
Series on Pesticides
No. 100

JT03445355
Annex 4 – Slides of Speakers’ Plenary Presentations

[PPT 1] Presentation on the OECD and the work of OECD-EGBP and general introduction to the seminar on ‘Test Methods for Micro-organisms’
Jeroen Meeussen (European Union Minor Uses Coordination Facility)

[PPT 2] Background and feedback from the OECD Survey on Data Requirements and Test Guidelines for Micro-organisms, and challenges for human toxicology
Marloes Busschers (Charles River’s-Hertogenbosch, The Netherlands)

[PPT 3] Test methods and the evaluation of micro-organisms: The EU experience
José V. Tarazona (Pesticides Unit, European Food Safety Authority (EFSA), Parma, Italy)

[PPT 4] Ecological risk assessment for microbial pesticides
Mark Whittaker (Applied Insect Science (APIS), Ripon, United Kingdom)

Bilgin Karaoglan (German Federal Environment Agency UBA, Dessau-Roßlau, Germany)

[PPT 6] Pollinators and test methods for micro-organisms
Emily McVey, Jacoba Wassenberg (Board for the Authorisation of Plant Protection Products and Biocides (Ctgb), Ede, The Netherlands)

[PPT 7] Microbes and Daphnia effects: False toxicity due to study design?
Maria Pilar Herrero (Valent BioSciences LLC, United States)

[PPT 8] The U.S. experience with long-term Daphnia testing
Shannon Borges (Environmental Protection Agency, Washington DC, United States)

[PPT 9] Experience with long-term Daphnia toxicity studies (OECD TG 211) for microbials and proposals for the amendment of the study design
Bilgin Karaoglan (German Environment Agency (Unweltbundesamt), Germany)

[PPT 10] How to develop and adapt OECD Test Guidelines for micro-biorganisms
Magdalini Sachana, Anne Gourmelon (OECD, Paris, France)

Alan Norden (Australian Pesticides and Veterinary Medicines Authority (APVMA, Kingston, Australia)

[PPT 12] Biological Pesticides in Japan
Hidetaka Kobayashi, Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan)

Shannon Borges (Environmental Protection Agency (EPA), Washington DC, United States)
Presentation 1

Presentation on the OECD and the work of OECD-EGBP and general introduction to the seminar on ‘Test Methods for Micro-organisms’
Jeroen Meeussen (EU Minor Uses Coordination Facility)
OECD

• A few words about OECD

• OECD Work on Biopesticides

• Today’s seminar: purpose, scope and structure

A few words about OECD

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

- Started after World War II
- Transformed in 1961 into the Organization for Economic Co-operation and Development with trans-Atlantic and then global reach
- Today the OECD has 35 member countries
- More than 70 developing and transition economies are engaged in working relationships with the OECD (Brazil, Russia, India, China and South Africa)

OECD – What is OECD?

A forum in which governments work together to:
- Co-ordinate and harmonise policies;
- Discuss issues of mutual concern;
- Work together to respond to international problems.

A provider of comparative statistics and economic and social data with more than 250 publications per year.
OECD-WGP (Working Group on Pesticides)

The objective of the OECD Programme on Pesticides and Sustainable Pest Management is to help governments co-operate in assessing and reducing the risks of agricultural pesticides and sustainably managing pests.

OECD

- A few words about OECD
- OECD Work on Biopesticides
- Today’s seminar: purpose, scope and structure
OECD-EGBP

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

- Since 2017: **Expert Group on BioPesticides (EGBP)**.

---

**OECD-EGBP**

**Biological Pesticides:**

- Macro-organisms
- Microbial pesticides
- Semiochemicals
- Botanicals
OECD-EGBP

The first tasks of the EGBP consisted of:
• reviewing regulatory data requirements for three categories of biopesticides;
• developing formats for dossiers and monographs for microbials, and pheromones and other semio-chemicals.

OECD-Publications (Ⅰ)

Registration requirements:
• for pheromones (Series on Pesticides, No. 12, 2001) revision (Series on Pesticides, No. 93, 2017)
• for microbial pesticides (Series on Pesticides, No. 18, 2003)
• for invertebrate biocontrol agents/IBCA s (Series on Pesticides, No. 21, 2004)
OECD-EGBP

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

OECD-Publications (II)

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

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

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

OECD-Publications (IV)

- Guidance Document on Storage Stability of Microbial Pest Control Products (OECD Series on Pesticides No. 85, 2016)
- Report of a Survey on the Need for Further Guidance on Data Requirements and Updated Test Guidelines to Support the Assessment of Microbial Pesticides (OECD Series on Pesticides No. 87, 2017)
- Guidance document for the assessment of the equivalence of technical grade active ingredients for identical microbial strains and isolates (OECD Series on Pesticides No. 96, 2018)
Workplan 2013-2016 and 2017-2020

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

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

**OECD-Seminars (I)**

OECD-Seminars (II)


OECD-EGBP workshops

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

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

- A few words about OECD
- OECD Work on Biopesticides
  - Today’s seminar: purpose, scope and structure

Seminar on Test Methods for Micro-organisms

The topic “Test methods for micro-organisms” was selected based on the results of an OECD survey conducted in 2012 to identify where existing test methods or guidance are not sufficient to generate data needed to assess microbial pesticides.

Micro-organisms used as pesticides are regulated in ways that are similar to chemical pesticides. However, the biological properties of living micro-organisms differ from the properties of chemical pesticides, and, hence, the test methods used may not be the same as used for a chemical pesticide.
Seminar - Scope

Discussion on:
- The applicability of existing test methods for micro-organisms.
- How to interpret results performed in tests for micro-organisms?
- Information on alternative methods and their results should be considered.
- Ensure that information on the biology of the active organism strain / species is considered when designing tests.
- Novel mechanisms of biopesticide action may require consideration of new or amended guidelines and test methods.
- Remove sewage treatment from data requirements for PPP?
- Is the current regulatory framework appropriate to register micro-organisms?

Seminar - Structure

Presentations focussed on:
- government, research and stakeholder experience and perspectives,
- followed by discussion after each set of presentations.
Seminar - Results

With the focus on "test methods for micro-organisms", the goals of this seminar are

1. for participants to promote a dialogue, and

2. to initiate a process to make recommendations for improvements to test methods for micro-organisms.
Seminar on Test Methods for Micro-organisms

I wish you an interesting and useful seminar!
Presentation 2

OECD survey on data requirements and Test Guidelines for micro-organisms, and challenges for human toxicology

Marloes Busschers (Charles River, The Netherlands)

Contents

- OECD survey
  - Background
  - Summary of results

- Challenges mammalian toxicology
Background

- OECD questionnaire submitted to member countries in December 2012

- To identify where existing test methods or guidance are not sufficient to generate data needed to assess _microbial pesticides_ before market approval

- ultimate goal: list of priorities for the development of new and/or amended test guidelines or guidance documents that are applicable to microbials

- additional guidelines or guidance is either _necessary_ or could be _supportive_
Survey

- Distributed to members of the OECD Bio-Pesticides Steering Group (BPSG)
- Questionnaire: table listing all of the data elements in the OECD Dossier Guidance for Industry Data Submissions for Microbial Pest Control Products and their Microbial Pest Control Agents (2004)
- The OECD Dossier Guidance common format and structure for applicants dossiers for active substance approval or plant protection product registration in OECD countries
- for each element: indicate if existing test methods for generating relevant data are sufficient, or if they need to be improved (e.g. lack of test guidelines, different interpretations of guidelines or of data points)
- A first compilation of responses was presented as a background document for an OECD/Swedish Chemicals Agency (Kemi/EU) workshop held in Saltsjöbaden, Sweden in June 2013, entitled Microbial Pesticides: Assessment and management of Risks (OECD, 2014)

Survey

- Part A (OECD data requirements for Microbial Pest Control Agents)
- Part B (OECD data requirements for Microbial Pest Control Products)
- Report Appendix 1 (responses to the survey)
2.6 Potential of the micro-organisms to produce metabolites that are of concern for human health and/or the environment.

EU:
This point should be clarified because to be considered as a.o. suitable for use as pesticide, it should not be able to produce metabolites that are of concern for human health and/or the environment.

CA:
PSMA DRD2001-02 Part 2.7.2 No.
OECD Yes. No microbial-specific guidelines.
U.S. EPA R5 1100 No.

As previously noted, dosiers are often lacking in details with respect to the production of potentially toxic metabolites. Additional guidance/strategies may be required to help applicants to properly address these concerns.

EU:
Y No guidance how to assess this.

EU:
Y No guidance how to assess this.

AN:
Y

The composition and structure of potential metabolites present in the product is extremely difficult to elucidate taking into account the complex nature of the growth media.

It would make sense to ask only for measurement of levels of toxic metabolites which are known from scientific literature. The existence of respective genes does not mean at all that toxicants will be formed during production or after application.

---

| OECD Annex | IM point | OECD data point number | Information, test or study according to OECD Boost Test Guidance Document, Appendix 4, Part 4 | Problems with Test Guidelines (Y/N)? | Overall Conclusion | Suitable method available without modification?
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>14.3.1</td>
<td></td>
<td></td>
<td></td>
<td>Y/N, please specify the Test Guideline number and describe the problem</td>
<td>SUCCESSFUL: Manual review and assessment of the data sheet and overlay documents; no further guidance needed.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No suitable method available with modifications.</td>
</tr>
</tbody>
</table>
Result of survey – guidance available

For some data requirements, guidance can be found in:

- test guidelines Series 885 of the US EPA, 1996
- in OECD issue papers or guidance documents:
  - OECD (ENV/JM/MONO(2011)43) on microbial contaminant testing
  - OECD (ENV/JM/MONO(2012)1) on environmental safety evaluation of microbial biocontrol agents

Result of survey – guidance needed

Identity

Necessary
- Equivalence:
  SANCO/12823/2012
  ENV/JM/MONO(2018)8
- Characterization of strain/serotype
- Identification metabolic by-products/impurities
- Secondary metabolites/toxins:
  OECD seminar ENV/JM/MONO(2017)5
  EU in prep
- Acceptable quality control/analytical profile

Useful
- acceptable range content MPCA
Result of survey – guidance needed

Biological properties

*Necessary*
- Natural occurrence (bridging)
- Mode of action
- Genetic stability (how to assess, relevance)

*Useful*
- Physiological properties (effect of light, pH, temp, humidity) – when non-GLP
- Resistance/sensitivity antibiotics

---

Result of survey – guidance needed

Analytical methods

*Necessary*
- Criteria for validate methods

*Useful*
- Storage stability:
  ENV/JM/MONO(2016)54
Result of survey – guidance needed

Mammalian toxicology

Necessary
- Sensitisation potential:
  OECD seminar ENV/JM/MONO(2017)8
  OECD survey ENV/JM/MONO(2016)37
- Unspecific effects in pulmonary study
- Genotoxicity

Useful
- Details on oral inf/tox OPPTS 885.3050
- Rationale for residue waiver
- Acceptability of qualitative Risk Assessment

Result of survey – guidance needed

Fate & behaviour

Useful
- Exposure scenarios related to way of application
  OECD seminar ENV/JM/MONO(2015)38
  OECD seminar ENV/JM/MONO(2011)42
- Rationale for non-submission of data
Result of survey – guidance needed

Ecotoxicology

*Necessary*
- Bee testing, incl brood testing
- Applicability of OECD tests

*Useful*
- Updating of several US EPA 885 tests, eg NTA
Challenges mammalian toxicology

OECD Survey

Mammalian toxicology

Necessary
- Sensitisation potential:
  OECD seminar ENV/JM/MONO(2017)8
  OECD survey ENV/JM/MONO(2016)37
- Unspecific effects in pulmonary study
- Genotoxicity

Useful
- Details on oral inf/tox OPPTS 885.3050
- Rationale for residue waiver
- Acceptability of qualitative Risk Assessment

Toxicology Test Guidelines

United States Environmental Protection Agency (EPA).
Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-96-318, 1996

Microbial Pesticide Test Guidelines, OPPTS 885 series.

885.3000 - Background--Mammalian Toxicity/Pathogenicity/Infectivity (February 1996)
885.3050 - Acute Oral Toxicity/Pathogenicity (February 1996)
885.3100 - Acute Dermal Toxicity/Pathology (February 1996)
885.3150 - Acute Pulmonary Toxicity/Pathogenicity (February 1996)
885.3200 - Acute Injection Toxicity/Pathogenicity (February 1996)
885.3400 - Hypersensitivity Incidents (February 1996)
885.3500 - Cell Culture (February 1996)
885.3550 - Acute Toxicology, Tier II (February 1996)
885.3600 - Subchronic Toxicity/Pathogenicity (February 1996)
885.3650 - Reproductive/Fertility Effects (February 1996)
Sensitisation

No validated (animal) tests, 885.3400 - Hypersensitivity Incidents (Feb 1996)

All EU products labelled as potential sensitisers: 'Micro-organisms may have the potential to provoke sensitising reactions'
  ➢ Often automatically PPE on label
  ➢ Not good for image of green product
  ➢ Problems with amateur use approval
  ➢ No ways to get rid of this "label"

Sensitisation

Dermal most probably less relevant compared to respiratory sensitization:
- M.o. will not penetrate intact skin -> no sensitization possible
- Then why gloves on label?
- If no respiratory exposure (liquids), no PPE on label needed
- Respiratory sensitizer if based on human evidence
Toxicology Test Guidelines

United States Environmental Protection Agency (EPA),
Office of Prevention, Pesticides and Toxic Substances (7101), EPA 712-C-96-318, 1996

Microbial Pesticide Test Guidelines, OPPTS 885 series.

