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WORKING DOCUMENT ON THE EVALUATION OF MICROBIALS FOR PEST CONTROL

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WORKING DOCUMENT ON THE EVALUATION OF MICROBIALS FOR PEST CONTROL

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FOREWORD

The OECD work on agricultural pesticides (i.e. chemical and biological pesticides) aims to help member countries improve the efficiency of pesticide control, share the work of pesticide registration and re-registration, minimise non-tariff trade barriers and reduce risks to human health and the environment resulting from their use. In support of these goals, the Pesticides Programme has undertaken work to: (i) identify and overcome obstacles to work-sharing; (ii) harmonise data requirements and test guidelines; and (iii) harmonise hazard/risk assessment approaches.

With the primary goal of facilitating the sharing of national review reports, OECD's work initially focused on ways to harmonise the format/structure of reviews that are exchanged. The OECD dossier and monograph guidance provide a general lay-out and standardised formats for industry reporting (dossier) and government reviews (monographs). They were developed with the aim of facilitating the exchange of reviews among countries.

The OECD BioPesticides Steering Group (BPSG) was established by the Working Group on Pesticides (WGP) in 1999 to help member countries harmonise the methods and approaches used to assess biological pesticides and improve the efficiency of control procedures. Biological pesticides include: microbials, pheromones and other semiochemicals, and invertebrates as biological control agents. The first tasks of the BPSG consisted of: (i) reviewing regulatory data requirements for the three categories of biopesticides; and (ii) developing formats for dossiers and monographs for microbials, and pheromones and other semio-chemicals. This was achieved in 2004.

The BPSG then decided to concentrate its efforts on scientific and technical issues that remain as barriers to harmonisation and work-sharing. Five areas have been identified: taxonomy; genetic toxicity; operator and consumer exposure; residues in treated food crops; and efficacy evaluation. The objective was to develop a document to assist government and industry risk assessors and scientists involved in the registration and regulation of microbial pest control products (MPCPs) and their active agents (MPCAs).

The present Working Document describes the views of different OECD countries on how they assess these scientific issues in the safety evaluation of MPCPs. It is intended to be used as guidance in the safety assessment of microbials, and the preparation and evaluation of the dossiers and monographs for microbial pest control products.

The WGP recommended that this document be forwarded to the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology, for consideration as an OECD publication.

The Joint Meeting agreed that it should be made available to the public. This document is published under its responsibility.

PREFACE

This Working Document is written for government and industry risk assessors, and for scientists involved in the registration and regulation of microbial pest control products (MPCPs) and their active agents (MPCAs). However, it can also be a useful tool in the assessment of microbial biocides.

The Working Document presents the views of the different OECD countries on how they address these scientific issues in the safety evaluation of MPCPs. It is intended to be used as guidance in the safety assessment of microbials, but its use is not a requirement. For example, it can be used for the 4th list of substances to be assessed by the EU, for re-registrations of microbials, for national authorisations. In this way, government safety evaluations of data submitted for registration can be improved. With this guidance, (i) companies should be better prepared to submit the relevant data for risk assessment and (ii) regulatory authorities should be better prepared to review the submitted dossiers and monographs.

SUMMARY

How to evaluate the safety of microbial pest control products

New pest control products with micro-organisms as the active agent are expected to enter the market. They need a proper safety evaluation as under particular conditions some micro-organisms may be infective or can produce serious detrimental toxicants. However, scientific and technical guidance on the safety evaluation of microbials is scarce. A safety evaluation should take into account the characterisation and identification of the micro-organism and other components of the product, the efficacy of the product and the emission to the environment during and after application. Subsequently, the exposure and effects (toxicity, infectivity) on humans and non-target groups as birds, bees or fish are assessed (risk assessment). This Working Document is intended to help industry prepare and governments evaluate such dossiers and prepare monographs on microbial pest control products.

INTRODUCTION

Micro-organisms, agriculture and safety

The use of microbial pest control products is an increasingly important approach for achieving a more sustainable agriculture, *i.e.* – amongst others – an agriculture that is less polluting and less dependent on synthetic chemical pesticides[1] (de Heer, 2000; van Lenteren *et al.,* 1992). Thus, in the coming years new MPCPs are expected to enter the market place. MPCPs are allegedly less persistent and hazardous than their synthetic counterparts[2] (van Lenteren *et al.,* 1992; Mensink *et al.,* 1998; RAFBCA (Risk Assessment of Fungal Biological Control Agents), 2005; Scheepmaker & Mensink, 2002). Various products have been used throughout the world without demonstrable negative effects to human health, wildlife and the environment; however some micro-organisms used in agriculture or horticulture may be infective or contain toxicants. For example, under particular conditions, strains of micro-organisms may produce toxins which have adverse effects on human health or the environment (Dewhurst, 2003). A proper safety evaluation of MPCPs prior to the placing on the market, is essential in order to guarantee their safe use by farmers and consumers (Dewhurst, 2003; KemI, 2001; Mensink *et al.,* 1998).

1 Together with substances of natural origin as pheromones and plant extracts, MPCPs are pest control products of natural origin (sometimes referred to as biopesticides or biological pesticides).

2 Synthetic pesticides which can be used against the same pest.

The cost associated with the research and development of a MPCP, the dossier prepared by industry, the evaluation, possible acceptance and approval by the regulatory authorities, and finally the marketing of MPCPs, can be significant (AgChemForum, 2001). In situations where a product can be placed in several markets, harmonisation across countries would facilitate the preparation and evaluation of necessary dossiers and monographs which have to be submitted for the same micro-organism.

Various initiatives are underway to harmonise the safety evaluations of MPCPs for regulatory purposes. The aim of these initiatives is that a dossier submitted to one member country (or monograph developed from that dossier) can be used by other OECD countries. This concept is described in an OECD paper entitles "*Vision for the future: A global approach to the regulation of agricultural pesticides*".

The most important initiatives in this respect are those from OECD s, the NAFTA (North American Free Trade Agreement) countries and the European Union. They are introduced below.

OECD

The OECD project on registration and re-registration of biological pesticides (including microbials, pheromones and semiochemicals, and invertebrates) helps governments work together to assess risks to man and the environment. The activity is conducted by the BioPesticide Steering Group (BPSG) under the responsibility of the Working Group on Pesticides (WGP). By working together, governments can evaluate a biological pesticide more quickly and thoroughly. This speeds up the process for approving safer new pesticides. The project consists of providing guidance for the submission and evaluation of biological pesticide data.

NAFTA countries

In 1997, the North American Free Trade Agreement Technical Working Group (NAFTA TWG) on Pesticides was established under the NAFTA provisions on Sanitary and Phytosanitary Measures to implement a core function of the North American Free Trade Agreement signed in 1994 – to serve as a focal point for addressing pesticide issues arising in the context of liberalized trade among the NAFTA countries. Since its creation, the TWG has maintained a primary focus on facilitating cost-effective pesticide regulation among the three countries through collaboration and work sharing, while achieving the environmental, ecological and human health objectives of NAFTA. Efficient work sharing requires a shared understanding of the responsibilities of each agency, as well as common procedures and timeframes. Cooperation has also covered a wide range of activities, such as sharing information, undertaking collaborative scientific work, forging common data requirements, collaborating on risk assessments, compliance methods, joint reviews and common NAFTA or international (e.g., OECD) standards (NAFTA, 2008).

In order to increase the number of biopesticides with NAFTA registrations, the Canadian Pest Management Regulatory Agency (PMRA) and the United States Environmental Protection Agency (EPA) established in 2002 (NAFTA, 2002) a process for the joint review of new microbial and semiochemical pest control products and recently extended these procedures to also include biochemical active ingredients other than semiochemicals. In the joint review programme the Agencies share the submission (dossier) review burden., prepare data evaluation reports (study review templates), peer review the other Agency's evaluations, conduct parallel risk assessments, work cooperatively to harmonize risk assessments and develop a common proposed decision on registration as well as prepare final review and risk assessment documents (monographs) together. Through the successful completion of joint reviews for five microbials and one arthropod pheromone, joint reviews have increased the efficiency of the registration process, facilitated simultaneous registration in Canada and the United States, and increased access to new pest management tools in both countries. Although there is limited scientific guidance available to evaluate the

safety of biopesticides within the NAFTA context, biopesticides are regulated in Canada and the U.S. on a case-by-case basis involving application of expert judgement and a tiered approach to requiring and assessing supporting test data/information (EPA, 2007; Health Canada 2001, Health Canada 2002; Health Canada, 2007).

European Union

According to the EC Directive 91/414/EEC, dossiers of micro-organisms must be reviewed and evaluated to conform to EU agreements[3]. Data requirements for microbials (European Commission, 2001) and the formats (OECD, 2004) for reporting these data are largely fixed. The EU Uniform Principles contain criteria for determining whether hazards or risks are acceptable (European Commission, 2005a). However, guidance for preparing and scientifically evaluating the dossiers and monographs is limited

An EU submission of a micro-organism must be accompanied by the dossier of at least one MPCP.

Once a micro-organism is evaluated and approved, it will be included in Annex I of the EC Directive 91/414/EEC (European Commission, 1991). Only then, the products containing this micro-organism can be approved for marketing in the respective Member States of the EU.

It must be emphasised that decisions on micro-organisms as pest control agents will be taken by the European Commission whereas the final decisions concerning the products which contain those micro-organisms will be made individually by the EU Member States.

The safety evaluation of a product has to comply with the amendment of Annex VI of the EC Directive 91/414/EEC. This amendment addresses the evaluation of MPCPs by the EU member states in order that they all use the same risk criteria (the Uniform Principles). The major objective of the EU safety evaluation of MPCPs is "...to identify and assess, on a scientific basis and until further experience is reached on a case-by-case basis, potential adverse effects on animal health and the environment of the use of a microbial plant protection product" (European Commission, 2005a).

"Existing" MPCPs will be re-evaluated in the 4th stage of the review programme of the EU. There is, however, little scientific guidance to evaluate the safety of these products.

DEFINITIONS

For the purpose of this document, the following definitions are used.

Micro-organisms: "microbiological entities, cellular or non-cellular, capable of replication and/or of transferring genetic material". The definition applies "to, but is not limited to, bacteria, fungi, protozoa, viruses and viroids"; nematodes are not included.

Relevant metabolite: "any metabolite that is of concern for human or animal health and/or the environment"; in this way, some toxins can be considered relevant metabolites.

Infectivity/infectiveness: ability of a micro-organism to invade and persist in a viable state and to multiply within or on an organism, with or without disease manifestation. The nature of an infection can vary widely with respect to severity, location and number of organisms involved. An infection may or may

[3] In the EU there is a jurisdictional division between "existing" (*i.e.* on the EU market before 25 July 1993) and "new" active substances (thereafter).

not result in overt disease. Any parasitic organism or agent that produces an infectious disease is a pathogen; its ability to cause disease is defined as pathogenicity.

Pathogenicity: ability of a micro-organism to inflict injury and damage in the host after infection; it depends on host resistance or susceptibility. A pathogen is determined by three characteristics: invasiveness, infectivity and pathogenic potential. Invasiveness is the ability of the organism to spread to adjacent or other tissues. Infectivity is the ability of the organism to establish a focal point of infection. Pathogenic potential refers to the degree that the pathogen causes morbid symptoms.

Toxicity: injury or damage in a host caused by a toxin including metabolites; infection, replication or viability of the micro-organism is not necessarily required (European Commission, 2005b, see also (European Commission, 2001/2005a).

In addition to the MPCP, various *co-formulantia* may be part of the product *e.g.* improve the adherence to leaves or increase the stability and thus efficacy. Generally, the micro-organism, the *co-formulantia* and growth medium residues form the end-use product that can be sold on the market.

Extracted, isolated and purified biochemicals of microbial origin are not addressed in this document. Both in the NAFTA and EU countries, such biochemicals are considered synthetic pesticides, thus requiring a full dossier and monograph. Guidance about these active substances is provided in 91/414/EEC (European Commission, 1991) and US EPA (2005).

Some technical terms are put in brackets as no clear definition is available. However, these terms may be useful in their context (*e.g.* indigenous, exotic and background level) and might be defined more precisely in the future. In this context, propagules are the micro-organism units for asexual or sexual replication. In this way *e.g.* hyphens, or conidiospores are or can be propagules.

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CHAPTER 1

Taxonomic Identification of Micro-organisms in Microbial Pest Control Products

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General view

Risk assessment of micro-organisms for use as microbial pest control agents in microbial pest control products is for several reasons not comparable to the risk assessment of chemical pesticides. Because of the diversity and complexity of micro-organisms, risk assessment has to be considered on a case-by-case basis.

It is a general view that the applicant should carry out detailed taxonomic descriptions and species affiliation of the microbial pest control agent (MPCA). Based on these taxonomic data, the MPCA should be evaluated and authorized by the competent authority at strain level. Consequently, the data requirements should be fulfilled at strain level unless the applicant can prove that this is not necessary for a particular strain. However, for the more general parts of the dossier, data for different strains of the same species might be used, when a high similarity within a species has been demonstrated. Strain-specific data remain necessary for the key issues, such as the mode of action and the possible production and effects of toxins.

Large parts of the dossier may consist of data coming from the literature. Such data have to be evaluated in the same way as other information. However, the regulating authorities in the OECD member countries might conclude that such information is not sufficient for risk assessment purpose.

In order to avoid duplication of work, applicants are encouraged to submit information that has already been assessed in earlier evaluations – if this does not interfere with the rules of data protection – e.g., by producing data on higher taxonomic level, exchanging letters for access to protected data, using the literature data, or using risk assessments performed in other countries.

Definitions

Bridging studies: Studies showing that two different sets of data are comparable (e.g. on different micro-organisms). Bridging studies will indicate whether or not studies of organism A can be used for risk assessment of organism B, and typically will be comparison studies including both organisms. Bridging studies will indicate to which extent familiarity can be used.

Familiarity: Familiarity is based on the knowledge and experience with an organism, product, trait, or environment. However, in addition to knowledge and experience, familiarity may include the use of similar micro-organisms, or a similar environment (OECD, 1995).

Genotype*:* The genetic constitution of an individual isolate. It is the basis for species differentiation of micro-organisms.

Isolate*:* An isolate is a pure culture derived from a heterogeneous, wild population of micro-organisms.

Microbial pest control agent (MPCA) (active substance in a MPCP): a micro-organism (*e.g.* bacterium, fungus, protozoan, virus, viroid, mycoplasma, algae, and rikettsia) and any associated metabolites, to which the effect of pest control is attributed.

Microbial pest control product (MPCP) (microbial plant protection product)*:* a product containing an MPCA that is registered or labelled with instructions for direct use or application for pest control purposes.

Phenetic*:* classification based on appearances (behaviour and characteristics) of organisms rather than on evolution from a common ancestor.

Phenotype*:* The phenotype of an organism is the class to which that organism belongs. It is determined by its morphology, development or behaviour as opposed to its genotype, which is the inherited instructions it carries.

Phylogenetic*:* classification based on an evolutionary development of organisms. When applied to bacteria at present times always based on genetic structures.

Species*:* For sexually reproducing organisms: a group of individuals not able to interbreed with other groups. For micro-organisms no clear-cut definition exists. Traditionally micro-organisms have been identified to species depending on morphological, physiological and phenotypic characters. Development in techniques and increased understanding of life cycles continuously change the taxonomy of micro-organisms. A thorough discussion of the definition of microbial species is included in the text.

Strain: A strain is a population of organisms that descends from a single cell or a pure culture isolate. Typically, it is the result of a succession of cultures ultimately deriving from an initial single colony.

Introduction and aim

An active micro-organism in MPCPs may cover different species as well as several strains of the same species. Recently, the EU Commission has developed a guideline on the taxonomic level of micro-organisms to be included in Annex I to Directive 91/414/EEC.

