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Working Document on the Risk Assessment of Secondary Metabolites of Microbial Biocontrol Agents

Series on Pesticides
No. 98

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Working Document on the Risk Assessment of Secondary Metabolites of Microbial Biocontrol Agents

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

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FOREWORD

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 OECD BioPesticides Steering Group (BPSG), which has been renamed to the Expert Group on BioPesticides (EGBP), was established by the Working Group on Pesticides in 1999 in order to help member countries to harmonise the methods and approaches they used to assess biological pesticides and to improve the efficiency of regulatory procedures. For the purposes of this group, biological pesticides include: microbials; pheromones and other semiochemicals; plant extracts (botanicals); and invertebrates used as biological control agents. The first tasks the BPSG undertook were: (i) reviewing the regulatory data requirements for three categories of biopesticide (microbials, pheromones and invertebrates); and (ii) developing formats for dossiers and monographs for microbials and for pheromones and other semiochemicals. After this was achieved, the BPSG decided to concentrate its efforts on addressing the scientific and technical issues that remain as barriers to the efficient regulation of biological pesticides.

This working document has been prepared to address the assessment of microbial secondary metabolites[1] (SM). This important issue was discussed in detail at the joint OECD/Swedish Chemicals Agency (KEMI)/ European Commission workshop on microbial pesticides held in 2013. This workshop recognised the complexity of the microbial SM issue and the gaps in knowledge and it concluded that further research and regulatory guidance were needed. Further discussion took place at the one-day BPSG seminar held on 18 May 2015[2]. This seminar considered methods for identifying SM and predicting their toxicity; regulatory approaches to conducting risk assessments; and industry views on the registration processes in some OECD member countries.

The main focus of this working document is the assessment of the hazards and risk of microbial SM produced i) during the manufacturing of MPCPs and ii) after application of MPCPs in the field. This document addresses a number of important topics for SMs, including concerns that MPCPs could be associated with known or as yet uncharacterised 'new' SMs.

This document may also form the basis of a future OECD Guidance Document on the subject. A background document has also been prepared to help provide clarity on issues and terminology related to microbial SM.

[1] Primary metabolites produced by microorganisms are involved in the growth, development, and reproduction of those organisms and are essential components for maintaining normal physiological processes. Secondary metabolites are biosynthesized from primary metabolites.

[2] See Report of the 6th Bio-pesticides Steering Group Seminar on Hazard and Risk Assessment of Secondary Metabolites Produced by Microbial Pesticides [ENV/JM/MONO(2017)5]

LIST OF ABBREVIATIONS

ALARA	As low as reasonably achievable
BMD	Bench mark dose
BPSG	Biopesticides Steering Group (renamed to Expert Group on BioPesticides; EGBP)
CCC	Counter current chromatography
EGBP	Expert Group on BioPesticides
EFSA	European Food Safety Authority
EP	End product
EtOAc	Ethyl acetate
GC/MS	Gas chromatography/Mass spectrometry
HPLC	High performance liquid chromatography
LPS	Lipopolysaccharide
ML	Maximum level
MPCA	Microbial pest control agent: (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.
MPCP	Microbial pest control product
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction *assay*
NOAEL	No observed adverse effect level
NRP	Nonribosomal peptides
OECD	Organisation for Economic Co-operation and Development
PDA	Potato dextrose agar
QSAR	Quantitative Structure–Activity Relationship
RAFBCA	Risk assessment of fungal biocontrol agents project
REBECA	Regulation of biocontrol agents project
SM	Secondary metabolite
TDI	Tolerable daily intake
TER	Toxicology/exposure ratio
TLC	Thin layer chromatography
TTC	Threshold of toxicological concern
WGP	Working Group on Pesticides

INTRODUCTION

This working document aims to help in the determination of whether microbial pest control agents (MPCA) could be associated with known or as yet uncharacterised 'new' secondary metabolites (SMs). It is also an initial step in providing guidance and a decision scheme that could facilitate regulatory decision making.

Definition of a secondary metabolite

Although not essential for their primary metabolic processes, microbes, particularly actinomycetes and fungi, produce various SMs, including compounds of industrial and economic relevance. SMs are chemically different naturally occurring compounds of relatively low molecular weight (in most cases < 3 kDa), and typically associated with individual genera, species or strains.

SMs are biosynthesised from primary metabolites in specialised pathways (i.e. polyketides or mevalonate pathways derived from Acetyl Coenzyme A, or amino acids) and some specific gene clusters. The expression of these genes appears to be controlled by one or more global regulators. SMs show biological activities that may be related to survival functions of the organism, such as competition against other micro- and macro-organisms, symbiosis, and transport of substances.

> ***Further Information*:** For a description of primary and secondary metabolites, see the introduction to Section 11 (Background document).

Definition of a relevant secondary metabolite

The regulatory data requirement for SMs essentially follows the regulation of chemical pesticides. However, it is not possible to copy these criteria of chemical pesticides to microbial pesticides as it would be difficult, or even impossible, to determine the amount of SMs. Instead, the criterion has been defined in a 2008 OECD Working Document[3] as "the relevant (toxic) metabolite".

It is a challenge to work with this criterion as 'the relevant metabolite' is also difficult to define. It seems reasonable that if a risk assessment is required for SMs it should parallel the requirements for chemical pesticides. This means that, like metabolites of chemical pesticides, SMs of microbial pesticides must also be acceptable to a certain extent.

An important difference between the two types of metabolites is that chemical pesticide metabolites are usually present wherever the parent substance has been applied regardless of the presence of the host/target, whereas in microbial pesticides, the SMs often appear where there is close contact with the host/target organism. Production of SMs can also be triggered by other biotic or abiotic factors. SMs might even be part of the mode of action. This difference can be to the advantage of microbial pesticides if the exposure of non-target organisms is more limited.

It is important to remember that micro-organisms only have the potential to produce a SM when they are metabolically active. When they are active, they may/may not produce one or more SMs at any stage and this may change over time. Each of these SMs may/may not be of concern. Also, although a single SM may be identified, it often does not occur alone and a series of SMs will often be produced together in a more or less complex mixture. This is similar to the situation for botanicals and the botanical guidance may give some useful approaches to the components of concern/metabolite issue (OECD, 2017).

[3] OECD Environment, Health and Safety Publications Series on Pesticides No. 43: Working Document on the Evaluation of Microbials for Pest Control.

The definition of a relevant SM that is used for this document is:

> ***Any secondary metabolite known to be formed by a micro-organism that can be of reasonable and justified concern to human health and/or the environment***.

Important groups of SMs that have been identified to date are mycotoxins, phytotoxins, exotoxins, and antibiotics.

> ***Further Information*:** For a description of each of these groups of metabolites, see the introduction to Section 11 (Background document) and for lists of each of these groups, see sections 8.2.1 to 8.2.4.

Enzymes and endotoxins are excluded from this working document: according to some regulations in OECD countries, enzymes and endotoxins may fall under the definition of residues. These substances are formed as a result of the use of a plant protection product (PPP) and include breakdown products, reaction products and SM. However, enzymes and endotoxins do not fall within the definition of an SM given above.

Enzymes are not considered to be SMs. They are necessary as catalysts for processes and it is recognised that some enzymes have a clear impact on pathogens.

Endotoxins are toxins produced by Gram-negative bacteria. A group of heat-stable lipopolysaccharide (LPS) molecules present in the cell walls of the Gram-negative bacteria have a characteristic toxic effect.

In *Bacillus thuringiensis*, the endotoxins are typically referred to as delta endotoxins, i.e., insecticidal crystal proteins. Biocontrol products with *Bacillus thuringiensis* are developed because of the toxic properties of these endotoxins, and these endotoxins are always included in the risk assessment as they are considered to be relevant for the toxicity of the product.

1.1 Decision scheme

A possible decision scheme regarding an approach for risk assessments is described below. It is made up of three main components: Basic information, Exposure and Persistence in the environment (see **Figure 1**).

The 'Basic information' box is the starting point of the evaluation. Section 2 further specifies what is included in this part of the evaluation. At this level, it is, in principle, possible to conclude that the micro-organism either produces no SM or only produces SMs that are not relevant based on adequate and reliable information as outlined in Section 2. The remaining boxes need to be evaluated independently. It may not be possible to design a decision scheme in which all interactions between the boxes are shown in detail. The present working document addresses each box individually.

This decision scheme also takes into consideration the proposal for the risk assessment of SM prepared by the EU-funded REBECA project (Ehlers, 2007). The starting point of their proposal is the evaluation of the relevance of the SM and the use of crude extracts (see Section 9.3). This approach almost immediately requires the identification of SM and a proper definition for 'relevant amounts'. This is a rather complicated approach and is not favoured in the decision scheme presented here. This current decision scheme will only ask for the identification of SM in cases where there is reason to assume that the product is potentially hazardous in relation to its SM. In these cases a thorough calculation of the maximum possible amount will be needed. This calculated maximum amount may already indicate whether an SM (toxic or non-toxic) might have potential to be of concern or not.

The decision scheme aims to provide a flexible approach to risk assessment. In the future, a guidance document may be developed and the scheme could be further elaborated and clarified.

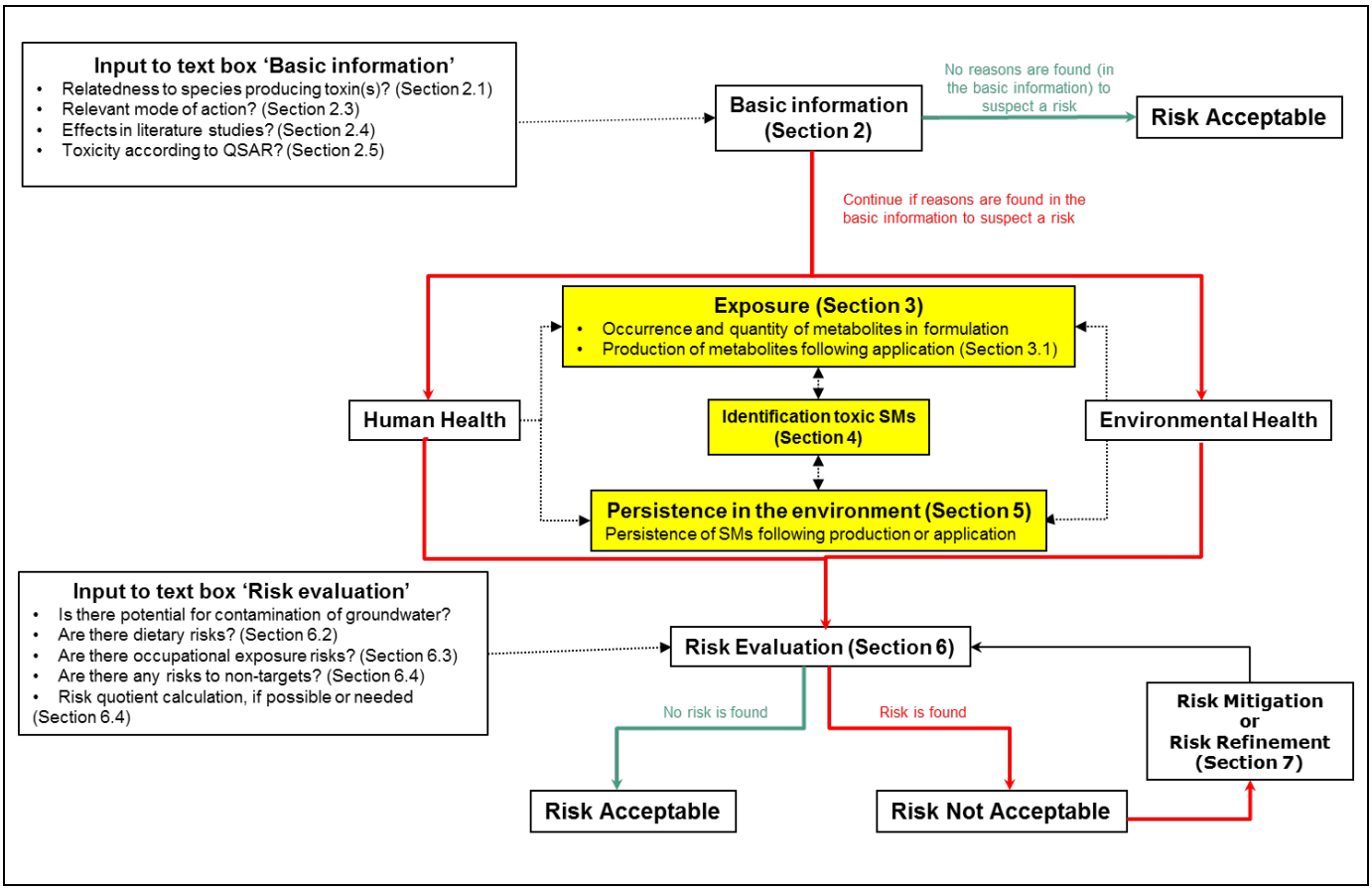

Figure 1. Decision scheme. Grey dotted lines indicate questions that should be posed in the 'Basic information' and 'Risk evaluation' boxes. Green lines indicate a negative answer to the questions posed in the boxes inputting into 'Basic information' and 'Risk evaluation'. Red lines indicate a positive answer to the questions posed in the boxes inputting into 'Basic information' and 'Risk evaluation'.

1.2 Groups of MPCA species

Groups according to concerns and risks were proposed in existing guidance document[4] for another type of biopesticides. In analogy to the existing guidance, three groups regarding SM could be distinguished. It is important to mention that these groups are based on the current knowledge of the species and not on the SM.

Group 1
MPCAs that are known within current knowledge to have no adverse effects on humans, animals and the environment.

Group 2

[4] Botanical guidance SANCO/11470/2012 rev. 8 and Guidance Document on Botanical Active Substances Used in Plant Protection Products. Series on Pesticides No. 90. ENV/JM/MONO(2017)6.

MPCAs for which the taxonomy and current knowledge indicates that they may produce secondary metabolites of possible concern for humans, animals and/or the environment. In this case these components should be identified and quantified.

Group 3
No reliable information available.

1.2.1 Group 1

MPCAs have been demonstrated that they:
a. are not known to produce toxic SMs;
b. are not related to known pathogens or toxigenic species (see Section 2.1.1.1 for the definition of close relatedness); and
c. have not demonstrated any effects during toxicity testing.

As a result, the likelihood that these MPCAs produce sufficient quantities of SMs to adversely affect non-target groups is low.

> ***Further Information*:** Background Document (Chapter 3) shows that *in situ* the expression of genes, and thus the formation of SMs, is strictly regulated. This means that any SMs produced affect target organisms locally only.

Assigning MPCAs to a particular group can be difficult. For entomopathogenic fungi, the issue of secondary effects (toxicity) has been raised by some regulatory authorities. However, feeding experiments with *Beauveria*- or *Metarhizium*-infected locusts (see Background Document, Chapter 4) did not show toxic effects in birds and reptiles.

Candidates for group 1:
- Some fungal entomopathogens
- Some bacterial biocontrol species belonging to *Bacillus, Pseudomonas, Streptomyces* (there might be exceptions for some *Streptomyces*). Certain strains of *Bacillus thuringiensis* are known to produce Beta-exotoxin, but this type of toxin is not considered to be an SM and its presence in formulations is regulated by authorities.
- Yeasts
- *Trichoderma* spp. as long as they do not produce mycotoxins

1.2.2 Group 2

a. No reliable or contradictory information is available on the MPCA.
b. MPCAs where taxonomy and current knowledge indicate that they may contain or produce SMs of possible concern for humans, mammals and/or the environment. In this case these components should be identified and quantified.

