
Fire blight epidemiology



Biocontrol – innundative vs. natural

- Flowers are essentially sterile when just opening
- Immigration of potential antagonists is gradual as flower ages (5-7 d), older flowers not at risk of fire blight
- Early pathogen colonization can overtake other microflora, *E. amylovora* is a relatively fast-growing Enterobacterium
- Effective antagonists are rare Require extensive screening to find
- Once registered, unlikely to convince industry to register a similar strain based solely on geographic origin

Swiss orchard trial example



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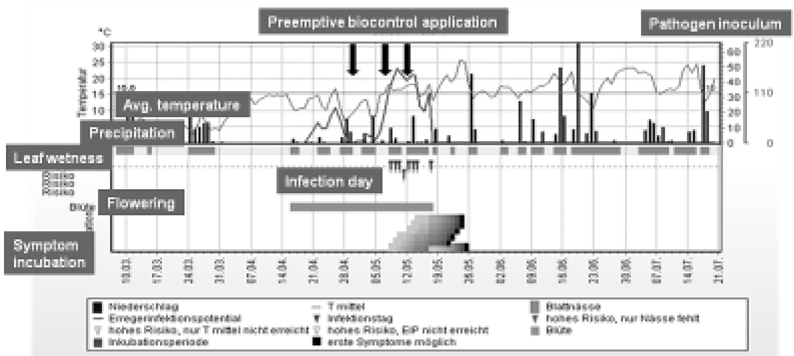
Environmental fate - establishment

- **Disease forecasting models used to also time biocontrol applications**
- ***Pantoea/Pseudomonas* mimic pathogen response to weather**
- *Bacillus* generally requires warmer temperature and time-lag

  Johnson et al., 2004, Plant Disease



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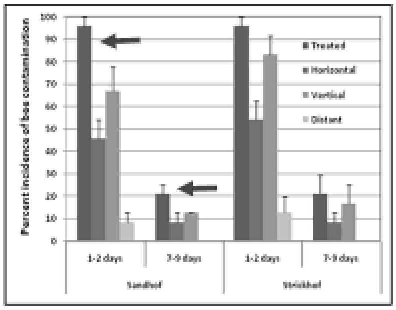
Environmental fate – secondary colonization

- Foraging insects are principal vectors
- Directly inoculated at hive with pollen traps - good
- Indirect contamination after visiting treated flowers – poor
- Thus need for repeat applications during bloom period ($)



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Bacterial biocontrol in phylosphere – B. Duffy 9

Environmental fate – secondary colonization

- High incidence on flowers opening just after application
- Reduced (inadequate) incidence on later-opening flowers

Secondary flower colonization within treated plots Swiss trials

	Sandhof		Strickhof	
	4-5 days	1-2 days	4-5 days	1-2 days
Incidence on flowers (SE)	11.1% (2.2)	55.6% (4.4)	24.4% (4.4)	71.1% (5.9)
E325 log cfu/flower (SE), all flowers	0.29 (0.05)	1.67 (0.19)	0.74 (0.12)	2.32 (0.27)
E325 log cfu/flower (SE), only positive flowers	2.62 (0.09)	2.99 (0.17)	3.06 (0.07)	3.24 (0.12)

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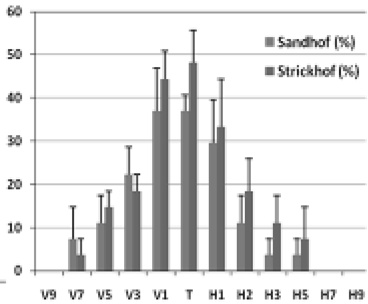
Bacterial biocontrol in phylosphere – B. Duffy 10

Environmental fate – dispersal

- **Low migration between rows within an orchard**
- **Not found outside orchards, or on alternate hosts**
- **Reflects bee flight patterns (vertical along row)**
- **Dilution effect as bees visit flowers**
- **Not recovered from beehives (2009), low in other studies**
  (Johnson et al. 1993, Nuclo et al. 1998, Johnson et al. 2006)

GAP: impact of beehive contamination not well studied
(other microflora/pests, colony behavior, metabolites effect/residues)?