The aim of this guidance is to help harmonize the evaluation of MPCPs regarding:

a. Which taxonomic level the micro-organism belongs to and how it should be identified?
b. Should the whole data package be provided for the 'exact' microbial strain under submission, or can data generated on closely related micro-organisms (species and strains) be used?
c. The concept of indigenous micro-organisms.

Taxonomy

The first step in the risk assessment of MPCAs is usually an evaluation of the identity and taxonomic affiliations of the micro-organism. Guidance would help the applicant submit the taxonomic

information that will be deemed adequate by the risk assessor. In addition, it would be desirable that the regulating authorities in the OECD member countries request the same level of taxonomic details for the micro-organism submitted to registration. Harmonization in this area is needed.

Below follows a brief introduction to the taxonomy of micro-organisms.

Taxonomy of fungi

Species differentiation is based on the following (Hawksworth *et al*., 1995):

1. Morphospecies are species delimited from other species by morphological and phenetic characteristics. This is the traditional approach of species identification of fungi.
2. Biological species based on cryptic and sibling characteristics are actual or potential interbreeding populations reproductively isolated from other such groups, whether or not they are distinguishable morphologically.
3. Phylogenetic species are delimited from other species by evolutionary characteristics based on measurable differences in biochemical, molecular or any other characters assessed by cladistic analysis. This is well suited for groups in which no sexual reproduction is known (e.g. mitosporic fungi[4]).
4. Ecological species are delimited by characteristics based on adaptation to particular niches rather than reproductive isolation (e.g. to particular hosts).
5. Polythetic species are based on a combination of characters, of which not all are necessarily present in each individual.

The morphospecies concept has predominated in mycology, although in the past mycologists often took the host of a parasitic fungus into account. Population studies and molecular data are, however, increasingly showing that many morphospecies comprise several biological and (or) phylogenetic species. Future taxonomic identification of fungi should more rely on genotypic methods in combination with the traditional identification techniques.

Morphological characteristics have a limited resolution power. A large array of molecular tools currently available should help provide information on the genotype and phenotype. The DNA sequence analysis of known genes, particularly portions of the ribosomal RNA genes (rDNA), has emerged as a powerful tool for identifying fungal species.

Mitosporic fungi are common among MPCAs and include the *Beauveria, Fusarium, Metarhizium, Paecilomyces, Trichoderma,* and *Verticillium* genera. Some of these genera do contain teleomorphs, but some strains in the MPCPs are mitosporic and their taxonomy is not resolved; it continuously evolves as soon as the sexual state is known and the molecular techniques reveal changes. Because of that, bridging studies and familiarity with closely related organisms are of little value. About fungal species for insect control, the current paradigm is that within the species, the (insect) host steers the structure of population. However, the selection of specific habitats and climatic zones might also be the driving force (Bidochka *et al*., 2001).

Taxonomy of bacteria

Several methods can be used for the taxonomic identification of bacteria; they are

[4] A large and heterogeneous group of imperfect fungi whose common characteristics are absence of a sexual stage. Mitosporic fungi are also called Hyphomycetes.

1. Phenotypic methods (e.g. morphology, physiology and metabolism) are comparable to the identification of morphospecies used for fungi. These methods are based on growth of the organism in pure culture under appropriate conditions.
2. Chemotaxonomic methods examine the phenotype by analysis of chemical constituents of the organisms, e.g. Gram-behaviour, composition and content of fatty acid and polar lipids, serotyping, toxins.
3. Genotypic methods, e.g. DNA base ratios and DNA hybridization, DNA-based typing methods, RNA-based typing methods, sequencing of house-keeping genes.

The term 'species' applied to bacteria is usually defined as a distinct group of strains having certain distinguishing features and for which the essential features of the cellular organisation closely resemble each other. This species concept is extremely subjective because it is not possible to accurately determine "a close resemblance", "essential features", or how many "distinguishing features" are sufficient to describe a species. Therefore phenotypic elements based on the potential functions of a bacterium have been developed to characterize/identify species. Accordingly, the morphological and biochemical properties of a bacterium may be used to characterize a species; generally 200-300 features are used. (based on Brenner *et al*., 2001).

Numerical taxonomic methods have further improved the phenotypic classification and permited to define species based on similarities between strains. However, even with a battery of 300 biochemical tests, only a very limited part (5-20%) of the genetic potential is assessed. It is recognized that phylogenetic is the most accurate basis for species-classification. Data necessary to agree on a definition of a phylogenetic species became available when the DNA hybridization technique was used to determine the relatedness between bacteria. Experience with a high number of strains from several hundred species led taxonomists to formulate a phylogenetic definition of a bacterial species (genospecies) as:

Strains with approximately 70% or greater DNA-DNA relatedness and with a 5°C or less ΔT_m (thermal stability) (Wayne et al., 1987)

Taxonomists further recommended that a genospecies should not be named if it cannot be differentiated from other genospecies on the basis of some phenotypic properties (Wayne *et al.*, 1987). Validity of the definition of species based on the DNA-relatedness has been questioned; critics are triple: (i) it employs an arbitrary cut-off for a species whereas evolution never stops; (ii) bacterial species are not real entities – they are useful but not meaningful from an evolutionary point of view; (iii) it does not allow to; obtain standard species; and (iv) the existing methods are difficult to implement and they are not comparable between them.

The best approach to the species issue is a pragmatic polyphasic taxonomy that integrates all the available data. Lately, Stackebrandt *et al*. (2002) recommended that the new developments such as sequencing (16S rDNA, household genes, and complete genomes), DNA typing methods, DNA arrays and characterization of cells by advanced physical methods, should be used for taxonomy. They encouraged searchers to propose new species based on these new methods, provided they can demonstrate a sufficient degree of congruence between the technique used and the DNA-DNA re-association.

The classification of bacteria has been a historical process during which the species concept has changed. The description of the existing species is not based on the same species concept; it is often based on the concept in use at the moment the species is described.

Additional information about taxonomy of bacteria in relation to risk assessment can be found in the OECD *Guidance document on the use of taxonomy in risk assessment of micro-organisms: Bacteria* (2003). This document describes the relevance of taxonomy in the risk assessment of bacteria as well as the methods used for the classification and identification of bacteria. It also includes several examples.

Taxonomy of viruses

Viruses are not living organisms, because they do not have the essential characteristics that define life, such as cellular organization, propagation by cell division and a cellular metabolism. They are biologically active systems and are dependent on other organisms, as they are infectious and intracellular parasites. A virus is composed of a capsid (protein) and a genome (nucleic acid under the form of RNA or DNA). The taxonomy of viruses differs from that of living organisms since the historic definition used for sexually reproducing organisms cannot be applied. However similar taxonomic levels, such as species, genus, family and order, are used. A virus species is currently considered a polythetic class of viruses that constitute a replicating lineage and occupy a particular niche (van Regenmortel, 2000). The criteria used for species demarcation may vary from a virus family to another.

The only viruses commercially used as MPCPs belong to the *Baculoviridae* family. Baculoviruses infect arthropods, primarily the insect orders *Lepidoptera, Diptera* and *Hymenoptera* as well as the crustacean order *Decapoda* (shrimp). They are divided into two genera, the Nucleopolyhedroviruses (NPVs) and the Granuloviruses (GVs). NPVs are characterized by the formation of polyhedral occlusion body embedding many virions consisting of single or multiple nucleocapsids. GVs have ovicylindrical occlusion bodies with virions containing only one (sometimes two) nucleocapsids. Recent research on genome sequencing revealed that NPVs isolated from Diptera and Hymenoptera have to be classified as separate genera since they are only distantly related to the Lepidopteran specific NPVs and GVs. In the literature more than 600 baculoviruses isolated from different hosts are described. The host range, DNA restriction profile and DNA sequences are presently used as species demarcation criteria. However, because there are no comparative data, these criteria are not clearly defined (Blissard *et al*., 2000, OECD 2002).

Comparison of genome sequence demonstrated that the gene sequence similarity between baculoviruses correlates with similarity in gene order and gene content (Herniou *et al*., 2003). Based on the whole genome and on single gene sequences, *Helicoverpa armigera* NPV and *Helicoverpa zea* NPV, and *Anagrapha falcifera* NPV and *Rachiplusia ou* NPV appear to be variants of the same species (Chen *et al*., 2002; Harrison and Bonning, 1999). The same situation is observed for many other baculoviruses isolated from different hosts. On the other hand, closely related viruses differing in only a few genes and encoding a very similar proteome may exhibit different host ranges and are thus considered different species. For this reason an unequivocal system of identification of virus that allows a taxonomic classification based on genetics is essential. A molecular approach to identify baculoviruses based on the sequence similarity of three highly conserved gene fragments (polyhedrin, lef-8 and lef-9) was recently presented by Lange *et al*. (2004). However, a molecular or phylogenetic definition of a baculovirus species is not yet established.

Due to the rapid knowledge in the gene and genome sequences of different baculoviruses, a better understanding of the relationship of these viruses and their taxonomy can be expected in a near future.

Conclusions regarding taxonomy

Genotypic methods are generally accepted for bacteria and baculoviruses, and a phylogenetic definition of species is in use. Fungi are usually identified by phenotypic (especially morphological) methods and in some cases molecular techniques are also employed. However, this difference in the

methods employed is due to the fact that more attention is given and more results for viral and bacterial molecular taxonomy are obtained compared with fungi; this is currently changing. Although a number of these methods can be used to identify a micro-organism, sometimes the best use of taxonomic methods fails to provide a definitive name. In that case, either the MPCA is considered a new species, or it is recognised that there are shortcomings in the methods used. The actual affiliation of a strain to a species is based on a number of characteristics, which can be phenotypic and genotypic.

A good taxonomic description of a MPCA allows its comparison with related micro-organisms described in literature. This may support a request for a data waiver if no related micro-organism has the characteristics the test is designed to measure, or, conversely, may indicate toxicity endpoints for which additional data may be required to demonstrate potential adverse effects, e.g. this may trigger a request for analysis of a specific toxin.

It is obvious from the discussion above that the definition of fungal, bacterial, and viral species is still under debate and will inevitably change in the future.

Evaluation of submitted data on the MPCA at strain or species level

The first step in the risk assessment of MPCAs is the evaluation of data on identification of the micro-organism. This is important for assessing the potential adverse effects and persistence of the organism and its by-products (e.g. toxins). A detailed taxonomic identification of the MPCA by the most appropriate and up-to-date methods, is necessary for the assessors to use properly the data submitted or found in literature (using a micro-organism for comparison purposes, i.e. the same species, but another strain). Such information can be used either to identify potential adverse effects and thus serve as a basis for further inquiries, or to demonstrate the safety of the MPCA. When bridging studies have been performed, these are considered acceptable only if the taxonomy of the micro-organisms is described in sufficient detail and the strains are adequately taxonomically related.

In summary, the identification of the MPCA should preferably be performed by at least two independent laboratories using the most up-to-date and standardized techniques available from the scientific community. The technique used will depend on the organism and possibly will also vary from a laboratory to another. Where a MPCA relates to different species, additional testing should be undertaken to definitely identify the MPCA and relate it to a species.

The MPCA in the Mycostop product illustrates the importance of a thorough identification of the belonging to a species. According to the producer, Mycostop contains *Streptomyces griseoviridis*; however, an evaluation of the product that included an identification of the MPCA (carried out by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and published by the Danish EPA, Winding, 2005) showed that the bacterium is a new species of *Streptomyces* and is not a *Streptomyces griseoviridis*. This result was obtained by using the DNA-DNA hybridization technique. Hence, the familiarity of this strain can only be at the level of the genus.

Six examples of important differences between strains or isolates of the same species are presented below (A-F). These examples clearly show that strains of one same species can have different activities e.g., produce different antifungal/antibiotic compounds, and therefore possess different modes of action and different toxicity vis-à-vis the targeted and non-target organisms. However, there are many examples of strains belonging to the same species having the same functions, potentials and pathogenic traits, for which bridging a gap between experiences and testing data is relevant.

Examples of differences between strains of the same species

A. Several MPCAs identified as *Pseudomonas fluorescens, P. chlororaphis* and *P. putida* are candidates to make MPCPs. As shown in Table 1, some strains of *P. fluorescens* produce different antifungal compounds. Obviously, the risk assessment of these strains has to be done at the strain level, especially when assessing effects (target or non-target) of the antifungal strategies as well as human health effects. There are however examples of strains where bridging studies will be appropriate as the antifungal compounds produced by several strains are identical; the *P. fluorescens* group that produces 2,4-diacetylphloroglucinol (DAPG) (F113, CHA0, Q2-87 and Q8r1-96) is an example.

Table 1: List of *Pseudomonas* species or spp. already marketed or potentially suitable as active micro-organism in MPCP.

Bacterial strain	Origin	Antifungal compound produced
P. fluorescens F113	sugar beet rhizosphere	2,4-diacetylphloroglucinol (DAPG), siderophore, hydrogen cyanide
P. fluorescens CHA0	soil suppressive to black root rot and with tobacco	DAPG, pyoluteorin (plt), hydrogen cyanide
P. fluorescens DR54	sugar beet	viscosinamide, cellulytic enzymes
P. fluorescens SBW25	sugar beet	unknown
P. fluorescens Pf29A	soil suppressive to take-all	unknown
P. fluorescens Q2-87	Wheat	DAPG
P. fluorescens Q8r1-96	Wheat	DAPG
P. aureofaciens TX-1*	Rhizosphere	phenazine-1-carboxylic acid (PCA)
P. chlororaphis MA342*	craw berry rhizosphere	2,3-deepoxy-2,3-didehydro-rhizoxin (DDR)
P. chlororaphis 3732	Unknown	unknown
P. putida WCS358r	Potato	siderophore
P. putida 06909	*Phytophthora citrophthora*	siderophore

*: marketed BCA (from Winding *et al*., 2004)

B. A number of *Bacillus thuringiensis* (*Bt*) strains are used as MPCAs for the control of insect pests. *Bt* forms a large set of strains, which produce crystalline inclusion bodies during sporulation. These crystalline inclusions contain insecticidal delta-endotoxins, which are encoded by plasmid-borne genes. Serotyping is the most commonly used method for differentiation of subspecies (serovars) within this species. In 1999, 82 different serovars were described (Lecadet *et al*., 1999), and since then this number has slightly increased. Most strains used as MPCAs are members of one of the three serovars: *kurstaki, israeliensis* and *tenebrionis* and are mainly used for the control

of lepidopteran, dipteran and coleopteran larvae, respectively. However, (i) the strains within each of the serovars differ in toxicity vis-à-vis specific insects and also in the range of targets, (ii) the target of specific strains within a serovar is not limited to a specific group of insects e.g. some *Bt-kurstaki* strains are active against dipteran larvae and some *Bt-israeliensis* strains are active against lepidopteran larvae, (iii) the occurrence of delta-endotoxin genes in specific strains is not unambiguously related to their serotype (Glare and O´Callaghan, 2000). Thus, it is not possible to base risk assessment on the affiliation of specific strains to serotypes, this should rather be based on the specific strain. Moreover, *Bt* strains selected for the control of bacterial plant diseases are under development (Cherif *et al*., 2003), and strains producing Zwittermicin A which have an activity against fungal diseases exist (Raffel *et al*., 1996). Many recent genetic analyses of *B. cereus* and *B. thuringiensis* strains have shown an evolutionary diversity resulting in groups of related strains that include both *B. cereus* strains, and *B. thuringiensis* strains, plus some strains from some other *Bacillus* species (e.g. Priest et al. 2004; Ticknor et al. 2001). In addition, these *Bacillus* species share many plasmids, which can be transferred among different strains. Since a number of significant traits, including the *B. thuringiensis* species-determining crystal insecticidal toxin trait, are found on these plasmids, the traditional species taxonomy is not sufficiently specific to separate these groups of *Bacillus* by their relevant traits (Hendriksen and Hansen, 1998).