Candidates for group 2:
- *Trichoderma* spp. that produce mycotoxins.

1.2.3 Group 3

a. MPCA is closely related to (myco)toxin producing species (see Section 8.2.1) or to bacterial species that are known pathogens in humans (see Section 8.2.2).
b. SMs produced by the MPCA are (known to be) stable. (Myco)toxins produced in food/feed could be stable as they are less exposed to environmental conditions (temperature, drought, UV light). However, stability may not be a suitable criterion as, theoretically, unstable very toxic SMs may have an instant toxic effect.
c. Products containing toxic SMs that are overexpressed or deliberately concentrated during production. These may be species producing phytotoxins which may be used in herbicidal products. As the efficacy of the product will (partly) depend on the presence of this SM, all relevant information on this SM should be available in the dossier.
d. Even if a micro-organism is potentially in a 'high risk' group, there is good reason to assume there is no risk associated with SMs if the genes responsible for producing the SMs of concern are known and shown to be absent in the strain being used.

Candidates for group 3 (theoretically, micro-organisms that are known to produce toxic SMs in biocontrol species that are also closely related to pathogenic species might fit into this category):

- Strains not producing mycotoxins. For strains developed as biocontrol agents that do not produce toxic SMs (e.g., non-aflatoxin-producing *Aspergillus* strains), adequate evidence should be given of the absence of these metabolites. There is good reason for assuming there is no risk associated with the SMs if the genes responsible for producing the SMs of concern are known and shown to be not expressed in the strain being used.
- Strains producing mycotoxins. Some plant-pathogenic species, such as *Fusarium oxysporum*, can be used as herbicides. The efficacy of bioherbicides can be greatly enhanced by selecting variants that overproduce and excrete amino acids that are inhibitory to the target plant. Thus, for efficacy, these amino acids may be more relevant than mycotoxins. As the genus *Fusarium* is known to produce mycotoxins, the greatest risk would be human and animal consumption of colonised plant material. The SM profile of *Fusarium oxysporum* should be determined using genetic approaches, e.g., gene probes, full genome sequencing (see Section 9.1.4) or RNASeq (see Section 9.1.5), and if mycotoxin genes are present, the risk should be assessed. *Fusarium oxysporum* is a large and diverse species complex and assumptions cannot be made about the production of mycotoxins. Each biocontrol strain should be assessed for its SM production. Other examples:
    - *Acremonium diospyri* may be used as an herbicide. A commercial product is not yet on the market. Some *Acremonium* species produce the mycotoxin crotocin.
    - *Clonostachys catenulatum* (formerly *Gliocladium catenulatum*) and *C. rosea* may be used against seedling blight and root rot (*Pythium, Rhizoctonia* spp.) in lettuce and cucumber. *Clonostachys catenulatum* produces several SMs, e.g. gliotoxin.

2 BASIC INFORMATION

To assess the likelihood that a micro-organism can produce relevant SMs, basic information should be available in the dossier that is prepared by the applicant (containing data and/or published literature) which may give a first indication of the potential for the microbial pest control agent (MPCA) to produce SMs. The dossier should be prepared according to the data requirements in the respective jurisdictions.

List of basic information
Basic information on the MPCA should be included in the dossier that is prepared by the applicant. If the subject is dealt with in the Background Document or in this document, reference to the exact location is given.

Microbial pest control agent: properties of the micro-organism

- Identification and taxonomic position of the MPCA (Section 2.1)
- Biology of the MPCA
- Colonizing abilities of the MPCA (primary/secondary colonizer)
- Life cycle
- Existing distribution/occurrence/exposure of the species in relevant areas (i.e. food, feed, environment)
- SM already known to be produced by the MPCA (Chapter 1.4 Background Document)
- Modes of action (Section 2.2)
- Host range
- Conditions for proliferation (where and how it proliferates and limitations to its growth)
- Production method (this may exclude the presence of a SM e.g. for washed conidia)

Microbial pest control product: properties (of the micro-organism) in the formulated product

- Formulation type and detailed composition of the product (Chapter 2 of the Background Document)
- Production method (this may exclude the presence of a SM e.g. for washed conidia)
- Detailed description of the intended uses, such as:
    - Site and method of application (e.g. seed, soil, foliar treatment; granular, spray,)
    - Treated crops and target organisms
    - Timing of applications (e.g., growth stages of crop plants, number of and time intervals between applications, pre-harvest intervals)
    - Application rates of MPCP and MPCA.

Properties of the SMs

- Name and structure
- Presence inside or outside the cell
- Presence of (known) SM-encoding sequences in the genome
- Stability (Section 5)
- Modes of action (including external and internal factors of the micro-organism necessary for the action) (Section 2.2)
- Effect on humans, mammals or other non-target species (Chapter 4 Background Document)
- Conditions (abiotic / biotic) under which the micro-organism produces the SM(s), especially any toxin(s) (Chapter 3 Background Document)
- Mechanism by which the micro-organisms regulate the production of the(se) SM(s) (Chapter 3 Background Document)
- Influence of the SMs on the micro-organism's mode of action.

Properties of the micro-organisms in relation to target organisms

- Intended use of the product (target organisms)

- Efficacy data

Properties of the micro-organisms in relation to non-target organisms
- Confirmation of absence from lists of effective antibiotics
- Confirmation of absence from lists of known pathogens in humans (Section 8.2.2)
- Toxicity/infectivity data on the MPCA,
- If available, health and medical reports
- List of non-target organisms, including humans, and the anticipated route(s) of exposure

This information should be sufficient to determine if there are concerns associated with the MPCA. If no exposure or other concerns can be identified, then the risks could be considered acceptable and the SM(s) is (are) of no concern. It is then deemed unnecessary for the evaluator to consider aspects in Sections 3 to 7 in more depth. To confirm, all aspects of the risk assessment should be undertaken for the micro-organism itself.

***Further Information*:** Additional information may be required to support registration on a case-by-case basis (Section 8.3) using the proposed decision scheme.

2.1 Identification and taxonomic position of the MPCA

The tables in Chapter 1 of the Background Document are prepared according to the current classification and provide information on known MPCAs and their reported SMs.

Some registered products contain these MPCAs and consideration of other products on the market may provide some orientation (see Table 6 in Section 8.3). However, to allow comparisons to be made, the MPCA/MPCP should be considered similar or equivalent qualitatively and quantitatively as some products might have been placed on the market under a regulatory regime with less specific/stringent data requirements. Also, the MPCP may differ in, for example, the strains they contain and the production conditions. These differences may lead to a difference in the range and quantities of the SMs.

2.1.1 Phylogenetic Relatedness

The crucial question is how to define relatedness? The phylogeny of fungi and bacteria, i.e., their evolutionary history, depends on the parameters chosen. For example, was it discrete morphological traits or nucleotide and protein sequences?

> ***Further Information*:** Check relatedness with pathogenic species; see Tables 5 to 24 in Background Document (Chapter 1). These tables also give an overview of which SMs are known to be produced by biocontrol species (registered products or under research) and pathogenic species.

Phylogeny based on SM production has not been successful so far. Background Document, Chapter 1, paragraph 1.4.5 shows that the same SMs can be produced in closely related or more distant families.

For example:
- Cyclosporine is produced in families within the same subclass of the Hypocreomycetidae.
- Bassianolide is produced in families of two related subclasses within the Sordariomycetes.
- Desmethyl destruxin B is produced in families belonging to different classes of the Ascomycetes.
- Beauvericin is produced in families belonging to both the Ascomycetes and the Basidiomycota.

Beauvericin is produced within

Ascomycetes
- subclass Hypocreomycetidae,
- family Cordycipitaceae by species within the genus of *Beauveria*
- family Nectriaceae by species within the genus of *Fusarium*
- family Clavicipitaceae by species within the genus of *Isaria*

Beauvericin is produced within
Basidiomycota
- class Agaricomycetes
- family Polyporaceae by *Polyporus sulphureus*

> ***Further Information*:** For production of other SMs in different families see Background Document, Chapter 1, paragraph 1.4.5.

Examples of biocontrol species that have pathogenic 'cousins' producing toxic SMs are:

- Two products containing *Aspergillus flavus* are non-aflatoxin-producing strains. These strains are derived from naturally occurring mutants. The non-aflatoxin-producing strains are applied to out-compete the pathogenic aflatoxin producing strains. The aflatoxin concentrations produced by these pathogenic strains are, by definition, higher than those produced by the biocontrol strains. In a case where the strain of the biocontrol agent produces a mycotoxin in lower quantities than the wild type, which can be proven with standard techniques, it is not necessary to take exposure and quantities produced into consideration.

2.1.1.1 Regulatory consequences of (no) phylogenetic relatedness

If the MPCA in question is related to another micro-organisms which produces SM (of concern), this may trigger a concern for the new MPCA and the risk of SMs needs to be assessed. If the new MPCA is not related to a known SM producing species, then it cannot be concluded without further assessment that the new MPCA does not produce SMs of concern.

2.2 Relevance of secondary metabolites in the mode of action

In order to better assess the possible hazards posed by SMs, it is important to know the mode of action of the MPCA (Sundh, 2012). The MPCA should be assessed on a case-by-case basis to determine whether the SMs play an important role in the mode of action. Many different modes of action exist, for example:

- Defence: these SMs act against the host immune system.
- Antagonism: these SMs have antimicrobial properties.
- Competition: these SMs enable the MPCA to compete for nutrients and space.
- Pathogenesis: SMs may be important pathogenicity determinants. They may kill or weaken the host.
- Hyperparasitism: the SMs can cause toxic effects or lead to infectious diseases in mammals or birds (see Section 8.2.1 for known mycotoxin producers and their mycotoxins) when coupled with infectious organisms. Some of the genera in this list contain species that are used as biocontrol agents (see Section 8.2.2 for known pathogens in humans). However, only four pathogens in this list produce toxins: *Clostridium botulinum, C. tetanii, Corynebacterium diphteriae* and *Shigella dysenteriae*. These species are not used as MPCAs.

Example 1:

> Researchers often try to maximise the production of SMs that play an important role in the suppression of fungal pathogens. In the fungal pathogen *Pythium ultimum* for instance, high iturin and surfactin producing strains have been developed. The mode of action for these lipopeptides is the penetration of the cell membranes of plant pathogens, thereby destroying the cells. MPCA species producing these SMs could be considered in group 1 and may not require further attention in the risk assessment of their SM production.

Example 2:

> Phytotoxins may be used in herbicidal products and the production may be upregulated. These phytotoxins may pose a risk to other non-target plant species if there is drift during application. MPCA species producing these SMs could be considered to belong to group 2. These SMs would likely require further attention in the risk assessment.

Some toxic SMs may simply be a by-product of fermentation and are not involved in the mode of action of the MPCA. Even if the SM is not involved in the mode of action, it is important that a risk assessment be conducted.

2.3 Reported incidences of infections or (geno)toxicity

A literature search investigating reports of infection and (geno)toxicity should be performed. However, it may not be clear whether any (geno)toxic effects reported were caused by the SMs.

Studies from the literature should be evaluated for:
- Type of exposure and which non-target group was exposed.
- Relevance of the concentration of the product application for the risk assessment.
- Accurate description of the active substances in the product.
- Overall reliability of the study.

The outcome of a study may raise concerns about SMs.

2.4 Quantitative structure–activity relationship models as a tool for predicting toxicity

The use of quantitative structure–activity relationship (QSAR) models could be helpful in conducting a risk assessment. However, an initial problem is that a CAS number, SMILES code or the exact structure needs to be entered into these models. This information may not be available as the SM is not known or there is a mixture of SMs.

QSAR models are regression or classification models used in the chemical and biological sciences and engineering.

One example of an expert system based on structure-activity relationships, relevant for assessing the effects of SMs is the Norine database [http://bioinfo.lifl.fr/norine/]. According to the website “The Norine database is a platform that includes a database of nonribosomal peptides together with tools for their analysis. Norine currently contains more than 1000 peptides. The name Norine stands for Nonribosomal peptides (NRP), with ‘ine’ as a typical ending of peptide names. For each peptide, the database stores its structure as well as various annotations such as the biological activity, producing organisms, bibliographical references among others. The database can be queried in order to search for peptides through their annotations as well as through their

monomeric structures. In the latter case, the user can specify the composition, the whole structure or a structural pattern (possibly including "undefined monomers") of the searched peptide".

Specific algorithms that compare structures are currently being developed. NRPs with similar monomeric structures have similar activity, so searching the monomeric structure of a NRP can help to predict its activity. In due time, Norine will provide more tools with which to study NRP activity or toxicity.

More QSAR software packages are available, e.g. DEREK, HAZARDEXPERT, TOPKAT or the OECD QSAR Toolbox. The predictive ability of these programmes for SMs has not been evaluated.

Users should evaluate if the QSAR program can predict chemically induced adverse effects of SMs, such as skin irritation or effects on reproduction.

Conclusions:

The Norine database already allows for the prediction of the toxicity of SMs. The use of QSAR programs can be helpful in identifying possible hazards of chemicals. However, these evaluations are preliminary. Positive and negative predictions should be further investigated (e.g. with the identification of a NOAEL).

3 EXPOSURE

Determination of exposure is crucial for a risk assessment of effects on human health and the environment. For human health assessments, worker, operator, consumer, residential and bystander exposures need to be considered. For environmental assessments, exposure to non-target groups must be considered, including non-target arthropods (in the soil and foliage), earthworms, soil micro-organisms, aquatic organisms, plants, mammals and birds.

The relevance of the exposure to SM for each of these non-target groups depends greatly on:
- Type of formulation (for instance WP, GR, WG, CP, SC, granule. See Background Document, Chapter 3)
- Concentration of the active ingredient in the product
- Treated crop
- Recommended rate of application
- Type/place/timing of applications (e.g. canopy/leaves, soil, seed, storage)
- Naturally occurring levels of SMs produced by closely related species or strains. If the environment and/or humans are naturally exposed the SMs and their levels are not significantly affected by the use of the MPCP, it may be considered to be of no concern.

Each of these factors should be considered and the combination of these and other factors (e.g., food consumption data for consumer risk assessment) will determine whether or not exposure is considered acceptable.

3.1 SMs present in the MPCA/MPCP or produced *in situ*

It is important to acknowledge that exposure to SMs can come from two sources:

a. SMs in the MPCP as a result of the production method. These may be products in which SMs have been upregulated. In other cases, the levels of SMs may be too low to cause any effects in non-target organisms (see Box 3 in Section 6.4 for calculation).
b. SMs produced in presence of precursor molecules and/or after contact of the MPCA with the pathogen/host:
   - *CanopyLeaves/Fruits*: Following application of the MPCP, SMs are produced by micro-organisms in contact with plant pathogenic fungi in the canopy, on leaves or fruits. This scenario could apply to fungal and bacterial control agents such as *Pseudomonas, Bacillus* or *Trichoderma* spp.
   - *Arthropods*: Following application of the MPCP, SMs are produced by micro-organisms in contact with canopy and soil dwelling arthropods. This scenario could apply to entomopathogenic fungal control agents such as *Beauveria, Metarhizium, Isaria* and *Lecanicillium*.
   - *Soil:* SMs are produced by MPCAs in contact with pathogenic fungi in the soil after application of the product, e.g. fungal and bacterial control agents such as *Trichoderma*, *Bacillus, Pseudomonas, Burkholderia* and *Streptomyces* spp. which live in the rhizosphere. SMs are formed in this micro niche. In bacteria, production of SMs is often regulated by quorum sensing upon reaching a certain population density (see Background Document, Chapter 3). In general, the soil is an extremely versatile ecosystem and, if they occur, effects on soil micro-organisms are generally transient.
   - *Consumption of diseased arthropods*: the main concern here is the secondary exposure to toxic SMs of some non-target organisms such as mammals, birds, amphibians or reptiles by eating diseased arthropods.

