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GUIDANCE ON THE RISK ASSESSMENT OF METABOLITES PRODUCED BY MICROORGANISMS USED AS PLANT PROTECTION ACTIVE SUBSTANCES

IN ACCORDANCE WITH ARTICLE 77 OF REGULATION (EC) No 1107/2009

COMMISSION STAFF WORKING DOCUMENT – DOES NOT NECESSARILY

REPRESENT THE VIEW OF THE COMMISSION SERVICES

This guidance has been developed in co-operation with the Member States. It does not intend to produce legally binding effects and by its nature does not prejudice any measure taken by a Member State within the implementation prerogatives under Regulation (EC) No 1107/2009, nor any case law developed with regard to this provision. This document also does not preclude the possibility that the European Court of Justice may give one or another provision direct effect in Member States.

Revision history

When	What
23 October 2020	First version of the document
21 March 2024 (Rev 1)	- Revision history is included. - Inclusion of the Annex III with the “overview table”. - Updates in order to align with most relevant definitions, terminology and wording of the Part B of the Annex to Reg (EU) 283/2013 as amended by Reg (EU) 2022/1439.

Introduction

Microorganisms are known to produce metabolites. Many metabolites are substances to which humans, animals and the environment may be exposed on a regular basis at low levels and that pose no concern. However, there are species of fungi and bacteria known to produce toxic metabolites that may pose a risk to human and animal health or the environment. Therefore, the potential risk caused by the production of metabolites is part of the evaluation of a microorganism on which the decision to approve its use for plant protection is based.

The data requirements and the uniform principles for microorganisms contain specific provisions on metabolites (Regulation (EU) No 283/2013 Part B and Regulation (EU) No 546/2011 Part B, respectively). Experience has shown that guidance is required in interpreting these provisions. Therefore, this guidance document aims to provide a practical approach on how the data requirements on metabolites can be applied in the approval of microorganisms as active substances at EU-level and the authorisation of plant protection products at MS level. This guidance document addresses metabolites present in the active substance[1] and the plant protection product and also those produced by the microorganism after application (*in situ* production). In contrast to metabolites of chemicals, which are breakdown products metabolites addressed in this guidance document are components produced by the microorganism. Thus, chemical and microbial metabolites are equivalent in name only. Therefore, data requirements concerning metabolites of chemical plant protection products would not be applicable to microbial plant protection products.

The approach described in this guidance document is based on the consensus reached by the EU Working Group on Biopesticides and endorsed by the Standing Committee on Plants, Animals, Food and Feed. The approach implies that the assessment of all metabolites produced by a microorganism through an evaluation as performed for chemical active substances is not required, not feasible and unnecessary from a risk perspective, however parts of such assessment are needed under certain circumstances described in this document. The approach ensures that applicants provide all available data on metabolites including any indication of hazardous effects of any of these metabolites. For those metabolites for which a hazard is identified, this identified hazard is followed-up on by generating additional data where needed for a focused risk assessment for those particular metabolites.

Implementation schedule

The guidance document will be evaluated, as soon as necessary, taking into account its suitability, its impact and proportionality based on the experience gathered by risk assessors, risk managers and applicants with concrete case studies for which a decision on their approval/non-approval could be reached.

Regulation (EC) No 1107/2009

If possible, relevant, and useful for metabolites, the standard approaches outlined in the relevant regulations and available guidance documents should be followed. This document will provide considerations for situations where these approaches are not appropriate or technically feasible,

[1] Including, where relevant, metabolites produced in the MPCA as manufactured or microbial PPP during storage.

without prejudice to the responsibility of the applicant to ensure that the information is sufficient to assess if the microorganism fulfils the approval criteria as set out in Article 4 of Regulation (EC) No 1107/2009. The data requirements and the protection goals as laid down in the uniform principles (Regulation (EU) No 546/2011) have to be respected.