C. *Erwinia carotovora* is an example of a bacterial species with varying pathogenicity. In the product BioKeeper, the MPCA is a non-pathogenic strain of *E. carotovora* isolated from Chinese cabbage. BioKeeper is used to prevent soft rot caused by the same species: *E. carotovora*, but a pathogenic strain.

D. According to the producer's information the MPCA in Vertalec and Mycotal, two MPCPs on the Danish market, are two different strains of *Verticillium lecanii*. However, changes in the taxonomy of this genus have changed the species affiliation. The strain in Mycotal is now identified as the species *Lecanicillium muscarium*, and the strain in Vertalec as *Lecanicillium longisporum* (Zare and Gams, 2001). This example clearly shows how important an up-to-date and best possible identification of the MPCA is for risk assessment. *Verticillium* belongs to the group of mitosporic fungi.

E. The mitosporic fungi *Fusarium oxysporum* and *F. moniliforme* are common causes of vascular wilts. However, a non-pathogenic strain of *F. oxysporum* is used as biological fungicide and commercialized under the name Fusaclean and Biofox C.

The examples above perfectly illustrate the importance of a detailed identification of the affiliation to species level of the MPCA as well as the need for submitting the results of detailed studies on thestrain concerned.

Indigenous *versus* non-indigenous micro-organisms

Applicants have to include information regarding whether or not the organism is indigenous or non-indigenous with respect to the area in which the MPCP is used. Canada has adopted the concept of indigenous and non-indigenous in its registration procedure for microbial pesticides, and because of its large geographical area, the country has been divided into five ecozones which are defined as large and very generalized ecologically distinctive areas based on the interplay of landform, water, soil, climate, flora, fauna and human factors. An organism may only be considered indigenous to a specific ecozone or ecozones from which its occurrence has been formally documented rather than to the whole country. However, a clear definition of the concept is missing. For animals and plants the concept is widely used to protect species and the environment in certain continents/countries. For micro-organisms there are different possible situations.

- To consider a micro-organism indigenous, one definition of a species or at least uniform tools for distinction of species and strains are needed to distinguish the micro-organism from the other micro-organisms already present in the environment; this is not the case for many micro-organisms.
- Due to the small size of micro-organisms, the geographical distance is often important:
  - For soil micro-organisms, the genetic distance between strains of *Pseudomonas* sensu stricto is related to the geographical distance, both at the scale of continents and for distances of less than 200 km (Cho and Tiedje, 2000). In biocontrol fluorescent pseudomonads that were isolated from different continents and produced DAPG (presence of the *phlD* gene), the diversity of the *phlD* gene is high. This shows a high level of polymorphism of fluorescent pseudomonads with biocontrol activities (Wang *et al.*, 2001). In hot springs cyanobacterial 16S rRNA was never identical in various countries (Papke and Ward, 2004). These results may partly be explained by the clonal origin of micro-organisms, combined with the fact that there is little transport within the soil matrix and between sites.
  - In contrast, transport by wind (either direct or attached to e.g. soil particles), soil, plants including seeds and vectors (e.g animals, man) clearly shows the potential of worldwide dispersal of some micro-organisms.
  - Whether micro-organisms introduced to new environments may have adverse effects on the residential microflora or will on the long run be out-competed in population size is difficult to assess.

Hence, some micro-organisms seem to stay in their local environment with low dispersal while continuing and maintaining their clone, while others disperse relatively quickly over long distances.

When the definition of a microbial species has not been agreed upon and when the MPCA is evaluated and authorized at the strain level, defining an MPCA as indigenous for a market, e.g., the EU, Canada or the USA is difficult. This makes the use of the concept of indigenous micro-organisms of limited value.

Recommendations

When submitting a dossier on an MPCP, the MPCA in the product needs to be identified at the highest possible level of detail, i.e., the lowest epithetic level. Where possible, the micro-organism should be specified at the strain level, and the strain should be deposited in an internationally recognized culture collection. When not possible (e.g., for viruses with mosaic genomes), the identification should be performed at the species level and include any additional information on distinguishing features. Specification at strain level and deposition in culture collections are prerequisites in order to obtain authorization for the MPCP in e.g. the EU, USA and Canada. All existing taxonomic details should be included in the dossier. Moreover, it is recommended that two independent laboratories determine the species affiliation of the organism.

The applicant should carry out detailed taxonomic descriptions and species affiliation of the MPCA. Based on these taxonomic data, the MPCA should be evaluated and authorized at strain level. Generally, the data package of an MPCP needs to be prepared on the basis of data generated on the exact strain of the MPCA. However, the regulating authority may accept that part of the data is generated on a comparison micro-organism and accept that such data replace data obtained on the exact strain of the MPCA (i.e., typically another strain of the same species). In order for the regulating authority to accept such data, the applicant should describe the familiarity of the two organisms and when appropriate should

present data of bridging studies. Since this will be a case-by-case evaluation, the competent authority should be consulted prior to submission of the dossier.

EU procedure for Baculoviruses

Based on the conclusions from the OECD Consensus Document on information used in the assessment of environmental applications involving Baculoviruses (2002), the EU has decided to include Baculoviruses (not genetically modified) on species level in Annex I of Directive 91/414/EEC, and to add the different isolates (after they have been evaluated) to a separate list, to be maintained in a separate Annex to the Review Report and that will be amended by taking note in the Standing Committee on the Food Chain and Animal Health.

New isolates would be submitted by a Member state (application for national authorisation). The member state should prepare a report on the properties of the new isolate and compare them with the reference isolate. The Standing Committee on the Food Chain and Animal Health will take note of this report.

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CHAPTER 2

Genetic Toxicity Assessment of Microbial Pesticides

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Introduction

Effective use of pesticides is an essential element of economical food production, and the use of agents with a high selectivity for pests and minimal toxic potential for humans and farm animals is the key to safe and effective use in pest control strategies (Montesinos, 2003). Microbial pesticides offer the advantages of higher selectivity and lower toxicity than conventional chemical pesticides, but evaluation of their safety may require modification of the traditional approaches used for chemical agents or may require unique tests for safety hazards that are specific to microbial agents. Thus, consideration must be given to the unique biological characteristics of microbial pesticides, and safety testing must be designed to address both general and specific potential hazards. Assessment of genotoxic potential poses special considerations, as the nature of the various test organisms employed in relation to the microbial pesticide must be taken into account.

General guidelines for assessment of the genotoxic potential of microbial pesticides have been implemented by the U.S. Environmental Protection Agency, Canada, and the European Community (USEPA6; Health Canada, 2001; EEC, 2001). These guidelines address the factors to be considered and testing approaches to be used to evaluate the potential for genetic damage from exposure to microbial pesticides. The Organization for Economic Co-operation and Development (OECD) has issued Guidance for Registration Requirements for Microbial Pesticides that is an important step in harmonizing registration requirements for microbial pesticides (OECD, 2003), and the Working Party on Chemicals, Pesticides and Biotechnology and the Chemicals Committee of the Environment Directorate have been working jointly to finalize OECD recommendations. It is noteworthy that the EPA, Canadian, and EU guidances on genetic toxicity testing used to be similar, but not identical, and this is recognized in the OECD Guidance for Registration Requirements for Microbial Pesticides of May 23, 2003. However, genotoxicity testing is no longer required by the revised US data requirements, effective Dec 26, 2007. Extension of the OECD guidance to include specific protocols for the testing of microbial pesticides that are accepted worldwide is very important.

[5] Originally, MacGregor issued a paper on December 9, 2005 at the request of Valent Bioscience. This paper reflected his personal comments and interpretation of Commission Directive 2001/36/EC. Based mainly on the comments received, revisions of data requirements, and the experience in the EU with 4th List microbials, it was decided to slightly adapt the originally paper, leading to a more general paper on genotoxicity testing of microbials, excluding the personal comments and interpretations of Directive 91/414/EEC of MacGregor.

[6] EPA Toxicology Data Requirements for Microbial Pesticides:
http://www.epa.gov/pesticides/biopesticides/regtools/guidelines/40cfr158_740c.htm

http://www.epa.gov/fedrgstr/EPA-PEST/2007/October/Day-26/p20828.htm

Safety Concerns

There is a need to differentiate data requirements for microbial pesticides from those for conventional chemical pesticides. This is due to differences in the characteristics of microbial agents from conventional chemicals. These characteristics may either increase or decrease concern for potential hazards of specific microbial agents relative to those for chemical agents. Depending on the nature of the biological agent, there may be concerns over potential infectivity, exchange of DNA from the microbe into mammalian species, or the potential for exo- or endotoxins to exert biological effects. Exo- or endotoxins can be appropriately tested in standard tests designed to detect genotoxic effects of small MW chemicals that can readily enter cells and interact with cellular macromolecules, but non-infective organisms that do not produce specific toxins are unlikely to be genotoxic either to mammalian species or in conventional mutagenicity test systems. It is thus critical to have a flexible approach to safety evaluation that takes into account the known biological properties of the specific microbial agent under consideration, and to customize the safety testing strategy to address those concerns that are most likely to occur based on knowledge of the characteristics of the specific organism.

Testing Requirements and Protocol Design

Recognition of the differences between biotechnology-derived products or organisms vs. chemicals has led certain authoritative bodies to conclude that the types of genotoxicity studies routinely conducted for chemical products are not applicable to these types of products and are therefore not needed for product registration. This is the position of the pharmaceutical community, which states in its guidance for safety evaluation of biotechnology-derived pharmaceutical products (ICH, 1998):

"The range and type of genotoxicity studies routinely conducted for pharmaceuticals are not applicable to biotechnology-derived pharmaceuticals and therefore are not needed. Moreover, the administration of large quantities of peptides/proteins may yield uninterpretable results. It is not expected that these substances would interact directly with DNA or other chromosomal material ....." Further, this guidance states that "The use of standard genotoxicity studies for assessing the genotoxic potential of process contaminants is not considered appropriate. If performed for this purpose, however, the rationale should be provided."

A similar conclusion was reached by a task force of the German-speaking section of the European Environmental Mutagen Society, the Gesellschaft für Umweltmutationsforschung (Goecke *et al.*, 1999), which addressed the needs for genotoxicity testing of biotechnology-derived products recognized however that certain specific proteins could potentially modify DNA metabolism or cellular growth. This group concluded that conventional genotoxicity tests may be appropriate under certain limited circumstances and that appropriate specific testing needs to be considered on a case-by-case basis. This group conducted a survey of approximately 30 companies that produce biological products and also reviewed the open literature to determine approaches that had been used to evaluate the safety of proteins and other biological products. References are provided to other existing guidelines for safety evaluation of gene therapy products, vaccines, and plasmid DNA vaccines that may have relevance to certain biotechnology products or organisms. A “decision tree” was developed to assist in the evaluation of appropriate testing needs based on the characteristics of specific products. Development of an analogous decision tree for the testing of microbial pesticides would be useful, and could be undertaken by a recognized group of international experts, as is discussed further below.

In those cases in which the conventional genotoxicity tests applied to small-molecule chemicals are appropriate for the evaluation of microbial products or lysates of microbes, careful consideration needs to be given to appropriate protocols that avoid uninterpretable or misleading results. Some examples of factors that could confound standard tests, when they are applied to the analysis of microorganisms or products derived from microorganisms, are provision of nutrients by lysates (*e.g.*, histidine, which would

allow growth of the auxotrophic tester strains in the Ames Salmonella assay), growth factors that may produce abnormal growth, growth inhibition, or DNA synthesis (*e.g.*, erythropoietin which causes micronuclei in bone marrow *via* induction of abnormal cell proliferation; lectins that may stimulate DNA synthesis in *in vitro* mammalian cell tests), enzymatic activity that could mimic endogenous activity in the test organism (e.g., kinase or phosphokinase activity in the $tk^{+/-}$ or *hprt* assays), or intracellular molecules with nuclease or proteolytic activity from *in vitro* lysates that would not normally have access to mammalian cells *in vivo*. Careful consideration to specific test design is needed to ensure that appropriate modifications of standard test protocols are made when necessary to avoid uninterpretable, false positive, or false negative results.

U.S. EPA CFR data requirements

EPA's data registration for microbial pesticides used to conditionally requires mammalian mutagenicity data (158.740) at Tier II level when:

- acute infectivity tests are positive in Tier I studies;
- adverse effects are observed in immune response studies;
- positive results are obtained in tissue culture tests with viral agents.

The revised data requirements, effective Dec 26, 2007, no longer included this requirement. However, the revised data requirements do permit the EPA to request studies that are not listed in the data requirements if an unanticipated need for them is identified in the course of evaluating the pesticide (or, a specific microbial toxicant). This is unlikely since EPA has never asked for a mutagenicity study on a microbial pesticide in the past.

EU Commission Directive 2001/36/EC

The EU data requirements are stated in EC Directive 91/414, amended by Directive 2001/36/EC:

EC Directive 91/414 5.2.3 Genotoxicity testing – Circumstances in which required
If the micro-organism produces exotoxins according to point 2.8, then these toxins and any other relevant metabolite in the culture medium must also be tested for genotoxicity. Such tests on toxins and metabolites should be performed using the purified chemical if possible.

If basic studies do not indicate that toxic metabolites are formed studies on micro-organism itself should be considered depending on expert judgment on the relevance and validity of the basic data. In the case of a virus the risk of insertional mutagenesis in mammalian cells or the risk of carcinogenicity has to be discussed.

The Directive focuses on flexibility in determining the need for testing and formulation of test strategies, the need to address the presence of metabolites with potential adverse health effects, and the need to address potential toxicities, pathogenicity, infectivity of the organism itself, and the need to discuss the potential for insertional mutagenesis by viruses. There is also a requirement to test purified or partially purified constituents from organisms known to produce exotoxins. Such testing should include any necessary test modifications suggested by the nature of the exotoxin expected (*e.g.*, whether it is a small molecule, a peptide, an enzymatically active protein, etc.).

Clear guidance is needed to define when *in vitro* and *in vivo* testing may be necessary, and the appropriate test methods to employ. For each test system anticipated to be used, there is a need for the development of protocols that include guidelines on appropriate dose routes, dosage forms, dose levels, and methods to be employed. As discussed below, it is highly desirable that these guidelines should be

developed through an international consensus process in order to avoid controversy over results or the need for unnecessary repetition of experiments in different regions.

Previous Experience with Complex Organic and Biological Samples

Many toxic, mutagenic, and carcinogenic secondary metabolites have been identified in microorganisms, particularly fungal species. Recognition of the potential health consequences of human exposure to such sources has resulted in much attention, beginning the early 1960s (Rodericks et al., 1977b), and a wide variety of biologically active molecules of diverse chemical structure, including carcinogens and mutagens, have been identified using various methods of isolation and bioassay (*e.g.*, see Steyn, 1977; Enomoto and Saito, 1972; Tazima, 1982; Rodericks et al., 1977a; Schrader et al., 2001; Sakai et al., 1992; Lakshmi et al., 2003). However, although specific products under consideration as microbial pesticides have been tested (*e.g.*, Genthner et al., 1998) it does not appear that a general method of screening fungi or other microorganisms for mutagenic activity has been developed. Although this has not been researched thoroughly, telephone contacts to a limited number of well-known scientists in these fields and a cursory literature search failed to identify reference to an established general testing scheme. The lack of reference to such a scheme in the EC Directive (2001), or the Health Canada (2001), EPA, or OECD Guidances (2003), support this conclusion.