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Survival post-bloom period

Leaves

- **E325 has low competitiveness as leaf epiphyte**
  On coronal leaves (highest exposure):
  At 1-2 days after application = 2.3-2.8 cfu/g leaf
  At 7 days after application = not detectable
  Strain P10c (NZ apple/pear) Vanneste et al. 2004**:**
  About 20% of leaf area contaminated, also declined rapidly

- **Leaves generally nutrient poor habitat**
- **Strain specific behavior expected, influenced by selection for nutritional use (?), host plant** (Pusey 2004, 2009)
- **Recent work with P10c on walnut suggests may survive longer on this host – AS REQUIRED**
  (Blum et al., IOBC Bull. 2009)

GAP: Few studies with *Pantoea* in phylosphere

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Survival post-bloom period

Fruit residues

- **E325 not recovered from fruit at harvest**
- **When found in other studies, at fruit set – not harvest**
- **Limited to calyx/sepal of fruit** (Pujol et al. 2006, AEM)

- **Rarely determined in *Pantoea/Pseudomonas* studies**
  Should consider pre-/post-washing when done

Persistence on fruit is the objective though for post-harvest biocontrol

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Bacterial biocontrrol in phylosphere – B. Duffy 13

Survival post-bloom period

Soil

- **Conventional sprayers have low drift;**
- **Objective is to cover canopy <u>without run-off</u> = low exposure risk**
- **No E325 recovered from soil under treated trees (2009)**
- Not generally a soil inhabiting taxa.
  Pseudomonas studies with introduced antagonists indicate decline over time

- **Gap: inadequate (?) study in soil (2010=worst-case studies)**
  No study has demonstrated recovery of declined populations.
  Remnant survivors would be a fraction of the microflora biodiversity

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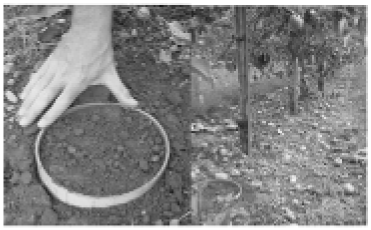

Survival post-bloom period

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  No study has demonstrated recovery of declined populations.
  Remant survivors would be a fraction of the microflora biodiversity

What would be a selection pressure to confer advantage (?)
Similar taxa are ubiquitous in nature.
Antibiotics produced in niches, low amounts – low exposure.
Isolates rare in original habitat – why would this change?
Major difference to pathogens – selection is artificially high!

Just a theory – but so is evolution……

Environmental impacts - plants

Biocontrol strains have no impact observed on target plant or any other plants:

- **Best *data* is the continued acceptance by growers.**
- **Contrary to previous reports - IAA genes are absent in *P. vagans* and *P. agglomerans.***
- **Biocontrol strains lack virulence plasmid characteristic of phytopathogenic pathovars**

- ***P. syringae* used for postharvest biocontrol on citrus:**
  Mode of action found to involve syringotoxin.
  Same mode of action used by pathogenic strains.
  <u>Potential</u> for non-target effects on plant prior to harvest.
  (Smilanick et al., Plant Disease 1996)

GAP: Insufficient data available on possible impacts.
Problem is ……. where to start?

