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Innovating Microbial Pesticide Testing: Conference Proceedings

Series on Pesticides
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OECD Environment, Health and Safety Publications

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IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
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Foreword

This publication constitutes the proceedings of the OECD conference on the “Innovating Microbial Pesticide Testing”. This event, organised under the auspices of OECD’s Expert Group on Biopesticides (EGBP), was held on 13-16 September 2022. A total of one hundred participants attended the conference, which was open to OECD delegates as well as external scientists, regulators and contract research laboratories.

The OECD EGBP, former known as BioPesticides Steering Group (BPSG), was established by the Working Party on Pesticides (WPP) in 1999 to help member countries to harmonise methods and approaches used to assess biological pesticides and to improve the efficiency of regulatory procedures. The first tasks the EGBP undertook were: (i) reviewing the regulatory data requirements for three categories of biopesticide (microbials, pheromones and invertebrates); and (ii) developing formats for dossiers and monographs for microbials, pheromones, and other semiochemicals. After tasks were concluded, the EGBP concentrated efforts on addressing the scientific and technical issues that act as barriers to the efficient regulation of biological pesticides by organising seminars and following up on the resulting recommendations.

The conference was built on the outcome of the 2018 OECD Expert Group on Biopesticides seminar [ENV/JM/MONO(2019)8] that identified a number of tests as the most problematic. The conference aimed to address the technical limitations and the difficulties in interpretating the results of current testing methods applied to microbial pesticides. The Conference also considered possible modification of existing testing protocols based on experiences gained in hazard and risk assessment of microbial pesticide ingredients and New Approach Methodologies (NAM) already used to assess the effects of chemical pesticides.

The conference resulted in recommendations for updated testing methods and will eventually lead towards setting or revising scientifically-based policies for pesticide testing, including clarity on when testing may not be needed and where there are opportunities to use non-animal testing methods. The implementation of recommendations from the conference is expected to contribute to efficient regulatory processes for microbial pesticide products by improving the reliability and applicability of hazard data that support microbial pesticide registration applications and their review and risk assessment by regulators. This document is published under the responsibility of the Chemicals and Biotechnology Committee.

The Conference on Innovating Microbial Pesticide Testing was sponsored by the OECD Co-operative Research Programme: Sustainable Agricultural and Food Systems, whose financial support made it possible for most of the invited speakers to participate in the Conference in person.

This report has been produced with the financial assistance of the European Union. The views expressed herein can in no way be taken to reflect the official opinion of the European Union.

***Disclaimer**: The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.*

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Christopher Lythgo, European Food Safety Authority, EU

The roles of the different actors in the submission and evaluation of dossiers to support regulatory decision making in the EU including the role of EFSA is outlined. The key legislation regarding data requirements, organism strain approval criteria and product authorisation decision making criteria is also outlined along with the guidance and support that is in place for those preparing dossiers and the competent authorities that have to evaluate the dossiers. The main decision-making criteria that the data from studies has to deliver information on are:
-Microorganism / virus identity, specifications and methods for ensuring specification compliance.
-Absence of pathogenicity to humans of a strain / viral isolate.
-For bacteria absence of known functional and transferable genes coding for resistance to relevant antimicrobial agents as defined in the data requirements.

-Absence of infectivity or causation of unacceptable health effects in humans or animals
-There being sufficient treatment options for humans and animals to be effective against the microorganism.
-Identifying metabolites of concern.
-Harmful effects on human or animal health arising from exposure to the microorganism and metabolites of concern remaining in or on plants or plant products.
-Residues occurring have adverse effects on animal health.
-Assessing groundwater exposure by metabolites of concern.
-Absence of pathogenicity to terrestrial vertebrates.
-Margin of exposure below toxic effects on terrestrial vertebrates originating from plant protection product use.
-Lack of pathogenicity to aquatic organisms, bees, arthropods other than bees or if pathogenic a risk assessment that under field conditions impact on these organism group populations would not occur.
-Margin of exposure below toxic effects on aquatic organisms, bees, arthropods other than bees originating from plant protection product use.
-When microorganism was not isolated from soil, lack of pathogenicity to meso-and macro-soil organisms or if pathogenic a risk assessment that under field conditions impact on these organism group populations would not occur.
-Margin of exposure below toxic effects on meso-and macro-soil organisms originating from plant protection product use
-When there might be effects on populations of non-target terrestrial plants if the microorganism has a herbicidal mode of action or is closely related to a known plant pathogen
Key criteria for study design / improved study design need to include:
-The issue of sensitisation potential (skin and respiratory) for humans, is anything possible.
-Exposure / microorganism dosing method to address pathogenicity and / or infectivity / clearance need to consider information on biology of the microorganism.
-Study design durations to address pathogenicity and / or infectivity / clearance need to consider information on biology of the microorganism.
-Which non-target test organism species / whether different test organism species might be better for investigations, considering ability to maintain them (both before testing and in the context of giving reliable control results).

-Which non-target test organism species / whether different test organism species might be better for investigations, considering their relationship to the active microorganism strain being commercialised and its biology.

1.2. Regulatory Requirements for Human Health Testing with Microbial Pesticides in Australia

Maria Trainer, Australian Pesticides and Veterinary Medicines Authority (APVMA), Australia

The Australian Pesticides and Veterinary Medicines Authority (APVMA) is the statutory authority responsible for the regulation of agvet chemicals up to the point of sale. There are legislated requirements that the APVMA must be satisfied that all actives and products are safe for humans, including through the consumption of residues in food and for the environment, are effective when used in accordance with label directions, and do not unduly prejudice trade between Australia and places outside Australia. Labels for chemical products must contain adequate instructions to use the products safely.

The APVMA takes a risk-based approach to regulation, and has limited prescriptive requirements for how these criteria can be met. We seek to ensure data packages and arguments are adequate to address the statutory criteria, taking into consideration both the underlying toxicity, and the likely exposure resulting from use in accordance with the label instructions. The risk assessment also considers effects on sensitive sub-populations.

The flexibility of data requirements in Australia allows an opportunity for the incorporation of new assessment methodologies as they become available. To assist in this process, a pre-application assessment process is available providing an opportunity for potential applicants to discuss proposed data packages prior to submitting, or even preparing, an application. We will take into consideration a range of factors in determining the required assessments for both conventional and novel pesticides, to ensure satisfaction in relation to the statutory criteria as well as an appropriate regulatory response.

1.3. Human Health Risk Assessments for Biopesticides in the US - Industry Perspective on Development of Alternative Methods

Leah Zorrilla, Bayer CropScience, United States

The regulatory requirements to register microbial pesticides in the United States were first established in 1983 and revised in 1996 (OPPTS Series 885). To specifically address human safety for these unique and diverse microorganisms, the required regulatory studies are designed to not just evaluate toxicity, but potential pathogenicity and infectivity of the microbe. These guideline studies, through various routes of administration, evaluate whether or not the microbe has the potential to cause infection or disease. In addition, standard acute "6-pack" studies, normally run with conventional pesticide active ingredients and their formulated products, are also required on the microbial formulation, which helps aid in categorizing hazard potential for the label and for safe use of the microbial pesticide product. Since the initial inception of these test methods, there have been efforts to reduce, replace, and refine the use of animal testing across industries. To that end, the US Environmental Protection Agency published a memo in 2019 outlining its goal to eliminate the request of and funding for mammalian studies by 2035. This goal is supported by the recent exponential increase in new approach methodologies (NAMs), which have the potential to better predict human health hazard for risk assessment purposes. As NAMs testing methodologies have been refined, several study designs have been validated and these alternative acute toxicity testing (OECD and EPA) guidelines are now accepted by regulatory authorities in many regions of the world. However, the validation of the alternative methods has mainly focused on single chemical substances, with limited testing of formulated conventional pesticides or microbial pesticides. This presentation will discuss some of the challenges for testing microorganisms in NAMs.

Biological pesticides (including microbials - bacteria, algae, protozoa viruses, fungi, pheromones and semiochemicals, macrobials/invertebrates such as insects and nematodes, and plant extracts/botanicals) have gained momentum in the past decade as alternatives to synthetic pesticide solutions. Some biological pesticides have been successfully used in integrated pest management systems, as well as provided farmers with alternatives to treat disease and pests which have developed resistance to synthetic pesticides. As regulatory agencies around the world have seen an increase in submissions for commercialization of biological pesticides, regulatory guidelines and guidance documents have been developed in some regions to address the unique characteristics of these alternatives.

The regulatory requirements to register microbial pesticides in the United States were first established in 1983 and revised in 1996 by the Environmental Protection Agency (OPPTS Series 885). In order to specifically address human safety for these unique and diverse microorganisms, the required regulatory studies are designed to not just evaluate toxicity, but potential pathogenicity and infectivity of the microbe. These guideline studies, through various routes of administration, evaluate whether the microbe has the potential to cause infection or disease. In addition, standard acute "6-pack" studies, normally run with conventional pesticide active ingredients and their formulated products, are also required on the microbial formulation, which helps aid in categorizing hazard potential for the label and for safe use of the microbial pesticide product. Since the initial inception of these test methods, there have been efforts to reduce, replace, and refine the use of animal testing across industries. To that end, the US Environmental Protection Agency published a memo in 2019 outlining its goal to eliminate the request of and funding for mammalian studies by 2035. This goal is supported by the recent exponential increase in new approach methodologies (NAMs), which have the potential to better predict human health hazard for risk assessment purposes. As NAMs testing methodologies have been refined, several study designs have been validated, and these alternative acute toxicity testing (OECD) guidelines are now accepted by regulatory authorities in many regions of the world. However, the validation of the alternative methods has mainly focused on single chemical substances, with limited testing of formulated conventional pesticides or microbial pesticides.

Currently, human safety assessments for biopesticides must be suited to the unique characteristics of the microbe that distinguish them from synthetic pesticide chemicals. These testing strategies focus on pathogenicity, infectivity and toxicity of the microbe, and we must evaluate the specific features of microbials in order to evaluate their safe use. Whether or not the microbe is living or dead, and whether or not the microbe relies on biological chemistry for its activity are critical to understanding and evaluating the safety of the microbe. The EU and EPA data requirements for microbial pesticides require a pathogenicity/infectivity/toxicity study via oral, dermal, pulmonary and intravenous routes, in addition to acute eye and skin irritation studies on the technical grade active ingredients (TGAI). If no adverse effects are observed in these "Tier 1" studies, no further studies are required for the TGAI; the acute 6-pack studies are required for formulated products that contain the microbial TGAI. If adverse effects are observed in the Tier 1 studies, additional animal data will be required to assess the potential hazard of the TGAI. In some instances, waivers are acceptable to meet these criteria, but it is dependent upon the characteristics of the microbe, the available data to support the waiver, and the regulatory jurisdiction. Many studies required for the registration of synthetic pesticide chemicals are irrelevant and unnecessary to understand the safety of the microbe, and others which have been developed as alternatives to animal studies for synthetic pesticides are incompatible with the physicochemical properties of microbes to satisfactorily use to evaluate human safety.

We have recently evaluated several alternative methods which were validated for synthetic chemistries, and also attempted to adjust existing NAMs guideline studies to meet the requirements of testing microbial pesticides. The two cases studies were summarized: 1) a series of *in vitro* genotoxicity assays and 2) an *in vivo* assay to assess the risk of respiratory sensitisation.

While there are no global requirements for genotoxicity assays for biopesticides, there is guidance available for testing in some instances. In the first case study, three genotoxicity assays were evaluated, the *in vitro* micronucleus, the Ames microplate assay, and the DDR DNA Damage kit. In each assay, significant issues were observed. In the *in vitro* micronucleus assay and the DDR DNA Damage kit studies, interference was observed with the microbial strains tested in the flow-cytometry portions of the assay. In the Ames microplate, interference was observed with the microbials tested, likely due to the bacteria that produce antibiotics. Unfortunately, after reviewing these and several other available genotoxicity assays, none could be successfully validated with microbes due the technical issues described above.

The next case study evaluated whether or not a respiratory sensitisation assay could be validated for microbial pesticides. Currently, there are no validated assays to assess the respiratory allergy potential of

biopesticides, resulting in a requirement that manufacturers include respirator use in labeling. This respirator label statement triggers the need for employers to implement a respirator program which is expensive and can discourage the use of microbial pesticides. However, an assay that could provide insight into a biopesticide's potential to induce respiratory allergy would benefit the US EPA in its regulatory capacity, as well as industry in potentially allowing less restrictive labeling for microbial pesticides. Thus, Bayer Crop Science and the US EPA established a cooperative research agreement to investigate the Rat Basophil Leukemia cell (RBL) assay for its potential to identify microbial respiratory sensitisers. Previous studies with the RBL assay have demonstrated its potential to assess the relative potency between various fungal microbes by providing an index of functional IgE induction. In the current studies, two biopesticides were evaluated in two strains of mice (to cover the genetic capability to respond to specific proteins). This investigation of these microbial pesticide results lacked clarity and further studies are needed to 1) identify commercially available allergy positive and negative controls; 2) evaluate reproducibility and transferability; 3) establish an acceptable cut-off for allergy induction by using a panel of known biological sensitisers using a standardized protocol prior to utilizing this study as an option to measure potential respiratory sensitisation.

In addition to the evaluation of existing testing paradigms for pesticides, we have closely monitored the development of NAMs-related OECD guidelines for pesticides to determine if any of these test guidelines would be applicable to test microbial pesticides. Similar to the challenges faced with validation of the genotoxicity assays, microbial pesticides will interfere with the common detection methods (fluorescence, opacity) for study interpretation, which could lead to misclassification, or, misinterpretation of the results. In addition, some NAMs OECD test guidelines require solid or water-insoluble materials, making test article administration to the test system a challenge, and again, leading to inaccurate or potential misclassification of the results. A few other test guidelines rely on *in silico* analysis and/or water solubility, LogP, vapor pressure and/or surface tension measurements, which cannot be conducted with microbial pesticides due to the nature of the test material. In addition, many of the contract laboratories that conduct the NAMs OECD assays do not routinely (or have ever) tested microbial pesticides, either due to not having permits to bring the material into the laboratory, or not wanting to have potential contamination issues. Furthermore, no microbials have been included in any of the validation of the alternative assays for which OECD alterative assays have been developed.

Additional detailed evaluations were conducted to review the test methods used in the *in vitro* skin sensitisation assays. The two animal models used to evaluate synthetic pesticides, the LLNA (OECD 429) and Buehler (OECD 406) test methods, are not validated for microbial pesticides, due to the route of exposure (dermal application) or the method cannot discriminate between foreign proteins and those that induce allergic sensitisation, respectively. As a result, a precautionary statement is placed on the label in most, if not all, regions reading "Contains <insert bacterial strain>. Microorganisms may have the potential to provoke sensitising reactions," (EFSA expert PRAPeR M3 meeting, 2009) or "Repeated exposure to high concentrations of microbial proteins can cause allergic sensitisation" (US EPA required statement). However, similar to the other *in vitro* alternative assays, there are several issues using microbial pesticides including physical-chemical properties (soluble test material and/or a known quantity of test material is required), detection methods (fluorescence or labeled detection methods), no true positive or negative controls. In addition, for this adverse outcome pathway, more than one study needs to be run for the evaluation of synthetic pesticides, and no single NAMs-based sensitisation test method has been validated with microbial pesticides due to the limitations listed above.

Lastly, we evaluated whether *in silico* tools could support safety assessments for biopesticides. However, microbial pesticides contain a biological matrix of 100's, even 1000's of chemistries in a complex mixture. In addition, they contain large macromolecules that are not part of read-across or training sets, as the training sets are focused on individual small molecules. If there are large macromolecules available for read-across, much less safety or toxicity data exists for the assessment. This leaves registrants with data gaps for this type of evaluation on microbial pesticides.

In conclusion, we need to address the unique characteristics of microbial biopesticides in order to develop strategies to effectively evaluate safety, and not rely on alternative solutions developed for pesticides. Many tools currently exist for safety evaluations of microbial pesticides including genome sequencing, biological chemistry identification, pathogenicity, infectivity and toxicity data, literature reviews (microbe and biological chemistry) and the ECHA CLP regulation for hazard assessments for formulations. Investments in publicly available searchable databases (similar to the COMPARE allergen database for protein analyses) may fill a gap until appropriate NAMs can be successfully validated for microbial pesticides. As of now, NAMs to evaluate synthetic pesticides cannot easily be modified to address the unique characteristics of microbials, however microbials should be included as NAMs are considered and validated in the future.

Acknowledgements: Elizabeth Webb, Nicolo Visconti, Lisa Ortego

1.4. Regulatory Requirements for Human Health Risk Assessment in the EU - Industry Perspective on the Development of Alternative Methods

José Carvalho, Certis Biologicals, Germany & IBMA Chair of the Microbials Professional Group

Re-writing entirely all chemical testing methodologies to be applicable to biological factories (microorganism) could be a long journey because the starting point will be often the host-microorganism interaction, making some toxicology testing redundant in the first place. Some agreements among OECD Member Countries on assessing microbial ecology and factoring in the host-microorganism relationship could help defining what actually needs to be tested (and which Guidance testing Documents need revision). The OECD Consensus Documents on commonly-used species in Biocontrol is a sensible step in this direction and it would prevent unnecessary (animal) testing.

On new NAMs methodologies: development should be driven considering the biological nature of the microorganism and its growth and germination conditions. Assessing pathogenicity and infectivity to humans and animals (feeding) needs a separate approach from the toxicity testing in use for Chemical Pesticides. The data/evidence from the microorganism ecological niche should drive the testing needs, even if changes are required in national legislation texts on the rules to conduct risk assessments: the microorganism-host relationship should define the testing needs, and which information is still missing from what we know.

1.4.1. Introduction

The EU Regulation for Plant Protection Products (Reg. EC 1107/2009) sets a common regulatory framework for Chemicals and Biocontrol technologies, including Microorganisms. Even if a different set of data requirements exist for microorganisms, the EU Legislation was developed with (single) chemical

substances in mind, does not account for the specifics of microorganisms' biology, and the fact that a microorganism is a "mixture" of substances. Testing methodologies to address the data requirements are still based on the concept of single entities being assessed – e.g., secondary compounds produced by the microorganisms are being dealt with as "metabolites" of the active substance, as if they were a degradation product from a chemical active substance and not a part of it. The EU Data Requirements for microorganisms and secondary metabolites are based on test methods used for chemical pesticides. Adaptation of some chemical testing guidelines is therefore necessary to address the data requirements but this is not always possible or suitable, particularly considering the biological mixture which is the test item (microorganism). A longer term goal is consideration of adapting data requirements to be appropriate for microorganisms.

The absence of appropriate methodologies has led to the potential rejection of approval or renewals of some microorganisms in the EU, or approval with label-impactful restriction based on data gaps because data cannot be generated due to a lack of appropriate methodologies. For example until testing methods are available to test microorganism for potential skin sensitisation, the revised EU data requirements cannot be met. Though experimental test methodology is currently unavailable, information regarding skin sensitising potential of bacteria and virus is available in the public literature (e.g. for most bacillus) and could be considered for the assessments.

Deviations from the current testing methodologies (developed for chemical substances) as well as alternative test methods should be agreed upon by experts to avoid repetition of animal testing. A microorganism is a biological mixture, which includes its secondary metabolites (SM), and therefore it should be recognised that after such mixture has been tested in in vivo guideline tests (with the MPCA/MPCP) there is not a need to also test individual SMs, which are part of the active substance to prevent repeating animal testing.

1.4.2. EU regulatory framework and industry perspectives

This talk focused on the EU regulatory requirements (for human health) aiming at identifying the data points for which methodology should be adapted/developed, and agreed upon, to avoid (the repetition of) animal testing, particularly in relation to secondary metabolites assessment, but also rethinking the assessment questions leading to the current approach to toxicity testing. The recent revision of the EU Regulations No 283 & 284/2013 Part B: Data requirements for Microorganisms including viruses (published 31st August 2022, and entering into force on 21st November 2022) opens the way to first consider the microorganism ecological niche, ahead of proceeding to any toxicological testing. This is an improvement in EU legislation, allowing applicants and regulators to agree upon what is logical to test, rather than having a prescriptive set of data requirements. Responses to the following questions can be used to determine testing requirements: What is the ecological niche of the microorganism? For well-studied species, is the species known to infect humans and other mammals? Is the animal model relevant for the testing purpose? Is the route of administration relevant for a microorganism? Given the exposure scenario, should a microorganism be administered into an aseptic compartment? Is the maximum concentration, usually considered the worst case in chemical testing, a suitable approach for microorganism considering inhibition and the challenges of delivering unrealistic concentration of a microorganism via the test vehicle?