885.3000 - Background: Mammalian Toxicity/Pathogenicity/Infectivity (February 1996)
885.3050 - Acute Oral Toxicity/Pathogenicity (February 1996)
885.3100 - Acute Dermal Toxicity/Pathology (February 1996)
885.3150 - Acute Pulmonary Toxicity/Pathogenicity (February 1996)
885.3200 - Acute Injection Toxicity/Pathogenicity (February 1996)
885.3400 - Hypersensitivity Incidents (February 1996)
885.3500 - Cell Culture (February 1996)
885.3550 - Acute Toxicology, Tier II (February 1996)
885.3600 - Subchronic Toxicity/Pathogenicity (February 1996)
885.3650 - Reproductive/Fertility Effects (February 1996)

Objectives Acute Toxicity/Pathogenicity Studies

- Provide initial information on the toxicity of a MPCA (Microbial Pest Control Agent) using a single high dose exposure to experimental animals of both sexes and an adequate post-exposure observation period
- Infectivity, pathogenicity and clearance of the MPCA are to be investigated
- Provide information on health hazards likely to arise from a single exposure

Unclassified
**Oral toxicity/infectivity/pathogenicity**

- OECD guideline not applicable
- OPPTS 885.3000/3050
- Lacks positive controls (pathogenic isolates): uncertainty whether a pathogenic isolate would give a response in the animal models used.
- Known human pathogenic Bacillus cereus strain did not give rise to illness or diarrhea in the rat study (Wicks et al., 2006).

![Image of a rat]

---

**Pulmonary toxicity/infectivity/pathogenicity**

- OECD guideline not applicable
- OPPTS 885.3000/3150
- Technically challenging:
  - Intratracheal: perforation of trachea or lungs
  - Inhalation: does MPCA survive the nebulization process, Air born MPCA, Risk of contamination, high amount of MPCA needed
- Slow clearance

![Image of a rat]

---
Iv/ip toxicity/infectivity/pathogenicity

- OECD guideline not applicable
- OPPTS 885.3000/3200
- Worst case study, not relevant humane exposure route
- Relevance of study? Waiving possible?
- Ip: no enumeration of m.o. in tissues

Acute toxicity/infectivity/pathogenicity

General points on the three acute studies

- High animal use, labor intensive, expensive, due to infectivity assessment
- Possible ways to simplify:
  - Waiving infectivity/pathogenicity part if m.o. does not grow at temperatures above 30°C
  - Tissue enumeration: start with port of entry (lungs, GI tract; exposure check) and draining lymph nodes. If lymph nodes are clean, why assess other organs?
- How to assess risk for immunocompromised individuals?
Critical performance factors and issues

MPCA
- Characterization MPCA: active ingredient, contaminating MO, 5 batch
- MPCA preparation suitable for analysis and toxicological studies (Continues production process: extraction of MPCA from MPCP)
- Bioanalytical method for MPCA enumeration tissues

Dosing
- Vehicle selection and stability
- Dose volume
- Physiological compatibility (intravenous)
- Penetration lungs, distribution lungs (intratracheal)

Sacrifice
- Days of interim sacrifice should be adequate to establish clearance pattern

Critical interpretation factors
- Collection of tissues in formaldehyde fixative, affecting enumeration.
- Enumeration method (cutting organ in half and pressing it on growth medium).
- No MPCA in any organs, not even at the first time point (exposure check).
- Significant signs of toxicity without infectivity.
- Unspecific effects, mortality (eg in intratracheal)
  - Stress
  - Anaesthesia
  - Overreaction of immune system
  - Overdosing (viscous liquid)
- MPCA found in certain organs in individual animals on individual time points.
  - Real effect?
  - Improper handling (contamination)?
Genotoxicity

- Usefulness if characterization data indicate potential production of known genotoxic metabolites
- How should this be addressed? Metabolites could be produced during different conditions in response to different environmental factors. Only tests with isolated and identified metabolites? Or crude extract or culture supernatant of MPCA/MPCP? Testing with plant extracts?
- Sensitivity of the test? Dilution of the extract
- Intracellular and extracellular metabolites
- Testing in vivo can be regarded as a waste of animals

- Not always easy to demonstrate the non-production of metabolites or toxins
Thank you for your kind attention
Presentation 3

Test methods and the evaluation of micro-organisms: The EU experience
José V. Tarazona (Pesticides Unit, European Food Safety Authority (EFSA))

OVERVIEW

1. Regulatory steps for micro-organisms
2. Reasonable expectations
3. Experience from EU assessments (EFSA peer-reviews)
4. Conclusions
1. EU Regulatory steps MICRO-ORGANISMS INC. VIRUSES

- Approval criteria
  - Regulation (EC) No 1107/2009
- Principles for evaluation
  - Regulation (EU) No 546 / 2011
    - EU Uniform Principles
- Data requirements
  - Regulation (EU) Nos 283 & 284/2013
    - Microorganisms including viruses & Preparations of microorganisms including viruses
  - Risk assessment guidance documents and guidelines
  - Test methods guidelines: USEPA 1996 Test Methods
  - Ad hoc studies and information gathering
  - Evaluation of each microbial active substance

Regulation (EU) No 283 & 284/2013: Data requirements

- Chemicals:
  - Focused concerns in the data requirements
  - OECD test guidelines under constant update
  - Prescriptive guidance documents

- Micro-organisms:
  - Tailored data requirements and case-by-case concerns
  - USEPA Test guidelines from 1996
  - Some general principles but no prescriptive specific guidance documents
1. EU Data requirements

- Identity of the active substance
  - At strain level

- Biological properties:
  - Natural occurrence, target organism, host specificity
  - Development stages, infectiveness
  - Relationships to known plant/animal/human pathogens
  - Genetic stability
  - Production of (secondary) metabolites
  - Antibiotics (production and resistance(s))

1. EU Data requirements, cont.

- Intended purposes: function, field of use, crops
- Methods of production, storage, decontamination
- Analytical methods
- Effects on human health
  - Including some basic studies, such as sensitisation, genotoxicity, short term toxicity and pathogenicity
- Viable and non-viable Residues in/on treated products, food and feed (nature and concentration over time)
  - Data on persistence and likelihood of multiplication in or on crops, feedingstuffs or foodstuffs
- Fate and behaviour in the environment:
  - Persistence and multiplication (competitiveness in soil, water, air)
  - Mobility
- Effects on non-target organisms (ecotox)
  - E.g. birds, other organisms
2. Reasonable expectations MICRO-ORGANISMS INC. VIRUSES

- The Applicant should always address:
  1. The assumed pesticidal mode of action and concerns associated to it, e.g.
     - Production of toxins/metabolites
       - Hazard, exposure and risk assessment for the metabolites
     - Pathogenicity to the pest
       - Full assessment for related non-target organisms
     - Competition
       - Population dynamics in the crop for intended use conditions
  2. Other potential concerns identified in the scientific literature
     - Other suggested modes of action
     - Production of toxins/metabolites by the strain or other strains of the same species

2. Reasonable expectations: pesticidal mode of action

- The dossier should be coherent
  - Good agricultural Practice
    - Proposed pesticidal MoA
  - Efficacy studies
    - Confirm efficacy for the assumed pesticidal mode of action

- Direct concerns for humans and environment should be addressed
  - Production of toxins/metabolites
    - What? Where? When?
    - If within natural backgrounds, clarify efficacy mechanism
  - Competition
    - Temporal dynamics in the crop supporting efficacy and dynamics in other compartments to demonstrate lack of persistency
2. Reasonable expectations: Other concerns

- Proper literature search at species level ... and this includes on the genus when the species is not identified
  - EFSA guidance applicable
- All concerns identified for the species should be addressed, there is flexibility for applicants when building up the justifications, but should be substantiated with experimental data or scientifically sound information
- Example: production of toxins/metabolites by strains of the same species, lack of relevance for the (active agent) strain can be demonstrated by, e.g.
  - Is a protein and the strain does not have the genes encoding the protein
  - Study(ies) have been reproduced and confirmed that the strain does not produce the toxins/metabolites under the same conditions
  - Toxins/metabolites monitored and not detected in field trials using the intended use patterns
  - Metabolites are produced and a risk assessment estimating exposure within acceptable levels is included

3. Experience from EU assessments

**Literature search**

- EFSA 2011 Guidance is not always followed
  - Guidance on the submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (EFSA, 2011)
- Information not in line with the systematic reviews published by EFSA
  - At least studies cited in EFSA documents!!!!!
- Identified concerns not addressed or assumptions not supported by information
- Justifications not coherent with other parts of the dossier
3. Experience from EU assessments

Evidence regarding metabolite production
- Lack of robust analysis of consequences of pesticidal mode of actions for humans and non-target organisms
- Lack of information on production of metabolites including other strains of the species, conditions of production and use
- If identified for the species (or genus without information on the species), lack of necessary follow up:
  - Evidence of absence in the (active agent) strain
  - Characterisation/measurement of levels expected on crops and environmental compartments following application
  - (eco)toxicological profile of the metabolites/toxins

Test design and consistency
- Insufficient level of evidence to demonstrate the lack of relationship to known pathogens
- Studies on pathogenicity or infectivity to non target species, not designed according to the biology of the active organism strain/species, particularly test duration
- Use patterns not sufficiently well defined for allowing a proper exposure assessment
- Studies on toxicity and ecotoxicity not ensuring the presence of possible toxins/metabolites
3. Experience from EU assessments

Other issues

- Lack of assessment of the potential for transfer/acquisition of genetic information to/from other species (primary concern usually plasmids between bacteria species)

- Insufficient information on competitiveness/multiplication to conclude if the organism will decline to background levels within a year

- No information on absence of interference of organism on prescribed methods of analysis for pathogens in drinking water

4. CONCLUSIONS

- Lack of prescriptive guidance and updated test guidelines is acknowledged

- Flexibility is needed:
  - Regulation allows justified deviations from standard data requirements
  - Alternative information and data can be provided

- Two “Reasonable expectations” must always be fulfilled:
  - Direct concerns related to the pesticidal mode of action
  - Proper literature search and follow up all possible concerns identified for the species
  - In the EU: Systematic literature review according to EFSA (EFSA, 2013;2015)

- In addition the dossier should be coherent, in particular
  - Demonstrate efficacy under the proposed use patterns (GAPs)
  - Demonstrate safety for humans and non-target organisms for the same uses
  - Design of test methods according to the biology of the organism
  - Exposure levels shall cover those anticipated for the proposed GAPs

Unclassified
Presentation 4

Ecological risk assessment for microbial pesticides
Mark Whittaker (Applied Insect Science (APIS), United Kingdom)

- A UK-based GLP contract research laboratory and regulatory consultancy for biopesticides.
- Founded on almost 20 years commercial and regulatory experience with biopesticides.
- Full range of terrestrial ecotoxicology studies undertaken, with particular expertise on bees.
- Experience of optimising OECD/ESCORT/OCSP studies for microbial test items.

OECD Expert Group on Biopesticides, 2018
Who we are

Work to promote biopesticide regulatory innovation in Europe and the US:

- UK Biopesticides Scheme.
- EU REBECA Project.
- Steering Committee member for IBMA Microbial Professional Group.
- Invited expert in EPA/BPIA and ICP-PR Microbial Working Groups.

OECD Expert Group on Biopesticides, 2018

Registering a microbial pesticide

There are only three problems to overcome:

1. The regulatory framework is inappropriate.
2. The study guidelines are inadequate.
3. Nobody can agree how to interpret the results.

OECD Expert Group on Biopesticides, 2018
Regulatory environment

- Biopesticides are regulated in Europe under (EC) No 1107/2009 in exactly the same manner as conventional agrochemicals.
- Microbial data requirements are a direct read-across from those for conventional agrochemicals.
- Some of the test species that have historically been used for chemical ecotoxicology testing are unsuitable for microorganisms.
- Where test species are suitable, the study guidelines often need to be adapted to take account of microbial modes of action.

OECD Expert Group on Biopesticides, 2018

Modes of action

Biological / physical
- Cuticle penetration.
- Dissemination in haemocoel.
- Hyphal tissue invasion and proliferation.

And / Or

Chemical
- Endotoxins or toxic metabolites.
- Toxic manufacturing impurities.

OECD Expert Group on Biopesticides, 2018

Unclassified
Dose response

- Ecologically, a microorganism will either be infective to a particular organism or it won’t, and once the minimum effective dose has been achieved there is usually no further dose-response relationship.
- Microbial studies are generally conducted as limit tests at the Maximum Hazard Concentration, which is 10 – 100x the maximum spray tank concentration.
- The active substance is usually tested rather than the formulation.
- The exception is aquatic studies, where the extreme hydrophobicity of spore powders makes the formulation easier to use.

OECD Expert Group on Biopesticides, 2018

Typical survival curves

OECD Expert Group on Biopesticides, 2018
Typical survival curves

- In common with agrochemicals, toxic manufacturing impurities or metabolites present in the TGAI are quick to manifest themselves.
- Metabolites produced during the infection process, or physical damage resulting from internal hyphal growth, typically take at least 3-5 days to show effects.
- Many standard Tier I studies are either too short to detect these effects, or the test species is unsuitable for microbial risk assessment.
Microbial study guidelines

- With no specific microbial study guidelines in Europe, applicants must either come up with their own proposals or go to a CRO with relevant experience.
- None of these methods have been validated or ring-tested for microbials.
- The US OCSP 885 series guidelines are specific to microorganisms, but are severely lacking in detail compared to ESCORT / OECD guidance and are therefore of little practical value.

OECD Expert Group on Biopesticides, 2018

Six examples from standard test species

1. Earthworm (*Eisenia fetida*)
2. Springtail / collembolan (*Folsomia candida*)
3. Parasitic wasp (*Aphidius rhopalosiphi*)
4. Honeybee (*Apis mellifera*)
5. Water flea (*Daphnia magna*)
6. Microalgae (*Raphidocelis* (previously *Pseudokirchneriella*) *subcapitata*)

OECD Expert Group on Biopesticides, 2018
Eisenia fetida (OECD 222)

- Worms are adapted to life in the most microbially challenging environment on Earth, and the robustness of their immune systems has been studied extensively by immunologists since the early 1960s.