It can be concluded that LC50 values obtained from contact and feeding studies using the MPCP, including SMs, can be useful where SMs are present in high amounts in the formulated product and are known to play

a role in the mode of action. This is certainly the case when the production of SMs in herbicides is maximised in order to obtain an improved efficacy.

3.2 Human exposure

Workers, operators, consumers, residents and bystanders can be potentially exposed to MPCPs. Operators can be exposed during mixing/loading and application if there are SMs in the product, and workers can be exposed following application of the MPCP to the crop if there are SMs in the product or if SMs are produced *in situ*.

Operator and worker exposure is usually greater than residential and bystander exposure. Residential and bystander exposure can occur through drift and contact with residential applications (e.g., applications to turf). Typically, detailed quantitative assessments for of residential and bystander exposure are only required if a risk has been identified for workers and operators. Consumer exposure can occur via consumption of treated crops.

3.3 Environmental exposure

For quantitative risk assessments related to SMs, the environmental exposure of various groups of non-target organisms should be considered. These groups of non-target organisms include birds, mammals, aquatic and terrestrial arthropods, earthworms, soil micro-organisms, aquatic and terrestrial plants, and fish. Details on the non-target organisms are given in the data requirements of the respective jurisdictions.

3.4 Exposure schemes

This section illustrates the connection between the type of application and the most likely non-target groups for soil treatments and for foliar applications. The figures are based on product information that is available in the Manual of Biocontrol Agents (BCPC, 2014).

When using spray applications, there is a risk that surface water could be exposed to the MPCP. Theoretically, aquatic species could be exposed to SMs/toxins if these substances are present in the MPCP and/or the organism is able to produce such substances in the aquatic environment. There may also be exposure of aquatic organisms if SMs/toxins are produced in the soil environment and there is run-off and/or subsurface drainage from the treated area. In this context, aquatic exposure was considered to be unlikely. Also, production of SMs may be unlikely in an aquatic environment if the MPCA does not encounter the target organism in this environment. Run-off and leaching from the treated area might also require consideration. Such considerations, however, can be complicated by the *in situ* production of SMs.
With regard to consideration of possible exposure of aquatic non-target organisms, Figures 2 and 3 (BCPC, 2014) do not consider possible exposure of aquatic non-target organisms.

Soil treatment, soil drench and seed treatment
Figure 2 illustrates the agricultural use of an MPCP for soil treatments by spray, granules, drenches or seed treatments. In most cases, the MPCP is a granule or wettable powder. The most likely non-target groups are confined to the soil: micro-organisms, earthworms and arthropods. Mammals and birds can be exposed by eating treated seeds or granules, by eating contaminated food items due to spray drift, or by uptake (drinking) of spray droplets. For human exposure, workers can be exposed, as can consumers of edible plant roots/tubercules (e.g. carrot and potato) as some micro-organisms are rhizosphere competent (i.e., able to live in the rhizosphere).

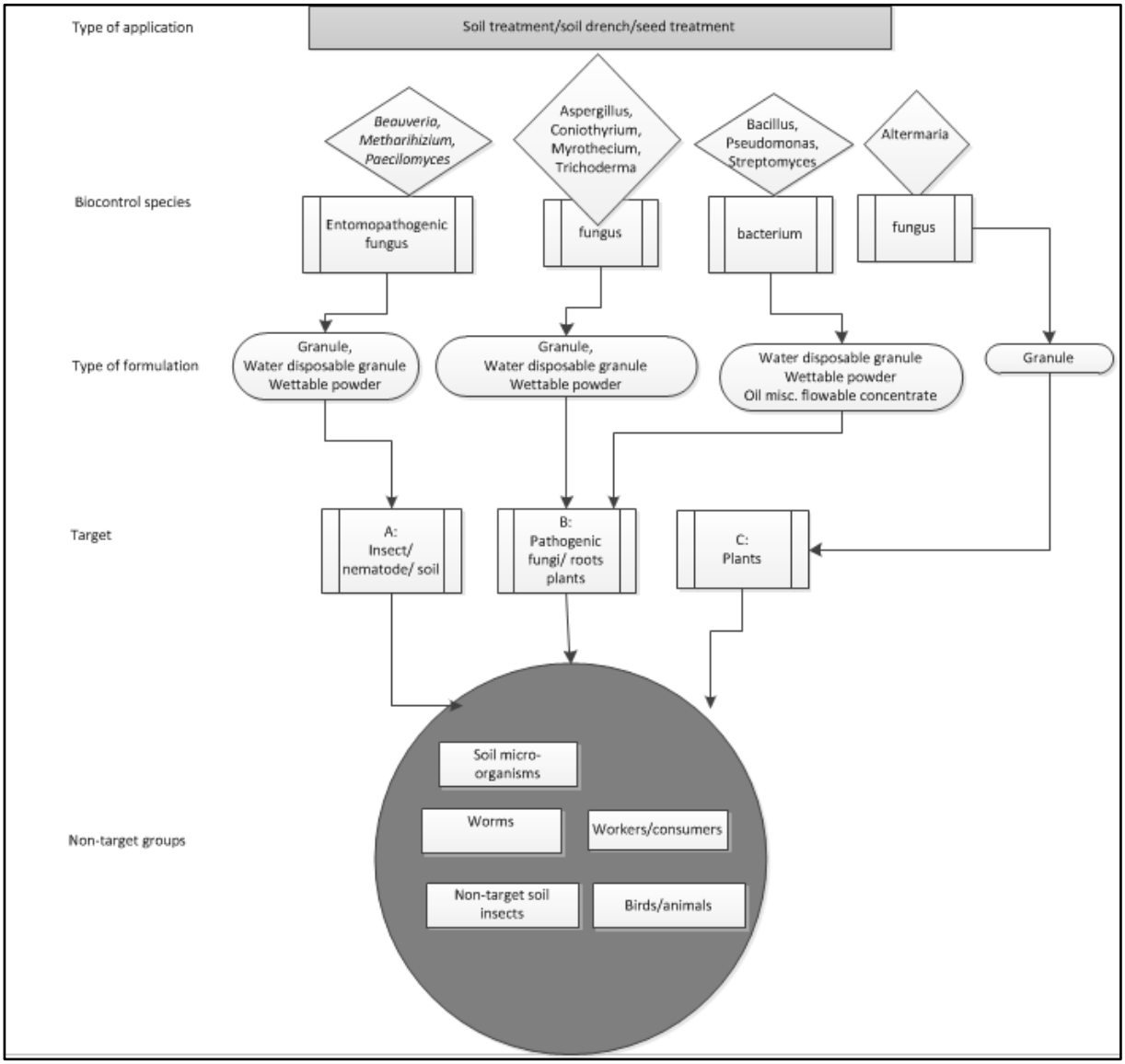

Figure 2. Most likely non-target groups for soil treatments/drenches and seed treatments, based on products present in the Manual of Biocontrol Agents (BCPC, 2014).

Foliar Spray

In Figure 3 the MPCP is a foliar spray, in most cases a water dispersible granule or wettable powder. The most likely terrestrial non-target groups are confined to the canopy/leaves/fruits: mammals, birds and arthropods. For aquatic non-target organisms, see above. Exposure to SMs through food/feed is possible as the MPCP is sprayed onto the canopy/leaves/fruits. Human exposure is possible for operators, workers, bystanders, residents and consumers.

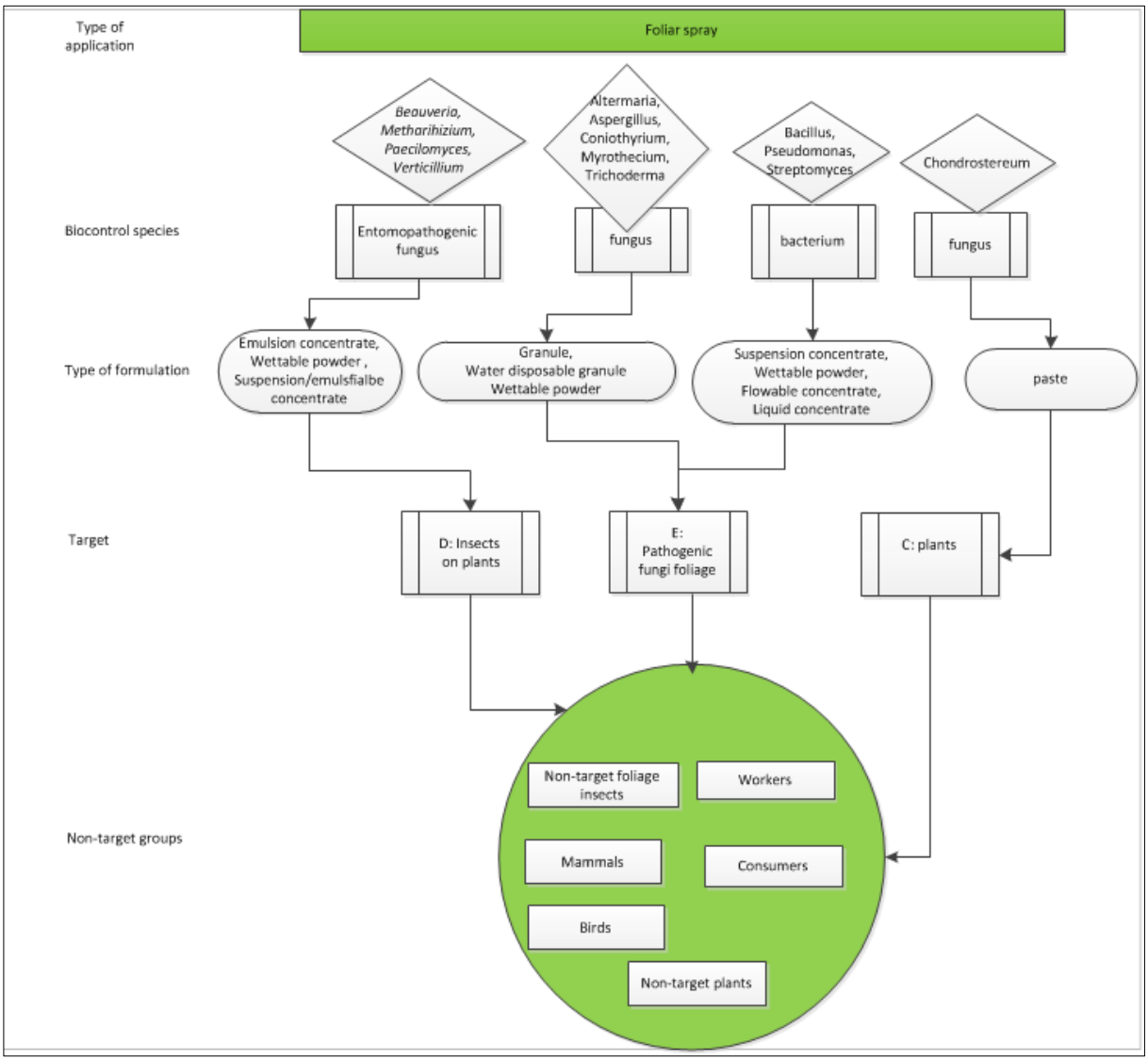

Figure 3. Most likely non-target groups when using foliar sprays, based on products present in the Manual of Biocontrol Agents (BCPC, 2014).

Summary

Table 2 summarises the most likely terrestrial non-target groups from figures 2 and 3. For aquatic non-target organisms, see above. Depending on the type of application, certain non-target groups are likely to be exposed. These non-target groups can be addressed first.

Table 2. Summary of non-target groups. √ indicates a likely non-target group and X indicates an unlikely non-target group.

Likely non- target groups	Type of agricultural application	
	Soil treatments/ drenches and seed treatments and granules	Foliar spray
Workers/Operators	√	√
Consumers	√	√
Bystanders/Residents	X	√
Mammals/ Birds	√	√
Aquatic organisms	X	√
Non-target soil arthropods	√	X
Non-target foliage arthropods	X	√
Earthworms	√	X
Soil micro-organisms	√	X
Non-target plants*	X	√

* In case of phytotoxins

4 IDENTIFICATION OF TOXIC SECONDARY METABOLITES

Background Document (Chapter 2) concludes that risk assessment of individual SMs may be necessary when they are present in relevant amounts but only when it is a relevant SM of concern.

4.1 Microbial pest control agent (MPCA)

Background Document (Chapter 1) concludes that SM production during the production process of the MPCA depends on both abiotic and biotic factors and may vary both temporally and spatially. Liquid fermentation has the potential to produce a vast range of SMs; the range depends on the media used and the growing conditions. Solid-state fermentation can also produce a range of SMs.

Whether or not any SMs that may have accumulated in the growing medium are still present in the MPCA depends on the way the MPCA is prepared. A well-chosen downstream processing method will limit or minimise the quantity of SMs present in the MPCA (Alabouvette et al., 2012). This can be achieved by selecting the downstream processing method which enables the most efficient separation of the viable propagules from the growing medium (Alabouvette et al., 2012).

Various information and data must be considered in the risk assessment methodology, including the presence of SMs in the MPCA and MPCP. If SMs are absent in the MPCA and MPCP or are only present in very low quantities, a detailed quantitative risk assessment for SMs may not be necessary.

In this document, a "low quantity" is defined as: 'Quantities lower than the lowest effect concentration for non-target organisms if available' (see Box 1 in Background Document, Chapter 4).

Possible analytical methods for the identification of SMs can be found in Section 9.

The identification of SMs should only be necessary in some cases:
- If exposure to humans or other non-target organism is anticipated. Exposure depends on the type of product (see Section 4.2) and the application method. Also refer to Section 3 on exposure; and
- If the hazard warrants a detailed quantitative risk assessment.

Examples regarding group 3:
- If a related strain is known to produce a toxic SM, genetic analyses such as gene probes, full genome sequencing (see Section 9.1.4) or RNASeq (see Section 9.1.5) could be requested for the MPCA under review. This seems a practical approach given the rationalisation of time and costs for the techniques used, and therefore the available data. Quantification of SM in liquid cultures (see Section 9.1.2), which is time consuming and very costly, is not favoured as it does not give information on production or accumulation under application conditions. Quantification of SMs in an *in vivo* situation is extremely complex as the production of SMs depends on the exact location of the MPCA and host interaction, the time and the environmental conditions (e.g., temperature). Quantification, however, may be required if no information is available on the genes implicated in the production of SM(s).
- If SMs are important in the mode(s) of action of the biocontrol species, the MPCP may contain high amounts of SMs due to enhanced production (e.g., this may be the case for some herbicidal biopesticides). It is probable that the producer will have invested in the identification of SMs early in the developmental program and so a full report on the identification of SMs could be available.

4.2 Microbial Pest Control Product (MPCP)

Applications on the plant canopy/leaves/fruits are mainly performed with suspendable concentrates, e.g., Water dispersible granule (WG), Suspension concentrate (SC), Oil miscible flowable concentrate (OF), Ultra-low volume suspension (ULV) and Oil dispersion (OD) formulations (see Background Document, Chapter 2).