Uniform Principles and data requirements

Regulation (EU) No 546/2011 on the uniform principles for the evaluation and authorisation of plant protection products states the following on determining the relevance of metabolites produced by microorganisms for the risk assessment:

"*Metabolism is inherent of all living organisms. If secondary metabolites that are known to be hazardous to humans or other non-target organisms have been identified during the assessment of the micro-organism, the evaluation of a plant protection product containing this micro-organism shall include an assessment of the risk due to exposures to such metabolites expected from the intended use.* "

The data requirements (Regulation (EU) No 283/2013, in particular point 2.8 of Part B) focus on identifying metabolites of (potential) concern and testing their toxicity (in Section 5. “Effects on human health” and in Section 8 “Ecotoxicological studies”).

The data requirements on metabolites of (potential) concern apply to both metabolites in the MPCA as manufactured or the microbial PPP and those produced *in situ*, unless otherwise indicated. For example, point 1.4 of regulation 283/2013 (“Specification of the microbial pest control agent as manufactured”) only concerns metabolites of concern contained in the MPCA as manufactured, and points 6.1 and 7.2.1 of Regulation (EU) No 283/2013 concerns also, where relevant, metabolites of concern produced after the application of the microbial PPP (i.e. *in situ* production).

Stepwise approach

To determine whether the microorganism is producing a metabolite of concern, the guidance document is organised according to a “step-by-step” procedure.

The structure of the guidance consists of 4 stages (see the figure in **annex II**):

- Stage 1: Determining the assessment type
- Stage 2: Collecting a basic set of information on metabolites, resulting in a list of metabolites of potential concern;
- Stage 3: Determining which of the identified metabolites are of concern, resulting in a list of metabolites of concern
- Stage 4: The risk assessment for metabolites of concern.

Each stage consists of several steps. These steps contain questions for the applicant and the risk assessor to guide them through the process of each stage.

This stepwise approach is summarized in the table in **annex I** which is intended to help to guide readers through this document.

Scope

Primary/secondary metabolites

For microorganisms a distinction can be made between primary and secondary metabolites as outlined in the OECD Working Document[2] on the Risk Assessment of Secondary Metabolites of Microbial Biocontrol Agents (OECD 2018).

Primary metabolites are directly involved in general metabolism required for basic life processes such as growth, development and reproduction of a microorganism and are typically key components in maintaining normal physiological processes. Primary metabolites are not metabolites of potential concern and are out of the scope of this GD.

Secondary metabolites are not essential for the primary metabolic processes of microorganisms and show several biological activities possibly related to survival functions of the microorganism, such as competition, parasitism or symbiosis and metal transport. In this document, metabolites should therefore be understood as secondary metabolites.

Metabolites are normally produced by the microorganism when specific physical and biological conditions are jointly in place. The capacity of an individual strain of microorganism to produce metabolites of concern depends on many environmental and genetic parameters specific for this specific strain. The absence of production of undesirable compounds for one or more strains does not necessarily lead to hypothesize the same for all strains within the same species.

Unknown metabolites only produced in situ

The approach described in this guidance document identifies metabolites based on information and toxicity studies on the microorganism at strain level, the mode of action, the analysis of the fermentation culture medium after harvesting the microorganism and analysis of the MPCA as manufactured and/or microbial PPP. This is a weight of evidence approach which relies on absence of data on metabolites of concern for microorganisms having a history of use in agriculture. This approach will not cover unknown metabolites not produced during fermentation and/or not related to the mode of action that may be produced *in situ*. Since they are unknown their existence cannot be reasonably verified for microorganisms isolated from nature. It can be expected that they are produced at low levels due to competition and limited resources and energy available to the microorganism. Moreover, for microorganisms that are considered ubiquitous, humans, animals and the environment can be expected to be naturally exposed to such metabolites. Therefore, unless indicated otherwise in appropriate investigations identified through an open literature review, toxicity studies and genomic characterization or other information on the microorganism, it can be reasonably assumed that such unknown metabolites would not constitute a foreseeable risk and do therefore not constitute "relevant metabolites" in the meaning of the legislation.

[2] OECD, Series on Pesticides No. 98, Working document on the risk assessment of secondary metabolites of microbial biocontrol agents. ENV\JM\MONO\(2018)33 https://one.oecd.org/document/env/jm/mono(2018)33/en/pdf

Antimicrobial metabolites

The two hazards relevant to microbial metabolites which are addressed in this guidance document are toxicity and antimicrobial activity. The production of antimicrobial agents is common among indigenous bacteria and fungi in soils and plant-associated environments worldwide (see Raaijmakers et al., 2002). However, application of antimicrobial agents to agricultural systems due to their presence in the formulated product may confer a risk to human and animal health. Therefore, if a microorganism is known to be able to produce a relevant antimicrobial agent, the presence of this compound in the formulated product should be assessed.