- They possess numerous cellular and humoral immune mechanisms, and produce several biologically active antimicrobial compounds.

- Viable test material is often recovered from the gut after studies where no adverse effects were seen.

OECD Expert Group on Biopesticides, 2018

Eisenia fetida (OECD 222)

- There are no known microbial pathogens of earthworms.

- Where adverse effects on earthworms have been reported they were shown to be due to the toxicity of co-formulants, or were induced at grossly unrealistic application rates.

- OECD Biopesticide Workshop (2013) concluded that an earthworm study was not necessary if the microorganism was naturally present in soil.

- Still an EU data requirement, and non-submission has to be justified in detail every time.

OECD Expert Group on Biopesticides, 2018
**Folsomia candida (IOBC/WPRS)**

- Soil-dwelling arthropods that graze on microorganisms, including mycorrhizal fungi – an activity thought to negatively affect the symbiotic establishment on plant roots.

- Feeding experiments reveal a preference for a wide range of common saprobic fungi over mycorrhizal fungi, with a concomitant increase in fecundity.

- Collembolans are thus unlikely to be sufficiently sensitive species for microbial ecotox testing – a conclusion borne out by experience at APIS.

OECD Expert Group on Biopesticides, 2018

---

**Aphidius rhopalosiphi (IOBC/WPRS)**

- The standard parasitic wasp species.

- Test design requires adult female wasps to be confined on treated substrate for 48 hours, after which a mortality assessment is made.

- Surviving females are then transferred individually to aphid-infested plants for 24 hours before being removed.

- Parasitism rates are assessed 10-12 days later.

OECD Expert Group on Biopesticides, 2018

Unclassified
**Aphidius rhopalosiphi life cycle**

- Female wasps mate within 24 hours of emergence.
- >90% of eggs are laid during the following three days.

---

**Time to effect is critical**

![Graph showing the effect of different treatments on bee population over time](image)

OECD Expert Group on Biocides, 2018
Aphidius rhopalosiphi life cycle

- Microbial pesticides rarely have directly fatal or through-the-female effects this quickly, so adverse effects on survival or fecundity are unlikely to be detected in this study.
- After the majority of eggs have been laid the cause of death – old age, predation or entomopathogen infection – is irrelevant.
- Microbial pesticides can have a negative impact on adult wasp emergence when applied to parasitized hosts, so there is invariably some collateral damage as a consequence of crop protection use.

Honeybees (OECD 213/214)

Study design

- Young worker bees removed directly from hive and caged in groups of 10.
- Test item applied directly to thorax, or provided in 50% sucrose solution for 4-6 hours.
- Mortality observations made for 2-4 days.
- Observation period extended for microbialis.
Honeybees, Tier I (OCSP 885.4380)

Test species
Testing shall be performed on the honey bee, *Apis mellifera*.

Age
When the MPCA may be expected to affect insect larvae, test insects should include honey bee larvae.

Route of exposure
When the MPCA may be expected to act by a dietary route of exposure or are particles of such a size that they might be carried back to the hive like pollen, the honey bees must be dosed orally. Testing in the hive may be necessary.

Controls
A concurrent control group is recommended and should be treated with microbe-free (or nonviable microbe) material from the culture system used for propagation of the MPCA.

Duration of test
Control and treated bees should be observed for at least 30 days after dosing.

OECD Expert Group on Pesticides, 2018
Chain of unintended consequences

- Extending observation period to 30 days entails rearing bees directly out of brood frame in an incubator, not picking them out of a hive.
- Diet then needs to be optimized, as sugar water alone is insufficient for adult development.
- Diet is known to affect pesticide resistance, and including pollen changes the expression of genes relating to detoxification.
- LD₉₀ values for toxic reference substances may then stray outside the study validity criteria.

OECD Expert Group on Biocides, 2018

Methodological questions and problems

- Are toxic references necessary for microbial products? The insecticide resistance status of the bees has little relevance to a product with a predominantly physical mode of action.
- Is 30 days really necessary? If the organism is pathogenic it will show adverse effects much faster than that.
- Standard 2-4 day study is known to induce stress in confined bees. How do we manage this in 30 day studies?
- Environmental conditions: optimised for bees or for the test item?

OECD Expert Group on Biocides, 2018
Methodological questions and problems

- Is the assumption that trophylactic feeding means all bees get an equal dose valid?
- How do we determine the cause of death?
  - Post-mortem saprophytic growth on cadavers is often incorrectly assumed to indicate fatal microbial pathogenicity.
  - Autopsy is time-consuming and is prone to ignorance of basic entomology (i.e. misidentification of thoracic flight muscle as hyphal growth).

OECD Expert Group on Biopesticides, 2018

Methodological questions and problems

How relevant are lab data to field conditions? Acute laboratory studies might overestimate effects on honeybees for a variety of reasons:

- Conditions are optimised as far as possible for growth of the microorganism and are not representative of hive conditions.
- Almost every mechanism by which bees in colonies limit microbial attack is negated in lab studies.
- A honeybee colony is a ‘super organism’ and not just 30,000 individuals.

OECD Expert Group on Biopesticides, 2018
A more fundamental problem

Are we even asking the right questions?

- The current test designs are completely irrelevant to managed honeybee colonies.
- Honeybees exhibit extreme sociality, living in high density colonies of several thousand individuals.
- High-density living favours pathogen spread, in response to which honeybees have evolved multi-layered defences, from colony-scale mechanisms down to individual immune responses.

OECD Expert Group on Biopesticides, 2018

A more fundamental problem

- Bees are not microbially sterile.
- Immune status is modified by commensal flora and their interactions with xenobiotic compounds.
- Some commensal gut bacteria can confer resistance to microbial infections.
- Individual immune status can show density-dependent plasticity.

OECD Expert Group on Biopesticides, 2018
Honeybee immune responses

Colony-level mechanisms:

- Removal of infected nest-mates.
- Self-removal when infected.
- Depositing corpses outside foraging range.
- Collection of antiseptic saps.
- Production of antiseptic enzymes (glucose oxidase).
- Brood fever (raising colony temperature to combat infection).

OECD Expert Group on Biocides, 2018

Individual-level mechanisms:

- Cellular responses (phagocytosis, encapsulation).
- Humoral responses (prophenoloxidase cascade $\rightarrow$ melanisation).
- The expression of some genes relating to these responses has been shown to change in response to pesticide exposure, although inconsistently.

OECD Expert Group on Biocides, 2018
What are the right questions?

Does the test item have the capacity to be pathogenic in lab studies?

If it does:
• How can we show that the test item was the cause of death?
• What effects are there in field colonies under natural conditions?

If not:
• Does it have non-pathogenic effects on colony health?

OECD Expert Group on Biocides, 2018

Establishing cause of death

• Sacrificial bees from the treatment group removed each day and haemolymph extracted by abdominal puncture.
• Assess test item load by qPCR and compare with control bees.
• If the increase in mortality is preceded by a corresponding replication curve in the sacrificial bees it would suggest pathogenicity.
• Thus reasonable to conclude that death was mediated by pathogenicity of the test item.

OECD Expert Group on Biocides, 2018
What happens in field colonies?

- In-hive sampling of larvae and adults following colony inoculation can help to elucidate patterns of transmission.
- Overwintering effects also need to be considered.
- RFID tagging of inoculated and control bees can show changes in foraging behaviour and field mortality as a consequence of infection.

OECD Expert Group on Biocides, 2018

RFID tracking following LPS injection

Immune stimulation by lipopolysaccharide injection results in increased foraging intensity.

• There is a trade-off between investment in immune response and longevity.

• A heavy investment in immune function, such as might be triggered by microbial invasion, can lead to a shorter lifespan, and would also be triggered by the inactivated test item.

• A significant foreshortening of longevity in the inactivated treatment group, coupled with an increase in antimicrobial peptides, would suggest a mechanism other than direct pathogenicity.

OECD Expert Group on Biopesticides, 2018

• \textit{Daphnia magna} are a useful indicator of the presence of toxic metabolites or manufacturing impurities when exposed to sterile filtrates, but they are also extremely sensitive to environmental stressors such as suspended particulate matter.

• Testing the TGAI invariably leads to rapid mortality, and this is always ascribed to the physical nature of the test item rather than to any intrinsic pathogenicity.

• Extreme hydrophobicity of most microbial test items often necessitates adjuvants at levels that would be immediately fatal to \textit{Daphnia}.

OECD Expert Group on Biopesticides, 2018
**Raphidocelis subcapitata** (OECD 201)

- Algal growth rate is usually negatively impacted by the addition of microbial test items.
- Increased turbidity reduces light absorption and hence photosynthesis and growth.
- All routine methods for deactivating the test item will reduce its suspensibility, thus making it useless as a negative control.
- Large concentrations of microbial material interfere with algal enumeration or biomass quantification.

OECD Expert Group on Biocidal Products, 2018

---

**Dose verification in aquatic studies**

“If any contract lab provides accurate dose verification from an aquatic study my first assumption would be that they’d made it up.”

– Ecotox evaluator from leading EU regulatory authority

- Microbial dose verification in aquatic studies is notoriously difficult due to the extreme hydrophobicity of microbial test items.
- Even if the formulation is tested, getting a homogenous distribution throughout the media is almost impossible, leading to wildly inaccurate results.
- Enumerating aged media is complicated by microbial contamination.

OECD Expert Group on Biocidal Products, 2018
Considerations for ecotox testing

- Microbial pesticides show strain-dependent variation in host specificity, and consequently also strain-dependent non-target effects.
- Some of these effects are due to direct toxicity from metabolites or manufacturing impurities, whilst others are due to pathogenicity.
- Microbial ecotox testing needs to address the biology of microbial test items and the selection of suitably susceptible test organisms.
- An opportunity to re-think the ecological risk assessment of microbial pesticides.

OECD Expert Group on Biopesticides, 2018

Current activities

APIS is involved in two current projects to improve the testing guidelines for microbial pesticides:

- EPA / BPIA project on improvement of OCSPP microbial testing guidelines.
  - This covers all studies, including mammalian toxicology, but the immediate focus is on NTAs.
- ICP-PR Working Group on the impact of microbial pesticides to bees.
  - Led by CTGB and EPA.

OECD Expert Group on Biopesticides, 2018
Test methods for micro-organisms and non-target organisms: Aquatic and terrestrial

Bilgin Karaoglan (German Environment Agency (Umweltbundesamt), Germany)

Contents

1 EU REGULATORY FRAMEWORK
   1.1 EU Data Requirements - What type of data can be used?

2 TEST METHODS FOR NON-TARGET ORGANISMS
   2.1 Deviations among tes guidelines / conclusions from OECD Survey

3 SOME ISSUES TO BE TACKLED

4 CONCLUSIONS
EU Regulatory Framework

EU Regulation 1107/2009:
“Substances should only be included in PPPs where it has been demonstrated that they are not expected to have any harmful effect on human or animal health or any unacceptable effects on the environment.”
Test methods for micro-organisms and non-target organisms: aquatic and terrestrial

**EU Regulatory Framework**

**EU Regulation 1107/2009:**
“Substances should only be included in PPPs where it has been demonstrated that they are not expected to have any harmful effect on human or animal health or any unacceptable effects on the environment”

**EU Data Requirement 283/2013**
(for micro-organisms) and 284/2013 (for PPPs), Part B:
“Information on toxicity, infectiveness and pathogenicity to [NTOs] must be reported.”

**EU Uniform Principles 546/2011:**
“No authorisation shall be granted if the micro-organism is pathogenic to [NTOs].”
Test methods for micro-organisms and non-target organisms: aquatic and terrestrial

**EU Data Requirements**

<table>
<thead>
<tr>
<th>No 283/2013 (microorganisms)</th>
<th>No 284/2013 (PPPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8. EFFECTS ON NON-TARGET ORGANISMS</td>
<td>10. EFFECTS ON NON-TARGET ORGANISMS</td>
</tr>
<tr>
<td>8.1. Effects on birds</td>
<td>10.1. Effects on birds</td>
</tr>
<tr>
<td>8.2. Effects on aquatic organisms</td>
<td>10.2. Effects on aquatic organisms</td>
</tr>
<tr>
<td>8.2.1. Effects on fish</td>
<td>10.3. Effects on bees</td>
</tr>
<tr>
<td>8.2.2. Effects on freshwater invertebrates</td>
<td>10.4. Effects on arthropods other than bees</td>
</tr>
<tr>
<td>8.2.3. Effects on algae growth</td>
<td>10.5. Effects on earthworms</td>
</tr>
<tr>
<td>8.2.4. Effects on plants other than algae</td>
<td>10.6. Effects on soil micro-organisms</td>
</tr>
<tr>
<td>8.3. Effects on bees</td>
<td>10.7. Additional studies</td>
</tr>
<tr>
<td>8.4. Effects on arthropods other than bees</td>
<td></td>
</tr>
<tr>
<td>8.5. Effects on earthworms</td>
<td></td>
</tr>
<tr>
<td>8.6. Effects on non-target soil micro-organisms</td>
<td></td>
</tr>
<tr>
<td>8.7. Additional studies</td>
<td></td>
</tr>
</tbody>
</table>

- What type of data can be used?
Test methods for micro-organisms and non-target organisms: aquatic and terrestrial

**EU Data Requirements - What type of data can be used?**

- **US EPA Microbial Pesticide Test Guidelines** (OPPTS/OCSSPP Series 885)
  “pending the acceptance of specific guidelines at international level”

- Test guidelines as described in Part A (e.g. OECD, IOBC-WPRS, ISO)
  “adapted in such a way that they are appropriate for micro-organisms”
Test methods for micro-organisms and non-target organisms: aquatic and terrestrial

**EU Data Requirements - What type of data can be used?**

- **US EPA Microbial Pesticide Test Guidelines (OPPTS/OCSSP Series 885)**
  “pending the acceptance of specific guidelines at international level”

- **Test guidelines as described in Part A (e.g. OECD, IOBC-WPRS, ISO)**
  “adapted in such a way that they are appropriate for micro-organisms”

- **Scientific peer reviewed literature**
  “Experimental data are normally required, unless it can be justified that an assessment of effects on non-target organisms can be performed with the information already available.”