In addition to fungi, complex organic materials reported to have been evaluated for mutagenic or anti-mutagenic activity include plants (Clark, 1982; Kaur et al., 2005), foods (Knudsen, 1986; Kaur et al., 2005), botanicals used as medicinals or cosmetics (Göggelmann and Schimmer, 1986; Cosmetic Ingredient Review Expert Panel, 2004), infected plant materials (Yahiaoui et al., 1994), and soil and water (Watanabe et al., 2005; Maruoka and Yamanaka, 1982). Experience with these materials points to those experimental factors that require consideration when testing complex materials, and provides some examples of modifications of conventional testing methods that have allowed these methods to be used successfully to test complex organic and biological materials. For example, preparation of samples for chemical analysis has been addressed for mycotoxins (*e.g.*, Boenke, 1995; Scott and Trucksess, 1977; Stoloff, 1977) and extensive analytical mycotoxin surveys have been conducted (see *e.g.*, Rodericks and Stoloff, 1977), and this experience will certainly be of value in identifying appropriate methods for preparation of samples suitable for mutagenicity analysis. Methods that have been applied to address the types of problems associated with mutagenicity analysis of complex organic samples include extraction procedures, the use of resins or binding substrates to isolate and concentrate active materials (Maruoka and Yamanaka, 1982; Scott and Trucksess, 1977), inclusion of enzymatic preparations that release conjugated forms of mutagenic constituents (Tamura et al., 1980), and destruction of nutrient constituents known to interfere with the assay).

Thus, there are numerous examples in the existing literature of methods used to evaluate complex mixtures of organic materials for mutagenic and/or anti-mutagenic activity. Many of the problems commonly encountered with the mutagenicity assessment of complex organic and biological substrates are expected to apply to testing of the classes of microbes used as pesticides. These problems include:

1) potential interference from toxic components,
2) the presence of nutrients that may interfere with certain assays (especially those based on mutation of genes required for synthesis or utilization of nutrients required for growth) (Nylund and Einisto, 1992),
3) the occurrence of potentially active constituents as bound or complexed forms (*e.g.*, as glycosides or other conjugates, or bound to macromolecular constituents) (Tamura et al., 1980), or
4) physical interference due to inactive bulk constituents and/or precipitates.

Other potential problems include biological activity of the test mixture, such as growth stimulatory or inhibitory constituents (such as lectins or other polysaccharide constituents that may stimulate lymphocyte proliferation, or agents with specific growth regulatory effects *in vivo* (Yajima et al., 1993). In each of these cases, methods have been successfully modified to allow reliable bioassays to be conducted. Although these modifications are necessarily specific to the organism upon which a particular assay is based and have generally been applied to a specific type of analyte, consideration of these analytical issues and their solutions would be a useful preliminary step in the identification of potential problems and potential solutions required for implementation of a generally applicable analytical strategy for assessment of the mutagenic potential of microbial pesticides.

Often, interference is evident from test results. For example, in the conventional Ames assay in which exposure occurs in top agar, the presence of histidine in the test sample is evident from excessive growth of the background lawn and should not lead to "false positive" conclusions as long as careful observations are made. The problem of histidine in samples for analysis using the Ames assay is well recognized, and methods of recognizing and avoiding this problem have been described (Aeschbacher et al., 1983; Busch and Bryan, 1987; Nylund and Einisto, 1992; Salmeen and Durisin, 1981). Likewise, excessive growth stimulation of the target cells in the *in vivo* micronucleus assay would result in an increased reticulocyte/erythrocyte ratio and in *in vitro* cell assays would lead to increased proliferation indices or altered growth kinetics. Since the nature of potential interference and appropriate indices for assuring that such interference has not occurred are dependent on the specific organism and testing protocol used, it is important that consensus guidelines on appropriate protocols and criteria for a valid assay are developed for suitable standard testing strategies by experts knowledgeable about the experimental variables involved. Moreover, the use of a suitable "positive controls" should be taken into account. For genotoxicity tests on biopesticides of fungal nature, cultures from the genus *Alternaria* may represent an adequate "positive control"; these molds are common in agricultural soils, well known plant pathogens, and have been demonstrated to contain metabolites which were found mutagenic in normal mutagenicity tests employing *Salmonella typhimurium* classical TA strains (Michael and Michael, 1986).

Development of Harmonized International Testing Approaches and Methodologies

As markets and products have become more global, international harmonization of required testing approaches and testing guidelines has become essential. In the area of safety testing, a number of organizations have played a significant role in facilitating the development of harmonized guidelines. These organizations have often either worked with the OECD, or have made their conclusions and recommendations available to the OECD, thereby extending the resources and activities of the OECD itself. The precedents established by these organizations provide a framework to consider in the present case, and the organizations themselves could potentially be engaged to assist in the development of harmonized guidelines.

The OECD is a key consensus-building body because its membership includes most industrialized countries and they have agreed by treaty to accept testing approaches and protocols that have been approved via the OECD consensus process. Thus, the OECD Guidance and its extension to more detailed protocol guidelines are very important.

Notable activities that provided a basis for harmonization of general genetic toxicology testing methods include the U.S. EPA GeneTox Program (Auletta *et al.*, 1991) and the International Workshops on Genetic Toxicology Testing (IWGT) (Kirkland *et al.*, 1994, 2000, 2003). Each of these groups brought together recognized experts in specific testing methods from around the world to review the existing literature, share their practical experience, and develop consensus recommendations on appropriate testing methods, interpretation of results, and/or testing strategies. The EPA GeneTox Program was supported by the EPA, and included representatives of OECD so that consensus recommendations could move quickly

through the OECD process. The IWGT was originally an "ad hoc" group but has now been formalized under the International Association of Environmental Mutagen Societies (IAEMS). There is a major meeting to address selected issues in conjunction with each of the International Conferences on Environmental Mutagens, which is organized by the IAEMS every four years, and working groups meet additionally as necessary.

Given the magnitude and importance of the implementation of harmonized international regulatory guidelines for genotoxicity testing of microbial pesticides, it would seem prudent that interested parties work to engage those organizations that can support focused collaborative international expert workshops to develop the necessary strategy and protocols. This might include development of an appropriate decision tree for selection of test methods, analogous to that described by Goecke *et al.* (1999) for biotechnology products, as well as development of appropriate testing protocols for the different types of microbial pesticides. This could be undertaken with joint international support from government bodies, industry trade groups, and non-government organizations, with consideration to engaging those experienced groups that have already contributed to the development of genetic toxicology testing methods for conventional pesticides.

Critical Elements of Testing Strategy and Recommendations for Development of Consensus

The characteristics of both the biological test systems employed and the specific biological matrix being tested need to be considered in order to assure that testing is conducted in a manner that allows appropriate exposure of the test organism and avoids interference by non-mutagenic constituents in the test material that could modify the normal response of the test organism. Both considerations require specialized technical knowledge, and since a general testing scheme will need to utilize several different specialized *in vivo* and *in vitro* testing methodologies to evaluate several different types of organisms and/or products, development of an appropriate approach that can be applied uniformly among global regulatory agencies will require a team of international experts with appropriate technical knowledge. The OECD and/or the IAEMS/IWGT[7] organizations would be appropriate to identify appropriate experts and to develop recommended approaches that would be adopted by global regulatory bodies.

The factors that need to be addressed in order to develop an appropriate consensus approach include definition of the tests required for specific classes of pesticide products and exposure scenarios, appropriate sample preparation and/or fractionation schemes, and appropriate protocols for testing in each individual bioassay. Appropriate positive and negative control conditions, specific positive control chemicals, and methods for demonstrating a lack of modification of the control responses by constituents present in the test fractions, will need to be defined. These factors are summarized below in Tables 1-3.

Conclusions

- Existing testing guidelines for chemical pesticides may not be directly applicable to testing microbial products, and specific guidelines for each test system and type of microbial pesticide to be evaluated, modified as necessary to avoid interference by constituents in the test samples, are needed

- A more comprehensive discussion, with references, of the available testing methodology and appropriate protocols, should be developed

[7] International Association of Environmental Mutagen Societies/International Workshops on Genetic Toxicology

- A "decision tree", analogous to that developed for the testing of biotechnology products by the GUM section of the European EMS, would be useful as a guide to appropriate testing strategies and methodologies

- Recommendations would best be developed by existing or specifically constituted working groups of international experts with experience in genetic toxicology assessment, testing of biotechnology and cellular products, knowledge of microbial secondary metabolism, and with specific experience in the use of the test systems to be proposed for use

- International harmonization of guidelines is highly desirable, and an international process that includes representatives from those countries and regions with existing guidelines should be used to ensure consistency of guidelines worldwide

- OECD guidelines based on the above are highly desirable as a means to assure harmonization of appropriate regulatory testing approaches throughout the world

Table 1. Testing Strategy: Need for consensus

- Products to be tested (incl. criteria for concern and extent of testing, such as relationship to species known to produce genotoxicants)
  - Fungal products
  - Bacterial products
  - Viral products (including integration into mammalian genome)
- Extent of testing
  - Specific tests
    - "Core" screening tests
    - Extended testing
  - Factors triggering extended testing
    - Exposure determinants (extent and route of human exposure)
    - Testing outcomes (*e.g.*, positive in initial screen, or level at which positive)
    - Relationship to microbial species known to produce genotoxicants
    - Other (SAR, class experience, etc.)
  - Factors influencing selection of follow-up tests
    - Nature of original response and/or mechanism (*e.g.*, bulky adducts *vs.* strand breaks, mutation *vs.* chromosomal aberration)
    - Knowledge of mechanisms (*e.g.*, involvement of reactive oxygen or nitrogen species, pool imbalance, spindle disruption, etc.)
    - Criteria for *in vivo* measurements (route of human exposure, tissues with greatest concentrations, metabolic activation requirements, etc.)
- Risk assessment criteria
  - Criteria for interpretation and for significant vs. insignificant or low risk
  - Relevance to humans

Table 2. Sample Preparation and Testing Protocols: Needs for Consensus

- Methods of lysis of intact cells (*e.g.*, physical disruption, detergents, solvents)
- Extraction and/or separation methods (*e.g.*, solvents, columns, adsorbents, filtration)
  - For liquid culture (*e.g.*, exotoxins)
  - For whole cells (intra-cellular constituents)
  - For cellular products
  - Release from bound forms (*e.g.*, conjugates and macromolecular binding)
- Removal of interfering substances
  - Toxic constituents (toxic to test organism)
  - Protein, fat, carbohydrates
  - Precipitates
  - Nutrients, growth factors or inhibitors (*e.g.*, histidine in Ames test, lectins in lymphocyte chromosomal aberration test, inflammatory agents in *in vivo* tests)
  - Enzymatic activity (*e.g.*, kinase or phoshoribosyltransferase activity in $tk^{+/-}$ or *hprt* assays)
- Protocols specific to each test system and class of microbial pesticide
  - Positive and negative controls
    - Appropriate positive control chemicals
    - Procedure to demonstrate lack of modification of response by inactive cellular constituents

Table 3. Test Systems to Consider, and Definition of Circumstances in which Appropriate

- *In vitro* gene mutation tests
  - Ames test (*his*$^-$ reversion in bacteria)
  - Mouse lymphoma $tk^{+/-}$ forward mutation assay
  - Chinese hamster *hprt* assay
- *In vitro* chromosomal aberration assays
  - Chinese hamster CHO or V79 assays
  - Human lymphocyte assay
  - *In vitro* micronucleus assays
  - Other
- *In vitro* DNA damage assays
  - Comet assay
  - Hepatocyte UDS assay
  - Microbial $rec^{+/-}$ or SOS assays
- *In vivo* chromosomal aberration assays
  - Erythrocyte micronucleus and bone marrow chromosomal aberration assays
  - Lymphocyte chromosomal aberration and micronucleus assays
  - Micronucleus or chromosomal aberration assays in other tissues
- *In vivo* gene mutation assays
  - Lymphocycte *hprt* or $tk^{+/-}$ assays
  - Transgenic mutation assays (*lac*I, *lac*Z, *gpt/spi*$^-$)
- *In vivo* DNA damage assays
  - Comet assay
  - UDS assay
  - DNA adduct assays
  - Alkaline elution assay
- *In vivo* germ cell assays
  - Transgenic mutation assays in germ cells
  - Spermatogonial cytogenetics
  - Sperm assays, incl. aneuploidy
  - Dominant lethal and heritable translocation assays
  - Mouse spot and specific locus tests
- Human assays

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CHAPTER 3

Discussion on Occupational, Bystander and Consumer Exposure and Risk Assessments for Microbial Pest Control Products

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General View

A qualitative approach to risk assessment should be used to evaluate occupational and bystander exposure to microbial pesticides. Three elements, the risk from exposure, the anticipated level of exposure, and the routes of exposure, are considered using information and data on the proposed use pattern, product characterization, toxicity and infectivity testing, and any available information on the environmental fate of the organism. For most microbial pest control products, the risk from exposure can be mitigated with appropriate labelling.

Introduction

During a meeting held on September-October 2003, the OECD Biopesticide Steering Group identified the need to develop a guidance document for regulators on assessing human exposure to microbial pest control agents (MPCAs). Microbial pest control agents present unique challenges to regulatory bodies accustomed to the scientific evaluation of chemical pesticides. To address these challenges, and to aid in the risk assessment of microbial pesticides, Health Canada's Pest Management Regulatory Agency (PMRA) has outlined its approach to occupational, bystander and consumer exposure and risk assessments for MPCAs as a model for future discussion and development among OECD member countries. The general guidance suggested herein is based solely on Canada's experience in regulating microbial biopesticides, as formalized (and harmonized) procedures have not yet been developed for this class of pesticides.

The approach outlined in this paper has also been used by EU Member Countries in preparing monographs for MPCAs which have been evaluated in the 4th stage of the review programme of the EU.

Exposure Assessment

Conventional chemical exposure models are not easily applied to MPCAs. For chemical pest control products, a quantitative approach is used that depends on adequate methods for the detection and quantification of the active ingredient in various environments, and on models addressing deposition during application and environmental fate. Current methods for monitoring micro-organisms in environmental samples do not afford the necessary sensitivity to adequately estimate exposures to MPCAs. Existing exposure models developed for chemical control products also require the input of an acute or chronic no observed adverse effect level (NOAEL) endpoint value from toxicity testing. Microbial hazard testing at Tier I is conducted at a maximum hazard dose or concentration by oral, pulmonary and dermal routes of administration, and by intraperitoneal or intravenous injection to assess infectivity in the absence of the innate host barriers to infection. Rarely is a minimum infective dose, the microbial equivalent to a lowest observed adverse effect level (LOAEL) identified. Instead, the Canadian approach to microbial

pesticide exposure assessment is qualitative, and considers populations that may be exposed to the MPCA by various routes of exposure, the anticipated level of exposure in each population by each route and the potential risk to humans from exposure to the MPCA by each route of exposure. Information and data submitted to support registration of the MPCA in Canada, in particular the characterization of the MPCA, results of human health and safety testing, and any information available on the environmental fate of the MPCA following application form the basis of this risk assessment. These inputs, elements of the risk assessment, and mitigative measures are summarized in Figure 1 (Appendix I).

Exposed populations

Exposure to an MPCA can occur during manufacturing of the active ingredient and formulation of the end-use product, at the site of application (commercial, residential or public spaces) during mixing and loading of the product, during application, and following application at a level and for a duration depending on the dispersion characteristics and environmental persistence of the MPCA. Exposure can also occur at sites distant from the site of application due to spray drift, runoff, natural mechanisms of dissemination of the MPCA, and from the consumption of treated foods, and treated or inadvertently contaminated drinking water. At these sites, those exposed can include manufacturing plant workers, professional pesticide handlers, other workers at the site of application, and members of the general public (domestic consumers of pesticides, bystanders at the site of application, consumers of treated food and drinking water and any who encounter the MPCA following environmental dissemination).