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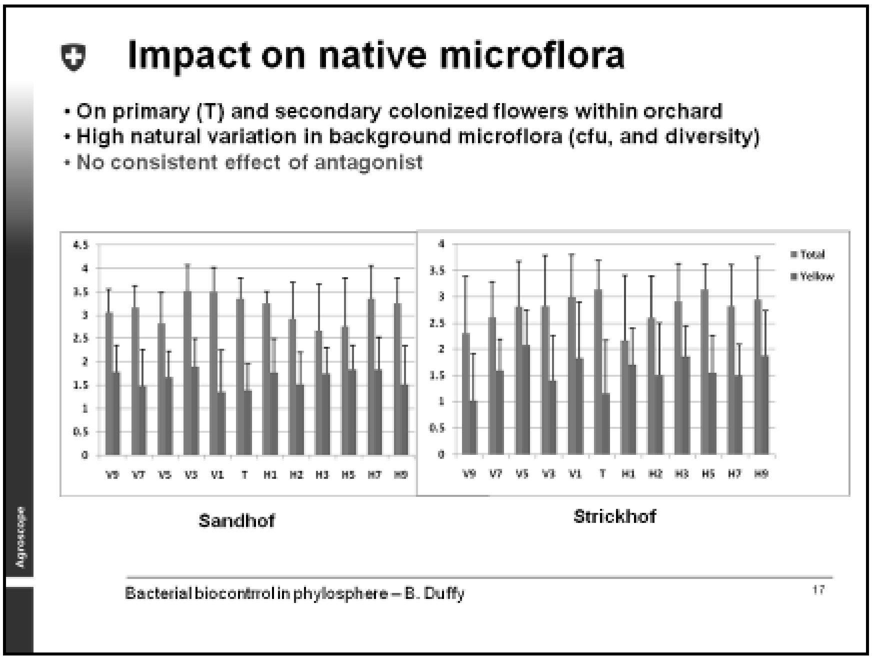
Impact on native microflora
• On primary (T) and secondary colonized flowers within orchard
• High natural variation in background microflora (cfu, and diversity)
• No consistent effect of antagonist
V9 V7 V5 V3 V1 T H1 H2 H3 H5 H7 H9
Total
Yellow
Sandhof
Strickhof
Agroscope
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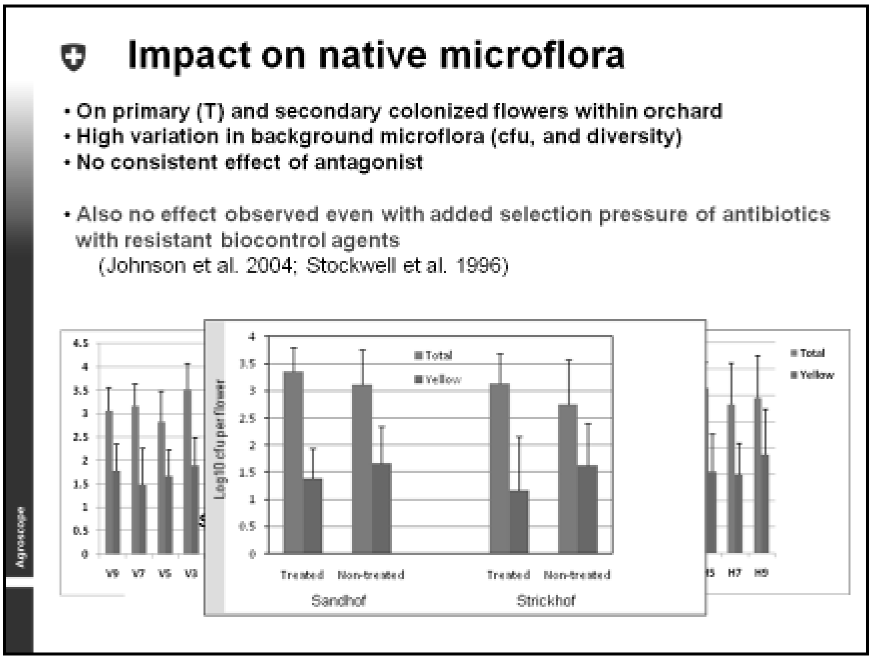
Impact on native microflora
• On primary (T) and secondary colonized flowers within orchard
• High variation in background microflora (cfu, and diversity)
• No consistent effect of antagonist
• Also no effect observed even with added selection pressure of antibiotics with resistant biocontrol agents
(Johnson et al. 2004; Stockwell et al. 1996)
Log10 cfu per flower
Total
Yellow
Treated
Non-treated
Sandhof
Strickhof
Agroscope

Impact on native microflora

Indications of potential for impact

- ***Pantoea*** **as a group has broad-spectrum activity** (fungal, bacterial diseases)
- **Peptides have broad-spectrum activity**

- **Non-target benefit of reduced pear russeting from IAA-bacteria/yeast indicates some effects can occur** (Lindow, Phytopathology, 2003)

Ps. fluorescens **A506 extracellular protease inactivates antibiotics of *P. agglomerans and P.vagans***

- **Reduces synergistic benefits of mixtures or rotating products**
- **A protease mutant has been developed, without negative effect**
- **Now this is used in IPM mixtures**

(Meadows et al. 2004)

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Impact on native microflora

But THIS IS THE POINT of effective biocontrol agents:
Relatively broad-spectrum activity = <u>multi-use</u>

Indications of little or no long-term effects:

- **Relatively high population is needed to have an effect on the target pathogen - highest exposed microbe**
  (Montesinos 2003, Intl. Microbiol)

- **Lack of a residual effect of prior releases on target pathogen**

- ***Ps. syringae*** **biocontrol strain (ice-negative) actually has lower long-term competitiveness compared to native ice-nucleating bacteria**
  (Buttner, AEM, 1989)

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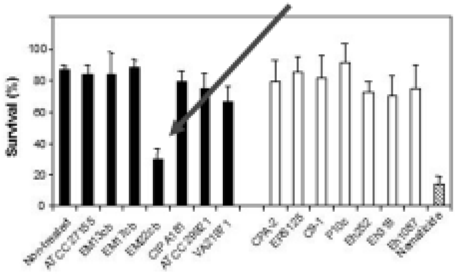
Bacterial biocontrrol in phylosphere – B. Duffy 20

Impact on nematodes

- Low expected exposure – not a soil bacterium
- No toxicity of cells or cultures (antibiotics, etc) on plant parasitic nemas
- No effect for any biocontrol strain, only one clinical strain



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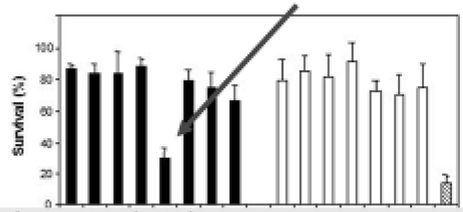

GAP: Natural soil populations not evaluated.

But what would this tell us?