- **MPCP data have to be used for risk assessments**
  “where it appears from available data that the plant protection product has a stronger effect than the micro-organism.”
EU Data Requirements - What type of data can be used?

- **US EPA Microbial Pesticide Test Guidelines** (OPPTS/OCSP Series 885)
  "Pending the acceptance of specific guidelines at international level"

  - How to achieve acceptance at international level?

- Test guidelines as described in Part A (e.g. OECD, IOBC-WPRS, ISO)
  "Adapted in such a way that they are appropriate for micro-organisms"

  - How to adapt such test guidelines?

- **Scientific peer reviewed literature**
  "Experimental data are normally required, unless it can be justified that an assessment of effects on non-target organisms can be performed with the information already available."

  - Data often not strains-specific! How to develop reliability/extrapolation criteria?

- **MPCP data have to be used for risk assessments**
  "Where it appears from available data that the plant protection product has a stronger effect than the micro-organism."

  - Data usually scarce! How to predict impact of co-formulants?
### Test methods for non-target organisms

<table>
<thead>
<tr>
<th>OECD Annex IIM point</th>
<th>Most obvious differences of US TG compared to OECD TG</th>
<th>Problems reported in OECD Survey (feedback from CAN, JP, EU, USA, IBMA)</th>
<th>Conclusions from OECD Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1 Effects on birds</td>
<td>OECD 223 (14 d) OPPTS B85-4050 (30 d)</td>
<td>• Several controls: (i) negative, (ii) attenuated, (iii) sterile filtrate oral doses daily for 5 days instead of single oral dose • 30-day observation period instead of 14 days</td>
<td>• OECD 223 not microbial-specific; test period too short to detect symptoms • OECD 223 suitable for MPCA assessment due to relatively long observation period, no guidance for determination of MPCA in test item</td>
</tr>
</tbody>
</table>

| 8.2 Effects on fish | OECD 203 (4 d) OPPTS B85-4200 (30 d) | • Two routes of exposure: (i) aqueous exposure, (ii) oral exposure use treated fish food or infected insects • additional sterile filtrate control • Maximum Hazard Dose (MHD) • 30-day exposure/observation • Evaluation of microorganism dissemination or replication in animal tissues, organs, or fluids. | • High microbial densities may cause turbidity and complicate study evaluation OECD test period too short to detect symptoms, no guidance for determination of the active ingredient in the test medium • Setting of the particulate MPCA at end of study | • OECD test guidelines for chemicals not suitable |

---

### Test methods for non-target organisms

<table>
<thead>
<tr>
<th>OECD Annex IIM point</th>
<th>Most obvious differences of US TG compared to OECD TG</th>
<th>Problems reported in OECD Survey (feedback from CAN, JP, EU, USA, IBMA)</th>
<th>Conclusions from OECD Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3 Effects on aquatic invertebrates</td>
<td>OECD 202 (4 d) OECD 211 (21 d) OPPTS B85-5100 (21 d)</td>
<td>• Several species considered: (i) planktonic, and benthic, depending on wise patterns and aquatic exposure; (ii) with close taxonomic relationship to the test host; (iii) likely to prey upon (or scavenge) the deceased target host organisms • additional sterile filtrate control</td>
<td>• High microbial densities may cause turbidity and complicate study evaluation OECD 202: no guidance for determination of the active ingredient in the test medium, short exposure period • OECD 222: General problem during prolonged exposure to e.g. bacterial or fungal spores; if effects are observed they are usually not related to pathogenicity of the microorganism but to mechanical interference of the spores with the feeding apparatus resulting in a decreased energy uptake</td>
</tr>
<tr>
<td>Test methods for non-target organisms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OECD Annex IVIM point</strong></td>
<td><strong>Most obvious differences of US TG compared to OECD TG</strong></td>
<td><strong>Problems reported in OECD Survey (feedback from CAN, JP, EU, USA, IBMA)</strong></td>
<td><strong>Conclusions from OECD Survey</strong></td>
</tr>
<tr>
<td><strong>test guideline (duration)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>8.4 Effects on algal growth and growth rate</strong></td>
<td>Positive controls are required for microalgal herbicides, or for MPCA's similar to known plant pathogens.</td>
<td>Turbid media associated with high solids concentrations may affect photosynthetic activity</td>
<td>OECD test guidelines for chemicals not suitable.</td>
</tr>
<tr>
<td>OECD 201 (3 d)</td>
<td></td>
<td>No guidance for determination of the active ingredient in the test medium, short exposure period</td>
<td>No specific guidance for microorganisms available, guidance required</td>
</tr>
<tr>
<td>OPPTS 885.4300 (3 - 14 d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>8.5 Effects on aquatic plants</strong></td>
<td>Positive controls are required for microalgal herbicides, or for MPCA's similar to known plant pathogens.</td>
<td>No guidance for determination of the active ingredient in the test medium, short exposure period</td>
<td>OECD test guidelines for chemicals not suitable.</td>
</tr>
<tr>
<td>OECD 221 (7 d)</td>
<td></td>
<td></td>
<td>No specific guidance for microorganisms available, guidance required</td>
</tr>
<tr>
<td>OPPTS 885.4300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(variable; comprising different species)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Test methods for micro-organisms and non-target organisms: aquatic and terrestrial**

<table>
<thead>
<tr>
<th>Test methods for non-target organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OECD Annex IVIM point</strong></td>
</tr>
<tr>
<td><strong>test guideline (duration)</strong></td>
</tr>
<tr>
<td><strong>8.6 Effects on terrestrial plants - No EC data requirement</strong></td>
</tr>
<tr>
<td>OECD 208 (21 d)</td>
</tr>
<tr>
<td>OECD 227 (21 d)</td>
</tr>
<tr>
<td>OPPTS 885.4300</td>
</tr>
<tr>
<td>(variable; comprising different species)</td>
</tr>
<tr>
<td><strong>8.7 Effects on bees</strong></td>
</tr>
<tr>
<td>OECD 213/214 (2 d - 4 d)</td>
</tr>
<tr>
<td>OPPTS 885.430C (30 d)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
## Test methods for non-target organisms

### Test methods for micro-organisms and non-target organisms: aquatic and terrestrial

<table>
<thead>
<tr>
<th>OECD Annex IM point test guideline (duration)</th>
<th>Most obvious differences of US TG compared to OECD TG</th>
<th>Problems reported in OECD Survey (feedback from CAN, JP, EU, USA, IBMA)</th>
<th>Conclusions from OECD Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>8.6 Effects on terrestrial plants</strong> - No EC data requirement</td>
<td>• Selection of test species depends on the type of MPCA (annual or plant-controlling MPCA); type of use (aquatic/terrestrial); Positive controls are required for microbial herbicides, or for MPCAs similar to known plant pathogens;</td>
<td>• If a MPCA relates to a plant pathogenic strain, the concurrent study using the pathogenic strain is necessary to investigate whether the MPCA has pathogenicity.</td>
<td><strong>Mixed answers:</strong></td>
</tr>
<tr>
<td>OECD 208 (23 d)</td>
<td></td>
<td></td>
<td>• Guidance OPPTS 855.4300 available; • Conditional data requirement if MPCA relates to a plant pathogenic strain; • No EU data requirement; • OECD test guidelines for highthroughput data available</td>
</tr>
<tr>
<td>OECD 227 (23 d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPPTS 855.4300 [variable; comprising different species]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>8.7 Effects on bees</strong></td>
<td>• Additional attenuated controls; Duration of study (50 days)</td>
<td>• Test positive data; Duration of study (50 days)</td>
<td><strong>New OECD Test guidelines available:</strong></td>
</tr>
<tr>
<td>OECD 213/214 (2 d - 4 d)</td>
<td></td>
<td></td>
<td>• OECD 223 Bee (Varroa) Test (4 d)</td>
</tr>
<tr>
<td>OPPTS 855.4300 (30 d)</td>
<td></td>
<td></td>
<td>• OECD 245 Honey bee chronic oral toxicity test (30 d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• OECD 246/247 Bumblebee, Acute Contact/Ocular Toxicity Test (2 d - 4 d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Conclusions obsolete?</strong></td>
</tr>
</tbody>
</table>

**Test methods for non-target organisms**

<table>
<thead>
<tr>
<th>OECD Annex IM point test guideline (duration)</th>
<th>Most obvious differences of US TG compared to OECD TG</th>
<th>Problems reported in OECD Survey (feedback from CAN, JP, EU, USA, IBMA)</th>
<th>Conclusions from OECD Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>8.8 Effects on terrestrial arthropods other than bees</strong></td>
<td>• Selection of leaf-dwelling test species depends on the type of MPCA (Viruses/Protozoa/Fungi/Bacteria); • OECD No. 67 states that US EPA provides no test guidelines concerning soil-dwelling arthropods; • No positive controls</td>
<td>• IOBC test methods do not take into account biological properties of the mPCA, mode of action and relevant routes of exposure (e.g., dietary uptake in the case of BB); • sometimes OECD is used which is in principle less suitable. Nevertheless the test can be used but BA is unclear • Different interpretations of results, test duration, conditions to evaluate side effects of PPP to non-target insects</td>
<td><strong>Mixed answers:</strong></td>
</tr>
<tr>
<td>IOBC Guidelines (version)</td>
<td></td>
<td></td>
<td>• IOBC guidelines do not take into account biological properties of the mPCA, mode of action and relevant routes of exposure; • OECD guidelines for chemicals are not suitable for the same reason.</td>
</tr>
<tr>
<td>OPPTS 855.4340</td>
<td></td>
<td></td>
<td>• OPPTS Microbial Pesticide Testing 855.4530 Non-target insect testing foresees application required with regard to exposure time</td>
</tr>
<tr>
<td>OECD 220 F; aculeifer 14 d</td>
<td></td>
<td></td>
<td>• The Canadian guidance document (Environment Canada, 2004) also gives useful advice on designing tests.</td>
</tr>
<tr>
<td>OECD 232 F; candida 28 d</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Test methods for non-target organisms

<table>
<thead>
<tr>
<th>OECD Annex H point</th>
<th>Most obvious differences of US TG compared to OECD TG</th>
<th>Problems reported in OECD Survey (feedback from CAN, JP, EU, USA, IBRA)</th>
<th>Conclusions from OECD Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.9.1 Effects on earthworms</td>
<td>- US EPA has no data requirement assessing the risk to earthworms.</td>
<td>- OBRA suggest following test design: ISO 13336-1 / ISO/DIS 17512-1. Instead of Eisenia fetida Dendrobaena is not a good choice.</td>
<td>- EU refers to OECD guidelines for chemicals. Short-term 14-day earthworm studies focusing on detecting lethal or sublethal effects (weight loss) are not suitable to prove the absence of infectivity or pathogenicity (OECD 67). CA registration procedure recommended test methodology. Test methodology required.</td>
</tr>
</tbody>
</table>

**8.9.1 Effects on earthworms (continued)**

<table>
<thead>
<tr>
<th>OECD 207 (14 d)</th>
<th>OECD 222 (56 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>US EPA has no data requirement assessing the risk to earthworms.</td>
<td>OBRA suggest following test design: ISO 13336-1 / ISO/DIS 17512-1. Instead of Eisenia fetida Dendrobaena is not a good choice.</td>
</tr>
<tr>
<td>Problems reported in OECD Survey (feedback from CAN, JP, EU, USA, IBRA)</td>
<td>Conclusions from OECD Survey</td>
</tr>
<tr>
<td>- EU refers to OECD guidelines for chemicals. Short-term 14-day earthworm studies focusing on detecting lethal or sublethal effects (weight loss) are not suitable to prove the absence of infectivity or pathogenicity (OECD 67). CA registration procedure recommended test methodology. Test methodology required. Recommendation from OECD/REACH/EU Workshop: Earthworm study not required unless the microbial is not naturally occurring in the soil.</td>
<td></td>
</tr>
</tbody>
</table>
Test methods for non-target organisms

<table>
<thead>
<tr>
<th>OECD Annex IIIM point</th>
<th>Most obvious differences of US TG compared to OECD TG</th>
<th>Problems reported in OECD Survey (feedback from CAN, JP, EU, USA, IBMA)</th>
<th>Conclusions from OECD Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td>OECD Annex IIIM point 8.10 Effects on soil micro-organisms</td>
<td>No OPPTS Test Guidelines available.</td>
<td>Relevance of carbon mineralization and nitrogen transformation tests according to OECD 216/217 appears to be low</td>
<td>There may be effects from almost anything added to the soil, but there is no valid way to interpret any results one might obtain from testing (OECD 67)</td>
</tr>
</tbody>
</table>

Some issues to be tackled

- Test item difficulties:
  - Extreme hydrophobicity of conidial spores (in contrast, blastospores lacking hydrophobins are hydrophilic)
  - Differences in study outcomes expected when testing TGAI or certain formulations/surfactants (spore attachment)

![Beauveria bassiana immediately after application to water (1.0 g of the test item was added to approximately 50 mL of deionised water)](image1)

![Beauveria bassiana five minutes after vigorous agitation](image2)

(Picture source: DAI Beauveria bassiana PPM 5539 Vol. 3, B&N, dated December 2016)
Some issues to be tackled

- Testing end-use products might be relevant in some cases:
  Effects of some inert carrier substances in microbial preparations have been discussed in the open literature:

  - **kaolin** (white clay mineral) in EPF-formulations:
    - affects water loss and longevity in bumble bees

  - **oil** in Btk-formulations:
    - negatively affected earthworms
    - oils as active substance are used as contact acaricides and insecticides (physical MoA). *What about other NTOs?*

  - **diatomaceous earth** in Btk-formulations:
    - negatively affected earthworms
    - can remove epicuticular lipids in arthropods

Conclusions I

**OECD Survey results:**

- Problems with study design encountered, especially in
  - prolonged *Daphnia magna* studies and
  - *Honey bee studies* → feedback is needed from ICPR[1] “microbial working group” given the new developments in bee testing
  - *Non-target arthropod studies* (leaf/soil-dwelling): Both IOBC and OECD test guidelines do not take into account biological properties of the mBCA

- Some testing methods appear questionable (e.g. earthworm test OECD 207 and carbon mineralization/nitrogen transformation tests OECD 216/217)

**Fundamental question:**

- Are we testing the right species? If yes, are the methods adequate?
- Resolve these issues by using the Canadian “centrifugal taxonomic approach”? (also termed “radial taxonomic testing” in Australia)

[1] International Commission for Plant-Pollinator Relationships
Test methods for micro-organisms and non-target organisms: aquatic and terrestrial

Conclusions II

- **Chemical guidelines** are not suitable for MPCA testing. However, they might be suitable with the following adaptations:
  - extending study duration (under consideration of control mortality)
  - adding further control groups (inactivated/sterile filtrate)
  - selecting species depending on the type of MPCA, biological properties, and use pattern
  - selecting exposure routes depending on the MoA (dietary exposure: feeding on infected host/treated food), contact exposure (topical/dipping)

- **OPPTS (OCPP) Guidelines** are generally recommend for testing MPCAs with slight modifications (e.g. test duration for adult bees; additional filtration in daphnid tests)

- **Canadian guidance document** (Environment Canada, 2004; EPS 1/RM/44) gives useful advice on test design!