Certain sectors of the exposed population are given special consideration. Infants and children interact differently with MPCAs, because of behaviours that may predispose them to exposure and due to the incomplete development of their immune systems. Residential, school, day care and recreational exposure is therefore considered separately from the general risk assessment. Increasingly large sectors of the adult population are also living with impaired immunity. The elderly, others in reduced health, persons living with HIV and those undergoing immunosuppressive therapy for cancer or organ transplantation are particularly vulnerable to microbial agents. Primary human pathogens are not registered as MPCAs, but many of the registered MPCAs have the potential to act as opportunistic pathogens in an immunocompromised host. The overall risk assessment must therefore consider the immunocompromised.

The product class (Restricted, Commercial or Domestic) determines, in part, the populations exposed to the MPCA. For example, Domestic products are available to members of the general public for residential use. Exposure to members of the household, including infants and children, and immediate neighbours is expected. Commercial products are registered for both agricultural and industrial uses. In addition to occupational exposure, the treatment of food crops may result in widespread exposure of the general public through the diet. Establishment of pre-harvest intervals and maximum residue limits mitigates exposure through treated foods. Restricted products are intended for licensed applicators, who are trained in the application of pesticides, and in the proper use of protective equipment and other mitigative measures, but these products may be applied to public spaces. For example, Restricted Class *Bacillus thuringiensis* subspecies *kurstaki* based-pesticides are sprayed over large Canadian cities, exposing the entire urban population, and Restricted *B. thuringiensis* subsp. *israelensis* based-pesticides are used to treat Canadian waterways, which, after treatment, provide drinking water to Canadian municipalities. Literature, advising potentially susceptible persons to stay indoors during urban spraying is made available by Health Canada's Pest Management Regulatory Agency. For Manufacturing class products, occupational exposure during formulation of the end-use product is considered, but members of the general public are unlikely to be exposed.

The application of the MPCA, including the use site, the method of application and application equipment, as well as the product formulation is also considered in determining which populations are exposed, and how. Dietary exposure is expected for food uses, as described above. Direct aquatic

application or inadvertent contamination of water bodies following application to sites where runoff is likely may result in drinking water exposure, if water treatment is insufficient to eliminate the MPCA. The method of application may also affect the populations exposed. Spraying may result in bystander exposure, but if protected by an enclosed vehicle cab or airplane cockpit, the applicator may be spared from exposure.

The life cycle and environmental fate of the organism determine its propensity to persist at the use site, and to disperse to different sites. These too may affect the populations exposed.

Route of Exposure

The commonly considered routes of exposure are oral (usually dietary), pulmonary or dermal exposure. Information on the formulation type, method of application and information on the environmental fate and life cycle of the MPCA help the reviewer to define the probable routes of exposure and populations exposed.

A product formulated for spraying, or as a fine powder may be more easily inhaled during handling, whereas a paste is likely to result in mainly dermal exposure.

The life-cycle and environmental fate of the organism may also influence the route of exposure. For example a fungal MPCA that is applied as a mycelial paste is most likely to result in dermal exposure to pesticide applicators only. However, if sporulation is expected after a period of growth in or on the treated pest host, the MPCA may become widely disseminated, possibly affecting a wider nontarget population, and inhalation becomes the most significant route exposure in humans (i.e., bystanders).

Level of Exposure

The amount of the MPCA encountered during application, in the environment following application, in treated food or contaminated drinking water, and the duration and frequency of exposure together determine the level of exposure, and must be considered for each exposed population. These factors are discussed as part of a description of the product use-pattern submitted to support product registration. The use-pattern includes the product class, formulation type, site and method of application, application equipment, the rate, frequency and timing of application and the concentration of the MPCA in the formulated product. The level of exposure is related to the route of exposure, in that the relative extent to which different populations are exposed is considered. As previously noted, the level of exposure is not quantified. Instead, the product use-pattern helps the reviewer assess relative exposure of different populations qualitatively.

Clearly, the concentration of the MPCA in the formulated product (guarantee), the rate and frequency of application partly determine the level of exposure to pesticide applicators and other workers at the site of application. This information can be used to predict the amount of the MPCA applied to treated food crops and released into the environment. Other factors may also affect the level of exposure and differential exposure between populations. Greater incidental exposure is expected from a product formulated as a fine powder or spray than from a paste formulation.

The site and method of application also influence the level of exposure. Application in the enclosed environment of a greenhouse is expected to result in a greater occupational exposure, but lesser bystander exposure at the time of application than outdoor applications. Food residues may be similar, or higher if the MPCA favours the temperature and humidity conditions in the greenhouse.

As live organisms, MPCAs have the potential to persist, and replicate, in the environment. The

life cycle of the organism, and its mode of action, may suggest the potential for environmental persistence, or dissemination to areas other than the site of application. The physical properties of the organism (e.g., susceptibility or resistance to degradation in sunlight or by dessication) may suggest a greater or lesser propensity for persistence. If submitted, data and information on the environmental fate of the MPCA may also be considered.

Risk from Exposure

The assessment of risk from exposure starts from the assumption that, regardless of toxic and infective potential, all MPCAs contain proteins that can cause allergic sensitization on repeated exposure. Standard precautionary statements and personal protective equipment requirements designed to minimize contact with the MPCA are required on all microbial end-use product labels. A detailed characterization of the MPCA and toxicity and assessment of infectivity test data, inform the regulator of all other risks from exposure to the MPCA. The results of toxicity and infectivity testing are the most directly applicable to this assessment. Evidence of toxicity or pathogenicity in surrogate laboratory animals strongly suggests the potential for adverse effects in humans, and may demonstrate variability in the level of risk by different routes of exposure, oral, pulmonary and dermal. The absence of findings in laboratory animals substantiates an overall assessment of lower risk, though due to the host-specific nature of pathogenic microbes, an element of uncertainty remains. Information on the characterization of the MPCA is therefore an important supplement to toxicity and infectivity test data. Characterization data are reviewed in detail prior to the initiation of the health and safety data review, and provide an overall picture of the MPCA that may alert the reviewer to potentially hazardous characteristics. The host range and mode of action of the MPCA as a pesticide, its natural occurrence, history of use and any reported adverse effects, its physical and biochemical characteristics, its relationship to known pathogens, and its potential for toxin production are all described under product characterization. Together, these provide a complete picture of the organism and its potential for toxicity or pathogenicity in humans.

Information on the pesticidal mode of action, including target pests, pest host range, mechanisms of infectivity or toxicity and the life cycle of the MPCA may alert the reviewer to the potential for toxic or pathogenic effects in humans. The pest and pest host range provides reviewers with a sense for the extent to which the mode of action is universal. For example, a herbicidal MPCA that infects only angiosperms might be considered less likely to cause adverse effects in humans than an MPCA with a broad animal host range. The mechanisms of infectivity or toxicity are determinants of host range. The reviewer may be alerted to a potential effect in humans if the host receptor, for an infective MPCA, or the toxin receptor, for a toxigenic MPCA, is common to the physiology of the target pest, and of humans. The entire life cycle of the MPCA must also be characterized. It is the life stage of the MPCA that is formulated into the end-use product that is usually used as the test substance in toxicity and infectivity testing, as this life stage is most relevant to occupational and bystander exposure during application. However, as a live organism, the MPCA may survive and replicate in the environment, and exposure to other life stages of the MPCA is possible some time after application. For example, *Bacillus* species applied as spores, may later germinate in the environment and could be encountered as vegetative cells. Alternatively, a product applied as fungal mycelia may sporulate and the spores may be released into the environment. The potential for these other life stages to pose an exposure risk must therefore also be considered in the review.

The natural occurrence and history of use of the MPCA is reported in the characterization of the organism, and applicants for registration are asked to submit reports of any adverse effects related to exposure during product development, manufacturing and use. In addition, the reviewer searches the scientific literature for clinical case reports involving the species in question. These elements, considered together can help the reviewer to predict the potential for harm. The absence of reported adverse effects from an MPCA known to be ubiquitous in the environment, or one that has been widely used as a pesticide in other countries, or for a non-pesticidal function in Canada substantiates an assessment of lower risk.

However, the absence of adverse effects reporting cannot be assumed to equate to product safety, particularly if the organism originates from a specialized niche, or does not have an extensive history of use.

The relationship of the MPCA to known pathogens and the potential for mammalian and genotoxin production are all discussed in the product characterization, and may alert the reviewer to possible mechanisms of pathogenesis to be aware of during the subsequent review. Mammalian toxicity is addressed in toxicity and infectivity testing. Evidence of genotoxin production may trigger new data requirements. An understanding of the physiology of the MPCA also helps the reviewer to predict its potential to act as a human pathogen. For example, the growth temperature is often considered; a psychrophilic organism is unlikely to survive or replicate at human body temperatures. Also considered are the metabolic requirements of the organism. Methanotrophs and sulfur-reducing bacteria are unlikely candidates for pathogenesis in humans whereas an organism with an obligate requirement for heme is likely to be a pathogen.

In addition to the active ingredient, the formulation of the pesticide may contribute to the risk from exposure. Potentially toxic formulation ingredients are identified during review of the product characterization, and considered in the risk assessment. The physical nature of the formulation is also considered. For example, dry powder formulations by their nature may be viewed as being potential eye irritants even if results of eye irritation tests using an aqueous suspension of the formulation shows it to be non-irritating. Canada considers all microbial products, regardless of formulation type, to be mild ocular irritants and requires cautionary language on all microbial product labels (e.g., CAUTION – EYE IRRITANT), unless definitive test data on the formulation (e.g., dry powder) are submitted showing that it is not. In addition, pesticide handlers (i.e., workers, loaders, and applicators) involved in the preparation and application of the product may be required to wear protective eyewear.

Another important consideration when evaluating occupational exposure and risks to MPCAs is the potential for allergic sensitization (hypersensitivity) to develop in handlers (mixers/loaders) and applicators as well as in early- or re-entry workers who enter into a treated area after pesticide application is complete, but before any restricted-entry interval for the pesticide has expired. Micro-organisms contain proteins and other antigenic substances that can elicit allergic or hypersensitive reactions in humans upon repeated exposure to high concentrations such as may occur in the operational use of microbial biopesticides. The degree of exposure to re-entry (or restricted-entry) workers will depend on the type of: (i) formulated product that is to be applied, (ii) method of application, (iii) application equipment employed (e.g., ground vs. aerial), (iv) area to be treated (e.g., greenhouse, nursery or field), and (v) crop treated which may require certain tasks to be performed during a restricted-entry interval (REI) such as scouting, pruning, watering, etc. Each of these variables will dictate whether an REI is advisable or appropriate and whether re-entry workers should wear some or all of the protective equipment as applicators to mitigate exposure to the MPCA because of allergenicity/hypersensitivity concerns.

Exposure Mitigation

The three elements, risk from exposure, level of exposure and route of exposure are combined in the final risk assessment, aggregate exposure is considered, and where necessary, mitigative measures are employed. Product (MPCP) label modifications are sufficient in most cases to mitigate any risk from exposure. MPCA labelling is conservative. That is, where there is uncertainty as to the level of risk from exposure, a hazard is considered to exist.

Hazard labelling on the principal display panel of the product label can alert workers to the level of risk on exposure. Personal protective equipment and measures for decontamination can also be identified as appropriate on the secondary panel of the product label under the heading “PRECAUTIONS”.

For example, because all microbial pesticides are considered to have the potential to cause hypersensitivity following repeated exposure, the signal phrase "POTENTIAL SENSITIZER" is always required on the principal (front) display panel for microbial products in Canada, and precautions to avoid contact, and minimal PPE requirements are required under "PRECAUTIONS" to limit repeated occupational exposures that may result in hypersensitivity. Where other potential hazards or uncertainties have been noted, (e.g., eye irritation, inhalation exposure) more extensive hazard labelling, precautions and PPE requirements are imposed, such as the wearing of eye goggles or a dust/mist filtering respirator designed for biological products. Canada typically requires precautionary labelling for microbial products to read:

> *May cause sensitization. Avoid contact with skin, eyes or clothing. Avoid inhalation of spray mists and dusts. All handlers must wear a long-sleeved-shirt, long pants, waterproof gloves, shoes plus socks and eye goggles, and a dust/mist filtering respirator (MSH/NIOSH approval number prefix TC-21C) or NIOSH approved respirator with any N-95, R-95, P-95 or HE filter for biological products, when handling, mixing/loading or applying the product and during all clean-up/repair activities. Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash before reuse.*

Other measures used in product labelling to mitigate risk include limitations on use, alteration of the product class, or changes to application directions. Limitations on use are occasionally imposed to protect bystanders who may be exposed after application, for example mosquito larvicides applied to aquatic environments carry a label warning to protect drinking water supplies: "Do not apply to treated, finished drinking water." In general, Canada considers a bystander to be a person who is located within or adjacent to an area where pesticides are being applied or has just been applied, but whose presence is quite incidental and unrelated to the application of the pesticide including around homes, schools, parks, playgrounds, playing fields, public buildings or any other areas where the general public including children could be exposed. As noted above, the product class imposes restriction on the user. Finally, the application directions may be modified to mitigate exposure risk. For example, standard aerial application instructions are very detailed, and include statements that are designed to reduce spray drift. Another regulatory option designed to minimize occupational exposure is to require an REI that reflects the underlying concern or degree of hazard presented by the MPCA. For example, an MPCA that may present a toxicological hazard via dermal exposure and is applied as a foliar spray to crops could require an REI of 4 hours to allow residues to dry before workers re-enter the treated area. Otherwise, any workers re-entering the treated area during the REI would be required to wear the same PPE as applicators, mixer/loaders and handlers, or a subset of the PPE as would be deemed appropriate (e.g., a respirator may not be required, but all dermal PPE might be necessary). The PMRA has established REIs only for those MPCAs that have an uncertainty of risk from a specific route of exposure. For example, if only supplementary pulmonary/inhalation test data are available that show toxic/adverse effects in test animals, then an REI of 4 (or up to 12 hours) may be necessary to alleviate concerns that the regulator may have for workers who may be exposed to airborne particles of the MPCA after spraying or dusting.

Even without toxicological or pathogenicity concerns, regulators may wish to mitigate exposure to the MPCA, and thereby lower the likelihood of hypersensitivity in mixer/loader/applicators and any workers involved restricted-entry (re-entry) post-application activities, by requiring the wearing of PPE. Personal protective equipment which limits dermal, oral, ocular and/or respiratory exposure (i.e., long clothing or coveralls, waterproof gloves, eye goggles and a respirator suitable for microbiological agents) may be warranted as a condition of registration/authorization for microbial biopesticides regardless of the health effects or hazards noted in animal studies. Depending on the MPCA in question and its other inherent characteristics or potential biological effects (e.g., acute toxicological concerns if inhaled or on dermal contact) that may make repeated exposures a concern to regulators, early-entry workers who enter a treated area within an REI may be required to wear sufficient PPE to minimize unnecessary contact or exposure to the MPCA. Re-entry workers could, therefore, be reasonably expected to wear the same PPE

as applicators, including respirator, if the MPCA is applied, for example, as a foliar spray or fine dust in a greenhouse or nursery where airborne particles may persist for several hours after application. Other use patterns where inhalation exposure would be minimal for re-entry workers (such as MPCAs applied by root dip), a respirator would not need to be specified as a condition of registration or authorization. However, other PPE such as long clothing, waterproof gloves and shoes plus socks may be warranted for these workers.