In contrast to an emission of antimicrobial agents resulting from their presence in the formulated product, the *in situ* production of antimicrobial agents by indigenous or applied microorganisms is limited to micro-sites for certain periods of antagonistic interactions. Even if human or animal pathogens present in the soil or plant environment are exposed to the antimicrobial agents during these direct interactions, this exposure would be restricted to short periods followed by long periods without exposure. Such circumstances would not favor the development and persistence of antimicrobial resistance. Therefore, *in situ* production of antimicrobial agents by microorganisms used for plant protection is not considered as a foreseeable risk to human and animal health. As a result, no assessment of the *in situ* production of antimicrobial compounds is necessary.

Definitions

For the purpose of this guidance document the following terms have been defined:

Closely related strains: Strains within the same species.

Metabolite: Any metabolic product of a microorganism, present or formed either in contact with target organisms, with non-target organisms or in the environment depending on biotic and/or abiotic factors.

Metabolite of potential concern: Metabolite potentially produced by the strain under assessment, where some indication of toxicity or antimicrobial activity has been identified in the initial assessment based on literature and experimental data (i.e., at the end of Stage 2 of the assessment described in this guidance). Further assessment is necessary to determine if a metabolite of potential concern is of actual concern.

Metabolite of concern: means a metabolite produced by the micro-organism under assessment, with known toxicity or known relevant antimicrobial activity, which is present in the MPCA as manufactured at levels that may present a risk to human health, animal health or the environment, and/or for which it cannot be adequately justified that *in situ* production of the metabolite is not relevant for the risk assessment.

Toxin: A toxin is any compound produced within living cells or organisms, that is able to injure or cause damage in an organism due to its toxicity.

Relevant antimicrobial agents: all antimicrobial agents important for therapeutic use in humans or animals, as described in the latest available versions at the time of submission of the dossier:

— in a list adopted by means of Commission Regulation (EU) 2021/1760[3] in accordance with Article 37(5) of Regulation (EU) 2019/6 of the European Parliament and of the Council[4] (n.b., the latest available list adopted at the time of the endorsement of the Rev 1 of the present guidance document is published under the Commission Implementing Regulation (EU) 2022/1255[5]), or
— by the World Health Organisation[6] in the lists of Critically Important Antimicrobials, Highly Important Antimicrobials and Important Antimicrobials for Human Medicine;

MPCA – AM (Microbial Pest Control Agent as manufactured): the outcome of the manufacturing process of the micro-organism(s) intended to be used as active substance in plant protection products, consisting of the micro-organism(s) and any additives, metabolites (including metabolites of concern), chemical impurities (including relevant impurities), contaminating micro-organisms (including relevant contaminating micro-organisms) and the spent medium/rest fraction resulting from the manufacturing process or, in case of a continuous manufacturing processes where a strict separation between the manufacturing of the micro-organism(s) and the production process of the plant protection product is not possible, a non-isolated intermediate;

Microbial PPP (microbial Plant Protection Product): A plant protection product containing a microorganism that is approved and labelled with instructions for direct use or application for pest control purposes.

"Natural" background level of a microorganism: Microbial population density(ies) that might occur in different environmental compartments in natural conditions and/or in other uses/situations such as food and feed (e.g. via plant edible parts), under conditions conducive to growth of the microorganism in question (e.g. presence of a host, availability of carbon and nutrient sources, etc).

"Natural" background level(s) of a metabolite: Level(s) of the metabolite that might occur in the different environmental compartment in natural conditions and/or in food and feed (e.g. via plant edible parts) under conditions conducive to growth for microorganisms known to produce the metabolite in question (e.g. presence of a host, availability of carbon and nutrient sources, etc).

***In situ* production**: The production of metabolites by the microorganism after application of the plant protection product containing the microorganism.