Thank you for your attention

Bilgin Karaoğlan
bilgin.karaoğlan@uba.de

www.umweltbundesamt.de
Presentation 6

Microbials and Bees
Emily McVey, Jacoba Wassenberg (Board for the Authorisation of Plant Protection Products and Biocides (Ctgb), The Netherlands)

Outline
- Risk assessment for pollinators in the EU
- Data requirements and available test guidelines
- Regulator’s delite
- Intro to ICPPR WG
- WP of WG
Biopesticides

- Micro-organisms

ctgb

EU Regulation 1107/2009:
Risk assessment

Uniform Principles EU: No authorisation shall be granted if pathogenic to bees

“Margin of Safety”

Maximum Hazard Dose/Concentration

c\text{tgb}

Data requirements

Commission regulation (EU) No 283/2013 and 284/2013, PART B

Effects on bees

- Information on toxicity, infectiveness and pathogenicity to bees must be reported.
- Experimental data required unless:
  - effect assessment possible with available information (public literature)
  - no exposure
Available Guidelines

- OECD 67
- Aupinel et. Al. (2005) (brood)
- Higher tier: EPPO 170 guidelines
- Adults:
  - OECD 213/214 (too short)
  - OCSP 885.4380 Honey bee Tier 1

US-EPA Microbial Pesticide Test Guidelines
OPPTS 885.4380
Honey Bee Testing, Tier I

1. **Test substance.** The actual form of the material to be regarded as the test substance is discussed in OPPTS 885.0001. In addition, any substances used to enhance virulence should be tested along with the test substance.

2. **Test species.** Testing shall be performed on the honey bee, *Apis mellifera*.

3. **Age.** When the MPCA may be expected to affect insect larvae, test insects should include honey bee larvae.
US-EPA Microbial Pesticide Test Guidelines
OPPTS 885.4380
Honey Bee Testing, Tier I

(4) **Route of exposure.** When the MPCA may be expected to act by a dietary route of exposure or are particles of such a size that they might be carried back to the hive like pollen, the honey bees must be dosed orally. Testing in the hive may be necessary.

(5) **Controls.** A concurrent control group is recommended and should be treated with microbe-free (or nonviable microbe) material from the culture system used for propagation of the MPCA.

(6) **Duration of test.** Control and treated bees should be observed for at least 30 days after dosing.

---

- Exposure route?
- Life stage?
- MHD?
- Study length?
- Test conditions?
- Species?
- Higher tier?
- Other effects?
- Alternatives for toxicity testing?
Some examples

- Timing: long enough to exclude infectivity = too long for controls!
- Humidity: optimal humidity for the bees = suboptimal for the test organism
- Temperature: optimal for bees = suboptimal for the test organism
- Modified colony tests for brood assessments?

Brief intro to ICPPR

- International Commission for Plant-Pollinator Relationships
- Non-profit, academia, industry and regulators
- Working groups (WG) OECD testing protocols
- Meeting every 2 years
- 1st meeting of WG on microbials 2017
Working group on microbiicals

- Under “Bee Protection Group” of ICPPR
- 49 members from regulatory authorities, academia and industry/CRO
- Smaller “core” group responsible for writing white paper (WP)
- Core group = (lucky) 13 members
- Three co-chairs (all three here today!)
- Initial draft WP presented to full WG at SETAC 2018

Outline of white paper

- Problem formulation
- Interim steps - Regulatory flexibility?!
- Future steps – New tests and models
The Pith

- Current guidelines not fully adequate
- New guidelines
- Modified existing guidelines
- New RA paradigms

Questions?

"Trust me, I'm an expert!"
Presentation 7

**Microbes and Daphnia effects: False toxicity due to study design?**

*Maria Pilar Herrero (Valent BioSciences LLC, United States)*

---

**The Daphnia Diet**

- Daphnia are largely nonselective filter feeders, which do not discriminate between food particles with regard to their nutritional quality (DeMott, 1986).
- Heterotrophic bacteria constitute a substantial part of the suspended particulate organic matter in many aquatic ecosystems.
- Isotope patterns and fatty acid biomarkers revealed that heterotrophic bacteria can contribute significantly to the nutrition of Daphnia species (Perga et al., 2006; Taipale et al., 2008, 2009; Wenzel et al. 2012).
- Martin-Creuzburg et al. 2011 highlighted the limitations of bacteria as a carbon source, cholesterol and/or the polyunsaturated fatty acid needed for good Daphnia growth.
- Wenzel et al. 2012 indicated a pure bacterial diet did not support survival, growth or reproduction. While a 20% share of Rhodomonas in the food allowed survival of daphnids, the occurrence of offspring on a 50% algal diet indicated that the threshold for successful reproduction was between those two proportions.
Bacteria + algae diets

- Pure bacterial diets always had detrimental effects on Daphnia.
  - But Flavobacterium sp. and E. coli (80-50%). In fact, diets containing small proportions of these heterotrophic bacteria (Flavobacterium 50%, E. coli 20%) even significantly increased Daphnia growth rates compared to pure algal diets, indicating a nutritional upgrading (Freece and Martin-Cresazburg, 2012).

Daphnia

- Daphnids, due to their small size and short generation times, respond rapidly to changes in algal food densities. One of the most important variables affected by changing food availability is the reproductive rate (ReIlstamb, 2006).
Egg production under different diets.

(Reese and Marlin-Creuzburg, 2012)

Example from a microbial Daphnia study.

- No-Observed-Effect-Concentration: 2.5 mg/L (0.73 X 10^5 CFU/mL)
- Lowest-Observed-Effect-Concentration: 5.0 mg/L (1.5 X 10^5 CFU/mL)
- Maximum Acceptable Toxicant Concentration: 3.5 mg/L (1.0 X 10^5 CFU/mL)
- 21-Day EC50 (Adult Mortality/Immobility): 13 mg/L (3.8 X 10^5 CFU/mL)
- 21-Day EC50 (Reproduction) 7.8 mg/L (2.3 X 10^5 CFU/mL)

Once daily during culturing and testing, daphnids were fed a mixture of yeast, cereal grass media, and trout chow (YCT), as well as a suspension of the freshwater green alga, Selenastrum capricornutum. During culturing, daphnids were fed 0.20 mL of YCT and 0.40 mL of algae per 80 mL of culture water. The same ratio of feed to water was maintained during the test. Each test chamber was fed 3.75 mL of YCT and 7.5 mL of algae per 1500 mL of test solution (Target algae cells in 3.5x10^7/ml). (5mL/L = 1.75 x 10^6 algae/ml)
Daphnia Feeding

- Thus for the Bt test above, the algae to bacteria ratio is approximately 1:1. Research would lead us to draw the conclusion that test is hampered by non-viable nutrient conditions.
  - Bacteria renewed repeatedly at times much more often than applications in the field
    - 9 vs 3–4 times

All microbes seem to be having issues with Daphnia.

- The minimum content of *B. amyloliquefaciens* strain FZB24 in the microbial pest control agent is $2 \times 10^{14}$ CFU/kg.
- 48-hour EC50 value $4 \times 10^8$ cfu/L

- *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747
- Nominal concentration $2 \times 10^{11}$ cfu/g
  - NOEC (reproduction): $2.84 \times 10^8$ cfu/L (EFSA) $\sim 1$ mg/L

- *Bacillus amyloliquefaciens* strain MBI 600
  - Daphnia magna: NOEC (immobilisation and reproduction) = $2.7 \times 10^7$ CFU/L (21 d; measured initial concentration at the lowest concentration, endpoint determined by comparison to the inactive substance (EFSA); physical effects observed
Consumption of Bacteria

- Pseudomonas chlororaphis strain MA 342
  - 21-day study on Daphnia magna was discussed during the Pesticides Peer Review teleconference 139. Considering that
    i) reproductive effects were not derived from the study;
    ii) 44% mortality occurred in the tested item group;
    iii) daphnids in the test item group were reported to be paler and smaller;
    iv) analytical measurements were performed on fresh samples only, the experts agreed that a data gap should be identified for a new 21-day study investigating toxicity (including reproductive effects), pathogenicity and infectivity of P. chlororaphis strain MA 342 to D. magna.

Spore count vs. mg/L and turbidity.

- Bacillus subtilis Strain QST 713:
  The 21-day LC50 was determined to be greater than $3 \times 10^7$ CFU/L (1.5 mg/L). The lowest observable effect concentration (LOEC), based on reproduction, mean length and mean dry weight was determined to be $1.5 \times 10^7$ CFU/L (0.75 mg/L). The NOEC was determined to be $7.5 \times 10^6$ CFU/L (0.38 mg/L).

The cause of death and whether pathogenicity was involved was not determined.
First Considerations

- Daphnia grazing needs to be remembered
  - Control with no daphnia to determine microbial counts???
- Bacteria/Spores can also be released by Daphnia
- Natural Pond water contains $10^4$ – $10^7$ cfu/ml of bacteria

Study Guideline/Protocol

- 21-day duration; start with neonates less than 24hrs. old
- Test media replaced 3 times a week, 9 renewals
- The concentration in the test water should, whenever possible, be at least $10^6$ units/ml or at least 1,000x the maximum calculated pesticide concentration in a 6-in layer of water
- Continuously mixed the test suspensions during the exposure (aeration or stir bars)

Results:
- Throughout the exposure period, newly prepared test media were observed to be cloudy and opaque, visual observations established that only dark coloured objects (e.g. those that were dark blue or black) could be observed through the newly prepared media (8ti; 50mg/L).
What Do We Know?

- *Daphnia* use a feeding mode that is described as filter feeding, ingesting particles in proportion to their abundance in the environment free-living algae, bacteria and fungi (Demott, 1982).
- Filter feeding invertebrates are generally less tolerant of turbid conditions than other aquatic species.
- Increases in suspended sediment concentrations (to 50 – 100 mg/l) decrease ingestion rates to potential *starvation* levels (Arruda, Marzolf and Faulk, 1983).

Daphnia Testing

![Daphnia Testing Image](image_url)
Settling

Bti tendency to adhere to and settle with suspended sediment and fine particulates. (Yousien et al. 1992; Ohana et al. 1987) B. subtilis is known as a bio-floculant (Vijayalakshmi & Raichur, 2003).

Turbidity Duration

- Robinson (2008)
  Many of the results observed can be explained by considering the major mechanism of suspended clay toxicity, which is a decrease in feeding efficiency. Exposure duration is more important than exposure concentration because for a range of concentrations the gut tract is filled with clay after some time being exposed and feeding is inhibited until they are placed in clean water.
Effects seen (12 vs 24hr exposure)

Robinson (2008)

- increase in days to gravidity, compared to controls
- significantly smaller than the controls
- differences in LC50

Literature indications

- Decreases in turbidity (range = 0.6–470 NTU) increased reproductive and moulting rates (Work and Gophen, 2001)
- Laboratory experiments have shown that natural concentrations and particle sizes of suspended sediments reduce the fecundity, survivorship and fitness of cladocerans (Arruda et al., 1983; Kirk, 1991a; Kirk, 1991b; Kirk, 1992; Kirk & Gilbert, 1990; McCabe & O'Brien, 1983; Zurek, 1982), especially when simultaneously exposed to low algae concentrations.
Daphnia Activity

- Georges (1983) A simple model based on surface irradiance, water turbidity and population density explained the basic pattern of vertical distribution throughout the season.
- The animals' light response *per se* ensured their aggregation at depths of maximum phytoplankton abundance.