In 2005, the U.S. EPA updated its pesticide Worker Protection Standard for agricultural workers. The U.S. EPA's WPS provides guidance on worker restrictions during and after applications and is a general regulation not specific to MPCAs, but rather to all pesticides. The primary objective of the WPS is to mitigate against exposure to pesticide residues in agricultural workers. In general, the WPS advises to keep workers out of a treated area during the REI. This restriction has only two types of exceptions: (1) early entry with no contact, and (2) early entry with contact for short-term, emergency, or specially excepted tasks that require publication in the Federal Register. Entry into treated areas during an REI is also allowed for workers to perform handling (including crop advisor) tasks as long as the persons entering such areas are trained and equipped as pesticide handlers and receive all other applicable WPS handler protections. Avoiding contact by using PPE does not qualify as no-contact early entry. Some pesticides have one REI, such as 12 hours, for all crops and uses. Other products have different REIs depending on the crop or method of application.

From a consumer safety perspective, limiting exposure to MPCA residues can also be achieved by requiring a pre-harvest interval (PHI) for a period of time after the last application of the MPCA to the crop that would result in the least or minimum amount of microbial residue deemed to be safe or, alternatively, below the limit of detection. In general, PMRA has avoided having to set PHIs for MPCAs, as doing so would mean that fate and expression data of the MPCA on plant surfaces would be needed to assess exposure. Such residue data would be difficult to interpret, as fate and survival of the MPCA may be specific to the environmental conditions at the time of the experiment and thus may not be representative of the climate (environmental) conditions for all uses and in all regions across Canada where the MPCA is to be applied. To date PMRA has not required PHIs for MPCAs, however, it remains a regulatory option to mitigate risk where health concerns may exist.

Conclusions

- Conventional chemical pesticide models used to quantitatively assess occupational and bystander exposures are not easily applied to MPCAs. However, it is recommended to use the available models to perform a qualitative risk assessment for the operator.

- Exposure assessments for MPCAs rely on qualitative parameters and consider the different populations that may be exposed to the MPCA through various routes of exposure, the expected level of exposure in each population by each exposure route, and the potential health risk(s) to humans from exposure by each route.

- Occupational exposure, regardless of the toxicological/pathogenicity hazard of the MPCA, should be minimized to a reasonable extent. There may be a risk for allergic sensitization by a variety of micro-organisms at repeated exposure, especially if inhaled.

- Labelling statements, customized to reflect the underlying health concerns associated with the MPCA to be registered/authorized, should be used to mitigate occupational exposure, including the wearing of suitable PPE and setting of REIs.

- Establishing PHIs is generally not recommended, or appropriate, for MPCAs, but does remain a regulatory option if health concerns warrant such a measure to reduce (or eliminate) consumer exposure to MPCA residues.

References

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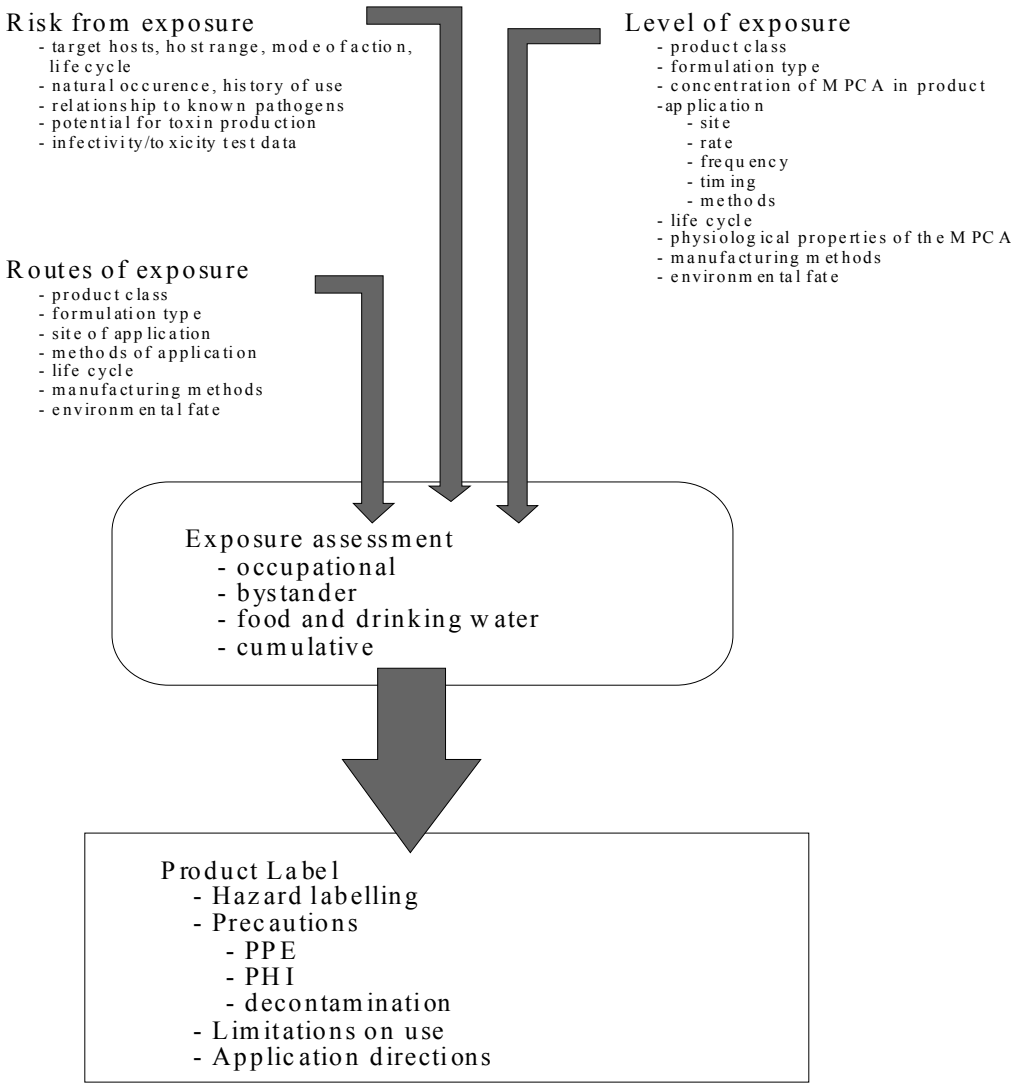
Appendix I

Figure 1. Submitted data parts informing the occupational and bystander exposure risk assessment, elements of the exposure assessment and risk mitigation.



CHAPTER 4

Discussion on Microbial Metabolite Residues in Treated Food Crops

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General Analysis

Unique challenges are encountered during the review of microbial pest control agents (MPCAs) and their associated end-use products (MPCPs) compared to conventional chemical pesticides. One such challenge is the potential for some micro-organisms to produce metabolites that could be harmful to humans if consumed. Currently, the extent of investigations on metabolites of MPCAs depends on what is already known in the published scientific literature or becomes apparent, sometimes by chance, during product development. Although regulatory authorities have the option to apply more stringent requirements for the identification of potentially toxic metabolic by-products produced by candidate MPCAs, such extensive requirements would effectively hinder the marketplace development of this class of reduced-risk, environmentally desirable pest control products. Consequently, there is no widely accepted guidance offered by regulatory authorities and applicants/notifiers can encounter new and unpredictable regulatory requirements that could ultimately delay or prevent registration/authorization.

Introduction

During a meeting held on September- October 2003, the OECD BioPesticide Steering Group identified the necessity to develop guidance documents for the identification and toxicological characterization of metabolites that may be produced by MPCAs and become residues in and/or on treated food commodities. This issue paper outlines the approaches adopted in North America by Canada and the United States to evaluate the risks of metabolites in food and feed crops treated with MPCAs as well as residues of the organism. The purpose of this issue paper is to promote a dialogue among OECD member countries on an appropriate regulatory approach for assessing potentially toxic metabolic by-products of MPCAs as relates to their dietary risks to consumers.

Canadian Approach to Microbial Metabolites

In Canada, before registering a pesticide under the *Pest Control Products Act* (PCPA) for use on crops or food-producing animals, Health Canada's Pest Management Regulatory Agency (PMRA) must determine that the amount of residue likely to remain in food, when the pesticide is used according to label directions, will not pose an unacceptable health risk to consumers. This amount is then legally established as a maximum residue limit (MRL) in Table II, Division 15, Part B.15.002 of the Food and Drug Regulations under the *Food and Drugs Act* (FDA). This applies whether or not the pesticide is already registered for other food or non-food uses. Maximum residue limits are also established for pesticides not registered in Canada that may be found as residues in imported food. If residues exceeding an MRL are found in food at the point of sale, the item is considered adulterated under the FDA and is prohibited from sale in Canada.

In Canada, the use of an MPCA on food also requires the establishment of an MRL unless its use is specifically exempted under paragraph 4*(d)* of the FDA respecting adulteration of food. Although the FDA applies to all MPCAs used on food, only MPCAs containing subspecies of *Bacillus thuringiensis* Berliner are currently exempted in Canada provided residue levels do not exceed 0.1 parts per million on treated food/feedstuffs. These exemptions are based on various key pieces of information and data submitted by the applicant in support of registration and are prepared by PMRA regulators. Canadian applicants for pesticide registration do not formally apply for this exemption. For other MPCAs proposed for use on food crops, the PMRA may conclude that there is no need for an establishment of an MRL if there are no adverse effects in an acute oral toxicity/pathogenicity study and there is no evidence that mammalian toxins or metabolites of concern are produced by the MPCA.

The hazard assessment of an MPCA is initiated during the review of product characterization data, for example, as outlined in Section 5.0, Part 2 of Regulatory Directive DIR2001-02, *Guidelines for the Registration of Microbial Pest Control Agents and Products*. In this submission or dossier element, detailed information and data on the origin, derivation and identification as well as the biological and ecological properties of the MPCA must be provided by the applicant/notifier. This essential information often relies on published scientific literature on species or strains closely related to the MPCA rather than on the MPCA itself. The most essential piece of characterization information is the identity of the MPCA, as this ultimately establishes the level or degree of familiarity that the regulator has to begin the hazard assessment. The taxonomic identification of the MPCA should be made at least to species, but preferably to the lowest epithetic level possible (e.g., strain, subspecies, forma specialis) to differentiate it from other phylogenetically close relatives, including known human pathogens (primary and opportunistic) and toxigenic species or strains. This information combined with synonyms and other superseded names associated with the MPCA can often highlight a particular metabolite or group of metabolites of concern. For instance, some fungi and actinomycetes are known to produce toxins and other metabolic byproducts that might have genotoxic potential. To help with the investigation, applicants are required to provide literature search results to specific search keywords (including synonyms and other superseded names of the micro-organism) and databases along with pertinent literature, if applicable. The applicant must also provide a discussion on the relationship of the MPCA to known pathogenic/toxigenic strains or species. The regulator, to ensure that all relevant data/information are captured for inclusion in the assessment, should also conduct an independent search of existing literature.

Few micro-organisms are so thoroughly described or their metabolic pathways so fully elucidated that a regulator can definitively draw conclusions on an MPCA's potential to produce mammalian toxins or other metabolic by-products that potentially present food residue concerns. Additional sources of information must therefore be investigated to determine the likelihood that a toxic metabolite can be produced by the MPCA and that it might potentially contaminate (adulterate) treated food crops. The origin of the strain and its biological properties such as natural occurrence, ecological niches and description of its life cycle can provide clues with regards to its potential to produce toxic metabolites either during product manufacture or post application if the MPCA is able to grow on treated plant foliage or in the soil rhizosphere where uptake by plant roots of extracellular microbial metabolites might be translocated to edible food portions (e.g., fruit or whole plant vegetable). Secondary colonizers, which invade ecological niches already established by other microbes and defend their domain once it has been established, are more likely to produce antibiotic or other toxigenic metabolites than primary colonizing microbes, which are known for their ability to grow rapidly in new niches. The MPCA's mode of action and descriptions of any unusual morphological, physiological, biochemical, pesticidal or resistance characteristics are also important when assessing the potential for toxic metabolite production. Micro-organisms with toxic or antibiotic modes of action and/or other unusual characteristic(s) are considered more likely to produce metabolites that are potentially toxic to humans than those with non-toxic modes of action.

In Canada, if characterization information shows no or minimal potential for the MPCA to produce mammalian toxins and the results of in vivo toxicology tests demonstrate no toxic effects (especially via the oral route of exposure), the MPCA will be either exempted from an MRL or the PMRA will simply not require the establishment of an MRL under the *Food and Drug Act* and Regulations. Otherwise, further investigation will be required as follows:

(*i*) If the presence of a mammalian toxin has been identified based on characterization and toxicological test results, the PMRA may recommend that the submission for registration be withdrawn or the applicant is advised to remove all of the MPCA's proposed food uses. If the applicant chooses to pursue registration of food use(s), the technical grade of the active ingredient is subject to the same registration data requirements as a conventional chemical pest control product, and all appropriate residue data are required to establish an MRL for the metabolite/toxin of concern, i.e., the toxin is identified as the residue of concern (residue definition).

(*ii*) In cases where characterization data indicate a potential for the production of a toxic metabolite, but no toxic effects are noted in toxicology tests, an analytical method and batch analysis data are required to detect the presence of the metabolite in the end-use product and/or in spent media from cultures grown under conditions known to produce the suspected metabolite of concern. Additionally, if the synthetic pathway for the suspected metabolite or group of metabolites is well characterized, the PMRA might also request molecular tests using various gene probes to determine if the toxin-producing genes are present in the MPCA's genome. If the metabolite is detected in the end-use product at levels that could pose a health risk, the PMRA will confer with the applicant to determine other regulatory options such as removal of all proposed food uses. As noted above, additional data are required if the applicant chooses to pursue registration of food uses. If the metabolite is not detected in the end-use formulation but it is detected in the spent growth medium following ideal conditions for production of the metabolite of concern or that the MPCA possesses the appropriate synthetic pathway to produce that metabolite after application to the crop (e.g., on treated foliage or in soil), the PMRA may request additional data or may request that the submission be withdrawn or the food use(s) be removed to avoid additional data requirements. The PMRA will not require the establishment of an MRL under the *Food and Drug Act* and Regulations if none of the tests indicate that the MPCA produces, or has the ability to produce, the metabolite of concern.

Since the test data required for registration depend so much on the identity and biological properties of the micro-organism, applicants are strongly encouraged to contact the PMRA for a presubmission or pre-registration consultation during the development phase of the product. Concerns such as the potential for metabolites in food are often identified during the presubmission phase and special recommendations may be communicated to the applicant at that time. These recommendations may include the addition of special test substances (e.g., sterile filtrate controls) to the toxicity testing protocols or of analytical tests that might prevent delays in the review of the application to register the MPCA because of toxic residue concerns.

U.S. Approach to Microbial Metabolites

In the U.S., pesticides are regulated similarly as in Canada, but there are some differences. Legislation in the U.S. requires that a tolerance or an exemption from the requirement of a tolerance be established by the U.S. EPA, as provided for under sections 406, 408, or 409 of the *Federal Food, Drug, and Cosmetic Act* ((FFDCA) 21 U.S.C. 346, 346a, and 348). This petition for a tolerance or for an exemption from the requirement of a tolerance must be submitted by the applicant as specified in 40 CFR 180.7 in connection with each application for registration of active ingredients where usage may result in residues in or on food for humans or feed for domestic animals used for human food.

For MPCAs, guidance on the requirement of residue data in the petition for a tolerance or for an exemption from the requirement of a tolerance is found in 40 CFR 158.740. These guidelines state that residue data are required as detailed in the U.S. EPA's Office of Prevention, Pesticides and Toxic Substances (OPPTS) 885, Group B (Residue Test Guidelines) when the MPCA is intended for use in or on food or feed, or is expected to result in residues in or on food or feed, and that the results of Tier I Toxicology studies conducted in accordance with the guidelines OPPTS 885.3050 through 885.3500 (mammalian toxicity, pathogenicity and infectivity studies), indicate that there may be significant human health concerns. Residue data, however, may not be required and an exemption from the requirement of a tolerance may be recommended for products intended for use on food feed crops or for uses expected to result in residues in or on food or feed, when the toxicology data developed from Tier I testing, in accordance with toxicology test guidelines OPPTS 885.3050 through 885.3500, indicate that there are no significant human health concerns. The exception to this is that a monitoring method will be required for each registered MPCA, even if exempt from tolerance. In considering exemptions from the requirement for tolerances, the U.S. EPA recognizes that microbial pest control agents do not necessarily pose the same potential hazards as conventional chemical pesticides and has provided the following characteristics justifying its position:

(i) The efficacy of the agent often depends upon its ability to replicate in the target pest, which is not likely to remain on the crop after harvest.