Antagonistic interaction: Interaction between a microorganism that suppress or interfere the normal growth and activity of another microorganism. Microorganisms demonstrating such antagonistic interaction can be used for pest control and may be predators, parasites, parasitoids, or pathogens that attack harmful insect, weed or plant disease or any other organism in its vicinity.

Future developments of this guidance document

The present guidance document may be further developed to comply with evolution of science and

[3] Commission Delegated Regulation (EU) 2021/1760 of 26 May 2021 supplementing Regulation (EU) 2019/6 of the European Parliament and of the Council by establishing the criteria for the designation of antimicrobials to be reserved for the treatment of certain infections in humans (OJ L 353, 6.10.2021, p. 1).
[4] Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC (OJ L 4, 7. 1.2019, p.43).
[5] Commission Implementing Regulation (EU) 2022/1255 of 19 July 2022 designating antimicrobials or groups of antimicrobials reserved for treatment of certain infections in humans, in accordance with Regulation (EU) 2019/6 of the European Parliament and of the Council (OJ L 191, 20.7.2022, p. 58).
[6] https://www.who.int/publications/i/item/9789241515528

increasing experience of the EU risk assessors and managers. Firstly, knowledge of microorganisms and microbial metabolites is expected to develop, resulting in the need to reflect such evolution in this document. New scientific and technical approaches supporting the risk assessment of metabolites would need to be incorporated. Moreover, the actual use of microbial plant protection products, and the number of applications concerning microbial active substances are increasing the experience of the EU risk assessors and managers which may trigger a revision of the guidance document.

Stepwise approach

Stage 1: Determine the type of assessment necessary for the metabolites of the active substance

Step 1: Role of metabolites in the mode of action of the microorganism.

Question 1.1: Is the principal mode of action of the active substance based on the presence of metabolites in the formulated plant protection product?

The purpose of step 1 is to determine whether the metabolite is the actual active substance present in the product, requiring a dossier in accordance with Part A of Reg 283/2013 (i.e. the data requirements for chemical active substances) in addition to the dossier for the microorganism. Information on the mode of action of the microorganism is important to identify metabolite(s) of potential concern. Therefore, the mode of action must be described in the dossier with as much detail as technically feasible and be backed up by scientific evidence so as to determine whether the identified metabolite is clearly involved in the mode of action (i.e. current data requirement outlined in Reg 283/2013 Part B, section 2.2.2). The information on the mode of action of the microorganism will indicate whether this case applies.

Specific cases where data in accordance with data requirements for chemicals may be required.

In the Introduction section to the data requirement for microorganisms, paragraph (viii), reference is made to two specific situations:

- *"If the plant protection action is known to be due to the residual effect of a toxin/metabolite, or*
- *If significant residues of toxins/metabolites are to be expected and not related to the effect of the active substance […]"*

If the mode of action is based on the residual effect of metabolites present in the product, meaning the application of the isolated metabolite would result in the same plant protection properties and can be regarded as the active substance, a dossier in accordance with the data requirements for chemicals (**Part A** of Reg 283/2013) is required. An example for which these conditions apply are products containing inactivated microorganisms and their microbial metabolites[7].

The "residual effect" of a toxin/metabolite is to be read in the sense of the conditions defined in Reg 283/2013, Part B, section 7 "Fate and behaviour "point (iv) of the introduction.

The conditions are: "*the relevant metabolite is stable outside the microorganism, its hazardous effect is independent of the presence of the microorganism, it is expected to occur in the environment in concentrations considerably higher than under natural conditions*". All must be met for Part A information to be required. The information on the mode of action will indicate whether this case applies.

When it is not clear whether Part A information is required in the dossier, the applicant should discuss this with the Rapporteur Member State (RMS) and EFSA, preferably in a request for a meeting or a pre-submission meeting.

[7] Meaning non purified metabolites, as they would then be considered as chemicals and be subject to part A of data requirement (ex. Spinosad)

For all microorganisms whose **mode of action is not based on secondary metabolites** present in the product, the assessment of the microorganism itself will cover the assessment of the metabolite of potential concern, therefore **proceed to step 2**.

Step 2: Exclusion of metabolite production.

Question 2.1: Is the microorganism a virus?