Revising existing OECD Test Guidelines can improve the assessment for microbial products, as long as the testing method provides relevant information. One example of a possible revision is an adjustment to the test concentration appropriate for the microorganism mixture under assessment, e.g., an acute toxicity test may be useful for the mixture (i.e., to assess the metabolites produced by a fungus strain), but the concentration selected should account for the microorganism capacity to reproduce, which seldom happen at the maximal dose. In contrast to a chemical substance, using high concentrations for microorganisms is rarely scientifically justified. Going forward, the adaptation of current OECD testing methods should consider: test concentrations, administration route, test vehicle preparation and the relevance of the host

species, based on the ecological niche of the microorganism. The host-microorganism relationship should drive the testing methodology, and this is specific to the microorganism type and species being tested.

The OECD Consensus Documents under preparation (i.e., on *Bacillus amyloliquefaciens* and *Beauveria bassiana*) take into account the evidence available and the ecological niche of the microorganism and seem to be the best way forward for well-known species, for which animal testing can be avoided. . Currently, animal testing may still be needed for the end-user product, to account for the overall end-user mixture which includes the microorganism (MPCA) and the inerts/co-formulants included in the formulation, though in the future, NAMs could be developed.

1.4.3. Summary

Adapting existing OECD Test Guidelines for toxicology testing (and ecotox) of microbial pest control agents could help applicants and regulatory agencies reach agreement on acceptability of data. Considering the recommendations in Guidance Documents such as 1) OECD recommendation paper/decision tree for a useful testing approach on what needs to be tested (dose, vehicle used, observation time, “clearance” assessment, etc.) and deviations allowed in OECD Testing Guidelines and 2) OECD Consensus Documents for well-known existing species used in biocontrol (e.g., *Bacillus subtilis/amyloliquefaciens/velezensis*, *Beauveria bassiana*, *Bacillus thuringiensis*) could help applicants and regulators agreeing on testing data needs on a species-by-species basis.

2 Session 2: Overview of New Approach Methodologies (NAMs) and Other Ongoing Research in Testing Methodologies

2.1. A Successful Case Study for NAM Development: Skin Sensitisation

Nicole Kleinstreuer, NIEHS, NICEATM, United States

In 2013, the OECD published an adverse outcome pathway (AOP) for skin sensitisation linking molecular initiating events and cellular and tissue effects in the sensitisation process to specific adverse outcomes. Several *in vitro* testing methods for assessment of dermal hypersensitivity mapped to key events in the AOP have been validated in international interlaboratory ring-trials, and combinations of these methods (so-called “defined approaches”) have been shown to provide superior performance to the existing animal tests when compared to human data. It is clear from these efforts that while no individual *in vitro* test can recapitulate the hypersensitivity response in its entirety, integrated strategies using varying combinations of *in vitro*, *in chemico*, and/or *in silico* methods could be highly accurate in identifying potential skin sensitisers. Tremendous efforts were put forth under the OECD project to curate high-quality reference datasets of unprecedented size, and to develop a novel defined approach (DA) guideline, the first phase of which covers DAs for hazard and potency category prediction. Other DAs for future consideration provide point of departure estimates that can be used for quantitative risk assessment. To further investigate the utility of these approaches, NICEATM solicited chemical nominations from ICCVAM federal agencies and ICATM partners. Just under 200 substances, including pesticides, formulations, excipients and industrial agents, have been tested to date and analysis of the data is

ongoing. A subset of this data has already been used by the US EPA in their 2020 “Draft Human Health and Ecological Risk Assessments for Several Pesticides for Several Isothiazolinones”. Recently initiated projects under the OECD workplan include substituting “me-too” assays as information sources in existing DAs, and refining and assessing the probabilistic Skin Allergy Risk Assessment (SARA) model under a partnership with Unilever to provide points of departure for quantitative risk assessment. This talk discussed the development and application of NAMs in regulatory decision making, and how defined approaches have been demonstrated to be more reliable and human relevant than the commonly employed *in vivo* test methods.

2.2. Human Health Risk Assessment in the EU and the Potential to Develop New Approach Methodologies

Norman Paege, Andrea Ziková-Kloas, Philip Marx-Stoelting, German Federal Institute for Risk Assessment (BfR), Germany

Risk assessment methodologies need to ensure a high level of safety for human health and the environment. At the same time, they have to be reliable and efficient to warrant that all partners in the risk assessment process, applicants and authorities can come to sound conclusions with appropriate resources and within a reasonable time.

New approach methods (NAMs) offer many advantages to achieve these goals. Moreover, the development of protocols to apply NAMs in risk assessment can reduce animal testing, which has only a limited value for some potential hazardous properties. Examples for the latter can comprise certain endpoints as well as regulatory contexts, such as aspects of immunotoxicity or microbial biopesticides.

Since there is an urgent need for approval of low risk active substances considering the requirements of the EU Chemicals Strategy for Sustainability, application of NAMs in the field of bio-pesticides seems to be very promising. However, for the various types of active substances used in bio-pesticides different endpoints need to be addressed by diverse NAMs and respective decision strategies.

Microorganisms as one type of active compounds used in bio-pesticides may be associated with hazardous properties including acquired antibiotic resistance, pathogenicity, infectivity, sensitisation, and the production of metabolites of concern. Here whole genome sequencing may be informative to assess these properties. In addition, an integrated approach

for testing and assessment (IATA) to detect sensitising properties has been developed that could be applied to address these hazards in a NAM based strategy.
In contrast, natural compounds as another type of active ingredients may be associated with similar hazards as other chemical substances. Here too, NAMs could help in hazard identification considering improvements in adverse outcome pathways (AOPs) and next generation risk assessment (NGRA). However, ab initio testing of chemical substances by NAMs is still under development in European projects such as RiskHunt3R or PARC and not yet available for regulatory purposes.
RNA as another type of potential low risk active substances is finding its way into pesticidal applications. For analysing potential hazards related to RNAi as a mode of action, bioinformatics may help as the sequence of the RNA used as active ingredient is usually very target specific. Hence, comparative sequence can support the conclusion on potential hazardous properties.
In summary, a combination of NAMs within a tiered testing and NGRA strategy, considering specific hazards associated with different types of active substances used in bio-pesticides, can help to ensure a high level of protection of human health reliably and efficiently.

2.2.1. Introduction

Pesticides only get authorization for use in the European Union if it has been demonstrated that they present a clear benefit for plant production and that they are not expected to have any harmful effect on human or animal health or any unacceptable effects on the environment [EC 2009] under the approved conditions of use. So called bio-pesticides, a group of active ingredients consisting mainly of microorganisms and viruses but also of natural compounds or RNAi are not per se less harmful than organic chemicals used as active compounds in plant protection products. The potential hazards associated with these microorganisms differ, however, in nature from those associated with their chemical counterparts. One main hazard results from the ability to reproduce and metabolize in the field after application. In addition, microorganisms may be pathogenic and or infective and have the ability to multiply after application. They may transfer information on antimicrobial resistance (AMR). Microorganisms may cause sensitisation in operators, workers, bystanders. They may produce secondary metabolites that are carcinogenic, mutagenic or toxic to the reproductive system (CMR) or show any other form of toxicity just as organic chemicals. Microorganisms may also be persistent in the environment. Hence, their hazard profile has to be analysed comprehensively prior to their approval.

Risk assessment of microbial active ingredients and other bio-pesticide active substances in the EU is based on Regulation 1107/2009 [EC 2009] and the respective data requirements are laid out in Regulation 283, Annex B [EC 2013] recently amended by Reg (EU) 2022/1439 [EC 2022]. Fulfilling the data requirements is mandatory to ensure microbial active ingredients are safe and consequently can get approval. Methodology suggested to fulfil these data requirements is, however, partially based on methods more appropriate for the analysis of chemical active compounds such as e.g., animal testing for short term toxicity. Such *in vivo* studies may not in all cases be the most adequate tool to address the hazards

associated with microorganisms, especially infectivity and pathogenicity but also sensitisation, also with regard to host specificity. Here NAMs may help to improve the quality of the hazard assessment.

The current manuscript is based on a presentation held during an OECD Conference on Microbial Pesticides in September 2022 in Paris. It summarizes suggestions for areas where a combination of NAMs within a tiered testing and NGRA strategy considering specific hazards associated with different types of active substances used in bio-pesticides, can help to ensure a high level of protection of human health reliably and efficiently. However, prior to implementation of NAMs a comprehensive evaluation and regulatory acceptance is needed. The framework and data requested by European regulations are at present the only legally binding requirements.

2.2.2. For which endpoints is evaluation by NAMs possible?

Microorganisms as one type of active compounds used in bio-pesticides may be associated with hazardous properties including antibiotic resistance, pathogenicity, infectivity or sensitisation and the production of metabolites of concern that may have genotoxic potential or other acute or chronic toxic effects. NAMs may be able to address some of these endpoints. NAMs comprise *in vivo* as well as *in vitro* and *in silico* tools. *In vivo* NAM refers to application of technologies such as transcriptomics or metabolomics that are used in addition to classical pathological techniques or clinical chemistry. *In vitro* assays are already available for a number of endpoints of concern including genotoxicity but also as part of an integrated approach for testing and assessment (IATA) for sensitisation. *In silico* tools may be combined with molecular tools such as whole genome sequencing of microorganisms in combination with database-evaluation if genes responsible for AMR or indicating an infectious or pathogenic potential are present. Further, *in silico* hazard prediction tools for microbial metabolites can be developed. In addition, a comprehensive analysis of literature and existing information on the respective microorganism is mandatorily required and can help to conclude on these endpoints. Table 2.1 below summarizes microorganisms that are considered to be used as pesticide active ingredients, potential concerns as well as an indicator if NAM based evaluation of that specific concern is possible and on which basis.

Table 2.1. Active ingredients, potential concerns and NAM based evaluation options

Microorganism	Substance / endpoint	Concern	NAM evaluation possible?
Beauveria bassiana	Beauvericin	Genotoxicity	Yes, *in vitro* battery (TG 471, 473, 476)
Pseudomonas chlororaphis	2,3-deepoxy-2,3-didehydro-rhizoxin (DDR)	Genotoxicity	Yes, *in vitro* battery (TG 471, 473, 476)
Purpureocillium lilacinum	Leucinostatin	Acute toxicity	No
Aspergillus flavus	Kojic acid, Infectivity/pathogenicity	Genotoxicity Infectivity/pathogenicity (aspergillosis)	Partially (yes for mutagenicity)
Metarhizium brunneum	Swainsonin	Neurotoxicity (Locoism)	No
Trichoderma ssp.	6-pentyl-2H-pyran-2-one (6-pentyl-2-pyrone)	Irritation	Yes

2.2.3. Other bio-pesticides

In contrast to microorganisms, natural compounds as another type of active ingredients may be associated with similar hazards as other chemical substances. Here too, NAMs could help in hazard identification considering improvements in AOPs and in AOP-based NGRA. However, ab initio testing of chemical substances by NAMs is still under development and not yet available for regulatory purposes.

RNA as a potential low risk active substance is finding its way into pesticidal applications. For analysing potential hazards related to RNAi as a mode of action, bioinformatics may help as the sequence of the RNA used as active ingredient is usually very target specific. Hence, comparative sequence analysis per se may allow for concluding on potential hazardous properties.

2.2.4. Conclusion

NAMs already play an important role in risk assessment of bio-pesticides. Depending on the type of bio-pesticide (microorganism, natural compound, RNAi) different endpoints have to be analysed and therefore different types of NAMs are required.

Depending on the endpoint comprehensive NAM batteries and accepted test-guidelines already exist (genotoxicity, sensitisation) or are under development or are still missing (pathogenicity, infectivity of microorganisms).

Several big EU-projects exist that will contribute to the development of NAMs (ASPIS cluster, PARC). Even though they are not targeted at microorganisms their outcomes will ultimately facilitate implementation of NGRA.

2.2.5. References

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EC (2013) COMMISSION REGULATION (EU) No 283/2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, available at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0283&from=DE

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2.3. Considerations for Developing In Vitro Studies as Alternatives to Acute Toxicity Testing: Understanding Variability in Reference Data

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Historically, toxicity testing has been conducted using *in vivo* test methods. Confidence in data from these methods is such that regulatory hazard classification and labeling systems have been designed around their results. To establish confidence in new approach methodologies (NAMs), we must demonstrate that they are as good as or better than the existing *in vivo* test method. For many toxicity endpoints there is no NAM accepted as a complete replacement for animal use because hazard categorizations based on data from the NAM do not always agree with hazard categorizations based on *in vivo* data for the same chemical set. However, discordance with *in vivo* results may not always indicate that the NAM is generating an incorrect prediction. To establish confidence in NAMs, it is critical to understand any variability inherent to the *in vivo* test a NAM is intended to replace, as this variability will directly affect the expectations for performance of NAMs that seek to replace it. This presentation discussed efforts to understand the reproducibility of several *in vivo* test methods and how variability in those results should be considered when comparing to a NAM.

2.4. New *in vitro* methodologies that can be applied for the study of biopesticides' hazard by the oral route

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The investigation of the toxicity of microorganisms used as biopesticides is challenging as *in vivo* testing has been described as inappropriate. Based on the evolution of toxicology to reduce and replace animal testing by New Approach Methodologies (NAMs), we designed simple *in vitro* assays to study the effects of microorganisms on the human intestinal barrier and applied them to a broad range of Bacillus cereus strains including several B. thuringiensis subspecies. This can be a first step of a strategy for screening the pathogenicity of microorganisms. Future developments are also described.

2.4.1. Introduction

Biopesticides include a wide range of active substance or agents such as microorganisms (Kumar et al., 2021). Their use in agriculture is expected to increase in Europe due to the farm to fork strategy and the new Regulation on the Sustainable Use of Plant Protection Products, with a target to reduce the use of chemical pesticides, in particular the hazardous ones, and to increase the use of non-chemical alternatives by 2030 (COM, 2022). Although, in most cases, consumer exposure to microorganisms used as biopesticide is not of concern, such occurrence cannot be fully excluded, especially following ingestion of treated crude products, and may increase with the broader use of biopesticides in the near future. Moreover, for biopesticides that are close to pathogenic microorganisms, concerns cannot be completely ruled out. As an example, Bacillus thuringiensis belong to the B. cereus group that is largely involved in foodborne intoxications (Jessberger et al., 2020). Some cases have been attributed to the presence of B. thuringiensis strains (McIntyre et al., 2008; Jackson et al., 1995) including some used as biopesticides (Bonis et al., 2022; EFSA, 2016 and for review Biggel et al., 2022).

Unfortunately, the investigation of microorganism toxicity is challenging due to the difficulty to demonstrate their virulence using classical toxicity testing. In fact, mammalian *in vivo* toxicological studies have been recognized as inappropriate for testing B. cereus enterotoxigenicity (BioHaz, 2018) and could not therefore be recommended for assessing the safety of microorganisms used as biopesticides. Moreover, as illustrated by the European position on animal protection (EU, 2010), numerous countries are claiming to reduce and to replace animal testing due to ethical issues. Concomitantly, hazard assessment is moving from animal-based testing to New Approach Methodologies (NAMs) described as “a broadly descriptive reference to any non-animal technology, methodology, approach, or combination thereof that can be used to provide information on chemical hazard and risk assessment” (US-EPA, 2021). NAMs include *in silico* methods, *in vitro* testing and omics.

In this paper, we propose a strategy using NAMs that can be implemented to investigate the toxicity of microorganisms including those used as microbial active substances in the biopesticides and we delineate some perspectives to improve the outputs.

2.4.2. In vitro models

Intestine, as the main organ of contact for food contaminants and pathogens, must be first considered for testing. Indeed, the symptoms that have been described during B. cereus foodborne outbreaks correspond to intestinal effects (Dietrich et al., 2021). Small intestine is a complex system integrating various cell types (Salvo-Romero et al., 2015) with an epithelial barrier regulating the transfer of compounds and agents from intestinal lumen to blood and lymph fluids. Along the intestinal epithelium, enterocytes are the most abundant followed by mucus cells. In the crypts, stem cells, Tuft cells and Paneth cells can be found. Finally, specific lymphoid structures called Peyer patches are mostly present in the ileal section. They are composed of microfold cells at the epithelium surface that are associated with immune cells of the lamina propria (Kobayashi et al., 2019).

Among the different cell types, toxicity is mostly investigated using enterocytes. The human Caco2 cell line is the most largely used model. Isolated from a colonic adenocarcinoma, these cells can differentiate after few weeks in culture, thus showing morphological and functional characteristics close to enterocytes including cell polarization, formation of tight junctions and secretion of intestinal enzymes such as sucrase isomaltase (Liévin-Le Moal & Servin, 2013; Fedi et al., 2021). This cell line is also recommended to study the human intestinal permeability of compounds. A guidance document for carrying out this assay has been established by ECVAM (ECVAM, 2013).

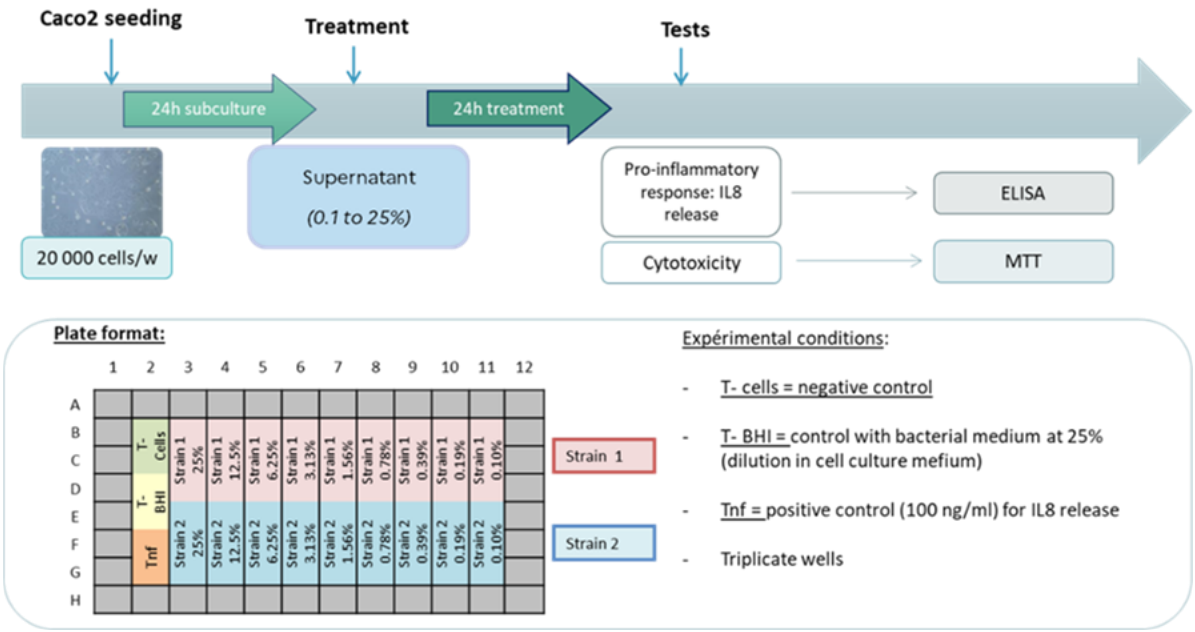
2.4.3. Strategy proposed for testing

Toxicity of bacterial supernatants

Supernatants from bacterial cultures at the beginning of the stationary phase (corresponding to the production stage of the major enterotoxins) are generated in Brain Heart Infusion (BHI) broth. They are further tested on human intestinal Caco2 cells as described in Figure 2.1. Two assays are carried on after a 24h treatment with various dilutions of the different supernatants in 96-well plates. First, the effect on cell viability is done by the 3-(4,5- dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, measuring the mitochondrial activity. Prior to the addition of MTT, the cell media are collected and are further processed to quantify by ELISA the release of interleulin-8 (IL-8), a pro-inflammatory marker. Negative and positive controls are included. Triplicate wells per experiment and at least 2 independent experiments are performed.

For the two endpoints (cell viability and IL-8 release), gradients depending on the level of response can be proposed, so to help classifying the virulence potency of the tested strains.