(ii) The living form of the agent in most instances will usually not replicate in the absence of the specific target pest (e.g. insect host).

(iii) Certain environmental conditions such as sunlight, rainfall, winds, humidity, and temperature often greatly affect the viability of the agent; therefore, the residues of living organisms are apt to be small or relatively insignificant shortly after application.

(iv) Data supporting currently registered MPCAs indicate that they would not likely pose a hazard to humans or other mammals.

(v) In many instances where and when a micro-organism is used as an MPCA, the micro-organism is already normally present in the environment and has demonstrated no adverse effects.

(vi) Residues of micro-organisms used as MPCAs that are capable of replication on food or feed -a very remote possibility- may be rendered nonviable or be removed by the usual processing of such foods and feeds (i.e., washing, drying, heat sterilization, and additions of sugar, salt, and other preservatives).

In addition to the data cited above, the U.S. EPA may request data derived from other tests as specified in 40 CFR 158.75 in order to make judgements regarding safety to humans, domestic animals, and other non-target organisms. Such data requests are only made under unusual circumstances and can be made if the U.S. EPA is presented with a toxicological mode of action, or a unique chemical or microbial property such as toxic microbial metabolites.

E.U. Approach to Microbial Metabolites

In the European Union, no authorisation shall be granted for a MPCA unless there is sufficient information for the MPCP to decide that there is no harmful effect on human or animal health arising from exposure to the MPCA, its residual traces and metabolites/toxins remaining in or on the plant or plant products (Part C, paragraph 2.6.2 of Council Directive 2005/25/EC).

Regarding the necessity of submitting an Annex II dossier for a toxin/metabolite the following is stated in the Commission Directive 2001/36/EC:

(viii) If the plant protection action is known to be due to the residual effect of a toxin/metabolite or if significant residues of toxins/metabolites are to be expected not related to the effect of the active substance, a dossier for the toxin/metabolite has to be submitted in accordance with the requirements of Annex II, Part A.

The most likely approach in the EU will be that the requirement for an Annex IIA dossier for a toxin/metabolite is considered not applicable in cases where the toxin/metabolite is only produced in the infected/target organism and/or where exposure is considered negligible.

Metabolites, especially fungal metabolites are probably only produced from active organisms *i.e.* during fermentation and/or in contact with the target organism. The main indication for the ability of a micro-organism to produce toxins usually comes from the characterisation part of the dossier, e.g. origin, derivation, identification, biological and ecological properties, the mode of action, toxin production in related species/strains. Sometimes toxicity tests might give a hint on that a toxic metabolite could be suspected; in that case it might be necessary to continue and identify the metabolite(s).

However, it should be kept in mind that few micro-organisms are so thoroughly described or their metabolic pathways so fully elucidated that a regulator can definitively draw conclusions on an MPCA's potential to produce mammalian toxins or other metabolic by-products that potentially present food residue concerns.

A possible way to handle (fungal) metabolites and their relevance to human health and the environment could be to discuss the exposure scenarios for humans and non-target organisms case-by-case and also do approximate calculations for such exposure scenarios. Most fungal metabolites when produced in relation to the use as a microbiological plant protection product would probably degrade quite quickly and would therefore not cause any residue problems. The survivability of the micro-organism has to be taken into account. Moreover, in contact with the target organism the levels would probably be very low and therefore extremely difficult to analyse due to detection limits in the analytical method. The uncertainties have to be discussed and handled. Known toxins could be used as reference for effect levels and known active substances on Annex I with unwanted properties could be used as examples for acceptance.

Hypothetically, the occurrence of metabolites/toxins in the fermentation medium could be screened. However, extraction and analytical procedures have to be developed, and the whole developmental process for analysis of unknown metabolites will probably be very expensive. Another option to find possible unknown metabolites is to test a crude filtrate from the fermentation medium in (geno)toxicity tests. There are questionmarks as to what will be the content of a crude filtrate, synergistic effects i.e. positive or negative interaction of the compounds in the crude filtrate.

Within the EU project RAFBCA experimental work has been done on fungal metabolites. Publications from this program could give input to the discussion on how to handle fungal metabolites. The overall conclusion of the RAFBCA project (http://www.rafbca.com/download/final_report_2004.pdf) was that the selected BCA metabolites evaluated did not enter the food chain nor pose a risk to humans and the environment. The metabolites were absent or, if present, then the levels were extremely low (i.e. concentrations were too low to pose a risk). Moreover, the project revealed that in many cases toxin production during fermentation was dependent on the method of production, and in the investigated fungi, only lab-scale production processes produced toxins, not the large-scale fermentations.

Conclusions

- Many MPCAs, primarily secondary colonizers, have the capacity to produce potentially toxic metabolites that can present a dietary risk to consumers if residues of these metabolites are found on/in treated food crops

- Risk of toxic metabolic by-products (toxins) to consumer safety must first be assessed qualitatively by investigating the MPCA in question, and its phylogenetically close relatives (species, strains, etc.), to determine the potential for it to produce mammalian-toxic substances

- If a known toxin-of-concern might be present based on the above analysis, the registrant can be given the option of analysing their isolate to determine if it might be one that does not produce that toxin

- Regulators must assess whether potential mammalian-toxic substances could be preformed in the formulated microbial end-use product prior to application as well as assess whether any such toxins might be produced by the micro-organism after application to crops and/or soil on which the crop is grown

- If toxins are present in the formulated product or can be produced by the MPCA post-application, then crop residue data may be warranted and exposure should be assessed

- If a mammalian-toxic substances has been identified and the applicant wishes to pursue registration/authorization for a food use of the MPCA, the microbial product should be subject to the same data requirements as a chemical pesticide, and regulatory authorities should require appropriate data to establish an MRL/tolerance.

References

Government of Canada, "Food and Drugs Regulations" (see Website http://laws.justice.gc.ca/en/F-27/C.R.C.-c.870/index.html for unofficial version), Division 15, *Adulteration of Food B.15.002* (see Website http://www.hc-sc.gc.ca/food-aliment/friia-raaii/food_drugs-aliments_drogues/act-loi/pdf/e_c-tabl es.pdf for unofficial version) of the *Food and Drugs Act* (see Web site http://laws.justice.gc.ca/en/F-27/index.html Regulations for unofficial version)

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CHAPTER 5

Efficacy Evaluation of Microbial Products

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Introduction

Biopesticides are micro-organisms, substances or extracts to be used as plant protection products. The focus of this document is on microbial biopesticides which are naturally occurring micro-organisms, including bacteria, algae, fungi, protozoa, viruses, mycoplasmae or rickettsiae, and related organisms. Genetically modified micro-organisms may also be used as biopesticides but the present text does not deal with the precautions specifically necessary for the field testing of genetically modified organisms.

Microbial biopesticides are generally derived via selection and culture of micro-organisms. There are two major types of pesticidal mode of action associated with micro-organisms a) direct toxicological or infective interaction with a pest, and b) pest population regulation through processes like competition for ecological niche (e.g. nutrients, habitat), induction of plant resistance, endophytic growth, root colonisation. The efficacy data required to support a microbial product is specific to its mode of action and the proposed label claims. For microbial products that exhibit a direct, measurable toxic or lethal effect on the pests, scientifically sound laboratory and field studies designed to quantify the susceptibility, dose response, time to mortality and residual control effect on proposed target pest are required. In the cases of population regulation, the performance data must help define any specific environmental conditions needed to maintain growth of the micro-organisms; these may need to be defined on the product label. Dose response behaviour should also be determined and the optimum dose recommended must be justified. The minimum dose required to achieve effective control, (or other defined benefit) should be established.

Background and Issues

The current pesticide registration requirements in many countries require the applicant to submit efficacy[8] data in support of the product (or provide a reasoned scientific case to waive or reduce data requirements). In some countries, it is a legal requirement that any label statement or claim made on efficacy must be supported by adequate data (field trials and/or scientific rationale), which is usually submitted as part of the registration package.

To support the registration of a pesticide product, the efficacy data may include:

- evidence of pest/weed/disease control to support the label claims
- evidence of safety to the treated crops

[8] In Canada, the term 'Value' is used to refer to both: (a) efficacy; (b) effect on host organisms in connection with which it is intended to be used; (c) health, safety and environmental benefits and social and economic impact.

- evidence of safety to subsequent crops
- dose justification (e.g. the Lowest Effective Rate (LER))
- evidence that yield and quality of yield will not be adversely affected
- consideration of the likelihood of pest resistance to the active substance developing
- evidence of biological compatibility (lack of antagonism) if tank mix is recommended

Also, depending on the nature of the active/product, the proposed label recommendations (but generally for herbicides only) and countries concerned:

- consideration of the need for special cleaning measures for spray equipment
- use in tank-mixes and spray sequences

Efficacy can be considered to be a balance between:

- the positive effects of treatment in performing the desired plant protection activity to fulfil the claims made on the proposed label, in order to achieve improvement in the quantity and/or quality of crop yield,
- the negative effects such as reduction of quality or quantity of yield/phytotoxicity, damage to beneficial organisms, damage to succeeding or adjacent crops, development of resistance; and
- other aspects of efficacy which, depending on the product, can be either positive or negative; these include effects on other non-target pests, length of time in which the plant protection product continues to be active, ease of its use, and compatibility with other cultural practices and crop protection measures.

The net result of the positive and negative effects should be a sufficient overall agricultural benefit in order to justify the use of the plant protection product. However, although not a view supported in many countries, it could be argued that there is no role for efficacy testing in pesticide regulation and market forces should rule. The alternative viewpoint is that pesticide application is so important to farmers that they need some basic level of reassurance that the product works. Built into this argument is also recognition for the need to minimize the impact of any pesticide use. An evaluation of effectiveness (and crop safety) as part of a regulatory process allows growers to use the product under optimal conditions, and ensures only the minimum necessary dose is applied. The problem for alternative control methods, such as the use of biopesticides, is that their performance is often low compared with conventional control methods, such as the use of synthetic pesticides. For example, conventional fungicides and herbicides may bring over 95% control of the pests (if applied correctly), while some alternative methods may bring reductions in pest numbers of less than 40%, which may also be of shorter duration. In other words, biopesticides may serve to reduce the pest pressure, but not to remove the pest entirely, which may be acceptable, particularly if it forms part of a programme of pest control. In fact, many countries do accept reduced use claims (e.g. 'can contribute to reduction in damage caused by the pest when used as a component of an IPM programme'). A lower level of efficacy can be acceptable if reasoned cases are judged as adequate to support its value in a pest control program or in unique settings (e.g. organic farming). The key issue is that the level of pest control/reduction achieved is providing a measurable benefit. Provided that is the case then a range of performance levels may be acceptable.

General principles of efficacy trials

Performance trials should be carried out under the conditions of use and the rates or doses proposed on the label must be tested in the treatments. In general, the baseline dose and specific condition(s) of use, i.e., temperature range, pH range, etc., should be determined in laboratory range finding study(ies). Field studies are then needed to establish the effective rate, the number of applications, the timing of application and confirm the condition of use under field conditions. Definition of the field dose should always take into account the mode of action of the microbials. In cases where no further growth of the microbial agent or product takes place before infection of the pest, justification of the dose must take into consideration coverage and survival over time, i.e., how long after application will the microbial agent or product be viable or infective. On the other hand, where the mode of action is based on processes involving growth of the microbial agent, i.e., competition for limited resources (nutrients, growth sites, etc...), justification of the dose should be based on growth parameters such as temperature, pH, relative humidity which can limit the growth or survival of the microbial. In some cases, parameter range may need to be defined on the label, e.g., a microbial may need high RH to germinate or to remain viable.

1.0 PERFORMANCE ASSESSMENT (TYPE OF DATA)

Laboratory and Growth Chamber Studies

For the purposes of this document, laboratory and growth chamber studies can be used for quantification, by appropriate means, of microbial activity on potential target pests or hosts, e.g., preliminary range finding tests, preliminary target pest screening. These might include conventional diet or foliage feeding bioassays, single plant or tree exposure, in vitro antagonism data, growth chamber or glasshouse data, etc., as appropriate to the nature of the end-use product (EP).

Laboratory studies should be designed to measure the intrinsic susceptibility, dose response behaviour, time to mortality, relative susceptibility of target pests or hosts, etc., as appropriate to the nature and activity profile of the microbial agent. The relationship of challenge doses used in laboratory studies to the expected exposure concentration of the microbials under actual use conditions should be considered and explained. Where relevant, laboratory studies should also address the susceptibility of various life stages of the proposed target pests or hosts.

For microbial agents that exhibit a direct, measurable toxic or lethal effect, scientifically sound laboratory studies designed to quantify the susceptibility, dose response behaviour and time to mortality on proposed target pests or hosts are normally required. Laboratory data should also incorporate studies that identify the pest host spectrum of activity of the microbial agents. These studies would normally be carried out at doses chosen in relation to the lethal concentration (LC) or lethal dose (LD) (i.e. LC50, LC95, LD50, etc.), of the most susceptible target pest, but alternatively these data might also be generated using challenge doses based on the expected exposure concentration of the microbial agents under actual use conditions.

The Technical Grade Active Ingredient (TGAI) or Formulation Intermediate may be used for laboratory bioassays, provided that any adjuvants or other constituents of the formulation which might be deliberately added to enhance toxicity or virulence are tested in combination with the microbials. Where more meaningful to the assessment of efficacy, the actual EP formulation itself should be used.

For new microbial agents, laboratory bioassay data will generally form the basis for preliminary target susceptibility screening and will contribute to evaluation of field performance. It is recognized that host or pest phenology, target pest behaviour and environmental constraints profoundly influence activity of microbials in the field. Where possible, information or data that describes the relationship of *in vitro* bioactivity, i.e., intrinsic susceptibility, to that expected or observed in the field should be provided.

These data submitted, in conjunction with information from *Environmental Toxicology* will be used in the assessment of potential environmental risk and are also important to the evaluation of claims regarding risk reduction and contribution to sustainable pest management.

For extension of use to closely related targets, laboratory or glasshouse data may, in some instances, serve to reduce or eliminate the need for field efficacy studies. In this case, it is essential that testing be carried out using the EP rather than the technical material. Applicants should consult the appropriate regulatory organization prior to testing when seeking label amendments.

Where laboratory bioassay data are not deemed appropriate or meaningful to the assessment of efficacy, the applicant may request a waiver of laboratory studies with the submission of a reasoned scientific case.

Field Studies

For the purpose of this paper, 'field studies' refers to testing of the proposed EP under conditions representative of and according to the use patterns proposed on the end-use label. ('field' in this context therefore includes use on glasshouse crops, protected crops and stored crops.)

Experimental field data should be developed and reported according to the principles outlined below and in the companion chemical efficacy guidelines. In general, the studies should be carried out using Good Experimental Practice (GEP), or a similar scheme if trials are conducted outside of the European Union. The data should demonstrate in a scientifically acceptable manner that when used according to label recommendations for rate, timing, number of application and any specific conditions, the EP will provide a meaningful benefit to the user. The acceptability of the organisation generating the data is subject to individual country requirements.