As according to the current scientific knowledge no indication exists that **viruses** produce metabolites of potential concern, **no further assessment is needed.**

For all other microorganisms: **proceed to stage 2**.

Stage 2: Collecting a basic set of information on metabolites

In Stage 2 different sources and data sets are combined to determine which metabolites may be produced by the strain under assessment and by closely related strains and to collect information on the hazard of these metabolites (i.e., toxicity or antimicrobial activity). Any metabolite(s) responsible for any observed toxic effect(s) should be identified. As part of Stage 2 three separate lists are prepared:

- Identified metabolites for both the strain itself and related strains (with their hazardous effects).
- Hazardous effects observed in (eco-)toxicology studies performed with the microorganism that may be caused by metabolites.
- (Eco)toxicology studies conducted with no effect observed.

Any metabolite(s) responsible for the observed hazardous effects in these (eco-)toxicology studies referred above should be identified. A separate literature search should be performed for all identified metabolites on these first lists. Subsequently, the two first lists referred above are merged to render a list of toxic metabolites of potential concern (including relevant antimicrobial agents). The information from the third list is relevant in the weight of evidence approach to carry out the risk assessment, in particular when the species is known to produce metabolites for which there is information of toxicity.

A first batch of information on the production and/or relevance of metabolites may come from **literature** in accordance with the EFSA guidance document[8]. While experimental data will provide strain-specific data, literature data may provide information on higher taxonomic levels (e.g., species). The relevance of information of not strain-specific data on the production of metabolites of potential concern for the evaluation of the microorganism, can for example be addressed by providing **genomic data** to be compared with information of relevant databases. In this way it can be determined if the microorganism contains the genetic information to produce a specific metabolite of potential concern.

In Stage 2, **Steps 3 – 5** described below are **mandatory while steps 6-8 are conditional on the outcome of step 5**.

[8] EFSA Guidance on submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2011.2092

Step 9 is an **optional** step while **Step 10** is a **conditional** step depending on the result of step 5.

Further details are provided below and explained in Annex I.

Step 3: Data from literature search performed for the microorganism and closely related strains.

Collecting information regarding the production of hazardous metabolites and metabolites with antimicrobial activity

A literature search for the active substance (i.e., the microorganism) is required (Article 8(5) of Regulation (EC) No 1107/2009) and should be performed in accordance with the EFSA guidance on the submission of peer-reviewed open literature (EFSA Journal 2011; 9(2); 2092). Literature retrieved from this search should be reported and included in the dossier.

For the literature search it is important to:

- Take into account previous taxonomic names.
- Justify the choice of taxonomic unit used in the search terms (e.g. genus, species).
- Use of the information on literature searches commissioned by EFSA (see for example EFSA external scientific reports by Mudgal et al. (2013) and by Hackl et al. (2015).

Question 3.1: Are metabolites identified for the strain under evaluation in the published literature?

All metabolites which are identified in literature for the strain under assessment should be listed.

Question 3.2: Are there any indications in the published literature that closely related microorganisms are known to produce metabolites of potential concern?

All metabolites of potential concern which are identified in the literature for closely related strains should be listed. The strains for which the information was retrieved should be indicated.

At this point it is not necessary to determine if the metabolites are in fact produced by the strain under assessment.

Question 3.3: Are hazardous effects identified for the strain under evaluation in the published literature?

All hazardous and antimicrobial effects which are identified in the literature for the strain under assessment should be listed.

Question 3.4: Are hazardous effects identified for closely related strains in the published literature?

All hazardous and antimicrobial effects which are identified in the literature for closely related strains (e.g. belonging to the same species) or species (e.g. belonging to the same genus or clade within a diverse genus) should be listed. The strains for which the information was retrieved should be indicated. At this point it is not necessary to determine if the metabolites are in fact produced by the strain under assessment.

Question 3.5: Are there any metabolites described to be produced by the strain under assessment, or closely related strains, without toxicological information available?

The metabolites that are described to be produced by the strain under evaluation, or closely related strains, without information about their toxicological properties or potential concern available, should be added to the list of metabolites of potential concern for further assessment.