New Btk study (2011)

<table>
<thead>
<tr>
<th>Nominal conc. mg/l</th>
<th>% adult survival @ 21 days</th>
<th>Mean length, mm</th>
<th>Mean Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>80</td>
<td>5.48</td>
<td>1.43</td>
</tr>
<tr>
<td>Att. Control, F</td>
<td>95</td>
<td>5.29</td>
<td>1.49</td>
</tr>
<tr>
<td>2.5</td>
<td>85</td>
<td>5.39</td>
<td>1.45</td>
</tr>
<tr>
<td>5.0</td>
<td>75</td>
<td>5.39</td>
<td>1.54</td>
</tr>
<tr>
<td>10.0</td>
<td>80</td>
<td>5.36</td>
<td>1.34</td>
</tr>
<tr>
<td>20</td>
<td>55</td>
<td>5.32</td>
<td>0.78</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40 – F/R</td>
<td>70</td>
<td>4.87</td>
<td>0.95</td>
</tr>
<tr>
<td>40 - F</td>
<td>60</td>
<td>4.68</td>
<td>0.72</td>
</tr>
</tbody>
</table>
Reproductive Parameters – Live Neonates

<table>
<thead>
<tr>
<th>N. Conc. mg/l</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 12</th>
<th>Mean/R. Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>8</td>
<td>187</td>
<td>124</td>
<td>18.3</td>
</tr>
<tr>
<td>Att. Control, F</td>
<td>12</td>
<td>223</td>
<td>83</td>
<td>17.5</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>165</td>
<td>149</td>
<td>19.0</td>
</tr>
<tr>
<td>5.0</td>
<td>3</td>
<td>156</td>
<td>91</td>
<td>20.2</td>
</tr>
<tr>
<td>10.0</td>
<td>2</td>
<td>144</td>
<td>191</td>
<td>18.5</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>120</td>
<td>124</td>
<td>11.1</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>50</td>
<td>6</td>
<td>2.6</td>
</tr>
<tr>
<td>40 - F/R</td>
<td>0</td>
<td>176</td>
<td>55</td>
<td>4.5</td>
</tr>
<tr>
<td>40 - F</td>
<td>175</td>
<td>71</td>
<td>5.1</td>
<td></td>
</tr>
</tbody>
</table>

Literature Indications

- Brackish lakes where Daphnia does not thrive tend to be turbid (Scheffer 1999)
- Multiple microcosm studies with Bti have shown no effects on Daphnia levels
- Daphnia have been shown to rapidly ingest Bti spores and loose 99% of the spores within 24 – 48 hours when removed from exposure (Vaishnav and Anderson, 1994).
Conclusions on one microorganism.

- Bts do not have direct toxicity to Daphnia.
- The high turbidity caused by the study concentrations are the cause of the effects seen. These conditions would not be present in nature, as the particulate matter will sediment to the bottom.

Actual known Parasitism of Daphnia

Six species of bacteria have been described parasitizing *Daphnia*. Several species of fungi have been observed parasitizing *Daphnia* and other *Cladocera*.

- *D. magna* with (left) and without (right) *S. cienkowskii* infection. The red color of the infected host is the best indicator of the bacterium. The females were collected from a natural rock pool population in southern Finland.

- *D. magna* with (right) and without (left) *P. ramosa* infection. The parasite can be seen as a dark cloudy mass filling the entire body. The brood pouch of the infected female is empty, whereas the healthy female carries a clutch of eggs.
**P. aeruginosa utilizes conserved virulence traits to kill D. magna.**

![Graphs showing survival of Daphnia over time with different strains and conditions](image)

**Toxicity occurring and seen very rapidly**

**Possibilities for redesigning the microbial Daphnia study**

- Limit to a 10 day study, which will show both mortality and reproductive effects.
- Filtration to remove larger particles which would settle more quickly anyway.
- Increase algae feeding levels
- Start with older Daphnia, who already have some food reserves
- Permit settling during the test; how often microbe need to be re-dosed; what does the microbe number decreasing indicate?
References

- Freese, H. and Martin-Creuzebry, Food quality of mixed bacteria-algae diets for Daphnia magna September 2012 Hydrobiologia 71%()  
The U.S. Experience with Long Term *Daphnia* Testing

June 18, 2018
The 9th Expert Group on BioPesticides Seminar
Paris, France

Shannon Borges
Branch Chief (Acting)
Risk Assessment Branch
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs

Common Issues

- From previous presentation, common issues were identified as:
  - High turbidity
  - Keeping test material suspended in water column
  - Clumping of test material (and surfactants used to reduce this)
  - Not following guideline and/or using poor technique:
    - Too few test animals
    - Lack of appropriate controls
    - Contamination
    - Failing to follow-up on cause of mortality or other adverse effects
Retrospective Sketch of Studies Submitted

- Most examined were acceptable or supplemental
- In some cases 48-hour and 21-day studies were both submitted
- Many were not limit dose studies, tested multiple concentrations
  - No clear dose-response in most cases
- Varied in types of controls
  - Negative controls always included
  - Usually a sterile filtrate was included
  - Few cases involved attenuated (e.g., autoclaved) controls
  - None included sterile filtrate and attenuated controls

Retrospective Sketch of Studies Submitted

- Renewal rates varied
  - 1-3 days, 3 times/week
- Endpoints varied
  - Most included mortality and reproduction
  - Some additionally included length and weight
  - A few observed mortality only
- Lower classification caused by
  - Incomplete reporting
  - Viability unverified
  - Unexplained problems with water quality
  - Contamination
  - Identity of test material unclear

Retrospective Sketch of Studies Submitted

- Turbidity
  - Not commonly reported; however...
  - When reported, it is a clear problem
  - In one case, inclusion of the attenuated control was helpful in identifying turbidity as likely cause of high mortality
- Clumping/suspension in water
  - Clumping reported more often than settling
  - Surfactant used to reduce clumping, caused significant mortality
  - Included appropriate controls
Retrospective Sketch of Studies Submitted

- Too few test animals
  - Occurred in a few studies
- Contamination
  - Uncommon, but obscured results in one case
- Not following up on cause of adverse effects
  - Inclusion of controls helped
  - Water quality was often an issue, no clear attempts to address it

Going Forward

- Expanded retrospective likely informative first step
  - Can be time consuming
  - Helpful to agree on useful parameters (reduce unnecessary details)
- From there, identify common trends/problems
  - According to type (fungi, bacteria, etc.)
  - According to methods used
- Explore modifications
  - What can be incorporated from knowledge gained in retrospective?
  - Identify and validate new/alternate methods
Experience with long-term Daphnia toxicity studies (OECD TG 211) for microbials and proposals for the amendment of the study design

Bilgin Karaoglan (German Environment Agency (Umweltbundesamt), Germany)

Toxicity of Bacillus spp. to Daphnia magna

No relationship between pesticide category and D. magna sensitivity
Experience with long-term Daphnia toxicity studies (OECD TG 211)

Toxicity of Bacillus spp. to Daphnia magna

No relationship between pesticide category and D. magna sensitivity

D. magna 21 d-NOECs [cfu/l]

- Bacillus Firmans 1.19E+04
- Bacillus thuringiensis var. aizawai A87S-1,887
- Bacillus thuringiensis var. israelensis ATCC 393
- Bacillus thuringiensis subsp. israelensis ATCC 393
- Bacillus thuringiensis subsp. israelensis (strain IG2DM2)
- Bacillus thuringiensis subsp. tenebrionis ARB5-32
- Bacillus thuringiensis subsp. tenebrionis NAB 175
- Bacillus subtilis (strain 62B)
- Bacillus amyloliquefaciens strain MV42KD
- Bacillus amyloliquefaciens strain MV42KD
- Bacillus amyloliquefaciens strain MV42KD
- Bacillus amyloliquefaciens strain MV42KD
- Bacillus subtilis (strain 62B)
- Bacillus amyloliquefaciens strain MV42KD
- Bacillus subtilis (strain 62B)
- Bacillus amyloliquefaciens strain MV42KD

- nematicide
- insecticide
- fungicide

14.06.2018 / 9th OECD Expert Group on Biodegradability Seminar 2018

Daphnia magna sensitivity compared to fish

Daphnia magna is usually the most sensitive test organism driving the aquatic risk assessment

Please note:
Endpoints were based on the lowest reported values from the EU LoR
- Daphnia values comprise 21-day NOEC values (based on reproduction or mortality)
- Fish values comprise LC50 and NOEC (based on mortality; test duration between 4-32 days); data includes „greater-than” values

14.06.2018 / 9th OECD Expert Group on Biodegradability Seminar 2018
### Table 3

**Description and purpose of treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
<th>Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Dilution water without test material</td>
<td>Any adverse effects not related to AgroAqua technical material</td>
</tr>
<tr>
<td>1</td>
<td>AgroAqua technical material at 100 mg/l dilution water</td>
<td>Toxicity of everything present in the technical material</td>
</tr>
<tr>
<td>2</td>
<td>Alternated cheesecloth: AgroAqua technical material at 100 mg/l dilution water</td>
<td>Toxicity of free metals and other components present in the technical material (dissolute, bioavailable components)</td>
</tr>
<tr>
<td>3</td>
<td>Paste thick slurry complex, obtained by the washing of the technical material, in the amount per litre required to produce 100 mg of technical material (40 mg/l dilution water)</td>
<td>Toxicity of insoluble de-esterified, the active ingredient in the technical material</td>
</tr>
<tr>
<td>4</td>
<td>A fermentation broth (0.22 µm filter sterilized supernatant) solution in the amount per litre required to produce 100 mg of technical material (40 mg/l dilution water)</td>
<td>Toxicity of soluble, bioavailable components present in the fermentation broth and potential ecotoxicity via leaching of bioavailable components</td>
</tr>
</tbody>
</table>

1-5: Test solutions were observed to be slightly cloudy with undissolved test material visible on the bottom of the vessel

(Source: DAR Bt azulene GC-91 dated May 2007 / RAR 2018)

### Experience with long-term Daphnia toxicity studies (OECD TG 211)

**Study investigating the origin of toxicity in a 10-day static renewal Daphnia study**

### Table 3

**Description and purpose of treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
<th>Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Dilution water without test material</td>
<td>Any adverse effects not related to AgroAqua technical material</td>
</tr>
<tr>
<td>1</td>
<td>AgroAqua technical material at 100 mg/l dilution water</td>
<td>Toxicity of everything present in the technical material</td>
</tr>
<tr>
<td>2</td>
<td>Alternated cheesecloth: AgroAqua technical material at 100 mg/l dilution water</td>
<td>Toxicity of free metals and other components present in the technical material (dissolute, bioavailable components)</td>
</tr>
<tr>
<td>3</td>
<td>Paste thick slurry complex, obtained by the washing of the technical material, in the amount per litre required to produce 100 mg of technical material (40 mg/l dilution water)</td>
<td>Toxicity of insoluble de-esterified, the active ingredient in the technical material</td>
</tr>
<tr>
<td>4</td>
<td>A fermentation broth (0.22 µm filter sterilized supernatant) solution in the amount per litre required to produce 100 mg of technical material (40 mg/l dilution water)</td>
<td>Toxicity of soluble, bioavailable components present in the fermentation broth and potential ecotoxicity via leaching of bioavailable components</td>
</tr>
</tbody>
</table>

1-5: Test solutions were observed to be slightly cloudy with undissolved test material visible on the bottom of the vessel

(Source: DAR Bt azulene GC-91 dated May 2007 / RAR 2018)
Experience with long-term Daphnia toxicity studies (OECD TG 211)

Study investigating the origin of toxicity in a 10-day static renewal Daphnia study

Table 3: Description and purpose of treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
<th>Determinant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Daphnia water without test material</td>
<td>Toxicity of everything present in the technical material</td>
</tr>
<tr>
<td>1</td>
<td>Agro technical material at 100 mg/l, daphnia water</td>
<td>Toxicity of everything present in the technical material</td>
</tr>
<tr>
<td>2</td>
<td>Alternated chain-strains Agro technical material at 100 mg/l, daphnia water</td>
<td>Toxicity of free viable and other components present in the technical material. (parameter: bead-labile components)</td>
</tr>
<tr>
<td>3</td>
<td>Purified spore cultural complex, obtained by the washing of the technical material, in the amount per litre present in 100 mg/l technical solution used for Treatment 1 (66.7 mg/l, daphnia water)</td>
<td>Toxicity of isolated delta-relating, the active ingredient in the technical material.</td>
</tr>
<tr>
<td>4</td>
<td>A fermentation broth (0.22 µm filter sterilized supernatant) solution in the amount per litre required to produce 100 mg of technical material (40 mg L-1, daphnia water)</td>
<td>LoF [2018]: „...heat labile components from the fermentation broth are carried over into the technical material. These components elicit the toxicity of GC-93 observed for Daphnia...“</td>
</tr>
<tr>
<td>5</td>
<td>An eliminated fermentation broth (0.22 µm filter sterilized supernatant) solution in the amount per litre required to produce 100 mg of technical material (40 mg L-1, bead-labile suspended L, daphnia water)</td>
<td></td>
</tr>
</tbody>
</table>

1:5 Test solutions were observed to be slightly cloudy with undissolved test material visible on the bottom of the vessel

Experience with long-term Daphnia toxicity studies (OECD TG 211)

Proposed approach for routine testing I

Short-term exposure (e.g., 2 - 6 days) to Bacillus spp. spores at high concentrations (MNCh/WHD)  →  21 d  →  Clean water + algae (e.g., 6 - 18 days)

Effect assessment: Immobility / reproduction / ... (treatment vs. control)

Notes: <24 h old daphnids can survive without food for 48 h and size of spores is similar to unicellular algae

Remark on turbidity: In many cases, no reports of turbidity in the test solutions. Several methods to measure turbidity are available, e.g., nephelometer (turbidity expressed in NTUs)
Experience with long-term Daphnia toxicity studies (OECD TG 211)

**Proposed approach for routine testing II**

**Microbial Pesticide Test Guidelines**
OPPTS 885.4240
Freshwater Aquatic Invertebrate Testing, Tier I

Draft version (2013):

"(b) For test substances which may confound toxicological testing due to turbidity or test organism uptake or ingestion, appropriate filtration of the test material may be appropriate in order to remove particulate matter resulting from fermentation and formulation of the MPCA."

"Filtration of the test material or medium to remove sediment and particulates prior to commencement of the test must ensure that the nominal concentration of the test active ingredient is not significantly altered during the process. An appropriate filtered control must also be included for comparative purposes."