N.B. Efficacy data submitted to support applications for approval in Member States of the European Union are only acceptable if they are generated by organisations 'officially recognised' as competent to carry out such trials work in the Member State(s) where they are performed. It is recommended that the regulatory bodies in individual Member States are contacted for specific details of official recognition procedures in each country.

2.0 PERFORMANCE CRITERIA

Since microbial agents are derived from a diverse spectrum of biological entities, they potentially include all types of pest management products. Field performance criteria and the specifics of experimental design will thus vary, depending on the biological characteristics of the microbials, the nature of the pest management problem and the goals of treatment. It is recognized that microbials may demonstrate performance benefits in a specific crop or production system in various ways, both direct and indirect. Examples include direct mortality to the target that results in control or suppression of pest populations to defined levels; plant growth promotion and prevention of disease establishment; induction of host plant resistance; and sublethal effects on pest targets that enhance natural biocontrol mechanisms or increase susceptibility to conventional control products. Criteria to measure and define performance may consequently differ substantially from those applied to conventional chemicals. In light of this, it is essential that the specific EP performance criteria employed be well defined and that the goal(s) of treatment be clearly stated.

For convenience, the key principles that should be observed in the design and execution of EP field efficacy trials are summarized below. Registration authorities in many countries have published extensive guidelines on the efficacy testing of chemical pesticides. Applicants should consult these

guidelines as many aspects will be very relevant to microbials, especially those microbials which have a direct toxic/lethal effect on the pest organism. The European Plant Protection Organisation (EPPO) (based in Paris) has also published an extensive range of guidelines on efficacy testing, including some general ones such as 'Principles of acceptable efficacy', 'Minimum effective dose' and 'Number of efficacy trials' (see the following link for more details: http://www.eppo.org/PPPRODUCTS/pp1.htm). These guidelines have been officially adopted by the EU and much of the information in these guidelines is relevant to microbial as well as chemical pesticides.

Test formulation(s)

Ideally, testing for efficacy and crop safety should be conducted using the EP formulation proposed for registration. Minor formulation changes between formulations tested may be acceptable, provided that a reasoned scientific case and/or appropriate bridging data to support equivalent performance claims are submitted. Major formulation differences must be supported by bridging data.

Biological activity

The biological activity and potency of each specific lot of EP tested should be confirmed, as per methods reported in *Product Characterization and Analysis* (as part of the dossier), and the results included in the efficacy report. The biological activity of the test substance at the time of testing should be representative of the product specifications and guarantee.

Storage stability information forms part of the chemistry information since shelf life may impact directly on the guarantee of the product. Some aspects of storage stability data such as viability of the microbials or retention of biological activity during storage, may be addressed by the submission of chemical or biological data as appropriate. An OECD issue paper concerning the storage stability of microbial biopesticides is currently being prepared by the BioPesticides Steering Group. Appropriate directions for correct storage of the product must be specified on the label.

Stability of a dry product once it has been rehydrated can be addressed through laboratory data to establish when the spray solution should be discarded after resuspension/hydration. Based on the results, appropriate label statements such as 'spray immediately', or other appropriate timescale might be required.

Microbials and Dose Justification

It is an important principle that the minimum amount of pesticide should be applied to achieve the desired effect. For chemical pesticides it is generally straightforward to generate dose-response data to support the Lowest Effective Rate (LER), which is defined as the minimum application rate required to provide effective control of a target pest, in terms of *level*, *duration* and *consistency* across a broad range of conditions in which the product will be applied. Typically, data from a number of dose-response field tests are used to establish the LER for a chemical pesticide product.

However, it is recognised that the impact of environmental factors can be greater on the performance of microbials so a less rigid approach to dose justification is acceptable. Data from preliminary laboratory tests will have been used to select the doses taken forward for field testing, and these data can also be used as the basis for justifying the recommended field dose. Confirmation that the recommended dose is more effective than reduced doses should then be demonstrated in field trials. This can most easily be achieved by including a reduced dose (e.g. 50% dose) in some of the field trials. Trials should be carried out under the conditions of application recommended on the label and support the rates or doses proposed on the label. In general, at least one field study aimed at showing that the reduced dose is less effective than the full dose is required for each main target pest. In all cases, the applicant should provide a reasoned scientific case supporting the proposed rate or rate range across the conditions in which the product will be applied.

Pest and host combination

Ideally, each pest and host combination on the proposed product label should be assessed separately. However, provided that the microbial mode of action, target specificity, pest behaviour and host phenology warrant such an approach, extrapolation between pest species and between crops may be acceptable, in lieu of data on every combination. Extrapolation would normally be from major pests or crops to minor pests or crops (e.g. aphids on one glasshouse crop to the same species on another glasshouse crop or *Bemisia* whiteflies to *Trialeurodes* whiteflies). An adequate reasoned scientific case is always required to support such extrapolation of claims. Laboratory bioassay or equivalent data may also be required on a case by case basis.

Note that the European Commission (EC) is currently developing proposals for extending and harmonizing efficacy and crop safety extrapolations to reduce the need for trials on minor crops (based on information supplied by the registration authorities of many MS). Although primarily concerned with chemical products, many of the proposed extrapolations may also be appropriate for microbial products. An EPPO standard on such extrapolations is expected to be published in Spring 2008.

Number of trials

As a general rule, refer to the efficacy guidelines from individual countries where registration is being pursued regarding guidance on the appropriate number of trials to be submitted. For outdoor uses, the studies should be conducted in the major geographical regions where the product is intended to be used. The studies should also take into account different pest population pressures and any environmental conditions that significantly affect EP activity. Artificially infested plots may be used where insufficient pest pressure exists. For glasshouse uses and other uses where environmental conditions are less variable, a reduced number of trials is generally acceptable.

Notwithstanding the point above, regional variability in climate, soil type, pest behaviour, host phenology, cultural practices, management goals, etc., may necessitate supplementary trials to adequately demonstrate performance claims under typical conditions of application and in representative sites and areas of intended use. Applicants may request waiver of some regional studies if:

-only a limited, regional registration is desired (or use in protected crops only); or
-reasoned scientific cases to support reduced regional testing are submitted.

Applicants may also request a reduction in the number of trials or a waiver of certain studies if:

-reasoned scientific cases supporting their proposals are submitted.

Reference treatment and untreated control

Trials must include an untreated check as an indicator of pest pressure and for comparison with treated areas. Untreated controls are always required unless field trials are conducted in an area or crop where the use of an untreated control may create a significant pest problem in the area or crop, and no other methods of controlling the pest are available. In such cases, a comparison with historical pest level would be required. Positive controls, consisting of comparison with reference products of known efficacy or normally accepted practice should always be included unless none exist. A reasoned justification for omitting a reference treatment would be required.

Whenever possible, the performance of the microbial must be compared to that of a reference product. This reference standard may be either a microbial product or a chemical product, but it must be an approved product whose efficacy against the pest in question is known. In some instances, it **may** be

helpful to include both types of standards in the same trials. It is recognised that if a chemical pesticide is used as the reference product, the efficacy of the chemical may be greater or quicker-acting but that does not reduce its value as a standard. The principle reason for including a standard in trials is to validate the trials by verifying that the results are in line with the expected results under the test conditions. Results for the standard may also assist in interpretation of the trials, bearing in mind that the main purpose of the trials is to demonstrate that a biopesticide provides a measurable benefit.

Even if the microbial product has a lower efficacy against the target pest than that of the reference product, its efficacy may still have value due to the following considerations:

- Use over a wider range of growth stages of the crop or the target pest
- Greater compatibility with cultural practices or other plant protection measures
- Lower potential of resistance development, or providing a useful alternative as part of the overall resistance management strategy for a particular target
- Fewer undesirable effects on beneficial organisms, other crops, etc.
- Acceptable for use in 'organic' productions
- Absence of residues

Performance measurements

Performance measurements should include direct counts of the target pest, disease incidence, disease severity, effects on the quality and where appropriate the yield (or components of yield) of treated crops, or effects on the quality of treated plant products. The possibility of adverse effects on transformation processes (if applicable) must be considered, as for chemical pesticides. Safety to treated crops (e.g. absence of adverse effects such as chlorosis or necrosis) must be assessed.

In some countries, safety to following crops, to adjacent crops and to plant parts for propagation must also be addressed, as for conventional pesticides, though reasoned cases may be sufficient in many instances for microbials.

Level of performance

As a general rule, microbials are more affected by environmental conditions than chemical pesticides because they are living organisms. As living organisms, they require more or less specific conditions to promote and maintain their growth, i.e., temperature, humidity, pH, specific growth promoting factors, presence or absence of the pest depending on the mode of action. As a consequence, their level of effectiveness is more variable than chemical product. Under the right circumstances certain microbials can be as effective as chemical products, while others, at their best, will only provide lower level of effectiveness. Because of the advantages associated with microbials (possible compatibility with IPM, acceptability in an organic production approach, absence of residues), levels of performance below those expected for regular chemical pesticides can be acceptable. However, biological control measures are not necessarily restricted to organic or integrated crop protection systems but may be useful for conventional crop protection as well. These diverse production systems require the use of clear and well-defined terminology on the product label to describe the expected level of control. Experience from the field trials can also be used to provide information and advice to growers on the optimal conditions under which the microbial products will work most effectively.

Adverse interaction from chemical pesticides applied to the crop is always a consideration and standard label warnings should be present on microbial labels where interaction can be expected (e.g. 'Chemical fungicides applied before or shortly after a fungal microbial may reduce its effectiveness.') unless data or a justification of non submission of data is submitted to avoid the need for the label warning.

Note that label statements such as 'Fungicides should not be applied within X days of application of the microbial.' carry an implicit claim that no adverse effects on the microbial will result from fungicide application just outside of this period. Specific evidence must be submitted to support such implicit claims of safety.

Types of label claims

In all cases, the efficacy package must demonstrate a product performance level at least significantly superior to the untreated control, i.e., that the use of the product is better than no use. Based on a demonstrated level of performance, and, when needed, a reasoned scientific case supporting its value to the users, the following examples of label claims can be made. (NB. The following are only examples. It is suggested that alternative label phrases can be used that are appropriate to the language in the country of use):

a) Control

A performance claim for 'control' of a pest must be supported by efficacy data which demonstrates that the product, when applied in accordance with the label directions, *consistently* reduces pest numbers or pest damage to a level comparable with a reference standard of known efficacy. The standard may be a microbial or convential chemical, providing the efficacy of the standard is known.

b) Moderate control or Suppression

A performance claim for 'moderate control' or 'suppression' of a pest must be supported by efficacy data which demonstrates that the product, when applied in accordance with the label directions, provides *consistent* control at a level which is not optimal but still provides commercial benefit.

c) Reduction

A performance claim for 'reduction' of a pest must be supported by efficacy data which demonstrates that the product, when applied in accordance with the label directions, can reduce pest populations or damage to a level significantly different from the untreated check.

N.B. The UK uses a 'gradation' system for chemical pesticides which is explained in detail for pests, weeds and diseases in section 3.3 of Chapter 8 of the 'Data Requirements Handbook' in www.pesticides.gov.uk and which could be used for microbials. However such a gradation system might require more precision than highly variable efficacy data could support.

d) Other

In some cases, microbial pesticides do not directly act to control or suppress the target pest. As an example, microbial pesticides with an action of Systemic Acquired Resistance (SAR) triggers plants' immunity to the target pathogen(s) and will not directly act on the target itself. Therefore, label claims such as control, suppression, and reduction may not be appropriate. In those cases, label claims can be a description of the actual activity of the microbial pesticides. For instance, 'promote immunity of the plants to the target pest', 'inhibit the growth of the target pest', 'promote plant growth', 'prevent disease establishment', etc.

3.0 COMPATIBILITY WITH CROP PROTECTION AND MANAGEMENT PRACTICES

Effects on Performance

Tank mixtures

Label recommendations regarding tank mixing with registered pesticides or use of the product with adjuvants, stickers, spreaders, diluents, etc., should be supported by specific compatibility and performance data. To do so, the data reviewed must include comparison of treatments with the tank-mix partners alone and together to demonstrate that no component of the mixture is antagonistic to any other component of the mixture.

IPM situations

Information on the efficacy and compatibility of the microbial when used in conjunction with other crop protection measures, particularly chemical pesticides, is highly desirable. This is especially important in crop production situations where current management practices involve use of control measures to which the microbial may be sensitive. In the latter case, laboratory and/or field data which assess compatibility of the microbial should be submitted. In some instances, cultural practices or other chemical components may also adversely affect EP performance. These and any other crop management practices that may negatively affect performance should be evaluated, where relevant to the nature of the EP and the production scenario. However, in the absence of appropriate evidence of compatibility, it is acceptable for the EP label to carry appropriate warning phrases.

Effects of the EP on natural biocontrol organisms

Claims regarding enhancement of, or compatibility with natural biocontrol organisms or IPM strategies should be supported by appropriate laboratory or field evaluation data relevant to the nature of the claim, the characteristics of the microbial and the proposed use pattern. Host spectrum data, and relevant non-target hazard testing data may be used to support claims regarding comparability and potential value to natural biocontrol and IPM. Where relevant to the specific claim, it is recommended that field performance testing includes observations regarding effects of treatment on natural biocontrols within the specific crop or system.

4.0 CROP OR RESOURCE PRODUCTION BENEFITS

To document the potential value of a new microbial in terms of crop or resource production benefits and sustainable management considerations, objective information in the following areas is required. It is the responsibility of the applicant to provide sufficient details to enable the registration authority to draw conclusions regarding the potential or actual benefits of the new EP. New EPs may demonstrate crop or resource production benefits or contribute to the implementation of more sustainable management practices or risk reduction in a number of ways. For example, new EPs might:

(*i*) enhance or complement more sustainable use of current management products by allowing growers to reduce the rate of conventional products or frequency of application required for conventional products;

(*ii*) provide a viable alternative to traditional products wherein significant problems or concerns exist, e.g., pest resistance, safety issues;

(*iii*) provide entirely novel approaches to pest management in situations where conventional tools do not exist or are considered unacceptable for a variety of reasons;

(*iv*) suppress pest populations to levels sufficient to promote commercially acceptable efficacy of more

sustainable, non-intervention management practices, e.g., crop rotation, physical barriers;

(*v*) encourage implementation of pest management practices such as scouting, timing and application of pest control products based on appropriate action or economic thresholds;

(*vi*) constitute essential, narrow spectrum tools that provide the foundation for development of IPM strategies;

(*vii*) enhance occupational or bystander safety in situations where significant exposure may be unavoidable, e.g., indoor uses, aerial application in residential areas.

5.0 RESISTANCE MANAGEMENT

Resistance is not an issue for most microbials acting through pest population regulation processes but it must however be addressed by applicants, possibly by means of a reasoned case. It can be an issue when the mode of action is based on direct toxicological or infective interaction with a pest. In the latter case, adaptation of the pest is much more likely to occur and resistance management strategies should be developed to minimize the selection for resistance. The same strategies (baseline population studies, alternation or tank-mix with different mode of action products, etc.) that are used for chemical pesticides can be adapted for use with microbials.

ABBREVIATIONS

BPSG	BioPesticide Steering Group (OECD)
CFU	colony forming unit
EC	European Commission
EP	End-use Product
EPA	Environmental Protection Agency (USA)
EPPO	European and Mediterranean Plant Protection Organisation
EU	European Union
GAP	good agricultural practice
LD_{50}	median lethal dose (the amount of an MPCA* to kill 50% of the test organisms)
LC_{50}	median lethal concentration (the amount of an MPCA* to kill 50% of the test organisms)
MPCA	microbial pest control agent (efficacious by intoxication and/or infection)
MPCP	microbial pest control product
NAFTA	North American Free Trade Agreement
OECD	Organisation for Economic Co-operation and Development
WGP	Working Group on Pesticides (OECD)