Question 3.6: Are there any studies reporting no effect observed for the strain under evaluation or metabolite of potential concern?

Eco-toxicology studies without toxicological or antimicrobial effects observed for the metabolite of concern or the microorganism can be useful to evaluate the impact of the hazardous effect described for the metabolite and provide useful information about the conditions necessary to provoke a hazardous reaction.

Actions resulting from Step 3:

Step 3 shall result in a list of all identified metabolites and hazardous/antimicrobial effects of both identified and unknown metabolites resulting from questions 3.1 – 3.5 in an overview table (see Annex III) and indicate whether this metabolite or hazardous effect was identified for the strain under evaluation or for closely related strains.

Step 4: Literature data for the metabolites identified in Step 3.

As part of this step, an additional literature search shall be performed for each identified metabolite as listed in the overview table.

For all metabolites listed in the overview table it should be determined whether there is an indication of hazardous or antimicrobial effect(s). To do so, an additional literature search should be performed for each listed metabolite. Note that at this point the objective is to determine if there is an indication for antimicrobial activity or hazardousness and if so, which type(s) of hazard and if reported the route of exposure leading to the hazard and the affected organism(s). At this point, it is not necessary to collect further information (e.g., physical-chemical properties, degradation rate) for the metabolite.

It is essential to list all types of hazards of the metabolite (e.g. toxic, mutagenic, genotoxic, carcinogenic, toxic for the reproduction, neurotoxic, or antimicrobial activity) and all affected organisms, as the type(s) of hazards to be observed for the metabolite and the organisms to which the metabolite is hazardous will orientate further assessment concerning the metabolite.

The search terms used for these additional literature searches should include the name of the metabolite and its synonyms (e.g., CAS numbers). As indicated in the EFSA guidance on the submission of scientific peer-reviewed open literature (EFSA Journal 2011;9(2):2092), synonyms may be identified based on the literature search; thus, the process of developing a search strategy may be iterative.

Search terms which may be used (but which are not limited to) to retrieve information on hazardous and antimicrobial activity are:

- tox*, mutagen*, genotox*, carc*, onco*, neuro*, Antimicrobial, Antibiotic, aneugen*

Question 4.1: For each metabolite, is there any indication of hazardous effects in the published literature?

To address step 4, a literature search shall be performed for all the metabolites identified as of potential concern, in compliance to the section 2.8 of the Commission Regulation (EU) No 283/2013. In particular the following aspects would need to be addressed:

- Any available information about the conditions under which the microorganism produces the metabolite
- Any available information about the expected quantities and the LOQ of the method used to determine/quantify the metabolite
- Any available information on the mechanism by which the microorganism regulates the production of the metabolite shall be provided
- Any available information on the influence of the produced metabolites on the microorganism's mode of action shall be provided
- Search terms (e.g. CAS numbers, excluding species name) applied in the research shall be provided

Actions resulting from Step 4:

The applicant and the risk assessor shall include the results of additional literature search in the overview table.

Step 5: Literature data. Is the genus of the strain under evaluation well studied?

Question 5.1: Is there enough published literature to assume that a literature search would provide sufficient information on metabolite production?

The body of knowledge of the strain and/or closely related strains or species should be assessed to determine if sufficient information is available to screen which metabolites of potential concern can be produced by the microorganism. The body of knowledge includes the history of safe use, the ecology of a microorganism in the agro-food chain or in other sectors, the scientific literature, clinical observations and reports (e.g. like infections in immunocompromised people where the microorganism has been isolated), industrial and/or medicinal applications, and other factors as considered appropriate. As a matter of principle, it is highly recommended to the applicant to conduct the search beyond the normally requested period of 10 years before the application, in order to gather all the possible relevant scientific literature to support the risk assessment.

Actions resulting from Step 5:

The applicant and the risk assessor shall state whether they consider that the literature search has been duly carried out in accordance with the EFSA guidance document on submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (see footnote 7) and draw conclusions on the body of knowledge regarding the metabolite production by the strain under evaluation.

In case it should not be the case, the assessment shall be pursued according to steps 6 to 8 and eventually also be addressing step 10 where relevant.

Otherwise the assessment stops at this step, if no toxic effects have been found in the literature.