Three important questions can be asked:

A. Are SMs being produced in the production medium?
Whether or not SMs will be contained in the MPCP depends on the type of manufacturing and downstream processes used to produce the MPCA/MPCA. Secondary metabolites can remain in the growing media. This information can be useful in evaluating occupational exposure during mixing and application. Liquid formulations might contain remnants of the growth medium whereas the granule formulations most likely only contain living fungal propagules/bacterial cells.

Production batches can be assayed for the presence of toxins. The requirement that SM concentrations are determined needs the development of analytical methods with high sensitivity for each SM that could occur.

If the answer to Question A is no → proceed to Question C
If the answer to Question A is yes → proceed to Question B

B. Are secondary metabolites removed from the production medium?
Products containing micro-organisms might not contain a significant amount of SMs as they may have been discarded during the production process, for instance as the result of the washing process. Some information is often provided already as part of quality control.

If the answer to Question B: yes → proceed to Question C
If the answer to Question B: no → provide data

C. Are metabolites being produced *in situ*?
Data may be required if significant quantities of SMs are produced *in situ*.

> ***Further Information*:** Background Document, Chapter 3 (Biology in relation to secondary metabolite production) shows that the expression and secretion of SMs is controlled by various genetic and cellular regulatory mechanisms.

Conclusions on the different types of MPCAs are given in Box 1.

Box 1 Conclusions on important species of MPCAs (see Chapter 3 in Background Document for more detailed information)

Fungi

Entomopathogenic fungi

- SMs of entomopathogens are not produced on the surface of the insect cuticle.
- Most SM production takes place in the insect host after its death (after proliferation of the fungus in the hemolymph).
- Smaller amounts of SMs can be released in the insect during penetration.
- Formation of SMs is controlled by several genetic and cellular regulatory mechanisms. Precursors need to be present in a pathway to initiate the production of SMs. Also, there is evidence that genes are differently expressed in the different infection stages of, for example, *B. bassiana* (aerial conidia, *in vitro* blastospores and submerged conidia).

Trichoderma

- Although a multitude of SMs are produced in *Trichoderma* spp. only a few seem to be produced in strains used for biocontrol. Production of the SMs, trichodermin and gliotoxin needs consideration in any risk assessment. SM production is a tightly regulated process in which SMs are produced in reaction to molecules that are released by stressed hyphae of the parasitised pathogen. SM quantities are sufficiently high to be toxic for the target pathogen. These quantities are not likely to affect non-target organisms.

Bacteria

Bacillus

- *Bacillus* spp. live in close association with soil (pathogenic) fungi, forming biofilms. Secondary metabolite production is regulated by quorum sensing and the mode of action of the SMs produced by *Bacillus* spp. (fengycin, iturin and surfactin) is limited to when there is penetration of cell membranes of the pathogen.

Pseudomonas

- *Pseudomonas* spp. live in close association with soil (pathogenic) fungi, forming biofilms. Secondary metabolite production is regulated by quorum sensing and the production of SMs by *Pseudomonas* spp. appears to provide a competitive advantage in the colonisation of the rhizosphere.

Burkholderia

- The biocontrol effects of *Burkholderia* spp. involve diverse mechanisms of action including rhizosphere competence, determining their population density on root surface, secretion of allelochemicals, including antibiotics and siderophores, competition for nutrients, and induced systemic resistance. The production of SMs is regulated by quorum sensing. As *Burkholderia* biocontrol species are related to *Burkholderia* species that are known human pathogens, their registration is complex and so far is limited to uses with no human exposure.

Serratia

- Only some species, such as *Serratia marcescens, S. plymuthica*, and *S. rubidaea* produce the SM prodigiosin, which has antimicrobial activity against fungi and bacteria. *Serratia* spp. with insect biopotency are not known to produce SMs.

Streptomyces

- *Streptomyces* spp. are known for the wide range of SMs they can produce, possibly explained by the fact that they are non-motile. The biocontrol agent *Streptomyces melanosporofaciens* strain EF-76 produces the SM geldanamycin, an antimicrobial which displays antagonistic activity towards several gram-positive bacteria and fungi.

5 PERSISTENCE IN THE ENVIRONMENT

The issue of persistence in the environment is an important component in the risk assessment. Stability is, for example, an important criterion in the EU (Commission, 2009b) (see Box 2).

Box 2. The EU regulation (Commission, 2013) setting out the data requirements for active substances, in accordance with Regulation 1107/2009 (Commission, 2009b), identifies stability as an important criterion (point 7, fate and behaviour in the environment).

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

- The relevant SM is stable outside the micro-organism; and
- a toxic effect of the relevant SM is independent of the presence of the micro-organism; and
- the relevant SM is expected to occur in the environment in concentrations considerably higher than under natural conditions.

Background Document, Chapter 5 on degradation in the environment shows limited data on the persistence of SMs.

Further Information: See Background Document, Chapter 5 on degradation in the environment

From the available data it was concluded that SMs of entomopathogens:
- quickly degrade in submerged media, hemolymph of infected living arthropods and hemolymph post mortem;
- in crops, they were not present in the plant material or at levels lower than the detection limit;
- are only stable when preserved under specific conditions in a vial. This contained situation is not relevant for risk assessment.
- *In situ*, degradation is caused by temperature and UV light, so SMs of entomopathogens are not considered to be stable when exposed to environmental conditions.

For *Trichoderma* spp. little information was found:
- Information is available on the stability of gliotoxin produced by *Trichoderma viride.* Gliotoxin stability was influenced by pH. Gliotoxin was below the limit of detection in non-sterilised soil.
- Trichothecene mycotoxin compounds (some are produced by *Trichoderma* spp.) are stable when exposed to air, light, or both but only when maintained as either crystalline powders or liquid solutions.

For bacteria very little information was found:
- Pyrrolnitrin produced by *Pseudomonas* spp. is highly unstable in sunlight.
- Information on geldanamycin produced by *Streptomyces* spp. could not be verified.
- Some data are available on the stability of iturins produced by several *Bacillus* sp. (*Bacillus subtilis*, *Bacillus amyloliquefaciens*).

Difficulties with stability and persistence

The stability criteria for SMs are not defined by a DT50/DT90 value. Risk assessments should consider both stability and persistence. Stability refers to the time where the SM maintains its activity whereas persistence is the net result of production and degradation. Environmental persistence of SMs can be difficult to assess since metabolically active MPCAs may produce SMs after application. Production of SMs is triggered upon sensing certain molecules released by the host/pathogen. As long as this trigger is present SMs can be produced and used/degraded at the same time.

In practice, testing persistence is difficult. Persistence testing is likely to run into the same issues as it does for botanicals, i.e., the SMs may already be common in the environment. Persistence testing for SMs only expressed *in situ* would have to account for these natural sources through the use of complex radiolabelling studies. In such cases, it may be easier to monitor the persistence of *in situ* SMs indirectly by measuring their levels in the environment and determining the period of time until their levels return to naturally occurring background levels.

It can be concluded that degradation of SMs is a challenging aspect of the risk assessment. Whether testing for stability/degradation of SMs ought to be a data requirement should be further discussed. In some cases, modelling could be employed to estimate environmental stability.

6 RISK EVALUATION

A detailed quantitative risk evaluation may be required if the steps in Sections 2–5 have concluded that there are strong indications that SMs pose a risk.

6.1 Toxicity tests

Regulatory agencies generally require acute studies to address human health and safety concerns. Depending on the country, industry may need to perform a 6-pack test for human health. The 6-pack tests (see below) are usually carried out using the MPCA and where the co-formulants are not inert or affect the toxicity, the MPCP is also tested.

Guideline series 870 contains six acute toxicity studies (the 6-pack test):
- Acute oral toxicity (OPPTS 870.1100)
- Acute dermal toxicity (OPPTS 870.1200)
- Acute inhalation toxicity (OPPTS 870.1300)
- Acute eye toxicity (OPPTS 870.2400)
- Acute dermal irritation (OPPTS 870.2500)
- Dermal sensitisation (OPPTS 870.2600) (No sensitisation testing is asked by EU, US EPA and Canada)

The use of these tests in SM risk assessments only makes sense when SMs are present in the growing media. These tests can be useful for testing herbicidal products as the SM production may be enhanced in some of them.

In Canada, studies are divided among MPCA and MPCP(s) (for more details on requirements for MPCP, see Regulatory Directive DIR2001-02[5]).

Common practice for the US EPA is that only SMs with established food safety testing endpoints or efficacy-related claims are independently assayed.

US EPA uses:
- Guideline series 885 that contains test guidelines on animal models to test for infectivity of the MPCA.
- Guideline series 870 that contains test guidelines on animal models to test for any toxic SMs in the manufacturing use product and to separately assess the toxicity of any inert ingredients added during manufacture of the MPCP.

In practice the specific test material used should be completely characterised, if possible, prior to testing so that SMs concerns can be adequately assessed in these studies. While this evaluation can be difficult to do a priori, it is highly recommended because repeating studies is costly, time consuming and animal intensive.

Evaluation of the test material should take into account:
- The form of the microbe;
- The fermentation medium used for growth;
- The length of time the microbe is fermented before extracting.

These standard tests are all acute tests. Acute tests are generally adequate for tier I reviews. However, higher tiered studies (e.g., chronic) may be required in cases where a quantitative risk assessment is required.

[5] http://www.hc-sc.gc.ca/cps-spc/alt_formats/pacrb-dgapcr/pdf/pubs/pest/pol-guide/dir/dir2001-02-eng.pdf

In environmental risk assessments, similar issues are encountered for MPCAs with SM(s). The use of acute tests in SM environmental risk assessments only makes sense when SMs are present in the growing media. Therefore, a tiered system involving acute, mesocosm, and field testing is warranted for micro-organisms. Higher tiered studies (e.g., mesocosm or field studies) may be required when non-target organisms are exposed to *in situ* production of SMs (e.g., after spray applications with *Bacillus, Trichoderma* or *Pseudomonas* spp.).

Conclusions

a. SM evaluations can be performed using toxicity test results with the MPCA or the MPCP in cases where the MPCP contains SMs and/or SM production is intentionally enhanced.
b. In other products where SMs are only produced following contact with the target organism, it is not particularly useful to evaluate the toxicity of the SMs since the SMs are not yet produced. Laboratory tests for these products may be useful. Testing with purified samples may be required for these products. Mesocosm and field tests may also be more appropriate for environmental SM assessments.
c. Distinction between the type of products has to be made:
    - In foliar applications, the MPCA may have been developed to enhance the presence of SMs;
    - In seed treatments, the SMs may not be produced at relevant concentrations *in situ*, and so toxicity testing is not useful as exposure to non-target organisms, except for micro-organisms, is absent.
    - For drench/soil applications it depends on the type of organism and it should be evaluated on a case-by-case basis.

6.2 Human dietary risks

A detailed quantitative dietary risk assessment for SMs should only be required if:
a. The basic information set as specified in Section 2 of this document leads to concerns about the human toxicity of SMs in the product or produced by the biocontrol agent in contact with the pathogen. If the MPCA is present on the list of fungal genera producing mycotoxins provided in Table 3 of Section 8.2.1, the SM should be evaluated for its risks in relation to human exposure as mycotoxins can be carcinogenic, mutagenic or reproductively toxic substances.
b. Species closely related to human pathogens should also be evaluated as some may produce toxic SMs that could explain their pathogenicity (see Section 8.2.2). For example, antagonistic species of the genera *Burkholderia, Enterobacter, Serratia* and *Staphylococcus* that are root-associated bacteria can interact with plants and humans.

If there is reason to believe that toxic SMs are formed go to Step 1.

Step 1: Determine gene expression

If the MPCA is closely related to a pathogenic species that produces the mycotoxins listed in Table 3, Section 8.2.1, the applicant should provide information showing that:
- The gene or gene cluster expressing this mycotoxin is not present in the MPCA. This information could consist of gene probing or full genome sequencing.
- If the gene is present but the MPCA is not producing the mycotoxin, the applicant should give an explanation for the lack of production.

If production of the toxic SM has been demonstrated go to Step 2.

Step 2: Determine exposure

Determine whether human exposure is likely when the formulation (granule, liquid) and type of application (seed dressing, foliage spray, soil treatment) are taken into consideration (see Section 3). If there is reason to believe that SMs do not pose occupational, bystander, or residential risks or risks from food, risk assessment can be stopped, otherwise, go to Step 3.

Foliar applications may lead to the greatest concern since plants could absorb these toxic SMs. Soil and seed treatments should also be considered but these use patterns rarely lead to concerns with dietary exposure. See OECD issue paper on microbial metabolite residues in treated food crops (Rochon and Belliveau, 2008).

Step 3: Demonstrate concentrations *in situ*

In this situation, some information on quantities or relative quantities of SMs may be required. In some cases, a simple plate assay (see Section 9.1.1) can be conducted on a known medium that supports the production of SM(s) to show that the (myco)toxin is not produced or is produced in much lower concentrations than in the closely related mycotoxin producer. A plate inoculated with this closely related species should be included in the test. However, a suitable medium for a simple plate assay may need to be developed first (see Section 9.1.1). If this is not sufficient or is not possible, the SM can be extracted from plant material and concentrations can be determined with LC/MS or GC-MS, HPLC or NMR (see Sections 9.1.2 and 9.1.3).

***Further Information*:** see Background Document, Chapter 5 on degradation in the environment.

It is stressed by Sundh (2012) that, although it is possible to detect and quantify SMs in the culture filtrate, it is more difficult and expensive to determine whether these molecules are produced *in situ* after application to the plant part to be protected. Moreover, the same species does not always produce the same SMs in the environment as SM production can depend on various factors, including the strain of the plant and the target pathogen (Marra et al., 2006).

The efficacy of the MPCP can also be considered in the assessment of SMs if the SM is relevant to the MPCA's mode of action. The SMs in MPCPs that rapidly lose efficacy and require frequent re-applications are likely to be rapidly inactivated following application.

Step 4: Determine toxicity

Toxicity data may not be available.

Maximum levels (MLs) for contaminants in foodstuffs can be used to determine whether the concentration of the mycotoxin in food is acceptable. Maximum level approach: A ML is the maximum concentration of a substance that is tolerable in a particular food.

For example, the EU Commission Regulation (EC) No 1881/2006 [6] sets maximum levels for contaminants in food, including for some mycotoxins. These levels are derived from monitoring studies and are based on feasibility: Lower MLs would not be practical, as a large part of the crop would have to be destroyed. The ALARA principle set out in EU Council Regulation 315/93/EEC[7] that lays down EU Community procedures for contaminants in food says: "contaminant levels shall be kept as low as can reasonably be achieved following recommended good working practices".

[6] http://ec.europa.eu/food/food/chemicalsafety/contaminants/legisl_en.htm

[7] http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:01993R0315-20090807

Some MLs are set for different foods (e.g. nuts, cereals, wheat, maize, rice milk, spices, coffee, wine, juices, bread, baby food, etc.) according to the ALARA principle. The range of these MLs is given in the list below (MLs for baby food are not included in the list below as they are always extremely low):

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

This list only serves as an example. Many countries have their own regulations for mycotoxins in food. A list with maximum allowable levels in food for individual commodities (e.g. cereals, coffee, dried fruit, milk) for the European Union, USA, China, Singapore, and Brazil can be found at the mycotoxins info site.

Many countries monitor food safety by measuring quantities of mycotoxins in feed and food. If quantities surpass the ML the whole lot is destroyed.