14.06.2018 / 9th OECD Expert Group on Biocides Seminar 2018

Experience with long-term Daphnia toxicity studies (OECD TG 211)

**Additional approach for further investigations**

aiming at a better understanding of the influence of food levels on the overall effects

- **spores**
- **algae**

**Effect assessment:**
Immobility / reproduction / ... (treatment vs. control)

Note: make sure that test conditions are optimal
- adjust light intensity?
- shorter exposure periods? (30 days is deemed sufficient)
Questions?

Bilgin Karaoglan
bilgin.karaoglan@uba.de

www.umweltbundesamt.de
Presentation 10

How to develop and adapt OECD Test Guidelines for micro-organisms
Magdalini Sachana, Anne Gourmelon (OECD, Paris, France)

HOW TO DEVELOP AND ADAPT OECD TEST GUIDELINES FOR MICRO-ORGANISMS

Magda Sachana and Anne Gourmelon
EGBP Seminar, 18 June 2018

OECD Test Guidelines (TGs)

• Developed for assessing the potential adverse effects of chemicals on human health and the environment.
• Internationally considered as standard methods for safety testing.
• Used in the testing and assessment of
  – industrial chemicals,
  – pesticides,
  – personal care products.
Recent experience to develop TGs for nanoparticles

- Nanomaterials have specific characteristics (volume or specific surface area, particle size distribution) that may determine some of their (hazard) properties
  - need specific TGs, but rather few
- Most other TGs are applicable, once adapted
  - adaptations can be described in guidance documents (GDs)

Nano Projects completed

- TG No. 318: Dispersion Stability of Nanomaterials in Simulated Environmental Media

Revised Inhalation TGs and GD to accommodate nanomaterial safety testing

- TG No. 412: Subacute Inhalation Toxicity: 28-Day Study
- TG No. 413: Subchronic Inhalation Toxicity: 90-day Study
- GD 39 on Inhalation Toxicity Testing
Nano Projects underway

**EFFECTS ON BIOTIC SYSTEMS**
- GD on Aquatic (and Sediment) Toxicity Testing of Nanomaterials

**ENVIRONMENTAL FATE**
- New TG on dissolution rate of nanomaterials in aquatic environment
- New TG for nanomaterial removal from wastewater
- New GD (Decision-Tree) on agglomeration and dissolution behaviour of nanomaterials in aquatic media
- New GD on assessing the apparent accumulation potential for nanomaterials
- GD to support implementation of TG 312 (Leaching in Soil Columns) for Nanomaterial Safety Testing

**HEALTH EFFECTS**
- GD on the Adaptation of In Vitro Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials

---

**Case of micro-organisms used as bio-pesticides**

- Living organisms (unlike chemicals)
- What’s the mode of action on target pest?
- Are there specific characteristics that determine (new) hazard properties?
  - Interaction with the host?
  - Infection? Immune response?
  - Release of toxic metabolites in host?
  - Accumulation/degradation and fate?
- Known target organs in animals?
Scientific basis for developing a Test Guideline

- Importance of understanding the interaction between the ‘test item’ and
  - the biological organism (e.g. knowledge about the initiating event, mode of action, toxic effect, response measured or endpoint) and how it relates to a hazard
  - the environmental media (e.g. behaviour in aquatic media, soil)

Scientific basis is important to develop a sound testing methodology

For new areas and new issues

- Start with Detailed Review Paper/State-of-the-art
  - Review what is known, scientific findings
  - Consider the different types of biopesticides
    - Are the issues identical for fungi/viruses/microbes?
  - Review current approaches/test methodologies
  - Determine if there are differences compared to current application of TGs?
    - In the preparation of the test item?
    - In the responses measured?
    - What drives the hazard in the pest and in the non-target host?
Proposals for new/adapted Test Guidelines and Guidance Document

- Only when the scientific basis is clear
- Process:
  - lead country/SPSF/ Expert Group established
  - experimental work for validation
  - draft documents for review
  - meetings and discussion to achieve consensus
Presentation 11

The Australian approach on test methods for micro-organisms
Alan Norden (Australian Pesticides and Veterinary Medicines Authority (APVMA, Kingston, Australia)

Overview

1. APVMA approach/background
2. Human health
3. Residues in food
4. Environment
5. Product chemistry
6. Challenges in risk/impact assessment
7. Questions
APVMA approach/background

- APVMA requires registration for a wide range of Microbial Pest Control Agents (MPCAs):
  - Similar types of products to most other regulators
  - Legislation requires a product is safe and effective
  - Assessment of human health, environment, residues, product chemistry, efficacy and target safety
  - Acknowledge OECD and other regulators guidance (US EPA)

- Beyond APVMA - other legislation - Gene Technology Regulator and importation of microbial agents

Human health

Identity of the Microbial Pest Control Agent

Presence and level of secondary metabolites and toxins

(OECD document in preparation)

Comments:

*Guidance to determine the presence and level of secondary metabolites and toxins will be very useful.*

*Particularly for the presence of potential genotoxic compounds (if structure and stability data is provided).*
Human health

Toxicological and Exposure Data and Information on the Microbial Pest Control Agent

Sensitisation potential

Comments:

Uncertain that tests using traditional protocols would provide meaningful results.

Given the size of MPCAs, skin penetration is unlikely using tests other than the Maximisation test. Conversely, the Maximisation test would involve induction through intradermal injection which is not reflective of exposure scenarios.

Human health

Toxicological and Exposure Data and Information on the Microbial Pest Control Agent

Intratracheal/inhalation infectivity, toxicity and pathogenicity

Comments: Pulmonary exposure studies are considered appropriate for hazard characterisation and guidance on the interpretation and on clearance of the MPCA would be useful.

Genotoxicity

Comments: Only likely to be required where characterisation of the MPCA identifies toxins/secondary metabolites of concern.

Traditional suite of approaches (ie. in silico, in vitro and in vivo testing) for determining genotoxic potential could be applied.
Human health

Toxicological and Exposure Data and Information on the Microbial Pest Control Agent

**Oral infectivity/toxicity**

Comments:

*This test is considered appropriate in the initial evaluation of the toxic or pathogenic characteristics of a MPCA. Further guidance on this test would be useful.*

---

Residues in food

Metabolism and Residues Studies on the Microbial Pest Control Agent

**Rationale for waiver of residue data**

Comments:

*Agree that in almost all cases a rationale for data waiver likely. Largely based on human health considerations/conclusions and where typically found in the environment. Estimation of dietary exposure may be necessary if metabolites of concern are identified.*

*EGBP should consult with Residue Chemistry Expert Group (RCEG).*
Environment

Fate and Behaviour Studies on the Microbial Pest Control Agent in the Environment

 Exposure scenarios related to way of application

 Rationale for the non-submission of data

Comments:

Agree with the difficulty and expense in establishing background concentrations.

We apply in part the USEPA requirements based upon the use pattern. We identify the hazards to non-target species as a first step (considering host range, infectivity and pathogenicity) which assists in defining which fate aspects need further consideration.

Environment

Ecotoxicological Studies on the Microbial Pest Control Agent (Effects on non-target organisms)

 Effects on bees, including brood testing

Comments:

In the absence of this information, we consider the adult bee endpoints as a surrogate.

Tier 1 guideline calls for higher tier testing when toxicity or pathogenicity is observed and the use pattern, host range, and other factors suggest potential for adverse effects.

Heavy reliance on expert judgement & scientific rationale is applied. Guideline for higher tier testing at this point could be useful (eg, testing of microbial product impacts on the hive).
Product chemistry

The APVMA uses or is in the process of adopting the following OECD guidance documents:

- *Guidance Document on Storage Stability of Microbial Pest Control Products*
  Series on Pesticides No. 85

- *Guidance Document for the Assessment of the Equivalence of Technical Grade Active Ingredients for Identical Microbial Strains*
  Series on Pesticides No. 96

- *Guidance for Registration Requirements for Microbial Pesticides*
  Series on Pesticides No. 18

Challenges in risk/impact assessment

- Test methods provide the results

- Risk/impact assessment requires interpretation and application (assessment methods and principles)

- Face challenges with MPCAs with potential wide host range

- Isolated locally from Australian environment

- Specific targeted use patterns

- Challenges in estimating likelihood of risk/impact, consequences and spread/infectivity beyond treatment area/target (to other non-target species)
Questions??

Thank you

Alan Norden
Executive Director - Registration Management & Evaluation
Australian Pesticides and Veterinary Medicines Authority (APVMA)
Biological Pesticides in Japan

Hidetaka Kobayashi, Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan

Biological pesticides in Japan

Hidetaka Kobayashi, Ph. D.
Deputy Director
Agricultural Chemicals Office
Ministry of Agriculture, Forestry and Fisheries

Biological Pesticides in Japan

- Microbial Pesticides
  - Bacteria
  - Fungi
  - Viruses
- Natural Enemy
  (No GLs)
  - Mites
  - Bees
  - Nematodes
Microbial Pesticides in Japan
- Registration system

- Registration shall follow the Agricultural Chemicals Control Act (Act No 82 of 1948)
- Same as chemical pesticides
- The law is to be revised
- Data requirements/ test guidelines for microbial pesticides were established in 1997

Microbial pesticide
- Number of products registered

<table>
<thead>
<tr>
<th></th>
<th>Bacteria</th>
<th>Fungi</th>
<th>Virus</th>
<th>Nematode*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insecticide</td>
<td>10</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>Fungicide</td>
<td>14</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Nematicide</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>14</td>
<td>4</td>
<td>2</td>
<td>45</td>
</tr>
</tbody>
</table>

*Virus parasitic on nematodes serves as insecticide
Microbial Pesticides in Japan

- Production of microbial pesticides in Japan: 205 t (Oct. 2015-Sep. 2016)
- Less than 0.1% of all pesticides produced (228,050 t)

GL for registration of microbial pesticides (outline)

- Biological properties
- Manufacturing process
- Analytical methods / quality assurance methods
- Efficacy studies
- Toxicity studies (human)
- Eco-toxicity studies
Evaluation: human risk

- Tier 1 – Acute toxicity
  - Oral, Dermal, Inhalation, Intravenous, Sensitization test
- Tier 2 – Subchronic toxicity
  - Oral, Inhalation
- Tier 3
  - Mutagenicity test
  - Reproductive test
  - Carcinogenicity test
  - Immunotoxicity test (only for viruses) etc.

Acute toxicity – Oral

- Single administration/ single dose (10^8 unit/animal)
- Rat or Mouse
- Observation: symptoms, body weight, excretion of microbes in feces
- Slaughter after 3, 7, 14, 21 days
- Count CFU in organs if symptom is observed
Acute toxicity – Oral (cont.)

- Tier 2 test is needed if
  - The animal is infected
  - The microbes survives in the body for a certain period of time
  - The pesticide is toxic to the animal
- Multiple administration repeatedly (1/day) for 90 days
- Further test may be needed: reproduction, mutagenicity test etc.

Acute toxicity (other pathways)

- Acute toxicity tests from other exposure pathways are also needed
  - Inhalation
  - Intravenous injection
- Consideration if Tier 2 test is needed: the same criteria as oral test
Acute toxicity
- Dermal exposure
  - Single administration/ single dose (10^8 unit/animal)
  - Rabbit, white
  - Observation: body weight, symptoms on the skin
  - Slaughter after 14 days
  - Consideration case-by-case basis if symptom is observed

Evaluation - Sensitization
  - Sensitization to eyes and skins
  - The guideline in Japan requires sensitization studies to evaluate the sensitization potential after repeated exposure to a microbial pesticide.
  - Requirement
    - Developed following the US EPA guideline “Pesticide Assessment Guidelines Subdivision M Biorelational Pesticide 152-36 Hypersensitivity study with microbial pest control agents”
    - However, USEPA GL no longer exists
Evaluation: Environmental risk

- Tier 1
  - Freshwater fish (effect, invertebrate test)
  - Birds
  - Plants, non-target
  - Insects, non-target
  - Honey bee
  - Silk worm
- Tier 2
  - Behavior/ fate in the environment
- Tier 3
  - Requirement determined case-by-case basis

Next steps

- The requirement/ GL was established more than 20 years ago

- We need to:
  - make the GLs updated
  - Make the GLs harmonized with international standard
  - Japan should contribute to the work in OECD for elaborating GLs etc.
The US experience with test methods for micro-organisms
Shannon Borges (Environmental Protection Agency (EPA), Washington DC, United States)

The U.S. Experience with Test Methods for Microbial Pesticides
June 18, 2018
The 9th Expert Group on BioPesticides Seminar
Paris, France

Shannon Borges
Branch Chief (Acting)
Risk Assessment Branch
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs

Data Requirements for Microbial Pesticides

- Data requirements in 40 CFR § 158 Subpart V
- Tiered System

Product Analysis
Toxicology
(Neurotoxicity, Human Health)
Non-target Organisms

Efficacy
Residue
Microbial Pesticide Guidelines

- Microbials have a separate set of guidelines (885 Series) due to unique properties
- Toxicology data requirements also include 870 Series guidelines for acute toxicity and irritation testing related to products
- Toxicology guidelines are more detailed than nontarget organism guidelines
  - Must allow testing with a wide range of test organisms and test material
  - Has resulted in a range of approaches to testing for certain guidelines

Microbial Pesticide Guidelines
Product Analysis and Toxicology

- Relatively few issues related to these guidelines
- Product Analysis
  - Increasing use of whole genome sequencing
- Toxicology
  - Identity of test material
  - Pattern of clearance from tissues and length of study
  - Appropriate tests for sensitization

- Guidelines are applicable
- Few problems with conduct and interpretation
- Do not foresee need for alternative methods (exception for sensitization testing)
- Low priority for new/amended guidelines