Figure 2.1. Experimental design for testing bacterial supernatants on non-differentiated Caco2 cells



Note: (TNF = Tumor Necrosis Factor α)

Bacterial adherence to intestine

It has been suggested that adherence of bacteria to intestine can be a marker of virulence (Andersson et al., 1998). However, for this endpoint, various experimental assays can be designed (Ramarao & Lereclus, 2006; Hansen et al., 2011) and it will be crucial to generate a harmonized protocol. For this investigation, differentiated Caco2 cells are in contact with the living bacteria. After several hours of contact followed by some washes, the bacteria and intestinal cells are counted by cell imaging using fluorescent dyes such as respectively 5-carboxytetramethylrhodamine (TAMRA) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

2.4.4. Application

This strategy was performed to investigate the toxicity of a large range of Bacillus cereus strains that are well-known food borne pathogens involved in human intoxications. The strains of the B. cereus group included also B. thuringiensis exploited or not as biopesticides as well as some strains involved in foodborne intoxications. Our results showed that, among the 10 strains of biopesticide tested belonging to the 4 subspecies (aizawai, israelensis, tenebrionis and kurstaki), an absence or a moderate cytotoxicity was observed while one subspecies did not show any increase of IL-8 release (data not shown).

2.4.5. Perspectives

Two main categories for future developments can be outlined:

- First, more complex intestinal cell models integrating various cell types can be used. Co-cultures of Caco2 cells with HT29-MTX mucus cells, with Raji B cells that induced Caco2 cell transformation into M cells, with THP1 macrophages as well as reconstructed intestinal epithelia and organoids (Maresca et al., 2018; Ghiselli et al., 2021) can be more relevant to study some effects. However, due to the cost and the throughput of these more complex models, they are less adapted to the screening of numerous strains for the moment and need further development.
- Second, additional endpoints can be investigated, whether using targeted methodologies with classical or high content analysis testing to measure for example oxidative stress, apoptosis, DNA damage, … (Li & Xia, 2019) or untargeted methodologies such as transcriptomics, proteomics and metabolomics (Canzler et al., 2020). The new data will enable to identify Key Events along a biological pathway from a molecular initiating event to an Adverse Outcome describing Adverse Outcome Pathways (AOP) (Ankley et al 2010) and providing additional information to the web-tool on AOP (Delrue et al., 2016). The pathways that are mainly involved in the virulence of microorganisms will therefore be more easily deciphered.

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2.5. Advancing reliable and relevant approaches for assessing pesticide products

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Thousands of animals are required to assess the toxicity of a single conventional pesticide active ingredient. Due to the limited reliability and relevance of many traditional animal test methods, the legal requirements in numerous regulatory jurisdictions to reduce animal use, and the ethical implications of testing on animals, the international regulatory and scientific community is shifting towards an increased reliance on new approach methods (NAMs). PETA Science Consortium International e.V. collaborates with regulators, academics and industry to harmonize international testing requirements, and develop and implement robust, fit-for-purpose NAMs. This presentation provided examples of our work in the area of conventional pesticide toxicity testing, including a focus on specific endpoints such as eye irritation, carcinogenicity, and avian dietary studies, as well as work conducted in collaboration with the Central Insecticides Board & Registration Committee in India to reduce animal use within the biopesticide regulations.

Building on previous efforts, this presentation also proposes a modern, flexible framework comprising five essential elements to establish scientific confidence in NAMs for regulatory use: fitness for purpose, human

biological relevance, technical characterization, data integrity and transparency, and independent review. The criteria presented here is broadly applicable across human health endpoints. Updates to the criteria are based on the recognition that (1) the relevance of the NAM results need not be determined through direct alignment with the results of the traditional animal test method, and instead may be determined through alignment with, or fidelity to, human biological understanding; (2) the NAM should not be required to replace the traditional animal test method one to one, nor produce the same information generated by the traditional animal test method; (3) the currently accepted levels of reproducibility in traditional animal test methods can be used to inform performance benchmarks for NAMs; (4) ring trials may not be necessary for the assessment of the reproducibility of a NAM; and (5) preferably before a NAM is developed, its purpose should be clearly defined and discussed amongst the method developer, regulators, and the regulated industry to ensure the production of NAMs that are fit for purpose. Universal uptake of this framework would facilitate the timely development and use of NAMs by the international community.

PETA Science Consortium International e.V. (the Science Consortium) is a not-for-profit non-governmental organisation funded by the international entities of People for the Ethical Treatment of Animals. The Consortium consists of 25 scientists working globally to promote regulatory use of robust non-animal methods, through funding the development of new methods, providing free training, workshops, and webinars on new approaches, and performing retrospective reviews on existing data.

2.5.1. Introduction

Microbial pesticides can be effective and target-specific, and are proposed to be less toxic to humans and the environment than conventional pesticides. However, many of the current OECD test guidelines that use animals are deemed unsuitable to assess the potential toxicity of microorganisms. Therefore, there is an urgent need to assess and adapt current non-animal methods, and develop new non-animal methods, where necessary, that will ensure the safety of humans and the environment.

One goal of the OECD Conference on Innovating Microbial Pesticide Testing was to investigate how microbial pesticides might be tested for potential toxicity and, in particular, how a non-animal testing paradigm may be developed. Here, we provide an overview of considerations for developing, adopting, or establishing scientific confidence in non-animal approaches that could be applied to the assessment of microbial pesticides.

2.5.2. Criteria for establishing confidence in new approaches

Some existing methods with broad applicability domains (e.g., reconstructed human corneal epithelium models or *ex vivo* eye methods) may be useful in their current state or when adapted. Additionally, it is anticipated that there will be a need to develop new methods for assessing microbial pesticides. The development of new, or adaptation of existing, test methods is slowed by lengthy validation processes. A recent publication proposed a flexible framework comprising five elements (fitness for purpose, biological relevance, technical characterization, data integrity and transparency, and independent review) that can be used to establish scientific confidence in new approaches (van der Zalm et al., 2022). The framework

was developed to follow the principles of validation outlined in OECD Guidance Document 34 on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (OECD, 2005), while updating the processes by which methods are validated. Below are some considerations that are relevant to the issues discussed at the conference.

- Resources should first be used to assess the applicability of existing non-animal methods, and then to adapt these methods when current methods have been demonstrated to be unsuitable for microbial pesticide assessments.
- If existing non-animal methods cannot be used or adapted, resources should be used to develop new non-animal methods where needed to provide information essential for regulatory decision-making that cannot be gained from current non-animal methods. In these cases, close collaboration between method developers and the end users will enable the most effective methods to be developed.
- Where new non-animal methods are being developed, there is an opportunity to create methods that are truly animal-free, using chemically-defined media, serums, and non-animal recombinant antibodies rather than those that require the use of animals.
- Reproducibility assessments are often time-consuming and expensive and should only be conducted on methods that have been demonstrated to be relevant and fit for purpose. Furthermore, full validation studies where a list of chemicals are tested in at least three laboratories may not be necessary for the assessment of the reproducibility of an approach, for example, when adapting a test method that has already been validated. Inter-laboratory reproducibility assessments may reflect laboratory quality rather than the quality of the new approach.

2.5.3. Training

Regulatory agencies around the world have made commitments to increase reliance on reliable and relevant non-animal toxicity testing methods (Bhuller et al., 2021; Escher et al., 2022; Craig et al., 2019; US EPA, 2021; Washington et al., 2022). In order for new approaches to gain traction in the regulatory arena, it is important to educate regulators and researchers of their uses, advantages, and limitations, as well as the limitations of the traditional animal test methods.

In particular, method developers, contract research organisations, non-governmental organisations, and standards making organisations should continue to provide training to end users on how methods address specific endpoints, how they are conducted, and how results can be interpreted for regulatory use. Additionally, training is needed on whether adaptation of existing *in vitro* and *in silico* test protocols, validated for standard chemicals, is needed to use them for microbial pesticides, and, if so, how to adapt them. Training can be provided through peer-reviewed publications, webinars, in-person workshops, or conferences. In addition, case studies that demonstrate how data can be integrated into reporting frameworks or risk assessments can help to establish trust in a method.

2.5.4. Standardization of common practices

To increase international harmonization, best practices and reporting standards for specific assays should be standardized. This will allow cross-laboratory comparisons and will facilitate the development of internationally agreed upon test guidelines. For example, interested researchers could collaborate to gain consensus on how to amend reconstructed human epithelial cell model platforms for microbial pesticide testing.

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3 Session 3: Regulatory Requirements for Non-target Organisms

3.1. Non-target Organism Testing with Microbial Pesticides in the US - Regulatory Requirements and Challenges

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Microbial ecotoxicity tests are used to provide information on the hazard of microbial pesticides to support the registration of new active ingredients, new uses, and review of currently registered pesticides. The non-target organism data requirements for microbial pesticides are outlined in the Code of Federal Regulations (CFR), volume 40 § 158.2150, and the most commonly submitted studies include tests on avian, aquatic invertebrates, fish, and terrestrial insects including bees. These tests are described in the OCSPP Series 885 guidelines for microbial pesticides. Because microbial pesticides are derived from a range of both naturally occuring and bioengineered microorganism that include bacteria, fungi, viruses, protozoa, and bacteriophage, the 885 guidelines to conduct these tests are not overly prescriptive and try to provide latitude to adapt tests based on the nature and hazards of the active ingredient in question. One of the many challenges when testing microbial pesticides is designing tests to adequately address both potential toxicity as well as pathogenicity. This paper provides an overview of the regulatory requirements and challenges with testing and assessment of microbial pesticides.

3.1.1. Introduction

Biopesticides are becoming an increasingly important tool for agriculture due to their less toxic human health and environmental profiles relative to many conventional pesticides. Microbial pesticides comprise

an important group within the registered biopesticides. In the U.S., microbial pesticides are registered as active substances/ingredients based on a particular isolate. The active ingredients may not necessarily be solely a living organism but may include also a mixture of spent fermentation media, metabolites or toxins produced by the microorganism and dead material. Thus, the effect of the microbial pesticide may be directly toxic as a result of the metabolites. Since most microbial pesticides are viable, it is not sufficient to test toxicity alone; the infectiveness and pathogenicity must also be evaluated. Infectiveness (or infectivity) refers to the ability of a microorganism to enter a host and multiply within that host, whereas pathogenicity refers to the ability to cause disease (i.e. harm) to the host. As such, it is possible for a microorganism to be infective without being pathogenic, whereas to be pathogenic, it must also be infective. In order to verify an acceptably safe environmental profile, hazard tests must assess both the toxicity and pathogenicity of the microbial active ingredient.

Data requirements for microbial pesticides are described in 40 CFR § 158 Subpart V. Microbials have a separate set of guidelines (885 Series) due to their unique propertieshttp://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-885-microbial-pesticide-test-guidelines. In addition to toxicity, infectivity and pathogenicity, application of microbial pesticides may result in variable exposure as a result of competition and transmissibility depending on the environmental in which they are applied. To accommodate these unique properties, non-target guidelines are much less prescriptive than those for conventional pesticides (850 Series). Microbial toxicity tests must accommodate a wide range of test organisms and test materials that may interact with testing matrices differently compared to chemical pesticides.

Microbial pesticides are tested in a tiered system progressing from highly conservative tests in Tier 1 to more complex testing in Tier 4. Tiers progress from acute toxicity / pathogenicity testing (Tier 1) to environmental expression testing (Tier 2) to chronic / range ecosystem testing (Tier 3), to semi-field and field testing (Tier 4). The most common non-target organism tests received include avian oral (885.4050), freshwater fish (885.4200), freshwater invertebrate (885.4240), non-target insect (885.4340) and honeybee (885.4380) toxicity/pathogenicity testing. Nontarget plant testing is generally only received if a microbial pesticide is taxonomically related to a known plant pathogen (as described in footnote 8 of 40 CFR 158.2150).

3.1.2. General Issues with Testing Microbial Pesticides

Microbial toxicity and pathogenicity Tier 1 tests are based on the maximum hazard dose. This dose ranges from 10 to 100 times (for terrestrial insects) to 1000 times (for aquatic taxa) the estimated environmental concentration that results from the maximum application rate of a given pesticide depending on the 885 guideline in question. These concentrations are not only meant to provide sufficient doses to detect toxicity and pathogenicity but are also meant to be overly conservative and to clear active ingredients that are not toxic. Unfortunately, the maximum hazard dose treatment is often the only treatment delivered in the test other than the negative and attenuated controls. Thus, if effects are observed during the test, there is uncertainty concerning the concentration at which effects would not be expected. The 885 guidelines stipulate that additional testing at multiple levels is recommended if adverse effects are observed; however, follow up tests are often not conducted. The inability to establish a lower limit of effects creates significant uncertainty about the impact of different exposure scenarios on the environmental risk profile of the active ingredient and may impact the consistency of the interpretation of test results.

Few environmental residue studies exist for microbial pesticides to inform the relationship between the maximum hazard dose and the duration and route of exposure to actual on-field pesticide residues. Many microbial pesticides optimally need to be ingested to be effective so understanding how the estimated environmental exposure, which is calculated as the microbial pesticide concentration that results from application at the highest rate, relates to actual exposure in the field through either consumption or contact would be particularly informative. For those active ingredients such as contact pathogens, that are effective

through contact exposure, improved understanding of the exposure threshold and duration needed for efficacy would help refine risk assessments. Tests attempting to achieve the maximum hazard dose often encounter problems achieving sufficient suspension and homogeneity in the test matrix that ultimately results in complications with how test species are exposed to the a.i. These challenges are particularly evident when conducting tests on fish and aquatic invertebrates as well as on terrestrial invertebrates and bees.

3.1.3. Challenges with Aquatic Testing

The nature of microbial pesticides presents several logistical challenges when conducting aquatic toxicity tests. Often microbial pesticides have low solubility which impacts exposure and test vessel conditions that may interfere with treatment responses. The high concentrations of microbes necessary to achieve a maximum hazard dose of up to 1000 times the estimated environmental exposure often results in high levels of turbidity within the test vessel. This increased turbidity potentially impacts the physical (light) and chemical (dissolved oxygen) parameters of the test. Elevated turbidity has also been associated with adverse impacts to survival and reproduction and can physically interfere with the test organism by adhering to carapaces of aquatic invertebrates and suffocating them or physically interacting with the gills of fish to stress the test organism. The potential adverse effects of turbidity make it difficult to discern whether the reduced survival and reproduction in a test were cause by high turbidity or pathogenicity/toxicity of the active ingredient. Aggregation of the test material may also make it difficult to maintain appropriate exposure levels and uniformity of exposure during the test. Clumping of the pesticide in test vessels may be reduced by use of surfactants, but surfactants may cause adverse effects and necessitate additional controls.

Better information and guidance are needed to improve aquatic testing. Improved understanding of when microbial pesticides are expected to persist in aquatic systems may inform when tests are needed, the duration of tests, and what concentrations need to be tested. When tests are conducted, additional examination of turbidity levels and their impacts on increased mortality or reduced reproduction could inform appropriate doses relative to the maximum hazard dose. In order to do this, testing multiple treatment concentrations rather than a one treatment limit test may be appropriate and inform concentration related changes in turbidity. Viability in the test vessel is also not often confirmed. Verifying viability would help inform exposure. Since viability may be difficult to verify in the test due to time and effort intensive assays, increasing the frequency of water renewal may improve exposure to viable test material. This information may improve interpretation of tests when observed effects cannot be conclusively attributed to the pesticide in question. Since keeping the active ingredient suspended and creating unrealistically turbid water columns when testing at the maximum hazard dose may be impractical, one potential improvement to aquatic testing in general may be to explore utilizing other test organisms, such as sediment dwelling invertebrates. Exposure in the sediment may be more appropriate when examining microbial pesticides that have a propensity to settle out and deposit in sediment environments. Finally, incorporating sufficient controls will help interpretation of the test. Both sterile filtrate and attenuated microbe controls as well as proper controls when surfactants are used will help interpretation of the test results.

3.1.4. Challenges with Nontarget Insect Tests and Honeybees

Nontarget insects and honeybees are exposed to microbial pesticides through both contact and diet in the field. For both nontarget insects and honeybees, conducting tests that challenge the organism at the appropriate treatment levels and exposure durations remains problematic. Improved information regarding environmental exposure and persistence would enhance the ability to parameterize toxicity tests. 885 test guidelines recommend testing insects at a maximum hazard dose of 10-100 times the estimated environmental concentration. Without better information about residues of microbial pesticides in the field,

the estimated environment concentration is typically calculated as the concentration that results from the highest application rate. While the recommended concentration is designed to be protective, it is unclear how close it approximates actual exposure for terrestrial insects either through dietary or contact exposure.

Many, but not all, microbial pesticides are only effective if ingested. The exceptions to these are contact entomopathogens where contact tests may be more appropriate but may not provide additional significant insight to effects as the mode of action, host range, and effects are well established. Contact tests may also be important in some cases where metabolites such as chitinases and biosurfactants are produced that may be active through contact exposure. For microbial pesticides that are only efficacious through consumption, dietary exposure when testing microbial pesticides can present challenges due microorganisms as test material are not soluble. Incorporating microbes into the treatment diets in ways that do not impact the palatability of the diet to the test organism is key to optimizing test exposure. Potential issues in palatability of the diet may impact the interpretation of the test when exposure is so long that organisms become stressed due to the inability to consume sufficient diet to maintain health. The consequent increase in growth, reproduction or survival from insufficient nutrition complicates interpretations of test mortality being due to pathogenicity/toxicity of simply husbandry issues.

Lack of information on environmental persistence of microbial pesticides also presents challenges when designing toxicity tests. In lieu of this information the duration of toxicity tests is designed to account for pesticide persistence and typically last 30 days. The longer test duration is also designed to provide sufficient time in order to detect possible pathogenic effects that take longer to manifest than direct toxicity to the test organism. There is little information in the 885 guidelines, however, concerning the exposure duration of test organisms, so tests have been submitted with exposure windows from several hours to several days to continuous exposure throughout the test. The conservatism of the longer and continuous exposure windows is preferred without more information concerning the persistence of the pesticide in the field but appropriately delivering the pesticide in a dietary matrix over longer time scales may be problematic and costly. The longer durations of exposure may also complicate the ability to assess if the true effects of the active ingredient result from pathogenicity or toxicity or are simply the result of the difficulty in maintaining organism health over longer durations. .

Most tests are designed as a limit test using only one maximum hazard dose concentration. While the 885 guidelines recommend additional testing at multiple levels if effects are observed at the maximum hazard dose, additional testing is often not conducted. In cases where effects are observed and additional concentrations were not tested, the uncertainty regarding how exposure relates to potential impacts in the field is difficult to characterize. Testing at the maximum hazard dose may overwhelm the test organism and cause toxicity effects before any observations of potential pathogenicity might occur. Additionally, the lack of solubility of most microbial pesticides complicates incorporation into the dietary matrix and complicated interpretations may be alleviated by testing at multiple levels. More routinely conducting tests using multiple concentrations may mitigate some of the challenges encountered in non-target insect and bee testing. Creating treatments with lower concentrations may allow incorporation of the pesticide into the dietary matrix with greater ease. Additionally, exposure to lower concentrations may also facilitate observations of potential pathogenicity when frank toxicity is less likely to occur over the course of the test.

The ability to detect pathogenicity depends on sufficient duration of the test and reduction of complicating factors such as lack of palatability to the test organism or potential direct toxicity to the test organism. The question of appropriate duration is particularly evident in toxicity tests with honeybees. The 885 microbial test guideline recommends a test duration of 30 days. This duration is difficult to achieve without unreasonable control mortality that calls into question the interpretation of the test. Tests with conventional pesticides, by contrast, have recommended test durations of 10 days due to husbandry issues beyond this time when insects are fed a sucrose solution. Questions remain as to whether 10 days is a sufficient test duration to detect potential pathogenicity should it occur or if the test duration should fall somewhere between the 10 day and 30-day test duration.

One additional complicating factor for non-target insect testing is the lack of standardization of which insects should be tested. The 885 guideline indicates that testing should be performed on three species of insects, representing at least two of the following groups—parasitic dipterans, predaceous hemipterans, predaceous coleopterans, predaceous mites, predaceous neuropterans, parasitic hymenopterans. Depending on the species selected, variations in protocols may exist that influence husbandry of the organism and delivery of the pesticide to that organism.

3.1.5. Conclusions

Additional information is required to improve toxicity testing with microbial pesticides, particularly with aquatic taxa and non-target insects and bees. Improved understanding of the environmental fate of microbial pesticides would help inform exposure and contribute to parameterizing complex toxicity tests. Ideally exposure matrices should be adapted to optimize exposure based on the microbe, exposure profile and dosage desired. Additional information concerning the duration of the tests and levels of dosage required to examine pathogenicity compared to those required to examine toxicity may help improve test design and interpretation to support assessment of potential environmental risks.