Tolerable Daily Intake (TDI) levels are calculated using no observed adverse effect Levels (NOAELs) or bench mark doses (BMDs) and a margin of exposure (generally 10,000 for genotoxic carcinogenic substances; 100 for other substances). In practice, the TDI levels are often surpassed for contaminants such as mycotoxins.

The threshold of toxicological concern approach.

Munro et al. (2008) state: "The TTC approach is based on the concept that reasonable assurance of safety can be given, even in the absence of chemical-specific toxicity data, providing that the intake is sufficiently low, i.e. that an exposure level can be defined below which there is no significant risk to human health. The approach is based on the knowledge gained from the general toxicity database that has been developed in the past 50–60 years. The TTC approach is a form of risk characterisation that balances uncertainties inherent in extrapolation of these data to an unstudied substance against the predicted or known low level of exposure".

TTC is related to human exposure dependent on the toxic potential and characteristics of the substance. For instance, for genotoxic carcinogens it is 1.5 µg/person/day or 25 ng/kg bw/day. Based on data on consumption, it is possible to calculate the tolerable quantities in food without surpassing the TTC. The TTC approach cannot be used for some very carcinogenic substances, e.g. aflatoxins.

The TTC approach is generally accepted in the risk assessment of naturally occurring substances in food and feed. This approach can be used as an example for the risk assessment of SMs produced by MPCAs in food and feed. If possible, concentrations of SMs can be compared with tolerable quantities in food/feed.

Suggestions for risk assessment

In the first instance "we must recall that the phytopathogenic micro-organisms that we are aiming to control also produce SMs and toxins, which are tolerated at low levels in feed and food. We should not be more restrictive on a biological control agent than we are for the pathogenic micro-organisms it controls" (OECD, 2014).

As a start, the production of mycotoxins produced by pathogens can be compared with production by MPCAs. For instance, Background Document, Chapter 1 shows that beauvericin is produced in high quantities (up to 3400 mg/kg) by *Fusarium* species while quantities produced by *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*) in arthropods are 1.6 mg/kg. The use of *Isaria fumosorosea* would not likely increase the natural background levels of beauvericin on plants.

In some cases, the ML values for the mycotoxin can be compared with quantities produced by the MPCA. A practical problem here is that MLs are only available for a very few very toxic mycotoxins. In Section 8.2.1, a list of fungal genera producing mycotoxins is presented.

An overview of relative toxicity of mycotoxins is not available in the regulation of contaminants. It seems impossible to develop a single standardised test for all pathogens, as pathogens grow under different conditions with different hosts and produce mycotoxins under different conditions.

A list of mycotoxins that have been reviewed for their presence in food/feed is provided in the Reference Section 10.2.

It should, however, be kept in mind that as part of the project Risk Assessment of Fungal Biocontrol Agents (RAFBCA, see Background Document, Chapter 5), field experiments showed that the main SMs that were analysed were not detected in any plant material.

In this project, several field (e.g. potato, maize and radish) and glasshouse trials (e.g. tomato, cucumber) were conducted under commercial conditions. Studies monitored major metabolites in the environment to see if they entered the food chain. Fungal applications were at 5x, 10x and 100x the recommended rate. Harvested plant material was analysed for some SMs:

- Oosporein: *Beauveria brongniartii* for cockchafer control in field crops (e.g. potato, barley, maize, carrot, lettuce).
- Elsinochrome A: *Stagonospora convolvuli* for bindweed control in strawberries.
- Gliotoxin: *Clonostachys catenulatum* (formerly *Gliocladium*) and *C. rosea* for control of seedling blight and root rot (*Pythium, Rhizoctonia* spp.*)* in lettuce and cucumber.
- Other MPCA/crop combinations were investigated with *Trichoderma harzianum, Metarhizium anisopliae* and *Verticillium lecanii*.

These trials found no SMs in the sampled plants (personal communication T.M. Butt). These findings suggest that the tested biocontrol species do not live endophytically or produce SMs endophytically below the limit of detection.

> ***Further Information***: See Background Document 5: RAFBCA field experiments.
>
> ***Information Gap*****:** Secondary metabolites produced on crops endophytically. The literature on endophytes has not been thoroughly explored (See Background Document, Chapter 3, paragraph 3.3.5 for a short review).

A short search (See Background Document, Chapter 3, paragraph 3.3.5) showed that a vast number of wild and crop plant species have been shown to harbour endophytes. Among these are biocontrol species such as *Beauveria, Acremonium, Cladosporium, Clonostachys*, and *Isaria* (formerly *Paecilomyces* (Vega et al., 2008)). Currently endophytes are of great research interest but they are not new in the environment or in crops – so there is already existing exposure.

Bacteria, such as *Pseudomonas* and *Bacillus* spp., are also well known endophytes. It is, however, not clear whether biocontrol species also share these capacities or that these are limited to specific species.

If some fungal entomopathogenic species occur as endophytes and exert their action against arthropods via the production of SMs, then they would be analogous to the mode of action reported for known entomopathogenic fungi. This raises the issue of whether inoculation of agronomically important plants with fungal entomopathogens might create a problem by producing SMs that could potentially enter the food chain (Vega et al., 2008). This area is not well studied and would need further studies. Endophytic association with several species of *Beauveria* and *Metarhizium* were shown to increase overall plant productivity (Behie and Bidochka, 2014), probably by translocation of nitrogen. To date, no convincing proof has been given that entomopathogenic fungi complete their life cycle inside the living plant and that endophytically produced SMs cause death of arthropods (Gurulingappa et al., 2011).

6.3 Occupational risks

Industrial workers and operators may be exposed to SMs during the manufacturing process and during mixing and application of the product, respectively. This exposure is expected to be primarily via dermal or inhalation routes, and to a lesser extent via ocular and oral routes. In principle, SMs should be assessed for human exposure when they are formed during production or are present in the MPCA or MPCP.

If the MPCA under consideration is related to one of the species mentioned in Section 8.2.1, it is advised that those mycotoxins that are likely to be produced by this MPCA are listed and the literature for dermal and inhalation toxicity of those mycotoxins is searched.

If there is high dermal or inhalation toxicity, a quantitative risk assessment should be performed. For such risk assessments, actual concentrations in the formulation are mandatory.

Occupational exposure assessments

For SMs that are present in MPCPs, the occupational exposure assessment can be conducted using existing approaches for conventional chemical pesticides, i.e., quantitatively evaluate occupational risk for various exposure routes using both exposure estimates and toxicity data.

Trichoderma spp. example
Trichothecenes produced by *Trichoderma* spp. should be taken into consideration (see Section 8.2.1). Trichothecenes are membrane-damaging toxins and can cause skin lesions and systemic illness without being inhaled and absorbed through the respiratory system.

Most products of *Trichoderma* spp. are wettable powders (BCPC, 2014) and are used as soil treatments and foliar sprays. *Trichoderma* biocontrol species may contain some mycotoxins such as trichothecenes (trichodermin and harzianum A) and gliotoxin (See Background Document, Chapter 1, Table 10). However, most *Trichoderma* biocontrol strains are not able to produce trichothecenes. Their toxicity is probably very low compared to other very toxic trichothecenes such as T-2 toxin, HT-2 toxin, diacetoxy-scripenol, nivalenol, deoxynivalenol, verrucarin A, roridin A and satratoxin H. Background Document, Chapter 4 only gives reliable toxicity data for gliotoxin (16 h LC50 for *A. salina* of 3.5 µg/mL (Table 40), 36 h LC50 for *A. salina* of 6.66 µg/mL (Table 42), 36 h LC50 for *D. magna* of 0.16 µg/mL (Table 43). These toxicity data are not comparable with the toxicity endpoints for undefined trichothecenes which were summarised in Background Document, Chapter 4, paragraph 4.3.1 (data provided at the IAQM website). These data showed that these trichothecenes were quite toxic via most exposure routes.

Fusarium spp. example
Fusarium oxysporum can be used as an herbicide. *Fusarium* species produce a wide range of mycotoxins (see Section 8.2.1). This list does not provide information on dermal and inhalation toxicity. Such endpoints

are necessary to assess whether aerosols of biocontrol species of *Fusarium* might cause inhalation toxicity or if workers that handle the product during processing and application will experience dermal toxicity.

6.4 Risks for non-target groups

Regulatory authorities typically evaluate the risk to various groups of non-target organisms, including arthropods (and honey bees), earthworms, soil micro-organisms, aquatic organisms, mammals and birds. These assessments are conducted on a hazard basis mainly using results of ecotoxicology studies on representative non-target species and gross estimates of environmental exposure to the MPCA and its associated SMs of concern.

The use of standard ecotoxicology studies in the qualitative risk assessment of SMs can be complicated for several reasons:

a. Ecotoxicology studies normally use the MPCA. However, the MPCA may not reflect actual concentrations of SMs that are produced *in situ*. The MPCA may contain SMs that are produced during fermentation of the microorganism. These SMs may not be produced *in situ* or at much lower concentrations (see calculation in Box 3).
b. It is not possible to prepare a standardised test medium that is based on *in situ* production (leaves or rhizosphere soil with the MPCA producing SMs). Although it is possible in theory to determine the quantities of SMs in the rhizosphere or canopy, analytical methods need to be developed or adapted for each situation. The presence of large quantities of organic matter can interfere with the recovery of SM, there is likely to be a mixture of substances and analytical methods need to be adapted. It is particularly difficult to standardise the extraction of SMs from the rhizosphere or canopy as the SM production will vary during the development of the colony and is influenced by many other factors and the data could not be reproduced. The resulting data are therefore less reliable. For such SMs, it may be preferable to conduct mesocosm tests or field studies to evaluate potential risks to non-target organisms.

The simple calculation shown in Box 3 could be used to show acceptable concentrations of SM in the MPCP of an entomopathogenic fungus.

This calculation is only possible in cases where data on the concentrations of SM in target arthropods are available, as is the case for *Metarhizium* and *Beauveria* secies which have been intensely studied. For other entomopathogenic fungi much less information is available.

Box 3. An example calculation of the acceptable concentration of metabolites in an entomopathogenic fungus based MPCP.

For this calculation, the lowest LC50 value for a sensitive target insect *Plutella xylostella* is used, i.e. of 17 µg destruxin E/mL (see Table 35 in Background Document 4).

In the field

In order to obtain these effects, the sprayed MPCA should contain at least 17 mg destruxin E/L. For this calculation, it is assumed that the spray solution is obtained by mixing 1 L of product in 1000 L of water.

In order to have a concentration of 17 mg destruxin E/L, the liquid formulation itself should contain 17,000 mg destruxin E/L. This can be compared with the concentrations of destruxin E as indicated by the applicant. Table 25 in Background Document, Chapter 1 gives a value of 97 ± 146 mg destruxin E/L in the production medium. This calculation shows that for the most sensitive target insect, contact or feeding mortalities are not expected to occur as a result of the presence of SMs in field applications when the MPCP contains 97 mg of destruxin E/L. In this example the levels of destruxin E in the application solution of the field application is 175 times lower than the lowest LC50 value for the most sensitive target insect.

Off-field

For the off-field area, decreasing exposure/hectarage should be considered as only a small percentage of the application rate is transported out of field via drift. If the off-field area is vegetated the vegetative structure should lead to a further dilution of drifted material (Barrett et al., 1994)

Remarks
- NTOs might be affected in a similar way if they are taxonomically closely related to the target species, they could, however, also be more sensitive than the target species
- Concentrations below the LC50 but above an NOEC can also adversely affect NTOs
- An assessment factor is usually considered in the risk assessment
- Some EPFs such as *Metarhizium* sp. are known to produce many types of destruxins. In this case, additive or even synergistic effects could be taken into account.

6.4.1 Example: risk of species of *Pseudomonas* and *Bacillus* to earthworms, soil arthropods, soil micro-organisms

Species of *Pseudomonas* and *Bacillus* applied as soil treatments, drenches or seed preparations, are only expected to form biofilms on the roots of plants. Exposure of non-target groups is therefore limited to earthworms, soil arthropods and soil micro-organisms. Since no test results with these non-target organisms were found in the published scientific literature, it can be assumed that there were no incentives to study the effects of SMs on these organisms.

Bacillus subtilis may produce the lipopeptides fengicin, surfactin and iturin A. These surfactants have a hydrophilic ring of seven amino acids and a long, hydrophobic hydrocarbon tail (See Background Document 3, paragraph 3.3.4.1). The hydrocarbon tail penetrates pathogen cell membranes, while the amino acid end stays in the soil solution. This action creates openings in cell membranes, inhibiting the growth of many pathogens. The same is true for the lipopeptides that are formed by *Pseudomonas* spp. (See Background Document, Chapter 3, paragraph 3.3.4.2).

Lipoproteins damage the membranes of competing (pathogenic) micro-organisms, thus potential effects on non-target earthworms and soil arthropods can be excluded. It is unlikely that these compounds would significantly affect environmentally or economically important species in soil since these micro-organisms are common, naturally occurring components of soil. Effects on micro-organisms other than pathogenic micro-organisms are expected to be temporary as SM production is under control of quorum sensing and also depends on the growth phase of the *Pseudomonas* and *Bacillus* species. (See Background Document, Chapter 3, paragraph 3.3.4.2).

6.4.2 Example: risk of entomopathogens to insectivorous mammals and birds

If feeding experiments are available, the effect levels can be compared with the levels in feed.

Example1. Consumption of infected arthropods
An example of the high theoretical risk for birds derives from Strasser *et al.* (2000b) and the EU DAR for *Beauveria bassiana* GHA (see Box 4).

Parameters used are:
a) concentration of oosporein in one larva (*Melolontha melolontha*) = 200 µg and
b) LD50 (chicken) = 6 mg/kg

Assuming an assessment factor of 10, the Toxicity Exposure Ratio (TER) for short-term toxicity should not be lower than 10. In this specific example, a risk was demonstrated when birds ate one larva (*Melolontha melolontha*).

Box 4. Risk assessment for insectivorous birds

Assuming an insectivorous bird having a body weight of 0.01 kg would eat only on larva:

$$TER = \frac{LD^{50}}{amount\ of\ oosporein\ in\ one\ larva}$$

$$TER = \frac{6x10^{-5} g\ oosporein}{0.2\ x\ 10^{-3} g\ oosporein}$$

$$\boldsymbol{TER = 0.30}$$

There are a few arguments that can be used to refine the SM risk assessment:

- Although experiments acknowledged that infected arthropods were readily eaten by the birds and reptiles (see Background Document, Chapter 4), it should be noted that most of these experiments were performed under no-choice conditions. Whether birds and reptiles would choose infected arthropods if they had a choice between dead and living arthropods has not been found in the literature. Predatory arthropods probably respond to movements and a dead insect may therefore not be noticed. Also predators may avoid non-fit hosts. However, alive but infested larvae were preferred compared to non-infested larvae.
- SM will be metabolised rapidly in the hemolymph. The DT50 of SMs is only a few days (see Background Document, Chapter 5).
- Many arthropods encountered on the foliage will be smaller (e.g. thrips are about 1 mm long) than the larva used in this calculation (*Melolontha melolontha)* so consumption per insect will be lower and many smaller diseased arthropods must be eaten to achieve the same amount of SMs.

Example 2. Consumption: mycosed granules
- Toxicity data: levels of 200 µg oosporein/g produced by *Chaetomium trilaterale* (Background Document, Chapter 4, Table 2) caused effects in broiler chickens (e.g. mucosal necrosis, proventicular, hepatic and renal inflammation). No effect at100 µg/g feed. No LD50 is available.
- Exposure data: only 3.27 µg oosporein/g grain is produced by *B. brongniartii (*Table 23 in Background Document, Chapter 1).