Microbial Pesticide Guidelines – Nontarget Organisms

- Relatively wide range of approaches to testing for certain guidelines
- Recurring issues involve:
  - Test material and controls
  - Exposure level
  - Exposure duration
  - Test duration
- Information is needed to improve testing for certain data requirements to achieve:
  - Consistency of approaches and interpretation
  - More efficient and useful outcomes
Nontarget Guidelines with Few Issues

- Avian oral testing is straightforward (885.4050)
  - Methods are clear, including dosing level
  - Dose level is sufficiently high
  - Test subjects fairly hardy in captivity
  - If there are problems, generally stems from incorrect dosing (i.e., not following the guideline)

  ➢ Guideline is applicable
  ➢ Few problems with conduct and interpretation
  ➢ Do not foresee need for alternative methods
  ➢ Low priority for new/amended guideline

Nontarget Guidelines that are Infrequently Used

- Avian Inhalation Toxicity/Pathogenicity (885.4100)
- Wild Mammal Toxicity/Pathogenicity (885.4150)
- Marine/Estuarine Fish and Invertebrate Testing (885.4280)
- Nontarget Plant Testing (885.4300)

  ➢ Applicability, Interpretation, Alternative Methods
    - Difficult to comment on this because so few submitted
  ➢ Low priority for new/amended guideline

Challenges with Commonly Used Guidelines
Aquatic Testing (Freshwater)

- Freshwater fish (885.4200)
- Freshwater invertebrates (885.4240)
- Common issues include:
  - High turbidity
  - Keeping test material suspended in water column
  - Clumping of test material (and surfactants used to reduce this)
  - Not following guideline and/or using poor technique:
    - Too few test animals
    - Test animals too small (fish)
    - Lack of appropriate controls
    - Contamination
    - Failing to follow-up on cause of mortality or other adverse effects
Aquatic Testing (Freshwater)

- **Applicability of guidelines**
  - Better information and guidance may be appropriate
  - Some methods need validation
  - Clearer guidance on when needed

- **Interpretation complexities**
  - Turbidity
    - Can cause mortality, reduce reproduction
    - Can be difficult to interpret the cause of mortality
  - Suspension in water column
    - Raises questions about actual exposure
    - Potentially testing on inappropriate organism

Aquatic Testing (Freshwater)

- **Interpretation complexities (continued)**
  - Clumping of test material
    - Raises questions about exposure level and uniformity
    - Surfactants added – can cause adverse effects
  - Ensuring stability of test material throughout study
    - Enumerations of test material from test water raises questions about reliability of identification
    - Can cause appearance of contamination if identified incorrectly
  - Too few/too small test animals
    - Raises questions of reliability

Aquatic Testing (Freshwater)

- **Alternative methods, improvements**
  - Turbidity
    - Include option to filter non-organic particles
    - Include both sterile filtrate and attenuated microbe controls
    - Test multiple concentrations
    - Validate maximum dose level (10^6 cfu/mL or 1000 X EEC, whichever is greater)
  - Suspension/clumping of test material in water column
    - Include stir bars or similar (fish)
    - Consider test with benthic test organism
    - Investigate what happens in nature, define testing needs
    - Include proper controls for surfactants
Aquatic Testing (Freshwater)

- Alternative methods, improvements
  - Ensure stability of test material
    - Focus on viability of test material
    - Ensure sufficiently frequent renewal
  - Validate duration (fish)
  - Standardize necropsy requirements (fish)

- High priority for improvement
  - However, clear guidance needed for when this study is required

Challenges with Commonly Used Guidelines

Nontarget Insects

- Required according to test note #8 under 40 CFR 158.2150
  - Point of testing is to understand unknowns
  - Growing body of literature challenges what we know about insect risks
  - EPA has flexibility in requiring data when needed

- Common issues:
  - Exposure level and exposure duration
  - Test duration
  - Control mortality
  - Age of test organisms
  - Exposure route

Nontarget Insects

- Applicability
  - Could use improvement to address common problems
  - Major challenge, though, is how to do this with unknown set of test animals combined with a range of types of microbes

- Interpretation complexities
  - Exposure level
    - 10X – 100X maximum application rate
    - No other guidance
    - How to convert from mass/area to cfu/volume?
  - Exposure duration
    - No guidance, but continuous exposure preferred
    - Otherwise difficult to ensure maximum exposure has been achieved
Nontarget Insects

- Interpretation complexities (continued)
  - Test duration and control mortality
    - Test duration may be too short to ensure observation of pathogenicity
    - Early control mortality raises concerns for validity of the study
  - Age of test organisms
    - May be too old, non-uniform
    - Mortality due to older age may be attributed to test material
    - Mortality may shorten test duration
  - Exposure route
    - Most microbial pesticides must be consumed to be effective
    - Contact studies may be inappropriate – cannot tell if test organisms meaningfully exposed

Nontarget Insects

- Alternative methods, improvements
  - No alternative guidelines available
  - Amended/alternative guideline may need to address:
    - Standardization of exposure level and duration
    - Test methods appropriate for biology of test animal and microbial pesticide or class of microbial pesticide

- High priority for improvement
  - Need clear guidance on when this study is required

Challenges with Commonly Used Guidelines

Honey Bees

- Required for almost all registration applications
- Common issues:
  - Many of the same as described for nontarget insects
    - Exposure level and exposure duration
    - Test duration (30 days)
    - Control mortality
    - Age of test organisms
  - Guideline requires larval testing when effects to larvae are expected, but no other guidance provided
  - Very little guidance
Honey Bees

➢ Applicability
  • Least applicability for obtaining useful information
  • Consider adaptation of guidelines for other types of pesticides microbiials

➢ Interpretation challenges
  • Exposure level
    • 10X – 100X maximum application rate
    • No other guidance
    • How to convert from mass/area to cfu/volume?
  • Exposure duration
    • No guidance, but continuous exposure preferred
    • Otherwise difficult to ensure maximum exposure has been achieved

Honey Bees

➢ Interpretation challenges (continued)
  • Test duration and control mortality
    • 30 days required by the guideline – unattainable
    • Early control mortality raises concerns for validity of the study
    • 20% cutoff for the study – too often viewed as validation to stop test
  • Age of test organisms
    • May be too old, non-uniform
    • Mortality due to older age may be attributed to test material
    • Mortality may shorten test duration, incomplete results
  • Exposure route
    • Most microbial pesticides must be consumed to be effective
    • EPA is receiving more contact studies

Nontarget Insects

➢ Alternative methods, improvements
  • Several possible alternatives
    • Adopt OECD guidelines/guidance documents for chemical pesticides
    • Use other guidelines to amend EPA's B85-A380 guideline
    • Test with a different species
    • Will need validation

➢ Highest priority for improvement
EPA Guidelines Studies – Best Practices

- Keep perspective - guideline studies provide data to inform the risk assessment, and quality is important
- Check the science – is it a good study?
- Consult EPA prior to conducting or submitting studies

Questions?
The Canadian Experience with Test Methods for Micro-organisms
Brian Belliveau (Health Canada, Ottawa, Canada)

Outline

- Existing test guidelines/protocols for microbials
- OECD Data Evaluation Templates
- Canada's regulatory experiences with microbial test methods
- Recommendations
Existing Guidance

• US EPA Guidance
  – OCSPSP Harmonized Test Guidelines Series 885 (Microbial Pesticide Test Guidelines)
  – OCSPSP Harmonized Test Guidelines Series 850 (Ecological Effects Test Guidelines)
  + Series 870 (Health Effects Test Guidelines) for chemical pesticides

• Canadian Guidance

• OECD Guidance
  – Publications on Biological Pesticides
    • EGBP Working Document, Guidance Documents, Seminar Reports
      http://www.oecd.org/chemicalsafety/pesticides-oecid/biological_pesticides.htm
  – OECD Guidelines for the Testing of Chemicals
    http://www.oecd.org/chemicalsafety/testing/oegdguidelinesforthetestingofchemicals.htm

Additional Guidance – OECD Data Evaluation Templates

• PMRA finalized OECD harmonized microbial data evaluation templates for TGAIs and EPs in January 2010; all can be obtained from:

• EGBP members agreed to include the templates in revised Microbial Dossier and Monograph Guidance Documents in March 2011
  – registrants can submit populated templates as OECD Tier II Summaries

• US EPA adopted most of the templates in 2011 with minor modifications to address EPA’s regulatory requirements; available online:
  https://www.epa.gov/pesticide-registration/oecd-data-evaluation-record-templates

• Templates contain embedded protocol details and testing criteria from relevant Canadian, US EPA and OECD guidelines
OECD Microbial Evaluation Templates — Characterization & Human Health

**Product Characterization & Analysis**
- Identity, biological properties, manufacturing methods, QA/QC

**Infectivity/Pathogenicity & Toxicity**
- Acute oral
- Acute pulmonary
- Intraperitoneal
- Intravenous
- Repeat dose inhalation
- In vivo mammalian cell
- Tissue Culture

**Toxicity, Irritation**
- Acute oral
- Acute inhalation
- Acute dermal
- Dermal sensitization
- Dermal irritation
- Eye irritation

**Other**
- Reporting of hypersensitivity incidences
- Genotoxic potential
- Sensitivity of detection

**Exposure**
- Occupational/Residential/Bystander
- Dietary

---

OECD Microbial Evaluation Templates — Environment

**Non-Target Organism Infectivity/Pathogenicity & Toxicity**
- Avian oral
- Avian pulmonary, inhalation or injection
- Wild mammals
- Terrestrial arthropods
- Honey bee
- Terrestrial non-arthropod invertebrates
- Terrestrial plants
- Benthic aquatic arthropods
- Pelagic aquatic arthropods
- Aquatic non-arthropod invertebrates
- Aquatic plants and algae
- Freshwater fish
- Estuarine fish

**Environmental Fate & Expression**
- Field studies

---
General Comments on Existing Test Methods

- Are the available guidance documents and guidelines on test methods perfect?
  - No, but individually and collectively, they are still valuable tools for industry, testing laboratories and risk assessors/evaluators
- Given their complexity and diversity, there is likely no one “standard” approach for testing that is suitable for all MPCAs; regulators and industry must accept that protocols will often have to be customized or tailored to suit the test material and the test organism
- Industry/contract testing laboratories should expect general guidance from regulatory authorities but they should also be expected to apply scientific judgement when designing/following test protocols/guidelines
  - Consult authorities as necessary to ensure that studies generated will address each data requirement appropriately
- Do not under value or under appreciate the microbial evaluation templates
  - Consolidate detailed testing and reporting requirements from U.S. EPA, Canadian and OECD guidelines
  - Ideal reference documents for study execution, data generation and study report preparation for industry

Experiences with Microbial Test Methods

In general, PMRA has found the existing guidelines/guidance documents on microbial test methods to be adequate based on many years of regulating MPCAs and their associated EPs

*Human Health*

- Studies conducted according to the US EPA Series 885 Group C Test Guidelines for assessing toxicity and pathogenicity/infectivity of MPCAs as well as EPA Series 870 or OECD Test Guidelines for assessing acute toxicity (TGAs and EPs) are the least problematic
  - No major issues/challenges with study results if guidance is closely followed
    - Interpretable test results are readily achievable
  - Challenge with some testing laboratories that do not perform a viability check of the test material prior to testing; Certificates of Analysis on potency/viability from the manufacturer are insufficient to demonstrate the test material contains live MPCA (and levels)
    - Cannot draw conclusions on infectivity or toxicity
  - Clarification on most appropriate administration route for pulmonary tests
    - Intratracheal instillation vs. inhalation of dry fungal spore preparations
Experiences with Microbial Test Methods

Non-target Organisms

- Studies following existing microbial guidelines for *birds, fish, aquatic arthropods/invertebrates, terrestrial arthropods and plants* are usually acceptable, but some challenges remain
  - Identification of test material and viability of the MPCA
  - Achieving maximum challenge dose/concentration vs. test system quality
  - Determining infectivity not always possible/practical
    - Study duration too short
  - Non-target terrestrial insect guidelines suggest suspension of MPCA (bacteria) in honey for dietary route
    - Honey has antimicrobial properties
  - Non-target terrestrial insect testing needs more guidance on dose levels especially for dietary routes of exposure
- Limited existing guidance on bee testing
  - Dedicated, updated guidance for microbiials needed
  - Currently being addressed by Bee Working Group of the International Commission on Plant-Pollinator Relationships

<table>
<thead>
<tr>
<th>Protocols &amp; Reports</th>
<th>Test product being proposed for registration (i.e., same manufacturing process, formulation etc.) or provide a rationale as to why data on a surrogate is relevant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clearly indicate whether test substance is MPCA, TGAI or EP</td>
</tr>
<tr>
<td></td>
<td>Use the test concentration or test dose recommended in guideline being followed</td>
</tr>
<tr>
<td></td>
<td>Report potency of batch being tested</td>
</tr>
<tr>
<td></td>
<td>Confirmation of viability and validation of recovery method required for infectivity studies</td>
</tr>
<tr>
<td></td>
<td>For infectivity studies, establish clearance or pattern of clearance</td>
</tr>
<tr>
<td></td>
<td>Include raw data and individual data</td>
</tr>
<tr>
<td></td>
<td>Justify any deviations from PMRA/EPA/OECD guidelines</td>
</tr>
<tr>
<td></td>
<td>Compare study report against PMRA’s data evaluation (DER) templates and ensure there were no errors in conducting the study before signing off on reports</td>
</tr>
<tr>
<td></td>
<td>Quality vs. quantity</td>
</tr>
</tbody>
</table>
Recommendations

• Important for authorities to offer *timely* presubmission consultations to industry and their contract testing laboratories
  – Review study protocols when significant deviations from standard test guidelines are expected for the MPCA
    • can help ensure studies are performed correctly

• Focus on updating/revising existing microbial test guidelines (e.g., U.S. EPA Series 885) or developing new guidance documents for *specific* studies where there is currently little or inadequate guidance/guidelines