3.2. Canadian Regulatory Requirements for Microbial Pesticide Non-target Organism Testing

Emma Babij, Pest Management Regulatory Agency (PMRA), Canada

Microbial pesticides are subject to the Pest Control Products Act and Regulations in Canada. In order to be registered, a product must demonstrate acceptable risk to human health and the environment when used according to its prescribed conditions of use. Under the Act, environmental risk is defined as the possibility of harm to the environment, including its biological diversity, resulting from exposure to or the use of the product. Canada's Pest Management Regulatory Agency takes a four-tiered approach to environmental testing of microbial pesticides, with higher tiered studies required if significant adverse effects are identified at lower tiers. The basis for the risk assessment is environmental toxicology testing on a broad selection of non-target organisms such as birds, mammals, fish, arthropods, non-arthropod invertebrates, microorganisms, and plants. Infectivity, pathogenicity, and toxicity are evaluated as adverse effects. Environmental fate data and additional testing such as acute toxicity, definitive toxicity, and chronic toxicity testing may be required as part of higher tiers. The submission of surrogate or published data, or robust waiver rationales based on sound scientific principles, may be considered to satisfy the regulatory requirements.

3.2.1. Background

Microbial pesticides are regulated by the Pest Management Regulatory Agency (PMRA), under the authority of the Pest Control Products Act and Regulations in Canada. In order to be registered, a product must demonstrate acceptable risk to human health and the environment when used according to its prescribed conditions of use. The registration requirements outlined in the Guidance for the Registration of Microbial Pest Control Agents and Products (PMRA, 2021) were developed to address the assessment of human health risk, environmental risk, and value of end-use products that contain naturally occurring and genetically modified bacteria, algae, fungi, protozoa, viruses, and related organisms, as the active ingredient. Because of the specific nature of these active ingredients, PMRA's assessment of these products includes an evaluation of aspects such as product characterization, manufacturing and quality control, which are distinct from those of conventional pest control products. The organisation of the information parallels that for conventional pest control products while taking into account unique aspects of these types of active ingredients, such as biological properties, host range, potential pathogenicity, infectivity and the abilities to persist, multiply and disseminate in the environment.

Because microbial pest control agents (MPCAs) represent a diverse range of microorganisms, not all studies or data requirements may be appropriate for a specific microorganism. The unique characteristics of a microorganism should be considered when addressing specific data requirements and protocols, and applicants are encouraged to consult with the PMRA before testing begins. In addition, waivers requests for certain data requirements will be considered when accompanied by a sound scientific rationale.

3.2.2. Environmental Hazard Testing

Part M9 of the Guidance prescribes the data requirements for assessing the potential environmental hazards of a microorganism intended for pest control registration. Specifically, environmental toxicology testing is required by the PMRA to predict possible adverse effects of MPCAs and added ingredients, (i.e., formulants) in end-use products, on such broad groups of non-target organisms (NTOs) as birds, mammals, fish, arthropods, non-arthropod invertebrates, microorganisms, and plants. Possible adverse effects can be expressed in terms of infectivity, pathogenicity or toxicity, and hypersensitivity. Infectivity describes the capability of an MPCA to invade and persist in a viable state or multiply within or on an organism, with or without disease manifestation. Pathogenicity or toxicity is expressed as direct injury to an organism of an acute, subacute, or chronic nature, as a result of the actions of the MPCA or its toxins. Hypersensitivity refers to the potential for an MPCA to initiate severe local tissue damage via the immunological consequences of exposure.

3.2.3. Tiered Approach

A four-level tiered approach to environmental testing of microbial pesticides is a feature of the environmental toxicology and fate testing requirements of the Guidance. The extent of the environmental toxicology testing requirements at each tier is dependent on whether the MPCA is indigenous or non-indigenous to the ecozone of intended use. Canada has been divided into five distinct ecozones representing the major agricultural and forestry land areas where microbial pesticides are most likely to be applied. The boundaries between ecozones are viewed by the PMRA as transitional areas, rather than distinct lines of demarcation. Ecozones are used by the PMRA to determine the extent and the nature of environmental testing required for both registration and experimental field trials. For example, a microorganism is considered indigenous if it has been isolated from or is known to occur in the ecozone(s) of intended use. Conversely, a nonindigenous microorganism is one that has not been isolated from or is not known to occur in the ecozone(s) of intended use. Genetically engineered microorganisms (e.g. modified through in vitro manipulation of genetic material) are generally classified by the PMRA as non-indigenous microorganisms.

Initial submissions from applicants for registration must address all testing requirements in Tier I. Tier I requires acute toxicity testing on up to seven broad taxonomic groups of non-target organisms.

Tier II requires environmental fate (persistence and dispersal) as well as additional acute toxicity testing of MPCAs. The Tier II data determines effects of the MPCA on susceptible non-target species, exposed at lower concentrations than in Tier I, and fate of the MPCA under laboratory or actual use conditions. In general, if significant adverse effects on non-target organisms are identified in Tier I tests with an MPCA that is non-indigenous to the ecozone(s) of intended use, then Tier II environmental toxicology and environmental fate testing are required.

In the case of indigenous MPCAs that produce significant adverse effects on non-target organisms in Tier I, environmental toxicology testing, but no fate testing, is required at Tier II. Fate testing of indigenous MPCAs, under field or actual use conditions, is required only if significant adverse effects on non-target organisms are observed during Tier II toxicology testing.

Tier III requires definitive toxicity testing (e.g., LC_{50}, LD50), as well as chronic toxicity (i.e., life cycle testing). Tier III toxicology testing (for example, definitive toxicity testing; chronic toxicity testing) is reserved only for non-indigenous MPCAs that produce significant adverse effects on non-targets in Tier II.

Tier IV requires experimental field testing of toxicity and fate. If results of concern are observed in Tier I, then the appropriate information from Tier II may be required, with progression to higher tiers as necessary. For any MPCA that produces significant adverse effects on non-target organisms in Tier II studies, Tier IV toxicology field testing is required to determine whether adverse effects are realized under actual use conditions.

3.2.4. Rationales to Waive Testing

The data requirements specified under Part M9 Environmental Toxicology of the Guidance apply to a wide variety of MPCAs with diverse biological properties, for example, viruses, bacteria, fungi, protozoa, algae, and as such may not always be appropriate for every MPCA. Some MPCAs may have unusual characteristics or atypical use patterns that would make particular data requirements inappropriate either because it would not be possible to generate the required data or because the data would not be useful in the evaluation of the MPCA's hazards. In cases where a data requirement appears inappropriate for the MPCA or end-use product to be registered, an applicant can request a waiver for that requirement.

To request a waiver, the applicant must submit a sound scientific rationale in lieu of a study. The request must specifically identify the data requirement for which a waiver is requested, explain why the data requirement(s) should be waived, describe any unsuccessful attempts to generate the required data, and, when appropriate, suggest alternative means of obtaining data to address the concern that underlies the data requirement. The waiver rationale must be supported by published literature search on toxicity/pathogenicity of the MPCA, and/or closely related microorganisms, to support claims of low risk to the specific non-target group (birds, fish, etc.). Environmental fate/expression data, for example, maximum growth temperatures, growth requirements, may also be used to support the scientific rationale if claims of low risk are based on an expected low level of exposure to the MPCA under operational conditions of use, for example, during application and post-application of the end-use product, or if the MPCA cannot grow at host body temperature. The PMRA will waive data requirements on a case-by-case basis in response to specific written requests from applicants.

3.2.5. References

PMRA (2021) Guidance for the Registration of Microbial Pest Control Agents and Products. https://www.canada.ca/en/health-canada/services/consumer-product-safety/reports-publications/pesticides-pest-management/policies-guidelines/guidance-registration-microbial-pest-control-agent-products.html

3.3. Where are we and where do we need to go? A Dutch perspective on the assessment of microbial pesticides

Emily McVey, Board for the Authorization of Plant Protection Products and Biocides (Ctgb), The Netherlands

This presentation focused specifically on the strategies for risk assessment and approval of products based on microorganisms, including viruses, of the Dutch Board for the Authorization of Pesticides and Biocides (Ctgb). In this context, the current data requirements, the evaluation of GLP, peer-reviewed, public, and grey literature and weight-of-evidence considerations thereof, were discussed.
Specific examples and strategies to address knowledge gaps and common problem points were given.

3.4. Regulatory Requirements for Non-target Organism Testing - Global Challenges

Roma Gywnn, Biorationale, United Kingdom

As microbial-based plant protection products become more established globally it raises the matter of whether the existing recommendations for non-target testing are taking into consideration the most appropriate organisms in all regions. For example, the soil fauna may be different in a tropical country and so the choice of species to test would be different to one used in a temperate country. So, if a dossier developed in the EU is submitted to Kenya and vice versa, for example, are the non-target organism types and species used in studies the most appropriate? How can evaluators from around the globe address this and what advice is available? It also has to be taken into consideration that expertise and resources available for evaluators to assess dossiers will be variable between countries, it is important to consider this when developing common approaches to work sharing reciprocity of data which are key components of a more streamlined regulatory system for microbials.

4 Session 4: Current Ecological/Non-Target Test Guidelines – Outlining the Issues

4.1. Aquatic Safety Studies with Microbial Pesticides – retrospective analysis and recent advancements

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Aquatic safety studies with microbial biocontrol agents (mBCAs) are frequently submitted in the context of regulatory risk assessments to provide information on potential toxicity, infectiveness and pathogenicity to aquatic organisms. In these aquatic studies the microbial test items are usually applied at relatively high concentrations. Due to the fact that microbes behave as particles that do not dissolve in test solutions, testing at high concentrations may cause several problems such as high turbidity or cloudiness (often in connection with physical effects) as well as water quality changes (e.g., drop of oxygen) which can negatively affect biological parameters of the test organisms. Quite often non-specific interference with physiological processes has been reported in prolonged *Daphnia* studies. This chapter provides a retrospective analysis of aquatic effect data submitted for regulatory purposes with particular focus on the choice of test species, testing issues and sensitivity to mBCAs. Furthermore, recent recommendations that emerged from the OECD background document on ecotoxicology are presented as well as recent findings from experimental *Daphnia* studies performed at UBA's aquatic testing facility.

4.1.1. Introduction

Over the last 10-15 years many problems became apparent in microbial pesticide testing with aquatic organisms. Testing issues in fish, algae, aquatic plants, and especially in prolonged *Daphnia* studies were

an important topic of discussion at EU and international level (OECD, 2017; OECD, 2019). In order to identify potential test guideline limitations and possible solutions, a retrospective analysis of aquatic effect studies has been performed using data from EU evaluations of active substances in plant protection products. One of the frequent observations in the analysis was that the high microbial doses commonly tested in aquatic tests caused several problems such as high turbidity which negatively affected biological parameters of the test organisms. This was especially notable in prolonged *Daphnia* studies, the most commonly studied test species. As daphnids are nonselective filter-feeders they may ingest large amounts of spores or suspended particles that have size ranges similar to algae. These physical effects in connection with turbidity are often mentioned as likely cause of adverse effects which complicate study interpretations, especially if proper controls (e.g., attenuated controls, sterile filtrates) are lacking. In order to overcome these problems and to develop methods better adapted to microbial pesticides, experimental *Daphnia magna* reproduction studies with an approved microbial biocontrol agent have been conducted in UBA's aquatic testing facility using a modified test method on the basis of OECD TG No. 211 (OECD, 2012). This chapter provides, firstly, a retrospective analysis of existing microbial effect studies on aquatic organisms. Particular emphasis is placed on the choice of test species, common testing issues and sensitivity to mBCAs with particular consideration of fish and aquatic invertebrates studies. Secondly, recent recommendations from the OECD background document on aquatic studies are briefly summarized. Thirdly, recent findings from *Daphnia* studies with an EU approved microbial pest control agent are presented, considering the recommendations made in the previous EGBP meetings and seminars to address common testing issues.

4.1.2. Retrospective analysis of aquatic effect data

Description of the dataset

Retrospective analysis of aquatic effect data was performed on the basis of EU approved microbial active substances (m.a.s.). Information on the approval status of m.a.s. was obtained from the EU Pesticides Database (EU-Pesticides-Database, 2022; last accessed on 8 August 2022). Available aquatic effect data submitted for each m.a.s. was collected from EFSA Conclusions including list of end points for the active substance and the representative formulation (e.g., *Beauveria bassiana* strain 203 (EFSA, 2020)). Detailed study information, particularly on testing issues, was obtained from the respective Draft Assessment Reports (DAR) and Renewal Assessment Reports (RAR).

Sixty-seven microbials (including viruses) were approved in the EU as active substances at the time of data collection in 2022, out of which 42 contained strain-specific ecotox data and thus were considered in the retrospective analysis. The remaining 25 microbials (including viruses) did not contain any strain-specific aquatic ecotoxicity data or ecotox data at all due to read-across, negligible exposure (e.g., trunk-injection treatments) or data waivers (e.g., plant viruses).

The 42 approved microbial active substances mainly belonged to fungi (n=31; 46%), followed by bacteria (22; 33%), baculoviruses (5; 7.5%), plant viruses (5; 7.5%), yeast-like fungi (3; 4.5%), and oomycetes (1; 1.5%). They comprised the following functions: fungicide (33; 49.25%), insecticide (33; 49.25%), viricide (5; 7.5%), nematicide (4; 6%), fungicide+bactericide (1; 1.5%), and insecticide+acaricide (1; 1.5%).

In total, the data set for the analysed 42 microbials (including viruses) contained 161 aquatic ecotoxicity studies, mostly performed in accordance with OPPTS and OECD test guidelines, including: aquatic invertebrates prolonged (n=38; 24%), algae (36; 22%), fish prolonged (28; 17%), aquatic invertebrates acute (28; 17%), fish acute (24, 15%), and macrophytes (7; 4%).

The analysis revealed that mostly rainbow trout (*O. mykiss*) studies are submitted (Figure 4.1), the only cold water fish (10-14°C) among tested fish species. Since the remaining eight species of warm-water fish (>20°C) are much less represented, further considerations might be required as regards potential microbial growth of mesophilic or psychrophilic mBCAs.

Among aquatic invertebrates, typically water flea (*D. magna*) studies are submitted (Figure 4.2). Very few studies comprised other taxa, namely grass shrimps (Crustacea), Eastern oyster (Bivalvia), mayfly (Ephemeroptera) and marine harpacticoid copepod (Crustacea). There was no indication of higher sensitivity to mBCAs when compared to *D. magna* study endpoints.

Except one study with mixed algal cultures, the green algae *P. subcapitata* (n=20), *D. subspicatus* (n=15) were the only species tested among algae. Macrophytes studies were available to a much lesser extent and the test species were *Lemna gibba* (n=5) and *Lemna minor* (n=2).

Figure 4.1. Number of acute (a) and prolonged (b) fish studies

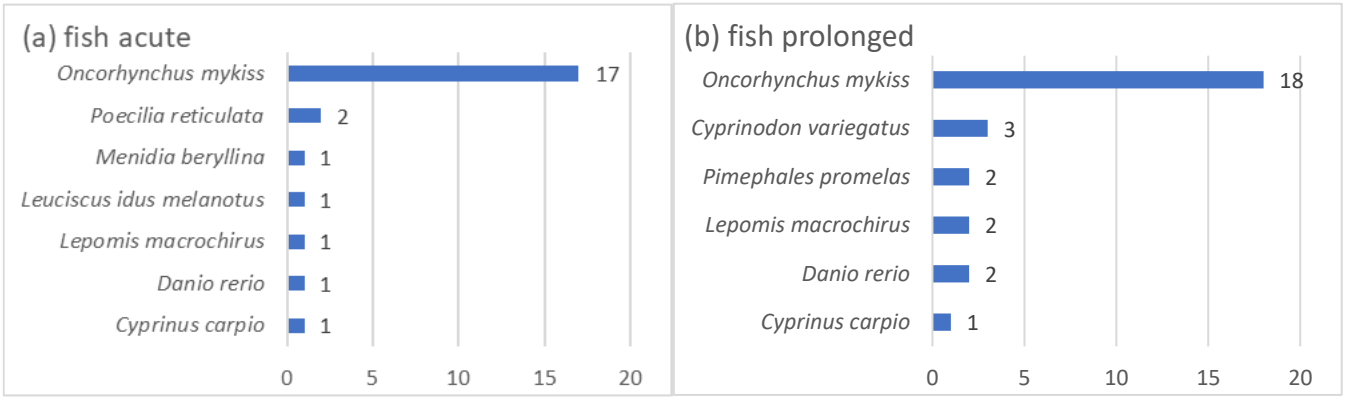
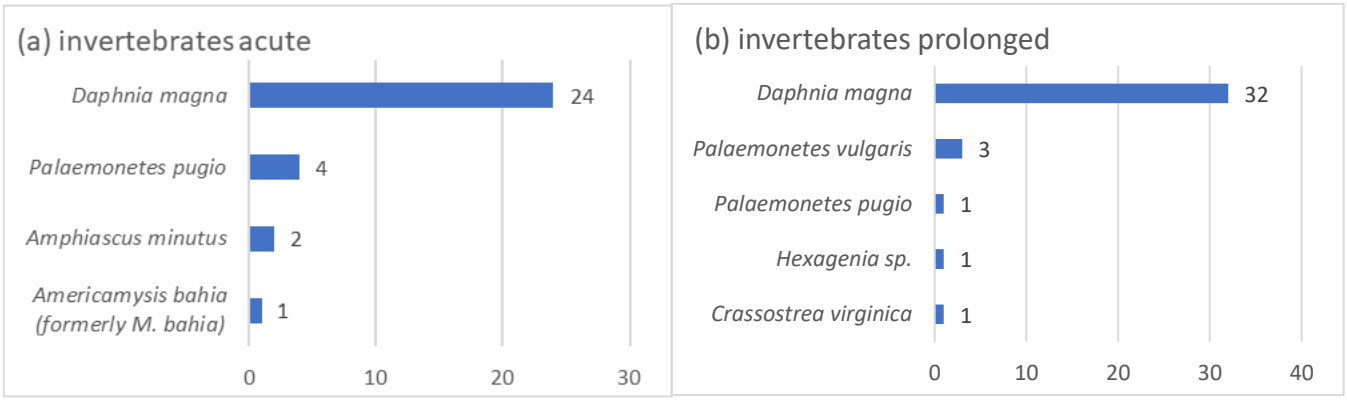


Figure 4.2. Number of acute (a) and prolonged (b) aquatic invertebrates studies



Based on the above, no reliable statements can be made regarding sensitivity differences among test species. For instance, there was no evidence for higher sensitivity in rarely tested species compared to the commonly tested species (rainbow trout or water flea). It is striking, however, that a few taxonomic or functional groups are completely lacking in the analysed dataset including benthic organisms such as *Chironomus riparius* (Diptera), *Hyalella azteca* (Amphipoda), *Lumbriculus variegatus* (Oligochaeta) or freshwater snails such as *Potamopyrgus antipodarum* and *Lymnaea stagnalis* (Mollusca). This is surprising, especially as OECD test guidelines are available for these organisms and exposure is considered likely in many cases.

Reported testing issues

According to the study summaries provided in the DARs and RARs, various testing issues were reported. However, it should be noted that the data collection on testing issues cannot be exhaustive in view of the varying degree of detailed reporting. The outcome of the present analysis on study limitations and testing issues can at least give clear indications or trends of general or test-specific issues related to aquatic studies.

In the first step of the analysis, testing issues or limitations were compared between prolonged fish and *Daphnia* studies. The reason for focusing on prolonged *Daphnia* and Fish studies was their main contribution in the data set and their particular relevance as regards infectivity and pathogenicity assessments. Testing issues or limitations reported in fish studies were mainly: lack of microbial controls (~45%), turbidity/cloudiness/coloration of test solutions (~31%), unknown cause of effects (~21%), highly variable CFU counts (~21%), and physical effects (~21%).

The situation was relatively similar in prolonged studies with *Daphnia magna* which reported the following issues or test limitations: lack of microbial controls (~33%), physical effects (~26%), poor suspensibility/inhomogeneous distribution/clumping (~21%), highly variable CFU counts (~21%), turbidity/cloudiness/coloration of test solutions (~15%), and unknown cause of effects (~13%).