A TER calculation cannot be made as an LD50 value is not available. However, using the available toxicity data it can be calculated that one chicken can consume 30.6 g of mycosed grain (100/3.27) without any apparent effects. A consumption of 61.2 g would be expected to cause mucosal necrosis, proventicular, hepatic and renal inflammation.

7 RISK REFINEMENT

Risk refinement should be performed when a risk has been determined. Risk refinement can only be done on a case by case basis and no recommendations are given in this working document.

8 ADDITIONAL RESOURCES

8.1 Databases and books on secondary metabolites

The Norine database
See Section 2.4.

The Accelrys Databases on metabolites and their toxicity
These databases on metabolites and on toxicity are linked but are not freely accessible. Without subscription, it is only possible to obtain information on the structure, molecular formula, etc. The metabolites database has entries for *Beauveria* and *Pseudomonas*, but not for *Metarhizium, Lecanicillium* and *Trichoderma*.

(see for FURTHER INFORMATION: Accelrys bioactivity presentation).
The following licenses would be necessary:
1x 2086XRT - Isentris Runtime for Content US$890/year
1x 1563X - Metabolite (In-House) US$2,240/year
1x Services (onetime)* US$4,000$

Handbook of secondary fungal metabolites (Cole and Schweikert, 2003)

8.2 Lists of secondary metabolites

8.2.1 List of known mycotoxin producers and their mycotoxins

The following table lists mycotoxins ordered by genera. Some mycotoxins are also produced in more than one genera. The genera mentioned 3 hold species that are known pathogens. Some of the genera also hold species that are used as biocontrol agents.

Table 3. List fungal genera producing mycotoxins (modified from (Lillard-Roberts) year unknown).

Genera	mycotoxin	Species
Acremonium	Crotocin	*Acremonium crotocinigenum*
Alternaria	Altenuic acid	*Alternaria alternate*
	Alternariol	*Alternaria alternate*
Aspergillus	Aflatoxin	*Aspergillus flavus, A. parasiticus*
	Aflatrem	*Aspergillus flavus*
	Austamide	*Aspergillus ustus*
	Austdiol	*Aspergillus ustus*
	Austocystin	*Aspergillus ustus*
	Brevianamide	*Aspergillus ustus*
	Citreoviridin	*Aspergillus terreus, Penicillium citreoviride*
	Citrinin	*Aspergillus carneus, A. terreus, Penicillium citrinum, P.hirsutum, P. verrucosum*
	Cyclopiazonic acid	*Aspergillus versicolor*
	Cytochalasin E	*Aspergillus clavatus*
	Destruxin B	*Aspergillus ochraceus*
	Fumagilin	*Aspergillus fumigatus*
	Gliotoxin	*Alternaria, Aspergillus fumigatus, Penicillium, Trichoderma virens*
	Malformin	*Aspergillus niger*
	Maltoryzine	*Aspergillus spp.*
	Ochratoxin	*Aspergillus ochraceus, Penicillium viridictum*

Genera	mycotoxin	Species
	Oxalic acid	*Aspergillus niger*
	Patulin	*Aspergillus clavatus, Penicillium expansum, P.roquefortii, P. claviforme, P. griseofulvum, Botrytis*
	Penicillic acid	*Aspergillus ochraceus*
	Sterigmatocystin	*Aspergillus flavus, A. nidulans, A. versicolor, Penicillium rugulosum*
	Tryptoquivalene	*Aspergillus clavatus*
	Verruculogen	*Aspergillus fumigatus, Stachybotrys chartarum*
	Viomellein	*Aspergillus spp. , Penicillium aurantiogriseum, P. crustosum, P. viridicatum*
	Viriditoxin	*Aspergillus fumigatus*
Botrytis	Patulin	*Botrytis*
Chaetomium	Chaetoglobosin	*Chaetomium globosum*
	Cochliodinol	*Chaetomium cochliodes*
Cylindrocarpon	Roridin E	*Cylindrocarpon spp.*
Dendrodochium	Roridin E	*Dendrodochium spp.*
	Verrucarin	*Dendrodochium spp.*
Eurotium	Xanthocillin	*Eurotium chevalieri*
Fusarium	1,4-Ipomeadiol	*Fusarium*
	1-Ipomeanol	*Fusarium*
	3-Acetyldeoxynivalenol	*Fusarium*
	4- or 15-Acetylscirpentriol	*Fusarium*
	4-Acetoxyscirpenediol	*Fusarium*
	4-lpomeanol	*Fusarium*
	7"-Hydroxydiacetoxyscirpenol	*Fusarium*
	8-Acetylneosolaniol	*Fusarium*
	Acetoxyscirpenediol	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Acetyl T-2 toxin	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Acetyldeoxynivalenol	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Acetylneosolaniol	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Anguidin	*Fusarium*
	Avenacein +1	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Beauvericin +2	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Butenolide	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Calonectrin	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Deacetylcalonectrin	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	deoxynivalenol (DON) or vomitoxin	*Fusarium*
	Deoxynivalenol deacetate	*Fusarium moniliforme, and F. nivale*
	Deoxynivalenol monoacetate	*Fusarium moniliforme, F. culmorum, F. avenaceum, F.roseum, and F. nivale*
	Diacetoxyscerpentriol	*Fusarium*
	Diacetoxyscirpendiol	*Fusarium*
	Diacetoxyscirpenol	*Fusarium moniliforme, F. equiseti, F. oxysporum*
	Diacetyldeoxynivalenol	*Fusarium*
	Diacetylnivalenol	*Fusarium oxysporum*

Genera	mycotoxin	Species
	Dihydroxydiacetoxyscirpenol	*Fusarium*
	Enniatins	*Fusarium moniliforme, F. avenaceum, F. roseum, F.solani, and F. nivale*
	Fructigenin +1	*Fusarium moniliforme, F. culmorum, F. avenaceum, and F. roseum*
	Fumonisin B1	*Fusarium moniliforme, F. culmorum, F. avenaceum, F. nivale and F. oxysporum*
	Fusarenon	*Fusarium*
	Fusarenon-X	*Fusarium oxysporum*
	Fusaric acid	*Fusarium moniliforme and F. oxysporum*
	Fusarin	*Fusarium moniliforme*
	Fusarinic acid	*Fusarium*
	HT-2 toxin	*Fusarium moniliforme, F. culmorum, F. avenaceum, and F. nivale*
	Ipomeanine	*Fusarium moniliforme, F. culmorum, F. avenaceum, and F. nivale*
	Lateritin +1	*Fusarium moniliforme, F. culmorum, F. avenaceum, and F. nivale*
	Lycomarasmin +1	*Fusarium moniliforme*
	Moniliformin	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Monoacetoxyscirpenol	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Monoacetylnivalenol X	*Fusarium*
	Monodeacetylanguidin	*Fusarium*
	Neosolaniol	*Fusarium moniliforme, F. solani, F. culmorum, F. avenaceum, F. oxysporum and F. roseum*
	Neosolaniol monoacetate	*Fusarium*
	Neosolaniolacetate	*Fusarium*
	Nivalenol	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Nivalenol diacetate	*Fusarium*
	Nivalenol monoacetate	*Fusarium*
	NT-1 toxin	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	NT-2 toxin	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. avenaceum, F. roseum, and F. nivale*
	Rd toxin	*Fusarium*
	Sambucynin +1	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. solani, F. avenaceum, F. roseum, and F. nivale*
	Scirpentriol	*Fusarium moniliforme, F. equiseti, F. oxysporum, F. culmorum, F. solani, F. avenaceum, F. roseum, and F. nivale*
	Solaniol	*Fusarium*
	T-1 toxin	*Fusarium moniliforme, F. equiseti, F. culmorum, F. solani, F. avenaceum, F. roseum, and F. nivale*
	T-2 toxin	*Fusarium moniliforme, F. equiseti, F. culmorum, F. solani, F. avenaceum, F. roseum, F. oxysporum and F. nivale*
	Triacetoxyscirpendiol	*Fusarium moniliforme, F. equiseti, F. solani, F. avenaceum, F. roseum, and F. nivale*
	Triacetoxyscirpenol	*Fusarium*
	Yavanicin+1	*Fusarium culmorum, F. graminearum, F. oxysporum, F. roseum, F. moniliforme, F. avenaceum, F. equiseti, and F. nivale*
	Zearalenol	*Fusarium*
	Zearalenone	*Fusarium culmorum, F. graminearum, F. oxysporum, F. roseum, F. moniliforme, F. avenaceum, F. equiseti, and F. nivale*
Monographella	Bentenolide	*Monographella nivalis*
Myrothecium	Roridin E	*Myrothecium roridum, M. verrucaria*

Genera	mycotoxin	Species
	Verrucarin	*Myrothecium verrucaria*
Penicillium	Citreoviridin	*Penicillium citreoviride*
	Citrinin	*Penicillium citrinum, P.hirsutum, P. verrucosum*
	Gliotoxin	*Penicillium*
	Islanditoxin	*Penicillium islandicum*
	Ochratoxin	*Penicillium viridictum*
	Patulin	*Penicillium expansum, P.roquefortii, P. claviforme, P. griseofulvum*
	Penitrem	*Penicillium crustosum*
	Rubratoxin	*Penicillium rubrum*
	Rubroskyrin	*Penicillium spp.*
	Rubrosulphin	*Penicillium viridicatum*
	Rugulosin	*Penicillium brunneum, P. kloeckeri, P. rugulosum*
	Sterigmatocystin	*Penicillium rugulosum*
	Verrucosidin	*Penicillium aurantiogriseum group*
	Viomellein	*penicillium aurantiogriseum, P. crustosum, P. viridicatum*
	Viopurpurin	*Penicillium viridicatum*
Rhizoctonia	Slaframine	*Rhizoctonia leguminicola*
Stachybotrys	Roridin E	*Stachybotrys spp.*
	Satratoxins, F,G,H	*Stachybotrys chartarum*
	Trichoverrins	*Stachybotrys chartarum*
	Trichoverrols	*Stachybotrys chartarum*
	Verrucarin	*Stachybotrys chartarum*
	Verruculogen	*Stachybotrys chartarum*
Trichoderma	Satratoxins, F,G,H	*Trichoderma viridi*
	Trichodermin	*Trichoderma viride, T. polysporum, T. sporulosum, T. reesei (Reino et al., 2008)*
	T-2 toxin	*Trichoderma lignorum(Reino et al., 2008), but isolation doubted by Nielsen et al. (2005)*
Trichophyton	Viopurpurin	*Trichophyton spp.*
Trichothecium	Trichothecin	*Trichothecium roseum*

8.2.2 Lists of known pathogens in humans

The following databases and lists can be consulted:

a. The American Biological Safety Organization Risk Group Database http://www.absa.org/riskgroups/
b. Public Health Agency of Canada maintains the Pathogen Safety Data Sheet and Risk Assessment database
http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php
c. National Institutes of Health of the USA Department of Health & Human Services issued the NIH guidelines which contains four risk groups:
Risk Group 1 (RG1) agents are not associated with disease in healthy adult humans.
Risk Group 2 (RG2) agents are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are *often* available.
Risk Group 3 (RG3) agents are associated with serious or lethal human disease for which preventive or therapeutic interventions *may be* available.
Risk Group 4 (RG4) agents are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are *not usually* available.
http://osp.od.nih.gov/sites/default/files/NIH_Guidelines_0.pdf

d. Liste BG Chemie Merkblatt B 0006. Sichere Biotechnologie: Einstufung Biologischer Arbeitsstoffe: Prokaryonten: A detailed German reference list of a large number of micro-organisms and their risk classifications (Baua, 2015)
e. Dir. 2000/54 EC on the protection of workers from risks related to exposure to biological agents at work. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32000L0054&from=EN

The list in Dir. 2000/54 EC classifies pathogens into four risk groups, according to their level of risk of infection:

- Group 1 biological agent is unlikely to cause human disease;
- Group 2 biological agent can cause human disease and might be a hazard to workers; it is unlikely to spread to the community; there is usually effective prophylaxis or treatment available;
- Group 3 biological agent can cause severe human disease and can present a serious hazard to workers; it may present a risk of spreading to the community, but there is usually effective prophylaxis or treatment available;
- Group 4 biological agent causes severe human disease and is a serious hazard to workers; it may present a high risk of spreading to the community; there is usually no effective prophylaxis or treatment available.

Only four pathogens in this list produce toxins. These are *Clostridium botulinum, C. tetanii, Corynebacterium diphteriae* and *Sigella dysenteriae*. Theoretically, it is possible that human pathogens also possess modes of action that could be used in plant protection. An example is *Burkholderia* spp. which is classified as Group 3. A related *Burkholderia* species with pest control properties was not registered as problems were encountered in the identification of the species.

8.2.3 List of phytotoxins

Table 4. Phytotoxins collected from the literature.

Metabolite	Produced by	Host specific Y/N	Crop or weed	Mode of action	Toxicity	Reference
AAL toxin (dimethylheptadecapentol ester of propane tricarboxylic Acid)	*Alternaria alternata* f.sp. *lycopersici*	Y	Tomato	Selective herbicide against dicotyledonous weeds; affects lipid synthesis Disrupts pyrimidine synthesis		(Moussatos et al., 1994), (Vey et al., 2001)
Acetylaranotin	*A. alternata* f. sp. *terreus*	Y		Inhibits plant growth		(Vey et al., 2001)
ACTG	*A. alternata* f. sp. *citri*	Y	Mandarine /oranges			(Vey et al., 2001)
AF	*A. alternata* f. sp. *fragariae*	Y	Strawberry			(Vey et al., 2001)
Aflatoxin	*Aspergillus* spec.					(Vey et al., 2001)
AK (ester of epoxy-decatrienoic acid)	*A. alternata* f. kikuchiana	Y	Japanese pear	Causes necrosis		(Vey et al., 2001)
AM (cyclic tetrapeptides)	*A. alternata f.sp. mali*	Y	Apple	Induces necrotic spots on leaves and fruit		(Vey et al., 2001)
Cercosporin	*Cercospora*	N		Photodynamic compounds	Affects the lipid bilayers of any cells including plants,	(Duke and Dayan, 2011)

Metabolite	Produced by	Host specific Y/N	Crop or weed	Mode of action	Toxicity	Reference
					mammals, bacteria, and fungi.	
Colletotrichin	*Cochliobolus nicotianae*	Y	Tobacco			(Vey et al., 2001)
Elsinochrome A	*Elsinoe fawcettimacro*			Photodynamic compounds	Same mode of action as Cercosporin	(Duke and Dayan, 2011)
Fusaric acid and related pyridine derivatives	*Fusarium*	N	Many weed species	growth inhibition, necrosis and chlorosis in	highly toxic to mammals	(Vey et al., 2001)
Gostatin	*Streptomyces sumanensis*			Inhibitor amino transferase	Potent phytotoxin	(Duke and Dayan, 2011)
HC (cyclic tetrapeptides)	*Cochliobolus carbonum*	Y	Specific to maize	Targets include plasma membrane		(Vey et al., 2001)
HV	Cochliobolus victoriae	Y	Specific to oats	Induces tissue leakage		(Vey et al., 2001)
Macrocidin (Cyclic tetramic acids)	*Phoma macrostoma*			Inhibition of carotenoid synthesis		(Duke and Dayan, 2011)
Monocerin	*Setosphaeria monoceras (Exserohilum monoceras* Inu	N	Barnyard grasses, Johnson grasses, cucumbers, tomatoes	Inhibition seedling growth		(Vey et al., 2001), (Lim, 1999)
Naphthazarins	*Fusarium*	N				(Vey et al., 2001)
Phytoxin	*Exserohilum/ Setosphaeria monoceras*					
Trichothecenes: T-2 toxin, Neosolaniol, Diacetoxyscirpenol Fusarenon-x, Nivalenol, Deoxynivalenol	*Fusarium*			Affects membrane function in plants	low toxicity to nontarget organisms such as mammals, vegetables, and fruit (Xu et al., 2013)	(Bennett and Klich, 2003) (Vey et al., 2001)
Trichodermin	*Trichoderma viride*	N	Bean and Corn			(Cutler and LeFiles, 1978)
T-toxin (linear polyketol)	*Cochliobolus heterostrophus*	Y	Maize	Mitochondria of susceptible maize cultivars become leaky		(Vey et al., 2001)
Ziganein	*Exserohilum monoceras* Inu		Barnyard grasses, Johnson grasses			(Lim, 1999)

8.2.4 List of antibiotics

Table 5 lists antibiotics (and SMs with other biological activity). This list is not exhaustive, e.g. antibiotics produced by Pseudomonades should be included: Phenazines, 2.4 diacetilphloroglucinol, 2.3 deepoxy 2.3- didehydrorhizoxin.