- **Hard to get more specific than look at total nemas, total bacteriophogous nemas, maybe family/genus.**
- **Study for effect on relevant or specific component of community <u>not realistic</u>.**
- **Nema age may influence response. Old nemas resistant to pathogenic bacteria.**
  (Garsin et al. Science, 2003)

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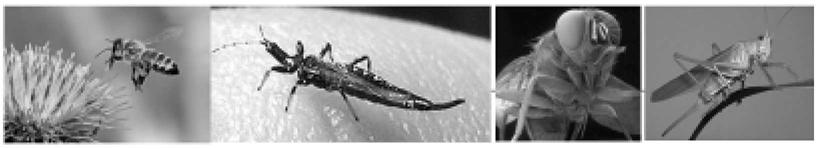
Impact on arthropods

- **NO adverse effect on honeybees.**
  Primary application method in NZ is with commercial beehives

- **Flurry of interest in 'symbiont' bacterial communities**
- ***Pantoea* frequently mentioned – problems though with ID reliability**
- **Beneficial impact on insect defenses: guaiacol pheromone in locust produced by gut *Pantoea* (Dillon et al. 2000)**
- **Age / nutrition dependent gut colonization (Dillon et al. 2010)**

Gap: confirm ID; determine if *Pantoea* passed in viable state and established in new habitat when moved by ingesting insect



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Impact on mammals / birds

Not studied beyond model test systems for registration.

Movement via birds unlikely to be relevant – it's not with *E. amylovora*

Virulence factors absent in *Pantoea agglomerans* and *P. vagans* genomes.

- **T3SS typical is absent, although a highly modified system does occur**
  (Mohr et al. 2008; Rezzonico et al. 2009)

Reports of immunostimulatory benefits from *Pantoea* cell components (EPS)

- **Potentiates antibody response to pathogenic *Salmonella***
  (Hebishima et al. 2010; Dutkiewicz et al. 2005)

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Bacterial biocontrrol in phylosphere – B. Duffy 24

Impact on humans

Pantoea vagans / P. agglomerans:
- **Hemolytic assays all negative**
- **Murine pathogenicity negative**

For biocontrol AND clinical isolates!
(Bonaterra et al., 2010 submitted)

Issue of inappropriate assumed link between isolation origin and biological behavior:
- **Most reports are of polymicrobial isolations from patients!**
- **No Koch's postulates; rarely conserve strains for further tests.**
- **Frequent misidentification as the composite species *agglomerans* (known pathogenic relatives typically misassigned)**

(Rezzonico et al. BMC Microbiology, 2009)

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Bacterial biocontrrol in phylosphere – B. Duffy 25

Impact on humans

- **Reports that exposure risk unlikely beyond farm.**
  Nasal swabbing failed to find *Pantoea* transfer to applicators
- **Antibiotic quantities and conditions for production in the environment not yet known – current work**
- **Antibiotic resistances found in genomes:**
  are few and non-transmissible
  not clinically relevant compounds
  nothing that isn't widespread in native microbe communities
- **Already have regular exposure to similar natural *Pantoea* e.g. on fruit, in public parks, gardens, etc.** (Venturini et al. 2002)

Gap: Effect on gut microbiome
- New metagenomic methods would make this useful to look at now.
- Antibiotics, probiotics, feed – shown to have (transient) impact.

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Model systems for testing

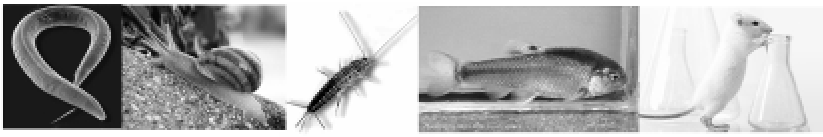
Necessary evil to limit the systems

- *Pantoea vagans* has passed the full gamut of tox/path testing
- *Pantoea agglomerans* has gone through reduced, but still extensive series of tests
- <u>Proving a negative is very hard to do, and to convince</u>

Relevance of these model systems?

Criteria for ecosystem relevant tests?

Put into perspective considering selection pressure for introduced strain and prevalence of native related bacteria.



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Environmental fate – establishment

- **Foraging insects are principal vectors of both pathogen and biocontrol agents**

Establishment of E325 on treated flowers sampled at 1-2 d and 5-7 d after application.

	Sandhof		Strickhof	
	1 day	5 days	2 days	7 days
Incidence on flowers (SE)	77.8% (6.4)	37.0% (3.7)	88.9% (6.4)	48.2% (7.4)
E325 log cfu/flower (SE), total flowers	1.95 (0.07)	1.36 (0.17)	2.16 (0.07)	1.86 (0.24)
E325 log cfu/flower (SE), only positive flowers	2.56 (0.31)	3.32 (0.14)	2.44 (0.10)	3.91 (0.30)
E325 population increase (log cfu/flower)		0.76		1.47

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