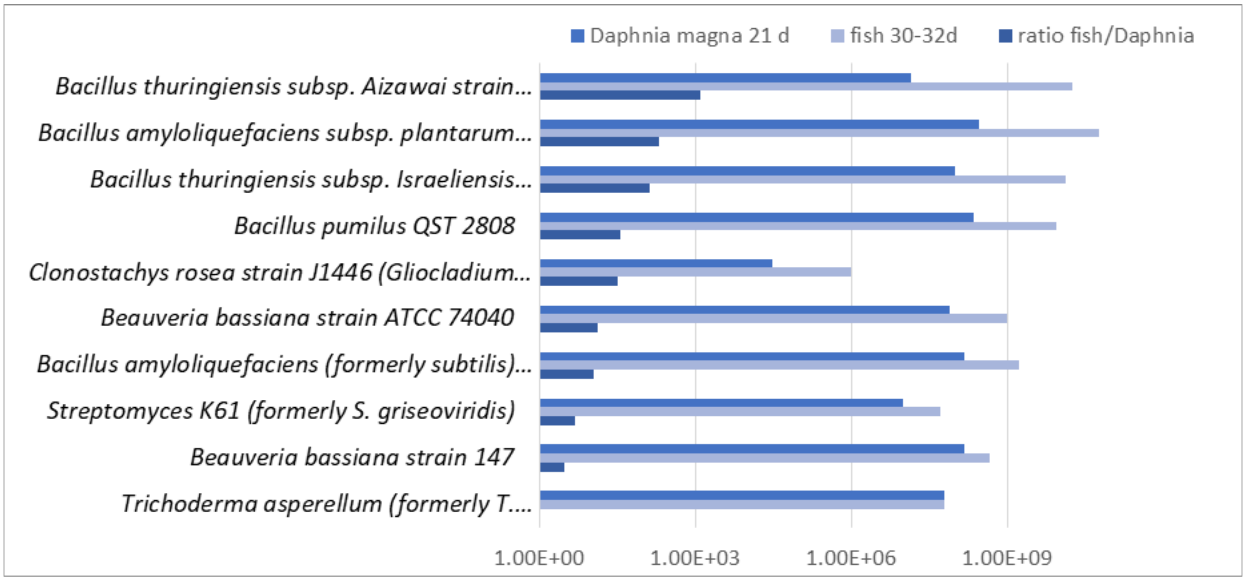
In the second step of the analysis, testing issues or limitations were compared between the main types of microbial biocontrol agents (mBCAs) approved in the EU, namely fungal and bacterial biocontrol agents. When the two types of mBCAs (fungi vs. bacteria) are compared, it can be seen that the reported testing issues and limitations were quite similar in terms of both type and extent. One striking difference, however, was the different outcome of highly variable CFU counts in both mBCAs types (6% in bacteria compared to 33% in fungi). This result might be explained by the fact that CFUs of bacteria can perhaps be more easily quantified using agar plates compared to fungal BCAs such as *Trichoderma* sp.

As a consequence of high turbidity associated with high test doses, the following issues were noted in algae studies: (i) Light decrease/Absorbance of photosynthetically active radiation by the test item, causing algal growth inhibition (e.g., reported for *Bacillus amyloliquefaciens*, *B. firmus*, *Beauveria bassiana*, *Metarhizium brunneum*), and (ii) competition of nutrients between algae and growing spores in the nutrient media (e.g., reported *for B. amyloliquefaciens*, *M. brunneum*).

Sensitivity to mBCAs (Fish vs. Daphnia)

Effect values from prolonged fish and *Daphnia* studies were compared in order to obtain information on the relative sensitivity of the non-target species towards various mBCAs. Based on the NOEC values available, *Daphnia magna* turned out to be more sensitive than fish in almost all cases (Figure 4.3). The results clearly show that *Daphnia magna* studies usually provide the decisive endpoints in the risk assessment for aquatic organisms.

Figure 4.3. Sensitivity to MPCAs (Fish vs. *Daphnia*). NOEC values are expressed as cfu/L.



4.1.3. Recent recommendations emerged from the OECD background document

The draft paper "Microbial Pesticides – Challenges and Future Perspectives for Nontarget Organism Testing" that was provided as a background document to the OECD conference on Innovating Microbial Pesticide Testing (13-16 September 2022) provided an overview on specific limitations in the current aquatic test guidelines when testing microbial pesticides. It was concluded that several test guidelines (whether OPPTS or OECD TG) were generally fit for purpose once the required modifications are made. In case of OPPTS TG, the need for expanded study descriptions and validity criteria was apparent. Since OECD TG were developed for testing chemicals, a number of adaptations have to made for microbial biocontrol agents, such as the inclusion of attenuated/sterile filtrate controls, appropriate methods for dose verification (cfu counts), methods for infectivity or pathogenicity assessments and dietary exposure.

4.1.4. Recent advancements in the field of aquatic testing, specifically Daphnia magna studies

Experimental *Daphnia* studies have been conducted with the test item *Bacillus thuringiensis* aizawai ABTS-1857 (XenTari Tech powder; provided by Valent Bioscience) taking into account recommendations made in previous EGBP meetings to address common testing issues such as physical effects. The aim of the project was to obtain a better understanding of the cause of effects in *Daphnia* studies.

The study was based on an adapted OECD TG 211 (*Daphnia* Reproduction Test), whereas the following adaptations were made: (i) Use of sterile filtrates and attenuated controls, (iii) increased food levels, (iii) coarse filtering prior to testing (i.e., filtering of fermentation residues in order to remove larger particles that would settle to the bottom anyway). The basic assumption was that increased food levels could compensate possible "starvation effects" in prolonged *Daphnia* studies. Another basic assumption was that coarse filtering may reduce physical effects caused by larger particles at high test doses.

A series of pre-tests were conducted focusing on production of stable suspensions, visual observations and cfu counting methods. The results are summarised below.

Results from the first pre-test indicate that increased food levels (i.e., 10 times higher than recommended in OECD TG no. 211) can be used for the definitive test (Table 4.1) as sufficient reproductive output was shown. However, as slight increase in mortality was noted with increasing food levels.

Table 4.1. Results from pre-test 1: Investigating different food levels

Test group	Mortality	Corrected Mort.	Reproduction	Inhibition
1 (standard food level) - control	5.9%	--	112.31	--
2 (5x standard food level)	20%	14.1%	148.38	-32.1%
3 (10x standard food level)	30%	24.1%	134.43	-19.7%
4 (50x standard food level)	30%	24.1%	84.71	24.6%

The second pre-test revealed that both autoclaving and sterile filtration can effectively be used in the definitive test. Furthermore, filtered test item group with a filter sizes of 10 µm and 20 µm can principally be used (Table 4.2).

Results from the definitive test showed high immobility (mortalities) regardless of the various adaptations made, i.e., increased food levels and coarse filtering (Table 4.3). Moreover, the influence of different feeding levels was not consistent between pre-test and definitive test: In the pre-test, immobility was 30% (too high) and reproduction was acceptable (higher than control). In contrast, the definitive test resulted in immobility of 20% (acceptable) and considerably lower reproduction compared to the untreated control. Increased food levels (10-fold) as possible adaptation step appears questionable due to high variability in reproductive outputs.

Table 4.2. Results from pre-test 2: Influence of filter sizes and heat-inactivation (autoclaving) on CFU numbers

Test group	description	results
1 (untreated control)	Stock solution at MHD	Viable CFUs (nominal values reached)
2	Autoclaving at 121°C	No viable CFUs
3	Sterile filtration with 0.2 µm	No (or negligible) viable CFUs
4	Coarse filtering with 5 µm	Significantly lower CFUs than in the untreated control
5	Coarse filtering with 10 µm	CFUs comparable to the untreated control
6	Coarse filtering with 20 µm	CFUs comparable to the untreated control

Table 4.3. Results from the definitive test: Impact of TG adaptations on biological parameters of *D. magna*

Test group	Feeding	Mortality	Reproduction	Length after 21 days
control-1	Standard	0 %	120.3	Mean 4.45 mm, CV 1.9 %
control-2	10-fold	20 %	41.4	Mean 4.20 mm, CV 6.7 %
test item-1	Standard	100 %	0.0	
test item-2	10-fold	100 %	0.0	
sterile filtr.-1	Standard	100 %	0.0	
sterile filtr.-2	10-fold	100 %	0.0	
autoclaved-1	Standard	10 %	20.3	Mean 3.99 mm, CV 7.4 %
autoclaved-2	10-fold	10 %	17.0	Mean 4.32 mm, CV 3.1 %

The biological results in the treatment groups including microbial controls (sterile filtrate/autoclaved control) suggests that adverse effects on survival was most likely due to toxic metabolites/toxins produced in the fermentate.

Reproductive effects in heat-inactivated (autoclaved) control were perhaps caused by an increase in toxicity after heat-treatment. This increase in toxicity can be explained by the fact that the heating procedure may alter the physical nature and ecotoxicological properties of the test item which complicates interpretation of test data (Borges et al., 2021).

Based on these findings, follow-up studies will be performed considering different microbial test items (e.g. *Pseudomonas chlororaphis* strain MA342) and possibly also different non-target species.

Taken together, proposals can be derived for improving test methods for aquatic organisms on the basis of future testing activities in conjunction with existing knowledge from regulatory aquatic safety studies.

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4.2. Testing Microbial Pesticides in Bees - Challenges and Current Advancements

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Several microbial plant protection products (PPPs) have been developed as alternative to chemical PPPs, since growing concerns regarding the adverse effects of chemical PPPs on environment and non-target organisms have been reported. In contrast to chemical PPPs, they present special challenges in non-target organism testing, including bees, and risk assessment depending on their modes of action include infectivity and pathogenicity rather than toxicity alone. As the current testing guidelines

are designed for testing of chemical pesticides, i.e. toxicity, they are not necessarily applicable to microbial pesticides without some modifications. Therefore, this presentation discussed several issues regarding the possible modifications of the current test guidelines. For instance, how the test duration or additional pollen feeding will affect the test results to detect the possible infectivity and pathogenicity. Otherwise, and due to the differences in colony and nest temperatures of different bee species as well as the different growth temperatures of the applied bacteria and fungi, some research results of our lab were interpreted on the effect of testing conditions, i.e. temperatures, on the effects of some microbial pesticides on different bee species. Finally, some possible approach was shown regarding the estimation of the exposure level and duration.

4.2.1. Introduction

Several microbial plant protection products (PPPs) have been developed as alternatives to chemical PPPs since growing concerns regarding the adverse effects of chemical PPPs on the environment and non-target organisms have been reported. In contrast to chemical PPPs, microbial PPPs are accompanied by challenges in non-target organism testing, including bees, and the risk assessment related to their modes of action, including infectivity and pathogenicity, rather than toxicity alone. As current test guidelines are designed to test effects of chemical pesticides, i.e., toxicity, they are not necessarily applicable to microbial pesticides without modification. Therefore, several issues regarding possible modifications of the current test guidelines were reported by Borges et al. (2021). However, different knowledge gaps were identified, including the environmental exposure level and duration as well the impact of field conditions compared to the in-hive conditions, which make the detection of pathogenicity a challenging process. On the other hand, Erler et al. (2022) reviewed the available literature and summarized the results of different experiments investigating effects of microorganisms on bees. They conclude that the experimental design, including application method, bees' age, study duration, nutrition of bees, and environmental conditions, can affect the outcome and should be considered when interpreting the results of microbial studies. Thus, an appropriate test procedure is required to ensure reliable and valid results, which can reduce uncertainties in the risk assessment.

In the following sections, possible approaches will be discussed depending on the research conducted at the Julius Kühn Institute. Furthermore, the needed research will be indicated, which will help to cover existing knowledge gaps.

4.2.2. Possible solutions to some specific issues

Test duration to detect pathogenicity

Our results show that a test duration of 48-96 h, which is typically used in acute toxicity testing for chemicals, is not sufficient to cover the pathogenicity of the microorganisms depending on the period needed for the reproduction of the microorganisms within the host and to result in detectable effects. The biological properties of the tested microorganisms play a critical role in defining the optimal test duration. According to OPPTS 885.4380, observation periods should be up to 30 days for the assessment of microbial pesticides, but specific information regarding the control mortality criterion is not provided. Depending on the short longevity of bees, this period may be related to adverse effects caused by the test

method itself, i.e., an elevated control mortality. Our results show that a test duration between 15-20 days can be sufficient to detect effects without method-related adverse effects. In this case, the control mortality should not exceed 20%. Further tests are needed to validate such criteria.

Exposure duration (acute vs. chronic) and test concentrations

Maximum hazard doses (MHD) or the maximum hazard concentrations (MHC), which are up to 10–100 times the recommended field dosage or tank mixture concentration, are recommended to be tested (OCSPP 885.4380; Environment Canada, 2004). However, toxic effects related to the high dose might limit the detection of pathogenic effects. Recently, a field study was conducted to investigate the exposure level of bees after the application of a *Bacillus thuringiensis* (Bt)–based product (Alkassab et al. 2022). The results show that chronic exposure can occur within the colony, as no reduction in the number of CFUs was observed over time. However, the detected concentrations were below the applied ones. This indicates that concentrations lower than MHC can be considered in the repeated exposure tests, which may be useful for detecting pathogenic effects. Our data also shows that an exposure duration of 10 days can be considered sufficient. In this case, a further observation period, i.e. at least 5 days, can be applied to cover a test duration of at least 15 days or until 20% control mortality is achieved.

Effect of diet during testing on adult bees

Special consideration should be given to enhance the survival of bees to meet the control mortality criterion. According to OECD Guideline No. 245, bees receiving sugar solution only can survive ten days with control mortality of ≤15%. An extension of the test duration can lead to an increase in control mortality, as the bees suffer due to a lack of proteinaceous nutrition. Our results show that until day 15, the mortality without pollen feeding is mostly below the acceptable threshold of 20%. Any further extension of the test duration needs additional pollen feeding to ensure a higher survival of tested bees and to meet the control mortality criteria.

Effect of temperature during testing on adult bees

Test conditions to which bees are subjected can affect the results. Therefore, special consideration should be given to the temperature during the test, which may affect the growth of the microorganism. In this context, different temperature ranges are recommended in OECD guidelines, where adult bees should be maintained at 25 ± 2°C (OECD, 213) or 33 ± 2°C (OECD, 245). These differences depend on the age of the tested bees. Whereas newly emerged bees are used in OECD Guideline No. 245, bees with an undefined age collected outside the brood nest are used in OECD Guideline No. 213. As the temperature can affect the growth of the tested microorganism, we investigated the response of bees under different climatic conditions after exposure to entomopathogenic fungi and bacteria. The results show that 33°C is a favour for the growth of bacteria but not for fungi. Thus, testing below 26 °C may be optimal for bees and tested microorganisms, but validation with further tests in different laboratories is needed.

Additional treatment groups

To enable a distinction between toxic and pathogenic effects, additional treatment groups have to be tested, e.g., inactivated test item groups, sterile filtrate groups, and negative control/vehicle control groups (Borges et al. 2021). Our results show that in some cases, autoclaving one time at 121 °C for 20 min is not sufficient to completely inactivate the test item. Autoclaving twice may be needed to ensure an inactivated test item, especially for bacteria.

4.2.3. Future required research activity

Testing of other bee species

Studied species by now are (*Apis mellifera and A. cerana*), a few species of bumble bees, very few stingless bee species, and only a single species of leafcutter bees (Erler et al. 2022). Thus, more research is needed to compare the exposure and response of solitary bees and evaluate if *Apis mellifera* is protective for a broad range of *Apis* and non-*Apis* bees.

Modelling approaches

Modelling methods can help to estimate the spread and persistence of microorganisms and their effects at the colony/population or community levels (Borges et al., 2021). Simulation models can account for dynamic environmental conditions such as climate, habitat, and additional stressors. However, more field data are needed to calibrate and validate such models.

4.2.4. Conclusion

The above-mentioned issues show that the current available OECD test guidelines, which were developed for testing of chemicals´ toxicity provide a useful basis for testing with microbials. However, several major modifications have to be considered, which help to maintain the validity criteria. Therefore, further tests with an adapted test design are required to set new validity criteria and define treatment groups, exposure level, and endpoints. The international commission on plant pollinator relations (ICPPR)-working group on microbials and bee testing is currently aiming to develop a new test design to address several issues related to the testing of microbials and bees. This, in turn, will provide reliable and valid results, which can reduce uncertainties in the risk assessment.

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4.3. Issues with Non-target Insect Testing with Microbial Pesticides - CRO perspective

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The current regulatory approach to ecotoxicology testing for microbial pesticides is identical in almost every respect to the approach developed for agrochemicals. It takes no account of the respective biology of either the microorganism or the test species, nor of the ecological relationships that have developed between them over evolutionary time. As a consequence, much of the standard ecotoxicology testing mandated for this class of plant protection product is entirely redundant from the perspective of meaningful risk assessment. This presentation looked at the extent to which the current regulatory approach to non-target arthropod testing for microbial pesticides can be considered truly scientific, and proposed some suggestions by which any genuine environmental risk posed by these products can be better evaluated.

The commercialisation of microbial pesticides in Europe has long been hampered by a regulatory framework that was designed for the evaluation of synthetic chemistry rather than for living microorganisms. Problems pervade every aspect of the process, from the well-publicised issues at the evaluation stage down to the execution of ecotoxicology studies and even the suitability of standard test species for environmental safety testing.

No genuine scientific thought was put into the development of the ecotoxicology requirements for microbial pesticides, and the test species and study guidelines are taken directly from the chemical requirements. Chemical pesticide legislation evolved to regulate substances that for the most part do not naturally belong in the environment, and to which non-target organisms would not normally be exposed. Microbial active substances, by contrast, are isolated directly from the environment and have coexisted with non-target organisms over evolutionary timescales. This is a non-trivial distinction which, together with a sound underpinning of entomology, ecology and microbiology, is key to identifying those studies that can be adapted for use with microbial pesticides and those that will never provide meaningful risk assessment data, regardless of how they are modified.

Ecotoxicology studies on microbial pesticides introduce a second living organism into the test system, and the extent to which a study guideline can be made to accommodate the slower development of microbial pathogenicity rather than the more rapid onset of chemical toxicity is usually dictated by the biology of the

non-target test species. At one extreme, honeybee workers are quite amenable to much longer study durations than the standard 48 to 96 hour OECD Test Guideline 213 or 214 design, and extending these for up to 30 days can be achieved relatively easily with a few basic adaptations to the existing protocol. At the other extreme, the short time span between adult emergence and the completion of egg-laying in parasitic wasp species means that no amount of adjustment to the protocol will make it suitable for the detection of microbial pathogenicity.

The test protocol for Aphidius rhopalosiphi exposes adult female wasps to residues of the test item for 48 hours, after which a mortality assessment is made, and the surviving wasps are confined individually on aphid-infested plants. They are allowed to lay eggs in the aphids for 24 hours before being removed, with parasitism rates assessed 10 to 12 days later. However, female wasps mate within 24 hours of emergence and lay over 90% of their eggs during the following three days. Microbial pesticides very rarely have directly fatal or through-the-female effects this quickly, so adverse effects on survival or fecundity are very unlikely to be detected in this study. Furthermore, once the majority of eggs have been laid the female wasp serves no further ecological purpose, and the cause of her death – whether it be old age, predation or entomopathogen infection – is irrelevant. In common with the majority of non-target organisms an adverse result on A. rhopalosiphi is a dead end, with no meaningful follow-up study.

There are also cases in which a standard study protocol is theoretically capable of detecting pathogenic effects, but the test species is insufficiently sensitive. Earthworms are wholly unsuitable as indicators of microbial pathogenicity, living in the soil and consuming a diet of bacteria and fungi, and whilst their long-awaited removal from the microbial data requirements is to be welcomed, the same arguments apply equally to other members of their functional group. In common with earthworms, collembolans such as Folsomia candida are decomposers and shredders, consuming and converting organic matter in the soil. They too are adapted for life in the most microbially challenging environment on Earth and have a diet consisting almost entirely of soil microorganisms. They are host to a diverse array of commensal bacteria, and are members of a functional group for which microbial ecotoxicology testing makes very little sense. In laboratory studies, collembola in microbial treatment groups often show better survival and fecundity than those in control groups, essentially because the study tests nothing more than their ability to survive on their normal diet. The ability of Folsomia candida to consume microbial pesticides with no adverse effects on survival or reproductive output has been widely documented in the scientific literature and underscores the extent to which the species is a wholly unsuitable candidate for microbial risk assessment.

The difficulty of confidently ascribing pathogenicity as the cause of death remains a current problem in ecotoxicology testing, and several unsatisfactory approaches to this have been proposed over the years. These include dissection of cadavers to look for hyphal growth, or incubating dead insects to see if the test item can be recovered. This invariably picks up saprophytic post-mortem growth, as many non-pathogenic microorganisms will grow readily on insect cadavers. Furthermore, a rapid review of hundreds of terrestrial ecotoxicology studies conducted at APIS over the last few years revealed that for most non-target species, most of the time, adverse effects in test item groups are also seen at some level in sterile filtrate or attenuated test item groups from which the living microorganism has been removed. Although the direct toxicity or physical action of matrix components does not mean that the microorganism itself is not pathogenic, it does result in the test species succumbing before pathogenicity has a chance to develop.