Table 5. Antibiotic compounds, producer strains with the important biological activities (Hassan et al. 2012).

	Compound	Producer	Biological activity
1.	AmphotericinB	*Streptomyces nodosus*	Antifungal
2.	Avermectin	*S. avermitilis*	Antiparasitic
3.	Anthracyclines	*S. galileus*	Antitumor
4.	Bafilomycin	*S. griseus* *S. halstedii*	ATPase-inhibitor of MO, Plant and animal cells
5.	Bialaphos	*Streptomyces hygroscopicus*	Herbicide
6.	DaunorubicinHCl	*Streptomyces sp*	Antitumoral
7.	Cephalosporin	*Cephalosporiumchrysogenum*	Antibiotic
8.	Chlortetracycline	*Streptomyces aureofaciens*	Antibiotic, growth promotant
9.	Chloramphenicol	*S. venezuelae*	Antibacterial , inhibitorof protein biosynthesis
10.	CyclosporinA	*Trichodermapolysporum*	Immunosuppressant
11.	DaunorubicinHCl.	*Streptomyces sp*	Antitumoral
12.	Doxorubicin HCl	*Streptomyces peucetius*	Antitumoral
13.	ErythromycinA	*Streptomyces erythreus*	Antibiotic
14.	Gentamicin	*Micromonosporapurpurea*	Antibiotic
15.	Hygromycin	*S. hygroscopicus*	Antimicrobial, immunosuppressive
16.	Kanamycin	*Streptomyces canus*	Antibiotic
17.	LincomycinHCl	*Streptomyces lincolnensis*	Antibiotic
18.	Mitosane	*Streptomyces caespitosus*	Antitumoral
19.	MitomycinC	*S. lavendulae*	Antitumor, Bindsto double-stranded DNA
20.	Oxytetracycline	*Streptomyces rimosus*	Antibiotic, feed additive
21.	Paclitaxel	*Taxomycesandreanae*	Antitumoral
22.	Penicillin	*Penicilliumchrysogenum*	Antibiotic
23.	Rifamycin	*Amycolatopsismediterranei*	Antibiotic
24.	Rapamycin	*S. hygroscopicus*	immunosuppressive,antifungal
25.	Salinomycin	*Streptomyces albus*	Growth promotant
26.	Spiramycin	*Streptomyces ambofaciens*	Antibiotic
27.	Streptomycin sulfate	*Streptomyces griseus*	Antibiotic
28.	Streptozotocin	*S. achromogenes*	Diabetogenic
29.	Teicoplanin	*Actinoplanesteichomyceticus*	Antibiotic
30.	Tetracycline HCl	*Streptomyces aureofaciens*	Antibiotic
31.	VancomycinHCl	*Amycolatopsisorientalis*	Antibiotic

S= *Streptomyces*

8.3 List registered products

In Table 6 products are listed that have been included in the Manual of Biocontrol Agents (BCPC, 2014). The exact regulatory status of the microorganisms and products mentioned in the list is not provided.

Metabolites have been mentioned for *Trichoderma*, *Clonostachys* (formerly *Gliocladium*) and *Bacillus* spp.

Table 6. List of products based on micro-organisms (extracted from the Manual of Biocontrol Agents (BCPC, 2014).

Active substance	Product	Secondary metabolites[1]
Bacteria		

Active substance	Product	Secondary metabolites[1]
Agrobacterium radiobacter K1026	Nogall	Not mentioned
Agrobacterium radiobacter K84	Galltrol – A	Not mentioned
Aureobasidium pullulans DSM 14940 and DSM 14941	Blossom Protect	Not mentioned
Aureobasidium pullulans DSM 14940 and DSM 14941	Boni Protect	Not mentioned
Aureobasidium pullulans DSM 14940 and DSM 14941	Botector	Not mentioned
Bacillus amyloliquefaciens D747	Double Nickel	Iturins
Bacillus amyloliquefaciens D747	Double Nickel 55	Iturins
Bacillus firmus I-1582	Nortica 5% wp	Not mentioned
Bacillus firmus I-1582	Poncho/Votivo	Not mentioned
Bacillus licheniformis SB3086	Roots EcoGuard (Lebanon Turf)	Not mentioned
Bacillus pumilus QST 2808	Ballad Plus	Not mentioned
Bacillus pumilus QST 2808	Sonata	Not mentioned
Bacillus sphaericus 2362 H5a5b	VectoLex	d-endotoxin
Bacillus subtilis KTSB	FoliActive	Not mentioned
Bacillus subtilis MBI 600	Subtilex NG	Not mentioned
Bacillus subtilis QST713	Cease	Not mentioned[2]
Bacillus subtilis QST713	Rhapsody	Not mentioned
Bacillus subtilis QST713	Serenade ASO	Not mentioned
Bacillus subtilis QST713	Serenade Max	Not mentioned
Bacillus subtilis QST713	Serenade Soil	Not mentioned
Bacillus subtilis subsp. *amyloliquefaciens* FZB24	Taegro	Not mentioned
Bacillus thuringiensis aizawai NB200	FlorBac	Toxic protein
Chromobacterium subtsugae PRAA4-1	Grandevo	Not disclosed
Clavibacter michiganensis subsp. michiganensis bacteriophage	AgriPhage	Not mentioned
Pseudomonas chlororaphis MA342	Cedomon; Cerall	Not mentioned

Active substance	Product	Secondary metabolites[1]
Pseudomonas fluorescens A506	BlightBan A506	Not mentioned
Pseudomonas spp. DSMZ 13134	Proradix	Not mentioned
Pseudomonas syringae ESC 11	Bio-Save 11LP	Not mentioned
Pseudomonas syringae pv tomato bacteriophage	AgriPhage	Not mentioned
Streptomyces griseoviridis K61	Mycostop	Not specified
Streptomyces lydicus WYEC 108	Actinovate AG	Not specified
Xanthomonas campestris pv vesicatoria bacteriophage	AgriPhage	Not mentioned
Entomopathogenic fungi		
Beauveria bassiana ATCC 74040	Naturalis; Racer	Not mentioned. Enzymes are the actives
Beauveria bassiana GHA	BotaniGard; Botanigard 22WP; Botanigard ES; Eco-Bb; Mycotrol; Mycotrol ES; Mycotrol O	Not mentioned Enzymes are the actives
Isaria fumosorosea apopka 97	PreFeRal	Not mentioned
Lecanicillium muscarium Ve-6	mycotal	Not mentioned
Metarhizium anisopliae BIPESCO 5/F52	Met 52 Granular; Pacer-MA	Not mentioned
Metarhizium anisopliae ESF1	*Metarhizium anisopliae* ESF1	Not mentioned
Metarhizium anisopliae subsp. *acridum*	Green Guard	Not mentioned
Metarhizium anisopliae subsp. *acridum* IMI 330189	Green Muscle	Not mentioned
Paecilomyces fumosoroseus Fe9901	NoFly ; NoFly WP	Not mentioned
Paecilomyces lilacinus 251	BioAct WG; Melocon	Not mentioned Enzymes are the actives
Paecilomyces lilacinus BCP2	PL Gold	Not mentioned Enzymes are the actives
Verticillium albo-atrum WCS850	Dutch Trig	No relevant metabolites
Fungi		
Alternaria destruens 059	Smolder; Smolder G	Not mentioned
Ampelomyces quisqualis M-10	AQ 10	Not mentioned
Aspergillus flavus AF36	*Aspergillus flavus* strain AF36	Non-aflatoxin-producing strain

Active substance	Product	Secondary metabolites[1]
Aspergillus flavus NRRL 21882	Alfa-Guard GR	Non-aflatoxin-producing strain
Candida oleophila O	NEXY0101	Not mentioned Enzymes are the actives
Chondrostereum purpureum HQ1	Limited or no product currently available	No relevant metabolites
Chondrostereum purpureum PFC 2139	Chontrol Paste; Chontrol Peat Paste	No relevant metabolites
Coniothyrium minitans CON/M/91-08	Contans WG	Not mentioned Enzymes are the actives
Clonostachys (formerly *Gliocladium*) *catenulatum* J1446	Prestop; Prestop Mix	Not mentioned
Clonostachys (formerly *Gliocladium virens* GL-21	SoilGard	Gliotoxin
Myrothecium verrucaria AARC-0255	DiTera	Not mentioned
Phlebiopsis gigantea (several strains)	Rotstop	Not mentioned
Pseudozyma flocculosa PF-A22	*Pseudozyma flocculosa* PF-A22	Not mentioned
Purpureocillium lilacinus	Biostat	Not mentioned Enzymes are the actives
Pythium oligandrum M1	Polyversum	Not mentioned
Trichoderma asperellum ICC 012	Bio-Tam	Not mentioned
Trichoderma asperellum T25	Tusal	Not mentioned
Trichoderma asperellum T34	T34 Biocontrol	Not mentioned
Trichoderma asperellum TV1	Xedavir	Not mentioned
Trichoderma atroviride I 1237	Esquive WP	Not mentioned
Trichoderma atroviride IMI 206040	BINAB TF WP; Binab TF WP	Not mentioned
Trichoderma atroviride LC 52	Tenet	Not mentioned
Trichoderma atroviride T-11	Trichoderma atroviride T-11	Not mentioned Enzymes are the actives
Trichoderma gamsii ICC 080	Bio-Tam	Not mentioned Enzymes are the actives
Trichoderma hamatum TH382	Incept	Not mentioned
Trichoderma harzianum ITEM 908	Trichoderma harzianum ITEM 908	Not mentioned

Active substance	Product	Secondary metabolites[1]
Trichoderma harzianum T-22 RIFAI (KRL-AG2	Eco-77; Eco-T; PlantShield HC; RootShield Granules; T-22 HC; Trianum-G; Trianum-P	Not mentioned
Trichoderma polysporum IMI 206039	BINAB TF WP; Binab TF WP	Not mentioned
Trichoderma viride	Ecosom-TV	Trichodermin, Gliotoxin, Viridin
Microsporidium		
Nosema locustae	NoLo Bait	Not known to be produced

1: When no information was found on SMs this was addressed as 'not mentioned'. In earlier dossiers information on SMs may not have been considered.
2: The EU dossier does make mention of metabolites for this strain - lipopeptides

9 METHODS FOR IDENTIFYING SECONDARY METABOLITES

Several methods for identifying SMs are available.

9.1.1 Simple plate assays

Some easy methods for identifying mycotoxins are described in detail by Stark (2010). Methods that involve expensive equipment or require highly trained experts are reviewed more briefly.

Plate assays supporting *Aspergillus* sp.
A plate assay supporting the growth of aflatoxin-producing *Aspergillus* sp. is described by Stark (2010). The *Aspergillus sp.* differential medium was specifically developed to support growth of *Aspergillus* sp. Aflatoxin production can be observed under 365 nm UV light. Other media supporting the growth of other biocontrol agents were not reviewed by Stark (2010).

Plate assay supporting *Trichoderma* sp.
Production of antibiotics by *Trichoderma* sp. can be triggered in plate assays. Fungal cultures of a pathogen and the biocontrol agent are placed opposite each other on a plate (Potato dextrose agar (PDA)) and incubated until contact occurs between the two mycelia. Plates can subsequently be extracted (with ethyl acetate (EtOAc)) and the organic layers dried and evaporated. The extracts can be analysed with thin layer chromatography (TLC) and liquid chromatography/mass spectrometry (LC/MS) (Vinale et al., 2006).

9.1.2 Isolation of secondary metabolites from liquid cultures

A method for isolating the major SMs from liquid cultures of *Trichoderma harzianum* strain T22 is cited in Vinale et al. (2006)":

> "*The filtered culture broth (2 L) of strain T22 was extracted exhaustively with ethyl acetate (EtOAc). The combined organic fraction was dried (Na_2SO_4) and evaporated under reduced pressure at 35C. The red-brown residue recovered was subjected to flash column chromatography (Si gel; 50 g), eluting with a gradient of EtOAc: petroleum ether (8 : 2 to 10 : 0). Fractions showing similar thin-layer chromatography (TLC) profiles were combined and further purified by using preparative TLC separation (Si gel; EtOAc : petroleum ether –6 : 4) or silica-gel flash chromatography (EtOAc : petroleum ether – 8 : 2 to 10 : 0). The culture filtrate of strain T39 was extracted with EtOAc as mentioned previously and the extract was separated by using vacuum liquid chromatography (VLC), with eluent gradient mixtures of petroleum ether and EtOAc (0–100% EtOAc). Homogeneous fractions were further purified by preparative TLC (Si gel; EtOAc : petroleum ether – 8 : 2 and 1 : 1)*".

The purification of SMs is often suggested as a straightforward way to identify SMs. Opponents of this method claim that purification is time consuming and requires the use of several analytical methods. In addition, only few of the several possible SMs produced by these organisms can be isolated. Therefore, a risk assessment investigation based on single SM is not feasible (Ehlers, 2007).

Instead, "REBECA proposes a tiered scheme for bacterial and fungal metabolites, based on the assessment of supernatants and crude extracts from cultures of the micro-organisms in question in bioassays apart from whole-animal testing. The microbial SMs may have additive or synergistic toxic effects. It is conceivable that the toxicological risk associated to a particular MPCA would be better foreseen by assaying mixtures of SMs on test organisms characterised by sensitivity to a large spectrum of different molecules, instead of assessing the toxicity of single SM. Sensible, high throughput and cost effective bioassay systems would allow the assessment several production batches, determining the toxicity range of a product. Moreover, such

test systems would allow the investigation of toxic SM production over a broader range of environmental conditions compared to animal testing".

Testing crude extracts

The REBECA position paper on SMs produced by micro-organisms (Ehlers, 2007, Strasser et al., 2007), proposes the use of crude extracts for toxicity testing as the cytotoxic activity of crude extracts represent a worst case scenario. Several pitfalls were mentioned:

- The crude extracts represent the 'worst case' scenario as the levels and spectrum of the metabolites being assessed are far higher than those occurring in nature.
- Crude extracts are hardly ever expected to show zero toxicity and so it is necessary to establish tolerance levels of toxicity in biological assays. This would need an evaluation of the probability that hazardous levels of SMs occur after *in situ* production, translocation and degradation.