The inclusion of attenuated treatment groups in general is also a frequent cause of unnecessary confusion. These consist of test items in which the living microorganism has been killed, leaving the physical properties of the material but without any biological activity. The most common method of producing attenuated material is by autoclaving, but the process can not only denature any heat-labile toxins present in the material but may also create new toxins in the form of thermal degradation products. It can also fundamentally change the physical properties of the material, making it difficult to pipette, impossible for non-target organisms to consume, or causing it to coagulate, disintegrate, sink or float when it didn't before. The clearest examples of how attenuated test item groups can lead to confusion are in algae and Daphnia magna, where adverse effects in test item vessels might be assumed to indicate toxicity or pathogenicity.

In the majority of cases, the particulate nature of the untreated microbial test item will increase turbidity, reducing photosynthetic capacity in algae and clogging the delicate feeding apparatus of Daphnia. The attenuated test item will often coagulate and sink to the bottom of the vessels as a consequence of autoclaving, leaving the water in these test units clear.

In contrast to the majority of non-target species we reviewed, the bumblebee (Bombus terrestris) and the honeybee (Apis mellifera) exhibited mortality in a high proportion of studies where direct toxicity or physical effects were easier to exclude as the mechanism of death. Both species are also amenable to higher-tier semi-field or field testing, making adverse effects in lab studies easy to investigate in more depth and under more realistic conditions of use, and both are suitable candidates for more sophisticated investigations of pathogenicity, such as tracking aspects of immune response via qPCR.

The extent to which appropriately designed studies on pollinator species are sufficiently protective of other non-target arthropods is worthy of further investigation as it potentially provides an opportunity not only to simplify the approach to microbial ecotoxicology testing, but to greatly improve the value of study data for risk assessment.

4.4. Issues with Aquatic Organism Testing with Microbial Pesticides - CRO perspective

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Contract Research Organisations (CROs) have extensive experience performing GLP aquatic toxicity tests with conventional chemicals, and to a lesser extent with microbial pesticides. Through experience with conventional chemicals, CROs have developed a keen understanding of how to conduct aquatic toxicity tests with conventional chemicals and maintain test concentrations for the duration of the test. Microbial pesticides have additional inherent challenges associated with conducting aquatic studies, specifically around delivery and maintenance of the test article in an aquatic system. For conventional chemicals, test concentrations are confirmed using analytical chemistry to quantify mean measured concentrations and guidelines have been published for setting and maintaining test concentrations. The physical/chemical properties and routes of exposure of conventional chemicals are key elements in successfully running a test and setting test concentrations. Microbial pesticides will need similar guidance but face unique challenges; you are no longer testing soluble chemicals in water, but dynamic living cells that act like particles in solutions. Concepts of solubility, stability and routes of exposure require new ways of thinking. Quantification and confirmation of the test item is more complex often requiring plating that yield results reported in number of colony forming units rather than mg product/L. In aquatic testing of conventional chemicals, the limit of solubility is often used to determine the highest test concentration. For many microbial materials,

solubility does not necessarily apply and the test material may actually grow and increase over the course of the test. Other issues with microbial products include limitations of carriers to aid in getting a product into solution, poor visibility in the test system, and low dissolved oxygen levels. Solvents are often used in aquatic testing to get materials into solution; however, such solvents can be highly toxic to microbial materials limiting their use. Reduced visibility can result when the product or carrier form cloudy water conditions making observations difficult during the test. Aeration may be required throughout the test to maintain oxygen levels in water due to increased oxygen demands of microbial products. Routes of exposure are also important considerations with microbial products (e.g. seed coating product vs. foliar applied product). As was done with conventional materials there is a need to work collaboratively among academia, government, and industry to develop better guidance for testing.

4.4.1. Introduction

There is growing interest in the testing of microbial pesticides (MCPA's) as their use has become more widespread, and because they are safer alternatives to conventional chemicals. The microbial pesticide guidelines for aquatic organisms were developed by the US in the mid-1990's and adopted by Canada and the OECD (OPPTS 850 series and Environment and Climate Change Canada 2016). While they are based on guidelines used for conventional chemicals, there are major differences that exist between conventional and microbial pesticides and those differences need to be acknowledged and incorporated into guidelines for MCPA testing with aquatic organisms.

One major difference between MCPA's and conventional chemicals is that microbes are particles (typically formulated as spores and cysts) that become a suspension in water, while chemicals are water-soluble and form test solutions. Testing methods for chemicals avoid testing particles (precipitates) and the water solubility limit sets the highest test concentration used for tests. Currently there is not a physical limit like water solubility to use in the setting of the maximum hazard concentration (MHC) for microbial pesticides. Conventional chemicals react in water by abiotic physical and chemical properties, while microbes are living organisms that have biotic processes that determine how they will react in water. Addressing these differences is necessary if MCPA testing is to be improved.

Some of the major endpoints reported in MCPA studies are inherently different than conventional chemicals. MCPA endpoints focus on toxicity, pathogenicity, and infectivity, while the endpoints for conventional chemicals are toxicity, growth, and reproduction (OPPTS 885.0001, OPPTS 850.1000, and OPPTS 885. 4200, 4240, and 4280). Current guidelines have little information on how to measure, analyse, and report pathogenicity and infectivity, or indicate when it is applicable. Further complications with MCPA's arise when calculating the MHC that relies on assumptions that are outdated, excessive, and not representative of current agricultural practices. Instead, the MHC should use assumptions based on more realistic exposure assessments under field use conditions.

Conventional chemicals require validated chemistry methods that need to meet performance criteria for a test to be acceptable. Results are usually expressed as mean measured concentrations and are used in the calculation of study endpoints to determine effects. Similar standards for method validation and

quantification of samples need to be set for MCPA's in water. The input of an experienced microbiologist is critical in designing and performing a valid study, and is necessary for determining the methods used for quantifying test concentrations.

4.4.2. Problems with Testing MCPA's

MCPA's are typically added to test systems as suspensions of particles in water that are made up of spores, cysts, or cells, and other components of an inoculum of formulation. The suspension of these particles reduces visibility and creates inherent stressors to aquatic organisms. For fish, these particles can clog gills creating additional physiological stress. For *Daphnia*, filter feeding through this suspension can create additional energy costs that can confound toxicity (OECD 2019 Seminar on Test Methods for Microorganisms). The suspension of particles make it difficult to separate out physical toxicity from general toxicity, pathogenicity, or infectivity. To overcome these challenges, in cases of excessive turbidity after mixing, it may be necessary to filter the test material prior to adding it to the test solution. If filtering of stocks or test solutions occur in preparing test solutions, then samples need to be taken before and after filtering to determine any effects filtering has on nominal concentrations.

MCPA's form suspensions that need to have experimental trials run to determine their homogeneity and stability in test solutions, which may be difficult to achieve. Their density is a critical physical property that determines whether they remain in suspension over time or separate by floating to the surface or sinking to the bottom of a test chamber. Homogeneity and stability may not be achievable and procedures need to be developed and standardized of how to deal with this problem.

While some MCPA formulations are exceptions, formulations are typically not good candidates for testing. Many MCPA's are formulated as spores or cysts with amendments added for storage stability and to improve application delivery. Amendments include oils, flour, diatomaceous earth, gels, and gums all of which are not very soluble in water (Bharti and Ibrahim 2020). Therefore, aquatic testing should be conducted with the MCPA as a stable powder or a broth with MCPA inoculum that can be tested in water, rather than the formulated product.

4.4.3. Differences in testing microbes (particles) vs conventional chemicals (solutions at or below solubility)

When testing conventional chemicals there are many GLP Chemistry Studies that can be used to design a test (OPPTS 830 guidelines). Water solubility trials are used to determine the maximum hazard concentration (MHC) for conventional chemicals. Hydrolysis, photolysis, and volatility of a chemical help determine if the test substance will degrade or evaporate from the test system, which can be used to determine what delivery system to use (flow through, static renewal or in the case of volatility a closed test system). The octanol-water partition coefficient (Kow) helps determine if the chemical will bio-concentrate, while the soil organic carbon – water partition coefficient (Koc) is an indicator of adsorption of the chemical to test system materials that include test chambers, tubing used in delivery systems, and food. All this information is extremely useful in designing tests with conventional chemicals, however, there is no such experimentally derived information for MCPA's in current guidelines. Preliminary tests to determine these types of physical parameters and necessary test conditions need to be developed and standardized to provide information that can be used to inform definitive studies.

Mixing trials are one way to determine if the MCPA floats, sinks, or remains suspended in test solutions. These trials can also be used to determine stability, homogeneity, the settling time before adding organisms, needs for aeration to overcome any loss of dissolved oxygen, and evaluating visibility and colour of test solutions. For 21-day *Daphnia* tests and 30-day fish tests with MCPA's, a static renewal test is typically run with test solutions being replaced on Monday, Wednesday, and Friday. With conventional chemicals, stability and homogeneity need to be demonstrated over the longest renewal period, which in

this example is 72 hours or 3 days, that covers the weekend period. Showing stability and homogeneity should also apply for MCPA tests through quantitative analysis of samples. Experimentally showing consistent counts of the MCPA over 72 hours in water will determine stability, while homogeneity in water can be evaluated by counting MCPA particles in multiple samples taken at different positions and/or depths at each sampling interval.

Mixing trials require quantitative measurements of samples based on validated analysis of samples. Since there is no formal guidance on how samples should be measured and quantified in current guidelines, there is a need for the development, standardization, and validation of quantitative methods. Several methods can be used to identify and quantify samples, but all need performance criteria assigned for acceptability of their use in studies. Several methods to consider are listed below (Madigan et al. 2022):

- **Standard or Viable Plate Count Method** – indirect measure of cell density that represents counts of only live bacteria expressed in colony forming units (CFU's). Should develop method with wide series of dilution factors to have final plate counts in the range of 30 to 300 colonies
- **Spectrophotometric (turbidimetric) analysis** – uses absorbance and optical density to indirectly reflect the number of bacteria both live and dead. Need high counts for method to work
- **Flow cytometry** – counts single cells and is used with stains to distinguish between live and dead cells
- **Bioinformatics** – gene sequencing of MCPA to identify MCPA's and used when there are no known culture methods for plate counting

As part of method development, it would also be advantageous to determine the viability of the microorganism in water. This can be determined by microscopic analysis of samples from mixing trials to determine viability (plate counts) and composition of the MCPA in water (spores, live cells, dead cells).

4.4.4. Setting Test Concentration(s)

MCPA Tier 1 testing is a limit test that has one test concentration set at the maximum hazard concentration (MHC). Tier 2 tests are the same guidelines tests as Tier 1 but are set up as a 5 concentration dose response test. For aquatic testing, the exposure route of the MCPA is both through water and through food.

The MHC for water is defined as 10^6 units/mL, or 1000 times the expected microorganism concentration in the aqueous environment, whichever is greater and readily attainable. The expected microorganism concentration is calculated by using the maximum calculated pesticide concentration in water immediately following a direct application of the MCPA to a 6-in layer of water and multiplying it by 1000.

The MHC for food is calculated using the same expected microorganism concentration in the aquatic environment used for water, multiplied by 100 instead of 1000. If after Tier 1 tests there are problems both unrelated to toxicity and pathogenicity and if the MCPA is unlikely to be harmful to the environment, another Tier 1 test can be run reducing the 1000 times the maximum application rate to 10 or 100 times this rate.

These MHC concentrations are based on guidelines established in the mid-1990's. This approach is very conservative and should be updated to reflect more current application methods and agricultural practices. Not all MCPA's will be applied directly to water. The process of selecting test concentrations should include an exposure assessment that factors in the following:

- intended use of the product (in-field or direct water application)
- the mechanism by which the MCPA will get into off-field bodies of water (spray drift, run-off, or evaporation)
- how the product is formulated (wettable powder, liquid formulation, granule)

- the application method (direct overspray, broadcast using a spray boom, air-blast sprayer, or plane, in-furrow, seed coating)
- how the MCPA interacts with microbial community both on- and off-target area of application (often the microbe will have a very specific niche and require a narrow range of environmental conditions)

The risk of an aquatic exposure is greatly reduced based on where and how the product is applied. The current calculation should be used for those products with direct applications to water (e.g., mosquito control), but new lower calculations for MHC's should be adopted for other application methods intended for in-field use only. Further reductions should apply for in-furrow applications and even further reductions for seed coatings.

4.4.5. Use of Controls in MCPA studies

All ecotox studies include a negative control to show that the test organisms remained healthy throughout the test under the environmental conditions of the test system. Negative controls are often untreated dilution water and serve as a control comparison to the treatment groups. They are also used to set performance criteria for tests. If a solvent is used to aid in the delivery of a test substance a separate solvent control is also included in the test design.

Positive controls are also used in tests to show that the response of test organisms is consistent with an expected response. If the response of organisms is less or more than expected, then organisms used in the test may be suspect. Positive controls are also used to confirm a known mode of action. For example, with fish endocrine tests an estradiol positive control group can be used to compare the results from treatment groups to the positive control to confirm both the mode of action and the strength of the responses.

Non-infectious controls (sterile filtrate) are useful to show that the observed response in the test is the result of the microbe being tested and not due to other amendments used for inoculation of the microbe into the test solutions. Inactivating microorganisms by autoclaving is one way in which these types of controls are prepared, but they may not be the best methodology since it kills cells and the test solutions now contain lysed contents of cells and cell fragments. Another method to use is a formulation or "inoculant" blank that adds the broth or other substances in the inoculum that do not include the microbe.

Ideally, we need a non-viable control with dead spores or cysts; however, research is needed in this area to develop a more standardized approach that is widely accepted.

4.4.6. Determining Pathogenicity and Infectivity

There is very little guidance on how pathogenicity and infectivity are to be determined in aquatic tests and procedures need to be developed to provide guidance so that standardized methodologies are used in these assessments. A similar problem existed with histopathology of fish gonads in endocrine testing which resulted in the issuance of an OECD guidance document in the *Series on Testing and Assessment*: Ecotoxicity Testing No.123 entitled "Guidance document on the diagnosis of endocrine-related histopathology in fish gonads" (Braunbeck et al. 2010). Such a document may be needed for MCPA testing.

Thorarinsson et al (2020) provides a potential starting place for standardization of an approach for scoring pathogenicity and infectivity for MCPA's. In that study, a DNA vaccine for Atlantic Salmon (*Salmo salar*) was evaluated. The authors scored the heart, skeletal muscle, and the exocrine pancreas of each fish separately for necrosis, inflammation, fibrosis, muscle regeneration, and tissue loss. The scoring system used three clearly defined categories for each organ and findings were scored from one to three based on the severity of the response. With each fish scored, the prevalence of responses was also reported. This

scoring approach for a DNA vaccine could be particularly relevant and readily adapted for MCPAs more broadly.

4.4.7. Having the Right Team of People

When aquatic ecotoxicological studies were first being developed, a lab only needed a biologist as a Study Director and a chemist to measure test concentrations for the calculation of ECx' values or NOEC's. When endocrine studies were added to the mix, the role of the Study Director became more complex and required coordination not only with a chemist, but also a statistician, histopathologist, and with a molecular biologist for the analysis of biomarkers such as VTG and genetic sex. The Study director needed to understand all of these different levels of biological organisation. MCPA's are similar to endocrine studies and require a good team of scientists that include the addition of a microbiologist. The microbiologist is needed to identify strains, determine taxonomy, as well as develop methods for quantitative measurement of test concentrations. Assessments of pathogenicity and infectivity are also likely going to involve the services and expertise of a histopathologist. Molecular biology may also be useful in identification of microbial strains based on bioinformatics. Once again, the Study Director will need to be familiar with all of these levels of biological organisation to inform the appropriate study design, delivery and assessment of endpoints.

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4.5. Aquatic Testing with Non-target Organisms and Microbial Pesticides - Challenges and Potential Solutions

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No abstract available.

4.6. Adventures in Ecological Testing of Microbial-Based Agricultural Products

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The interest in and use of biological materials in crop production is increasing globally at a rapid pace. Part of the interest is that these technologies are viewed as safer alternatives to conventional chemicals. While establishing the safety of these materials is as important as for conventional chemicals, there are important distinctions between them. Microbial pesticides are unique materials, particulate by nature, often in the form of viable spores, and they require unique testing and assessment approaches as compared to chemicals. For example, microorganisms need to be evaluated for their pathogenic potential which generally means longer exposure or observation periods, additional control groups, and where dose confirmation is required, quantifying exposure using plate counting methods to enumerate colony forming units. And there is an additional challenge of the test material being a particle that does not dissolve in aqueous media like dilution water for aquatic animal testing or sucrose diet used for bee testing.

Over the last several years we have explored some of these testing challenges and would like to share our experiences regarding chronic *Daphnia magna* testing, chronic honeybee testing and bumble bee testing, and larval bee testing. I would also like to introduce some aspects of testing

and assessment that we have not focused on for microbial pesticides and the benefit that might result from considering new approaches.

There are lots of questions to consider when starting an aquatic test with a microbial pesticide. Among these

- What types of control groups do I need?
- How do I achieve the maximum hazard concentration?
- How do I manage high levels of particulates and turbidity in the test system?
- How do I distinguish between toxic/pathogenic/non-specific effects?
- How will I confirm the test levels analytically?

Testing at the maximum hazard concentration (MHC) has its purpose. The MHC is a test level defined by US EPA that results in a very high, environmentally unrealistic exposure, usually a multiple of the application rate. It was intended to provide a margin of safety in case the microbe replicated and increased its population after application. However, levels approaching the MHC can result in turbid, particulate-laden water, or test diet that is not palatable to test organisms. For aquatic testing, we often cannot test at the MHC because of the resulting poor water quality. Even at test concentrations below the MHC, the particulate nature of the test material is such that it can result in tank fouling, entrapment of the test organisms, nutrient depletion, and gill or intestinal obstruction. *Daphnia magna* do not tolerate these conditions very well, and the effects observed are often the result of non-specific, physical effects of the test material. We have attempted to separate the *Daphnia* from the bulk of the particulate matter by creating an exposure cup with a mesh bottom inside the larger exposure beaker. The material is kept suspended by placing the whole container on a stir plate and using a slowly rotating stir bar. This has helped separate the *Daphnia* from larger aggregates, but often the water is uniformly turbid, so the advantage may be limited. It may be helpful to explore sediment test organisms such as *Hyalella azteca*, that may be more tolerant of poor water quality.

Another challenge is distinguishing between toxicity, pathogenicity and non-specific effects. Multiple controls are often used to aid interpretation. We commonly employ a sterile filtrate, an attenuated control, and a dilution water control. Findings in the sterile filtrate indicate when toxicity might be the driver of any effects observed. If there are effects in the test vessels but not in the sterile filtrate or attenuated control, pathogenicity might be the cause. Heat and pressure (autoclaving) are typically used to attenuate the sample. However, it has been our experience that autoclaved samples result in greater effects than the test material. It is unclear whether physical or chemical changes (or both) are being introduced by the process, but the outcome is that the autoclaved sample may not be a good control match for the test item. There may be alternatives to attenuate the sample, such as gamma irradiation or ultra-violet treatments. We've been exploring the use of gamma-irradiation; however, more research is needed to find the best attenuated control match for the test sample.

One of the most important gaps in our testing guidance is the lack of recommended methods to investigate infectivity/pathogenicity. This is true across all of the taxa.

An important aspect of aquatic testing is analytical confirmation of the test concentrations. For microbial pesticides, this is typically accomplished by plate counting. However, the method has been variable in our experience. Some factors that contribute to the variability are uniformity of the test solution, lack of sterile conditions of the test system (sterile aquatic test systems are just not practical), and the test item is a living organism. We've investigated some PCR methods that look promising. Some methods used for soil may be applicable (e.g., Mendis et al. 2018). However, it may be worthwhile to consider whether analytical

confirmation should be required or at least required with the same rigor as for conventional chemicals. Could endpoints based on nominal levels be sufficient for microbial pesticides?

Considering the challenges for pollinator testing in adult honey bees, palatability of the test diet and survival for the duration of the chronic test have been key issues. The particulate nature of the microbial pesticide can result in clogging of the feeding tube restricting food access and difficulties in maintaining homogeneity of the test material in sugar solutions used as bee diet. US EPA pathogenicity testing guidelines (OPPTS 885.4380) recommend a study duration of 30 days. A literature search by Bayer suggests that 15 days may be the minimum duration required to evaluate pathogenicity. However, honeybee survival for study duration greater than 10 days can be challenging. Our pollinator researchers at Bayer have been experimenting with the test design, examining factors that may contribute to longevity of the bees. The factors investigated include source colony, wax and pollen supplementation, cage type and number of bees per cage, *Varroa* infestation rates, and presence of the queen. So far pollen supplementation did not appear to provide benefits, although dosing via pollen could improve palatability of the test diet when dosed via the pollen rather than sucrose solution. Source colony seemed to be the largest contributor to longevity in our experiments. Additional work examining seasonality, source colony and queen pheromone is ongoing.

Larval bee testing guidelines for conventional chemicals [OECD (Organisation for Economic Cooperation and Development) Guidance Document No. 239] also create challenges for microbial pesticide testing. It appears that the optimal timing of an infectious agent for larval bees is less than 2 days post hatch (at least for *Paenibacillus larvae larvae,* Brødsgaard et al. 1998), but exposure to the test item in this testing protocol does not occur until days 3-6 post-hatch. In addition, the temperature and relative humidity of the test system may not be suitable for all microbes (may be true for adult honeybee testing as well). We have performed some limited tests that demonstrated that the royal jelly, known for its anti-microbial properties (Blum et al. 1959; Fujiwara et al. 1990; Fontana et al. 2004; Romanell et al. 2011; Bilikova et al. 2015) inhibited the growth of several common biopesticides and a known bee pathogen (*Paenibacillus larvae*) (Schmehl et al. 2019). Some additional work is necessary to determine if the larval bee test system is appropriate for microbes. In lieu of the larval bee assay, targeted genomic analysis looking for genes important in bee pathogenicity could be a useful assessment tool. Colony tests may also be useful in determining larval health if *in vivo* methods are needed.

In addition, research is needed on appropriate suspension agents and concentrations to prevent settling of the microbial pesticide test item in sucrose solution feeding tubes. Some options to consider regarding bee test diets include directly dosing the bees using a pollen-based diet or feeding the bees a pre-determined quantity of treated liquid sucrose diet for a limited number of days and then supplementing with clean diet. Methods are needed to distinguish palatability from true gut/feeding effects. Are bumble bees an option as a surrogate? They could extend the treated pollen exposure route and infection window in the studies. As with the aquatic organisms, validated methods are needed to distinguish between infection/pathogenicity and toxicity.

To address some of these challenges, Bayer continues to work on improving longevity in adult bee pathogenicity assays, with the potential for collaboration within the International Commission for Plant-Pollinator Relationships (ICPPR) Bee Protection Group and OECD.

What is missing from our toolbox for the testing and assessment of microbial pesticides? Testing guidelines are needed for environmental expression. There can be many objectives to this type of testing including, survival of bacteria in the environment (e.g., water), monitoring lateral or downward movement in the environment, persistence of an introduced genetic element, potential for endophytic activity, and disruption of native microbe communities. Lab or field testing may be fit for purpose. Regulatory requests are increasing for these types of data, and we need to develop standardized and reliable approaches to generating these data. And such data would be necessary if we want to explore the potential of environmental modeling the exposure to microbial pesticides.

Here are some concluding thoughts. The unique characteristics and safety questions (i.e., pathogenicity) call for specific test guidelines for microbial pesticides. While there are many challenges, focusing on aquatics, pollinators, and environmental expression/modeling would help to address many issues including those with other taxa as well. While specific testing guidance/guidelines are important, it is equally important to have guidance on when it is necessary to test, and when it is not. If we are to realize innovation related to microbial pesticide testing, we need to develop guidance and guidelines that are suited to the testing and assessment of these materials.

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5 Session 5: Current Ecological/Non-Target Test Guidelines – Addressing the Issues

5.1. Challenges in Testing with Microbes in Insects - Considerations for Test Improvements

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Microorganisms used in pest control have specific qualities as biological entities, which make them different from conventional chemical pesticides. Some microorganisms produce toxins as active principles, others are pathogens which would need to start an infection of the target pest organism; others function through competition or antibiosis. Microorganisms used as biocontrol agents may have different roles in the concept of integrated pest management; some may be used in a preventive or inoculative manner to avoid development of pests and diseases, whereas others, such as baculoviruses, are applied in a curative sense to control an existing pest population. Using the example of baculoviruses, intrinsic difficulties for cell culture testing are addressed as well as improving methods to determine background of naturally occurring viruses.

5.1.1. Introduction

Baculoviruses are dsDNA viruses which infect specific taxa of Lepidoptera, Hymenoptera and Diptera (Harrison et al., 2018). In general, a given baculovirus has a narrow host range, often infecting only a single or few host species. Due to their high virulence for target insects they are used as biological control agents. During their replication cycle baculoviruses produce two virus phenotypes: The occlusion derived virus (ODV) is embedded in a proteinacous occlusion body (OB) and serves as disease transmitting agent from insect to insect, whereas the budded virus (BV) transmits infection from cell to cell and tissue to tissue within an infected insect.

5.1.2. Testing viral activity

ODVs exhibit *per os* infectivity but low infectivity for cultured cells, whereas *in situ* host insect cells or cultured insect cells are permissive for BVs rather than ODVs. As a result, different methods for activity testing of both virus phenotypes are required. Activity of ODV/OB against a specific insect is tested by feeding insect larvae with a given dose or concentration of OBs. Then, the mortality is scored and median Lethal Dose (LD_{50}), median Lethal Concentration (LC50) or median Survival Time (ST50) is calculated. Activity of BV is usually determined as median Tissue Culture Infective Dose (TCID50) and calculated as Plaque Forming Unit (pfu) (Eberle et al., 2012). Determining a TCID50 requires a permissive host cell line, which may not be available for many baculoviruses used as biocontrol agents, especially not for granuloviruses (=members from the genus *Betabaculovirus*). There is also no standardized susceptibility of cell lines to BV and it might be vary considerably from virus to virus or host to host.

Bioassaying the activity of ODVs/OBs is not less complicated and needs specific adaptation to each virus-host combination. In such bioassays insect larvae are fed with virus OBs. Thus, the design of such bioassays depends on whether natural host plant tissues are needed as a matrix for OB administration or if some semi-artificial diet can be used. It further depends on larval stage, microscopic enumeration of OBs, and duration of bioassays which depends on whether as a slow or fast killing baculovirus is tested (Federici, 1997). As a consequence, bioassays need to be adapted for each baculovirus-host system and cannot be standardized between such combinations.

5.1.3. Consequences for cell culture studies

In many OECD countries, cell culture studies are required for intracellular replicating agents, such as viruses. The currently most widely used guideline is OPPTS 885.3500 Cell Culture. It depends on availability of TCID50 or LD50 values of a given virus. Considering the above mentioned properties of BVs and ODVs/OB and the lack of standards to determine a LD50 value, it is highly difficult if not impossible for most baculoviruses to fulfill the test requirements and achieve the aim of the *in vitro* study, especially when a susceptible insect cell culture is lacking for a given virus.

On the other hand, no toxicity, no infectivity and no transformation capacity was demonstrated for all tested baculoviruses so far, e.g. Autographa californica multiple nucleopolyhedrovirus (AcMNPV), Heliothis zea single nucleopolyhedroviruses (HzSNPV), Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) and several others (OECD 2002). Because baculoviruses cause no hazards to non-target organisms, they have been assigned a QPS (qualified presumption of safety) status in the European Union (EFSA, 2005). Due to their non-toxicity and non-infectivity to human cells, baculoviruses, such as AcMNPV, are used in human cell therapy without any adverse effect (Airenne et al., 2013). It is therefore questioned if an impropriate or incomplete cell culture test which appears to be intrinsic to the biological properties of baculoviruses can contribute to their safety evaluation. Because of the available evidence on the lack of transformation, infectivity and toxicity of baculoviruses to mammalian cells, it should be considered to waive cell culture testing for new baculoviruses.

5.1.4. Methods for testing environmental prevalence

Another aspect is the identification of background levels of microorganisms in the natural environment. Since microbial strains used as biocontrol agents are isolated from nature, the same or similar microorganisms may preoccupy and persist already in the field. To assess the efficacy as well as the fate of a sprayed microorganism, strain specific identification methods are required, especially when microorganisms are used in an inoculative manner, aiming at establishing the MO in an environmental compartment. Thus, strain specific methods for identification and quantification are required to distinguish between sprayed microorganisms and natural background. Such natural populations often exists a mixtures of genetically heterogeneous individuals (Fan et al., 2021). In a proof of concept, we researched

a method for amplicon-based determination of single nucleotide polymorphisms (SNPs) to characterize the genetic structure of natural populations of Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) and to distinguish it from a commercial product. Based on comparisons of available whole genome sequences of LdMNPV isolates, multiple genome loci with specific SNPs were identified and used as targets for PCR amplification. High throughput Illumina sequencing of the amplicons allowed the identification and determination of the quantitative distribution of a family of specific SNPs, which represents the genetic structure of the whole genomes. Out of about 500 samples, about 15% revealed LdMNPV-specific amplicons which clustered into three groups with eight specific SNP patterns (unpublished data). About half of the LdMNPV-specific samples showed quantitatively homogenous patterns indicating their genetic homogeneity, whereas the other half of the isolates consisted of genotype mixtures. Interestingly, related SNP patterns typical for the commercial product were found in the environmental samples, although the individual SNP patterns of all samples could be clearly distinguished from that of the commercial product. After further validation, this methodology could be used to distinguish natural specimen from sprayed ones and it might be extended to other non-viral microorganisms, such as bacteria or fungi.

5.1.5. Further considerations

It needs to be stated that current registration requirements of microbial organisms mainly address inundative use of microbial biocontrol products. Some characters of microorganisms, however, especially their specificity for certain disease agents and pests, delimit their field of use to a limited number of indications, in certain cases of baculoviruses even to a single target in very few crops. Such products may be infrequently need depending on pest prevalence, but they are of extraordinary value for a specific control of cycling pest populations or in emergency cases of local epizootics. For societal and environmental reasons it must be ensured that the registration process deal with such agents which might be used less frequently and on much smaller areas than broad-spectrum chemicals.

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5.2. Working with Invertebrate Pathogens in the Laboratory - Considerations for Improving Non-target Organism Testing

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Registration of microbial control agents in Australia must face some of the most rigorous risk assessment regimes in the world. Requirements include not only safety and efficacy data but must satisfying strict legislation on import of non-native organisms.
Laboratory bioassays attempt to address these requirements but face challenges in determining the potential harm from and exposure to these biological agents that would be found in field condition. The present provided examples from the registration and release of microbial control agents, and discussed limits and potential improvement of laboratory risk assessment.

This paper offers some perspective on points of difference in testing of microbial insecticides. One is the difference between research and regulatory testing. Research is essential and may inform large shifts in our understanding (the impact and interactions of fungicides and insecticides in bees, for example), but for regulation purposes tests must be relevant to the product and the environment of application, not a handicap to the registration of microbials.

There challenge in adapting testing and regulation designed to assess chemical pesticides to microbial controls has been well discussed, but there are further challenges in differentiating tests for replicating organisms (viruses, fungi, some bacteria) from those more suited to assessment of biorationals. Several speakers have raised questions about the validity of extrapolating laboratory tests to impacts in the field, and it is especially difficult to extrapolate when evaluating replicating organisms with complex ecological interactions, and in predicting risks (or lack thereof) from secondary metabolites that may be produced during interactions with hosts and the environment.

Australia has demanding standards of evidence required for the import and release of new organisms: we make TV shows about stopping biological material getting through the airport. The regulatory requirements for microorganisms typically include extensive evaluation of risks to endemic species and non-target host range testing, and are a significant obstacle to the import of any biopesticide based on replicating microorganisms (viruses, fungi, live bacteria) that are not known to occur in Australia. It is important to differentiate between biosecurity risk assessment and registration, so that once organisms are approved for release, processes for issue of permits for trials and for registration should be no more onerous than that required for chemical insecticides.

Useful questions to ask before testing an insecticide are: what are we testing? Is it 'alive' (a replicating pathogen or beneficial organism) or a biorational toxin: a toxin derived from an organism? How does the target acquire the active? What is the 'mode of action'? And how long do we need to keep the test subject alive after exposure?

Recent studies using multiple intergenic sequences discriminate between closely-related 'cryptic' species of entomopathogenic fungi, and whole genome sequencing is becoming (or is already for baculoviruses) the standard to identify a microorganism of economic interest. Simple Basic Local Alignment Search Tool (BLAST) searches of single short marker regions are no longer sufficient. Organisms should be identified using multi-locus or whole-genome phylogenetic analysis including well-curated reference sequences.

Laboratory assays for efficacy and non-target effects do not always allow for between chemical pesticides, biorationals, and replicating pathogens. Most laboratory assays developed to determine efficacy and risk are based on simple LD_{50} or LC_{50} bioassays developed for fast-acting gaseous neurotoxins, some of which have been adapted to test new chemistry such as systemic chemicals. They are typically applied in easily determined and controlled doses with short exposure times (a few hours) and can be used for both target and non-target testing without placing undue stress on untreated controls. These assay methods have been adapted to determine efficacy of microbially-derived biorational toxins with different routes of exposure, mode of action, and speed of kill. LC_{50} of spinosyns and spores or proteins derived from spores of *Bacillus thuringensis*, for example, can be determined by application to semi-artificial diet. This both exposes the target insect to the toxin through ingestion or contact, and feeds the target after exposure to allow for the slower action.

Sometimes results from laboratory assays can be completely unpredictable. Fungal entomopathogens are typically described as causing infection following adhesion of conidiospores to the insect cuticle, germination, and penetration leading to proliferation and death. In our laboratory direct application of the entomopathogenic fungus *Metarhizium anisopliae* causes infection and death in green vegetable bug, *Nezara viridula*, but in the field it proved almost impossible to infect the bugs even with very high application rates. Green vegetable bugs climb to the top of the plant in mid-morning and bask in the sun. Like desert locusts, they are probably inducing a 'fever' that prevents infection by the fungus, a defence against infection that isn't replicated in laboratory assays. In contrast, we were initially unable to infect the stored grain pests *Tribolium castaneum* and *Rhizopertha dominica* in assays using direct application of conidiospores of either *Beauveria bassiana* or *Metarhizium* species. Gas chromatography mass spectrometry found high levels of anti-fungal chemicals in the cuticle of the adult beetles. We even tried rolling the beetles in vials of spores: a very few died. We subsequently found that dose response and secondary transmission assays in adults and larvae could achieve high levels of infection by incorporating fungal spores into food, or by placing infected cadavers into flour with larvae. The fungus presumably infects following ingestion of spores, probably through the buccal cavity.

Efficacy assays are usually conducted on animals of the target life stage (larvae, for example) from colonies adapted to mass rearing in laboratory cultures, typically on semi artificial diets. Assays become more difficult and less reliable when testing non-target organisms, particularly in near-species host range tests: a 'high bar' for microbial biopesticides that demands we attempt to work with species that are unsuited to bioassays conditions. Many of these non-target organisms cannot be adapted to laboratory rearing (larvae of species requiring specific host plants for example), or have to be forced to take up the active or suffer trauma during assays (leaf miners), or are maintained in entirely artificial conditions (bee larvae), or must be kept alive in laboratory containers for a week or longer (when testing fungal pathogens). In these assays the stress on the subjects will affect their susceptibility to the test organism, or result in high mortality in untreated controls. We recently attempted to conduct assays of granuloviruses in near relatives of the diamondback moth, *Plutella xylostella*, many of which are leaf miners, have high specificity for host plants (citrus blossoms, for example), or could not adapt to laboratory conditions: most of the controls also died.

Much excellent work to develop assays that reduced the stresses on test subjects was conducted for risk assessment of transgenic microorganisms in the 1990s and early 2000s. These included using F1 progeny of field collected insects maintained on their host plants, infected with baculoviruses) by droplet assay or diet contamination following starving for 24 hours, then reared for several days on fresh leaves washed briefly in dilute bleach to remove contaminating pathogens. My team developed assays that standardised application to food plants using a low volume spinning disc applicator: treated leaves were then picked into food containers and insects introduced for 24 hours before removal to a clean food source and rearing through to death or pupation.

It is particularly difficult to extrapolate the effects of laboratory assays to in-field effects on non-target beneficial insects (predators and parasitoids). Behavioural interactions between beneficial insects and fungal entomopathogens have been reviewed by Baverstock et al (2010). Predators and parasitoids show a range of responses to fungal pathogens, sometimes avoiding infected prey or hosts, sometimes not. Our work has shown that larvae of green lacewings, *Mallada signatus*, avoid walking on areas filter paper treated with conidiospores of *Metarhizium* sp.: they prefer to walk on areas treated only with the carrier, even when aphid prey were placed on the area with fungus.

Overall, the value of bioassays of pathogens on near relatives is more relevant to biosecurity than registration of products. There's little value in testing susceptibility of a rare leaf roller from the tropical rainforest to a bacterial toxin intended for use in canola. Treatment effects on non-target species in the laboratory can be hard to differentiate from death due to stress, malnutrition, or humidity. Susceptibility in the field cannot be reliably extrapolated only from laboratory results. We need to pick our targets, and our tests, to represent conditions in the field in which products will be applied, and to test against subjects that are not artificially stressed, malnourished, or dying.

The successful registration of microbial controls in Australian has been driven by insecticide resistance management strategies (IRMS) for both chemical insecticides and transgenic crops. Australian IRMS are based on principles of integrated pest management. In the late 20th century the Australian cotton and sorghum industries were faced with widespread resistance to all available chemical insecticides, particularly by the lepidopteran pest *Helicoverpa armigera*. They developed IRMS that avoided the early-season use of 'disruptive' chemicals that particularly affect beneficial predators and parasitoids found in those crops, and promoted the use of non-disruptive microbial insecticides, particularly baculoviruses of *H. armigera*. This have now been adopted over hundreds of thousands of hectares of commercial cotton and grain production.

These programs included a significant body of fundamental work including testing impacts of chemical insecticides on beneficial insects in both laboratory and field. As a consequence, field efficacy testing of new microbial insecticides (baculoviruses and fungi) automatically included assessment of impacts on beneficial insects. Quantitative assessment of beneficial insect numbers was included alongside sampling of target pests in multiple replicated trials of products and rates in several crops (cotton, sorghum, maize, mungbeans and soybeans) over wide geographic separations, (coastal, inland, tropical and subtropical regions).

This work demonstrated conclusively that the pathogens tested (baculoviruses of *Helicoverpa* species, and three fungi: *M. anisopliae*, *B. bassiana, M. rileyi*) had no or negligible impact on field populations of non-target insect predators. Thirteen separate, replicated, 5x5m plot trials in mungbean and cotton compared multiple rates of *M. anisopliae* and *B. bassiana* in Propar®12 to a water-only control, a Propar 12 (carrier) control, and Dimethoate. The fungal controls and Dimethoate all had significant impacts on target (mirid) abundance, but not on abundance of 10 species of predatory insects (bugs, ladybeetles and lacewings), ants, or spiders. Impacts on beneficial insects were seen in only two treatments in 2 trials. *M. anisopliae* applied at the extreme high rate of $5x10^{13}$ cfu/Ha had a significant impact (compared to controls) on total predatory bugs that was not significantly different from the effect of Dimethoate. *M. anisopliae* applied at $5x10^{13}$ cfu/Ha had a significant impact (compared to controls) on spiders that was not

significantly different from the impact of Dimethoate on spiders. All other treatments including *B. bassiana* at $5x10^{13}$ cfu/Ha and lower rates *M. anisopliae* were not significantly different from the water control (Knight, 2013). In our field trials of *M. rileyi*, parasitoids were shown to emerge successfully from *H. armigera* larvae that survived exposure, boosting overall mortality of the pests by up to 20%.

Finally, some comments on assessment of secondary metabolites. The colonisation of plants (particularly the rhizosphere) by beneficial microorganisms is an essential part of healthy productive soils, protecting crops and pastures from insect pests and pathogens. This is achieved part by production of toxic secondary metabolites, some of which are registered insecticides (Ivermectin, Spinosad, Avermectin). Toxic secondary metabolites also include the insecticidal and nematicidal toxins produced by *Xenorhabditis spp* (bacteria) in *Steinernema spp* (nematodes).

Evaluating the parvome, the "humungous microbial world of small (secreted) molecules of great structural diversity" (Schreiber, 2005) is a challenging field for regulation. Predicting the potential to produce secondary metabolites and non-ribosomal peptides and peptaibols (produced by *Trichoderma* and many other species of fungi) from genome sequences is not yet possible. Non-ribosomal peptides are not directly encoded in the genome but are produced by 'assembly lines' of non-ribosomal peptide synthetases (NPRS) which are encoded by sets of chromosomally-adjacent genes in a 'biosynthetic gene cluster'. However, the order of NPRSs in an assembly line may differ from the order of genes in this cluster. Genome mining with machine learning can predict some of the amino acids associated with each NPRS in the assembly line, but the core NRPSs are often assembled in a non-linear fashion, may undergo post-assembly modification, and may associate with more than one amino acid. Combine genome mining with mass spectrometry might be able to achieve some level of prediction of potential products - eventually – but cannot not predict under what conditions, and at what levels these might be produced (Behsaz *et al,* 2021).