These are serious draw backs that are considered to unnecessarily complicate the risk assessment and so it was decided not to incorporate crude extracts into the decision scheme of this working document.

Canada recommends the use of crude extracts for certain tests, e.g., to determine if a specific SM is produced by a particular micro-organism. For instance, crude extracts can be very useful in plate bioassays. Although it is true that these studies could be viewed as "worst case" scenarios if not properly conducted, there are ways to ensure that the tested concentrations represent relevant concentrations in cultures. For instance, if the volume of the extracted medium were recorded, the collected extract could be re-dissolved to relevant concentrations. Crude extracts, however, would not likely be sufficient if detailed toxicity studies were required for the SM to support registration (i.e., like conventional chemical pesticides). These studies would require purified samples.

9.1.3 Detection and determination of (myco)toxins in the analytical laboratory

The following methods are mentioned by Stark (2010):

- High performance Liquid Chromatography (HPLC)
- HPLC-Mass Spectrometry (LC-MS)
- HPLC-tandem Mass Spectrometry (LC-MS-MS)
- Enzyme-linked immunosorbent assay kits
- Lateral flow Immunochromatography

Methods not mentioned by Stark are NMR and GC-MS.

9.1.4 Full genome sequencing

A complete and concise examination of the genomic sequences of micro-organisms is now a straightforward process at a fairly low cost. However, currently this genomic sequence data are not always available (Blumenthal, 2004). Canada does not require full genome sequencing. Gene probing, however, could be required in order to support registration. Full-genome sequences could be submitted, if available.

If the analysis shows that the strain lacks the gene / gene cluster, there would be no concern for this SM.

When the gene is present it should be determined whether or not it comes to expression. Indeed, on some occasions, the gene is not expressed as the expression of genes is generally tightly regulated. In Canada, the presence of genes alone is not considered to be definitive evidence that a micro-organism produces a specific SM since the gene(s) could be dormant or not fully expressed. The presence of gene(s) in a micro-organism,

however, would trigger additional requirements, specifically additional characterisation data to demonstrate whether the SM is expressed under growing conditions that are known to support its production in other species or related micro-organisms. Any information on the regulation mechanisms or conditions in which expression of the gene of concern occurs (or not) will be useful for the risk assessment.

9.1.5 RNASeq

RNAseq is newer than full genome sequencing and is becoming a popular technique for genome wide ecological transcriptomics (Alvarez et al., 2015). RNAseq uses next-generation sequencing methods to characterise RNA transcripts using high-throughput sequencing of a cDNA library to generate hundreds of thousands of fragments of DNA sequences.

If RNAseq is performed, conditions have to be specified as gene expression is very sensitive to change of environmental conditions.

Yu et al.'s study (2011) used RNA-Seq technology to demonstrate that the profile of the *Aspergillus flavus* transcriptome depends on temperature. Thus, as the result of actual expression of genes, RNASeq is able to show the transcriptome under different environmental conditions. Therefore this technique overcomes the disadvantage of full genome sequencing which shows that the gene is present but does not give information about the actual expression of the gene.

9.1.6 Gene probing

Gene probing as method used in molecular biology for locating a particular gene on a chromosome. It involves pairing a known short segment of deoxyribonucleic acid or ribonucleic acid with a matching sequence of bases on a chromosome.

9.1.7 Microarrays

The sequencing of a genome facilitates the construction of microarrays, which can be used to study transcriptional regulation of the biosynthesis of a particular SM in different environmental conditions. While microarrays are a robust tool for genome-wide gene expression analysis, they have been plagued by high background and low sensitivity problems (Yu et al., 2011). For regulatory genes with a low level of expression, microarrays often fail to provide meaningful information about their expression levels. For background information, see this flash animation On DNA microarray methodology: http://www.bio.davidson.edu/courses/genomics/chip/chipQ.html

9.1.8 Metabolomics

Metabolomics is a next-generation technology which enhances the sensitivity and speed with which specific elements of the cellular metabolome can be identified and quantified. According to Wikipedia (http://en.wikipedia.org/wiki/Metabolomics) metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind", and the study of their small-molecule SM profiles. The metabolome represents the collection of all SMs in a biological cell, tissue, organ or organism, which are the end products of cellular processes.

9.1.9 Biochemometrics

In some cases it might be useful to establish the SM profile. For this, Inui et al. (2012) propose biochemometrics, which enables the assignment of biologically active constituents, both structurally known

and unknown, in complex natural product mixtures without the risk of losing actives during the separation. The method consists of four steps:

- Step 1: High resolution preparative fractionation of the complex metabolomic mixture, e.g., ethnobotanical crude extract, by countercurrent chromatography (CCC);
- Step 2: *In vitro* biological evaluation of all resulting fractions and generation of a high-resolution biochromatogram, derived from the high-resolution CCC fractions;
- Step 3: GC/MS analysis of all CCC fractions and subsequent building of a 3-dimentsional CCC-GC/MS data matrix;
- Step 4: Data processing and chemometric analysis, establishing links between the deconvolved biochromatogram (Step 2) and the 3D CCC-GC/MS data matrix (Step 3).

9.2 Biological test systems

When toxic effects are observed in bioassays, it is not known if these toxic effects will be produced under natural environmental conditions. It is possible that the local concentration of SM will be insufficient to exert the effect observed *in vitro*. It is also difficult to predict how adsorption, degradation and interaction with other SMs will modulate its toxicity *in situ*. It may not be possible to determine or control all these factors.

9.2.1 Lepidopteran cell line (SF-9)

Fornelli (2004) used a lepidopteran (*Spodoptera frugiperda*) cell line (SF-9) to determine the toxicity of several fungal SMs by Trypan blue dye exclusion and MTT[8]-colorimetric assay, after 48 h of incubation.

By MTT assay, the cytotoxicity ranking was: fusarenon X (IC50 0.3 µM)=diacetoxyscirpenol (IC50 0.5 µM)=beauvericin (IC50 2.5 µM)=nivalenol (IC50 5.3 µM)=enniatin (IC50 6.6 µM) ≥ gliotoxin (IC50 7.5 µM) > zearalenone (IC50 17.5 µM) > deoxynivalenol (IC50 47.6 µM).

By Trypan blue dye exclusion, the cytotoxicity ranking was: fusarenon X (CC50 0.4 µM)=diacetoxyscirpenol (CC50 1.1 µM) beauvericin=(CC50 3.0 µM)=gliotoxin (CC50 4.0 µM)=enniatin (CC50 6.7 µM) ≥ nivalenol (CC50 9.5 µM) > zearalenone (CC50 18.3 µM) > deoxynivalenol (CC50 45.0 µM).

Conclusions
- A cytotoxicity ranking is useful in determining the relevant toxicity of the studied SM.
- The ranking may show slight differences depending on the technical characteristics of the assay.

9.2.2 Other test systems

Several other assays can be found in following references.
(Skrobek et al., 2006, Mao et al., 2010, Skrobek and Butt, 2005, Seger et al., 2004, Dudley et al., 2004, Butt et al., 2009, Strasser et al., 2000b, Strasser et al., 2007, Samuels, 1998, Safavi, 2013, Poprawski et al., 1994, Li et al., 2003, Favilla, 2006, Sundh, 2012).

9.3 Earlier decision schemes

The following scheme was designed by RAFBCA experts. In this scheme, risk assessors should argue which of the tests and results are essential for MPCA registration on a case by case basis.

[8] tetrazolium dye

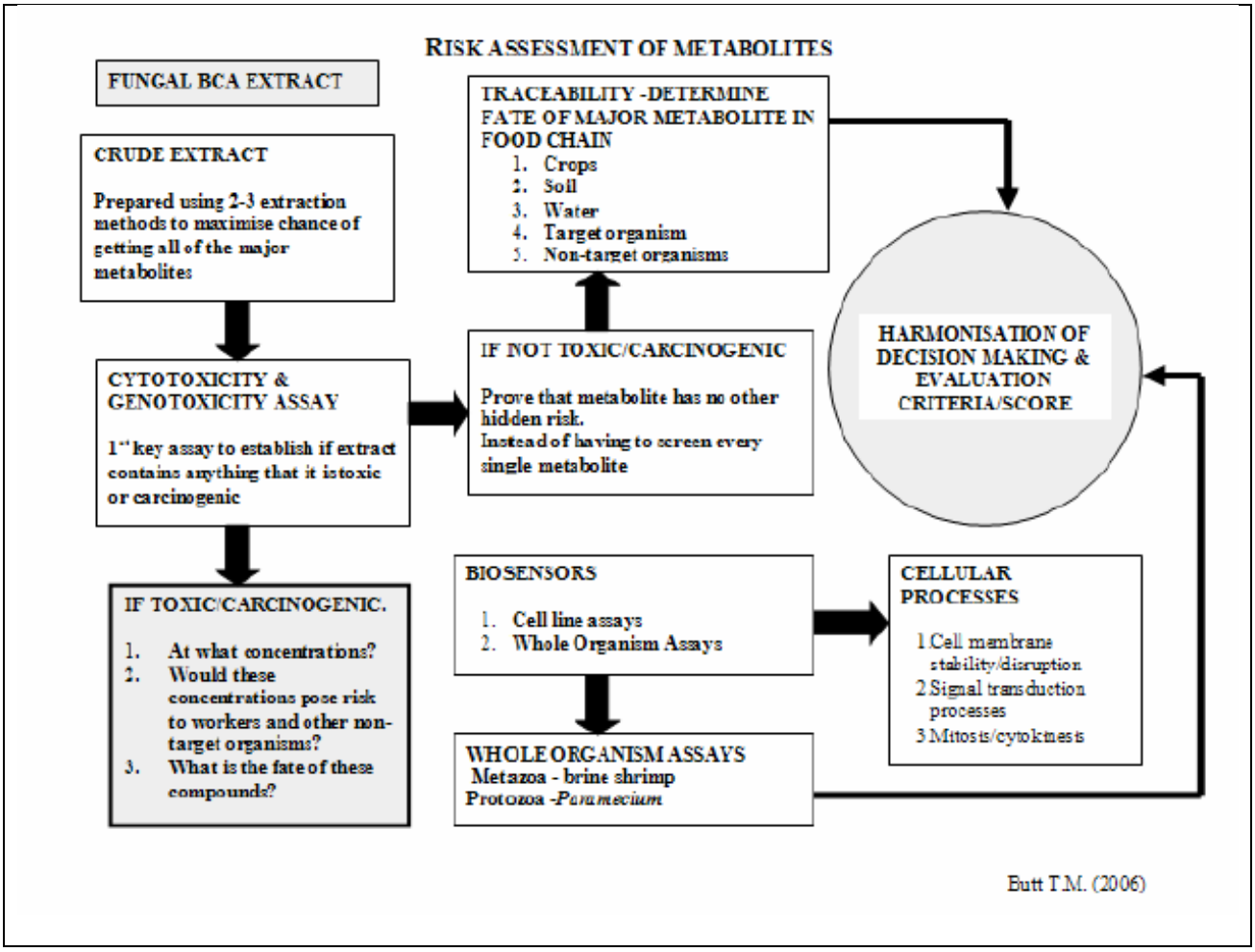

Figure 4. RAFBCA Scheme for risk assessment of potential relevant secondary metabolites in fungal BCA extract.

The scheme below was proposed in the REBECA deliverable 10 (Ehlers, 2007).

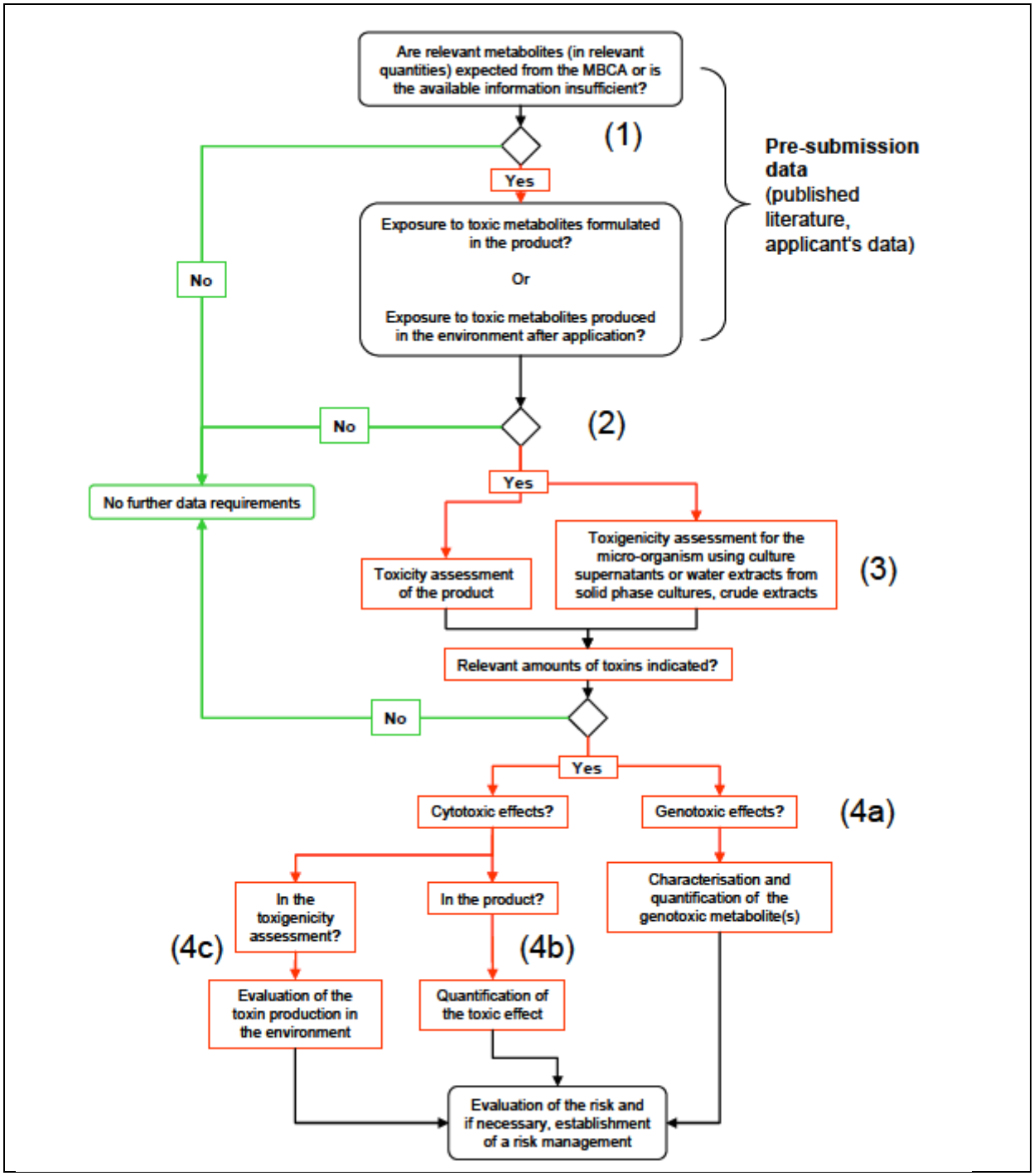

Figure 5. Scheme for assessment of potential relevant secondary metabolites of MPCAs (Ehlers, 2007).

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Working document for information and use in discussion related to contaminants and toxins in the GSTCFF.

11 BACKGROUND DOCUMENT

The background document is available as an annex to this report under reference ENV/JM/MONO(2018)33/ANN1.