Between 2011 and 2022 the pasture mealybug *Heliococcus summervillei* has caused 'pasture dieback' in over half a million hectares beef production pasture, estimated to have cost the Queensland beef industry A$2billion. We recently conducted extensive field isolation of fungi associated with the rhizosphere of pasture grasses across Queensland and northern New South Wales. We also conducted glasshouse assays to determine the effects of the seed-born fungal endophyte *Epichloë festucae* and some of the rhizospheric fungal isolates on the mealybug.

E. festucae occurs as a seed-born endophyte in perennial ryegrass in New Zealand and expresses around 60 bioactive metabolites, particularly indole-diterpene alkaloids, one of which, Lolitrem B causes 'staggers' in livestock when abundant at high temperatures. Other alkaloids (ergovaline, peramine) protect from insects, while different metabolites protect the grass from pathogens. Commercial isolates of *E. festucae* have been selected to provide crop protection but not induce staggers, and have been essential in managing the pasture mealybug *Balanococcus poae* in NZ. Our glasshouse assays showed that the commercial and 'wild type' endophytes significantly reduced the abundance of *H. summervillei* in rye grass compared to the same rye grass variety without endophytes, particularly the 'wild type' endophyte that produces high levels of a wide range of secondary metabolites including Lolitrem B.

Our field surveys isolated over 1,200 fungal isolates from 17 sites. Amongst these, 36 species from 5 genera of rhizospheric / endophytic fungi were significantly associated with pasture tolerance and recovery from mealybug: *Penicillium* spp., *Trichoderma* spp., *Clonostachys* spp., *Purpureocillium lilacinum*, and *Beauveria* sp.. Soils with greater resilience have more fungal abundance and diversity. Circular patches of recovered grasses in severely damaged fields were associated with presence of these beneficial fungi. Laboratory tests that inoculated grasses with cultures of the fungi were able to identify those with significant activity against *H. summervillei*, reducing mealybug abundance.

Symbiotic fungi are abundant and widespread in healthy pastures and crops. They contribute to natural crop protection and can be used as inoculants to improve pasture resilience. How can we determine which are expressed when, which are hazardous, and which are beneficial? We can't yet determine this *in silico*,

but mealybugs, it turns out, are excellent lab rats with which assess the presence of at least some bioactive secondary metabolites.

In short: testing for registration should conduct assays that are appropriate to the microorganism (a toxin or a replicating organism) and with healthy, minimally-compromised target organisms that are relevant to the expected patterns of use. Where possible, obtain field data.

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5.3. Importance of problem formulation for the risk assessment of biopesticides in general and for microbial metabolites in particular EU

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The risk assessment for microbial active substances includes the assessment of the risks that may result from the production of metabolites by the microorganism. For the EU approval assessment this has proven to be complex, elaborate and time consuming. Due to the fundamental differences between microbial metabolites and degradation products (metabolites) of synthetic chemical substances, it is crucial to avoid the pitfall of applying the approach to metabolites of microbial and chemical substances. Instead, the risk assessment of microbial metabolites should include a problem formulation step in which foreseeable risks are determined. Based on the outcome of this problem formulation step, microbial metabolites should be identified or excluded as being of concern in the EU assessment. The EU guidance on the risk assessment of metabolites produced by microorganisms used as plant protection active substances aims to improve and harmonise this problem formulation step, as not properly performing this phase may lead to unwanted outcomes such as continuing requests for more data. An appropriate problem formulation step can shorten timelines for the assessment and make the outcomes more predictable. This approach may be extended to the risk

assessment of all biopesticides to help meet the goals set by the Farm to Fork strategy.

5.3.1. Introduction

For a microbial active substance to be approved for use in plant protection in the EU, the microbial active substance needs to be assessed as being sufficiently effective and not causing harmful effects on human health or unacceptable effects on the environment (Regulation (EC) No 1107/2009). For this assessment the foreseeable risks caused by the use of the plant protection product (PPP) containing the microorganism should be evaluated (Regulation (EU) No 283/2013 and No 284/2013). Foreseeable risks may be caused by one of several hazards which may apply to microorganisms. These hazards are for example pathogenicity, antimicrobial resistance and also toxicity of metabolites produced by the microorganism. Therefore, the assessment includes the metabolites produced by the microorganism. This paper describes the importance of performing a problem formulation step to assess the risks that may result from the production of metabolites by a microbial strain used in plant protection.

5.3.2. Microbial metabolites

Metabolites produced by microorganisms are grouped into two main categories: primary metabolites which are involved in normal growth and reproduction and are not considered to be of concern. Secondary metabolites have another function, such as nutrient sequestration, communication and defence against other microorganisms. The production of secondary metabolites by microorganisms is part of their normal biology and should not be confused with the production of degradation products of synthetic chemicals which may result from biodegradation (which are also referred to as metabolites).

The assessment of microbial metabolites for the EU approval assessment of microbial active substances has proven to be complex, elaborate and time consuming. The main cause for this is the inherent complexity of biological entities such as microorganisms. Bacteria and fungi will produce many different secondary metabolites; e.g., for the genus *Streptomyces* thousands of metabolites are known (Bérdy, 2005). Furthermore, it is not possible to obtain a single test item containing all the metabolites of a microbial strain to be able to test for toxicity. This is because secondary metabolites are only produced by the microorganism under certain conditions (e.g., when iron is limiting growth or during interaction with a certain plant or microorganism). In addition, many secondary metabolites will only be produced in very low amounts to exert their function in microsites – sufficient quantities for testing may not be available. As a result, quantitative information on the effects of secondary metabolites and on exposure of humans and the environment to secondary metabolites is often not available for the risk assessment. While dealing with this lack of quantitative information, it is crucial to avoid the pitfall of applying the same approach to microbial metabolites as for the degradation products (metabolites) of synthetic chemical substances.

For chemical substances, a worst-case approach can be used in the risk assessment which is based on a maximum level of exposure. This maximum level is determined by the use rate of the active substance. Obviously, the maximum exposure to a microbial metabolite cannot be determined by the use rate of the microbial active substance, as microorganisms may multiply upon application and can produce the metabolite in situ. As determining the hazard properties and the exposure of humans and the environment for each metabolite that may be produced by a microorganism is not possible, a worst-case approach as used for degradation products of chemical active substances will inevitably lead to data gaps and issues that cannot be finalised in the risk assessment. Examples are statements such as ‘The production of secondary metabolites cannot be excluded and therefore the risk assessment cannot be finalised’.

If applying the same quantitative approach as for chemical active substances is not appropriate for the risk assessment of microbial metabolites, which approach should be taken? A crucial first step to determine the correct approach is to acknowledge the fact that a risk assessment should not aim to assess any risk that might occur under any circumstance, but to assess the foreseeable risks. Therefore, as part of the risk assessment, a distinction should be made between these foreseeable risks and any risk that may potentially occur, but which is negligible. This step of the risk assessment is part of the problem formulation. Due to the differences between chemical and microbial active substances the problem formulation step for their risk assessment is markedly different, as will be explained below.

5.3.3. Problem formulation

As described by Wolt and colleagues (2010), problem formulation for a risk assessment is based on:

- Problem context, which identifies the parameters and constraints for the risk assessment. For the risk assessment of a PPP these are given by the regulatory framework of regulations, guidances and guidelines.
- Problem definition, which takes into consideration the specific attributes of each active substance.

For synthetic chemical active substances, the problem formulation step can rely heavily on the problem context: what are considered to be foreseeable risks for these substances is largely specified in the regulatory framework. The regulatory framework for synthetic chemicals has evolved based on actual observed effects due to their use (e.g., bioaccumulation, toxicity), exposure scenarios for humans and the environment are available and quantitative thresholds are in place to delineate the risk assessment to include only foreseeable risks. For example, for the degradation products of chemical active substances, a threshold value is set for the formation percentage below which in principle further information is not considered needed (Regulation (EU) No 283/2013). As a result, even if the problem definition step is not explicitly performed, the risk assessment of a synthetical chemical active substance will focus on the foreseeable risks.

The effect of not performing an explicit problem formulation step becomes more apparent when instead of synthetic chemical substances, natural chemical substances are assessed. For these natural substances such as plant extracts, the problem context is similar to the problem context of synthetic chemical substances as the same legal regulatory framework applies. However, while the hazards which are addressed by this framework for synthetic chemicals are as a rule all relevant for synthetic substances, this is not per se the case for natural substances such as plant extracts. For example, while for synthetic chemical substances it is highly relevant to determine the route of degradation (for example to determine if persistent degradation products are formed), for plant extracts consisting of primary metabolites such as carbohydrates, lipids, proteins and nucleic acids the routes of degradation are known and do not pose a hazard. Not acknowledging this difference in the problem formulation step will lead to requiring and assessing data which is not relevant for the risk assessment.

For the assessment of microorganisms the problem formulation step may be even more relevant. For these substances, the problem context is different as separate data requirements and criteria are used for microorganisms. Many of these data requirements are conditional based on the biological properties of a microorganism and no quantitative thresholds are defined, thereby requiring problem definition to determine if the data requirement is relevant for the specific microorganism. For the hazards due to the production of secondary metabolites, the data requirements indicate that for all microorganisms except viruses (which do not have a metabolism) information is needed on the production of metabolites. Based on this information, metabolites should be identified or excluded as being of concern. Regarding toxicity, a metabolite of concern is defined as a metabolite which is produced by the microorganism with known toxicity and which is present in the microbial active substance as manufactured that may present a risk to human health or the environment and/or for which it cannot be adequately justified that in situ production

is not relevant for the risk assessment. In effect, the data requirement for metabolites (i.e., point 2.8 of Regulation (EU) No 283/2013) asks for the outcome of the problem definition step of the risk assessment for microbial metabolites.

Guidance on how this problem definition step for microbial metabolites can be performed is given in the EU Guidance on the risk assessment of metabolites produced by microorganisms used as plant protection active substances (SANCO/2020/12258; 2020). Based on the body of knowledge of the microorganism and taking into account the principles of microbial ecology, the guidance document describes a step-by-step process on how to determine if any metabolite produced by the microorganism should be considered as a foreseeable risk (a metabolite of concern). The assessment to exclude or identify metabolites as being of concern follows a qualitative approach, for example by using information on the natural occurrence of the microorganism or the expected background levels of the metabolite. For well-described microorganisms, the problem definition step focuses on known metabolites with known toxicity. For less well-described microorganisms more experimental data may be needed.

5.3.4. Considerations for non-target testing

In case experimental data is needed to conclude the risk assessment of metabolites, It is important to note that the problem definition step is needed to inform what should be tested and how; performing a standard set of tests may not lead to the needed information. When testing is needed, a case-by-case approach should be followed, depending on the characteristics of the microorganism and the product. Information relevant for the problem formulation step regarding non-target testing is for example whether relevant levels of metabolites are present in the product. Furthermore, to design appropriate tests for the specific microorganism regarding microbial metabolites, it is relevant to first determine if tests are also needed for the assessment of pathogenicity and toxicity.

5.3.5. Conclusion

By providing guidance on the problem definition step, the EU guidance document for the assessment of microbial metabolites aims to improve and harmonise the problem formulation phase and thereby shorten the timelines for the assessment. Not correctly performing the problem definition step in a risk assessment may lead to ‘continuing requests for more data, disproportionate risk mitigation measures and miscommunication of risk findings’ which result in ‘increased concerns about the environment and leads to delayed decision-making’ (Wolt et al., 2010). Therefore, the guidance aims to provide a pragmatic method in order to reduce the number of data gaps and issues not finalised in the risk assessment and to speed up decision making.

5.3.6. Outlook

The effectiveness of the guidance document on the assessment of metabolites can be improved by future efforts. For example, the body of knowledge on microbial species used in plant protection can be made more easily accessible (e.g., as part of consensus documents or species reviews), which would facilitate a focused assessment for strains within the species. To increase the effectiveness of the risk assessment of all biopesticides including microorganisms and natural chemical substances (fermentation products, plant extracts), it is necessary to acknowledge the need for a more explicit and formalised inclusion of the problem formulation step in the risk assessment. Subsequently, harmonised agreements on how to perform and include problem formulation in the risk assessment of these substances are needed. In fact, the European Commission is currently exploring the role of problem formulation to justify in a harmonised and transparent manner when information is not required for the risk assessment due to the nature of the plant protection product or its proposed uses. This approach is applicable in particular to biopesticides. In this way, the focus, relevance and quality of the risk assessments of microorganisms and natural chemical

substances will improve and concurrently the timelines will be shortened and the outcomes more predictable – both are necessary to meet goals set by the Farm to Fork strategy.

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5.4. Environmental Risk Assessment of Entomopathogenic fungi - Considerations for Non-target Organism Testing

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The value of a plant protection product, chemical or biological, depends on its effectiveness against a target species, as well as its safety for the environment. Risk assessment schemes have therefore been developed to facilitate classification and regulation. These guidelines however are directed towards chemical substances and are in many cases not suitable for the specific requirements of biocontrol organisms. Here the non-target risk assessment of entomopathogenic fungi is discussed on the example of *Metarhizium brunneum*, one of the most widely used fungal biocontrol agents. Modifications for test protocols considering the fungal biology are recommended in terms of duration, end points and quality control.

5.4.1. Introduction

Entomopathogenic fungi (EPF) have a long tradition as biological control organisms (Lord 2005). Already in 1835 Agostino Bassi suggested the use of fungus infected insect eggs to control pestiferous caterpillars (Steinhaus 1957). First attempts in mass producing the EPF *Metarhizium anisopliae* also date back to the 19th century, when Elie Metchnikoff and IM Krassilstschik used this fungus against the sugar beet weevil, *Bothynoderes* (*Cleonus*) *punctiventris* (Krassilstschik 1888; Lord 2005). Today many commercially available biocontrol products are based on EPF as active substance (de Faria and Wraight 2007; Maina et al. 2018).

In general, EPF are considered to be of low risk for the environment (Zimmermann 2007a, Zimmermann 2007b). Nevertheless, as the environmental safety of all plant protection products, whether chemical or as in this case microbial, is of great importance (Brühl and Zaller 2019; Scheepmaker et al. 2019) also these products undergo assessment schemes before they can be used by farmers (Gwynn 2017). One of the aspects of these risk assessments is the impact on non-target organisms.

Standardized methods previously used for the assessments of the active ingredients of plant protection products often did not discriminate between chemical and microbial agents, like EPF. They thus failed to consider differences in modes of action and target specificity, as well as dissimilarities in environmental behavior and the ability of microorganisms to multiply (Chandler et al. 2008; OECD 2019). As a result, data required for the registration of microbial products could often not be provided, complicating and delaying the process, and causing an increase in costs of developing new microbial products (Köhl et al. 2019). Despite big efforts by the OECD to tackle the issue, by for example publishing a general guidance protocol to evaluate the environmental safety of microbial control agents (OECD 2014), the problem still exists. Distinct assessment approaches and detailed guidelines for specific tests are yet lacking (Arora et al. 2016; Sundh and Goettel 2013, OECD 2016).

In 2021 Reinbacher et al. proposed a guideline for non-target test of soil biota to soil-applied EPF used against insect pests and highlighted main aspects for designing new assessment methods on the example of the EPF *M. brunneum.* In the following these findings are summarized and discussed, and further steps to improve risk assessment of EPF are proposed.

5.4.2. The biocontrol organism Metarhizium brunneum

Metarhizium spp. are distributed in soils worldwide in various climates (Zimmermann 2007a). Species diversity and abundance is especially large in grasslands, however they are also readily found in arable land (Fernández-Bravo et al. 2021). Some *Metarhizium* species like *M. album* or *M. acridum* have a very narrow host range while others like *M. anisopliae*, M. *robertsii* and *M. brunneum* can infect a broad range of arthropods (Hu et al. 2014). Nonetheless also within these species fungal isolates may differ in host range. Due to a strong selective pressure created by the need to overcome the insects' cuticle barrier and to interact with distinct immune defense mechanisms, fungal isolates of the same species may be highly specialised (Ortiz-Urquiza and Keyhani 2013). The infection process of *M. brunneum* starts when a susceptible arthropod comes in contact with conidia, the fungus' asexual spores. Through hydrophobic interactions, the conidia attach to the arthropods cuticle and germinate when the biotic (e.g. insect hydrocarbon composition) and abiotic conditions such as temperature and humidity are suitable (Boucias and Pendland 1991; Ortiz-Urquiza and Keyhani 2013). Usually this means high humidity (>90%) and warm temperatures, the optimal temperature for germination and growth for most *Metarhizium* isolates lies between 25 and 30°C (Zimmermann 2007a). The fungus then starts penetrating its host by the formation of an appressorium. Beneath this hold-fast structure a penetration peg is formed and several enzymes are produced to pass through the hosts cuticle (Butt et al. 2016). Inside the hosts body, fungal hyphae transform into yeast-like blastospores (Prasertphon and Tanada 1968; Wang and Leger 2007). They multiply in the haemolymph and deplete the nutrients in it (Zhao et al. 2006). Once the host has died, the fungus re-emerges on the surface of the insect cadaver and produces new conidia (Cherrie-Lee and Bidochka 2005). Some *Metarhizium* isolates also produces destruxins, secondary metabolites that facilitate the pathogenesis (Schrank and Vainstein 2010). *M. brunneum* one of the most commonly used fungal biocontrol agents (Lacey et al. 2015).

5.4.3. Aspects of fungal biology to consider in non-target testing

Perhaps the most apparent difference between biocontrol agents, such as EPF, and inanimate plant protection agents is that efficacy depends on their viability as well as their virulence. To achieve these two characteristics and keep them stable is one of the major challenges for EPF as biocontrol organisms. Viability and virulence can be readily affected by environmental conditions such as temperature, UV exposition or the nutrients available to them (Kim et al. 2019). For *M. brunneum* growth rates and virulence, among other factors, can vary considerably depending on the temperature (Bugeme et al. 2008; Li and Feng 2009) or for example soil type and moisture levels (Jaronski 2007). To ensure the quality of the tested

product it is thus necessary for non-target testing to carry out viability tests as well as to confirm virulence of the fungal isolate in the given circumstances i.e. the experimental setup (Reinbacher et al. 2021).

A second element to consider in non-target testing when looking at the environmental effects on the biological activity of EPF is the test duration. The time it takes for infection and eventually host death to occur may vary depending on incubation temperature, fungal isolate (Ekesi et al. 1999), and host species. As a consequence, the exposure period of non-target organisms to the infective propagules may need to be prolonged, considering also the life cycle of the non-target species.

Furthermore, inanimate substances degrade over time, whereas EPF after killing their host may produce resting structures or further infective spores for the proliferation to a new host (Roy et al. 2006). This could potentially lead to build up their populations, and promote further secondary infections (Meyling and Eilenberg 2007). Reinbacher et al. (2021) therefor proposed to add mycosis as an additional endpoint in non-target risk assessments as mycosed non-target organisms may delay the decrease of fungal inoculum over time.

To summarize: When thinking about modifying non-target test setups flexibility is necessary to adapt them to for the diverse requirements of EPF and microbial biocontrol agents in general. In addition to new or adapted test protocol it may also be necessary to clearly define acceptable risks of the data generated with them. A task that may require the cooperation of expert groups, policymakers, and stakeholders.

5.4.4. Conclusions

The substitution of hazardous chemical pesticides is one of the key elements towards more sustainable plant protection in agriculture (Pretty 2018). To reduce environmental risk it is recommended to prioritize biological control organism such as EPF over chemical plant protection agents (Möhring et al. 2020). However, high prices of microbial insecticides in comparison to chemical insecticides are a major threat to the successful implementation of microbial control. Depending on how much emphasis is given to sustainable pest management by policy makers, governments can play a key role in this issue (Ravensberg, 2011). One aspect of political influence is determining the data requirements, like non-target risk assessments, for the registration of microbial insecticides. This strong impact was evident in Brazil when the adoption of a simplified registration process for biopesticides in 2009 led to an upsurge in available products (Mascarin et al. 2019). Removing obstacles for microbial pesticide registration is thus highly important. In the short-term this could be attempted by standardized protocols created specifically for microbial control agents and sharing information on adapted protocols that are already in use. In the long-term fast-track authorization systems of low-risk products with lower data requirements as suggested by Möhring et al. (2020) could be highly beneficial.

5.4.5. References

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