Step 6: experimental (eco-)toxicology data with the microorganism

Question 6.1: Are there any hazardous effects in the available toxicity or eco-toxicity studies that indicate that the strain under evaluation produces metabolite(s) of concern?

The toxicity and eco-toxicity studies performed on the MPCA-AM in accordance with section 5 and 8 of the data requirements for microorganisms may give indications of toxicity due to metabolites. According to the US-EPA test guidelines[9], the tiered toxicity studies on the MPCA-AM are designed to detect mainly acute toxic effects of biological or non-biological components of a MPCA-AM (and those produced in the test animal during the test). According to the test guidelines, a control group treated with the inactivated[10,11] microorganism (considering also the thermo-stability of toxins) may prove useful to evaluate hazardous properties of the metabolites. If hazardous effects are observed in these studies, in the absence of signs of infectivity or pathogenicity, this is an indication of the presence of metabolites of concern.

The detection of acute or -if tested- sub-lethal toxic effects of biological or non-biological components of a MPCA-AM (and those produced in the non-target organisms during the test) may give indication of hazardous effects due to metabolites. According to the test guidelines, a control group of non-target organisms treated with the inactivated microorganism (considering also the thermos-stability of toxins) may prove useful to evaluate (eco-)toxic properties of the metabolite(s). The metabolite(s) responsible for the effect need(s) to be identified and further studied (see Question 6.2 and Step 8). Information on the possible analytical methods for identification can for example be elaborated in accordance with the OECD working document on the risk assessment of secondary metabolites of microbial biocontrol agents (OECD No. 98, 2018)[12]).

Question 6.2: Is the identity of the metabolite which is responsible for the effects observed in the toxicity or eco-toxicity studies known?

It is of major importance to determine the identity of the metabolite which is considered as responsible for the effects observed in the studies screened under step 6, in order to relate its chemical structure to other compounds for which more information on hazardous or eco-toxic effects would be available for the risk assessment of the metabolite of concern. The information on identity would eventually help setting residue levels, where necessary.

Actions resulting from Step 6:

All identified metabolites and their respective hazardous effects (if known) shall be listed. In case the identity of the metabolite(s) is unknown the assessment shall **proceed to step 7** and following for the identification of the metabolite of potential concern. In case it can be established that the strain under evaluation produces metabolite(s) of concern this shall be listed in the overview table and proceed with the next steps.

Step 7*:* Experimental data – chemical analysis

Question 7.1: Is analytical chemistry data available which indicates that the strain under evaluation

[9] See: https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-885-microbial-pesticide- test-guidelines

[10] For the inactivation of microorganisms and their endospores, sterilization by autoclave system at pressure of 1 atm, temperature 121ºC, for 20 min can be recommended. For thermostable endotoxins inactivation can be made following the same process during 3 consecutive days. In addition a sterile filtrate of the TGAI as an additional control group can be tested to detect potential effects of metabolites to avoid degradation of metabolites during the sterilisation phase.

[11] US-EPA test guidelines refers to inactivated microorganisms with the meaning of "rendered incapable of reproduction or germination"

[12] http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2018)33&doclanguage=en

produces metabolites of potential concern?

Available databases are reporting the identity of metabolites typically produced by microbial species. The presence in the MPCA-AM/product of these metabolites of potential concern can be tested with various analytical methods. Analytical methods from various levels of complexity can be used, e.g. starting from a simple plate assay up to isolation and purification of liquid cultures or crude extracts and then analytical characterization by various analytical techniques (HPLC, Gas Chromatography combined with mass spectrometry, or any other combinations) as described in chapter 8 and 9 of the OECD working document on the risk assessment of secondary metabolites of microbial biocontrol agents (OECD No. 98, 2018).

Actions resulting from Step 7:

Based on this analytical screening and identification data, the list of all identified metabolites will be included in the overview table.

If the data on identity are not available **proceed to Step 8**.

Step 8: Identification of unidentified metabolites with hazardous effects

When hazardous effects are reported in published literature (Step 3, questions 3.3 and 3.4) for which the responsible metabolites are not known, a conclusion on the relevance of this toxic effect for the strain under evaluation should be presented and the overview table updated. This conclusion may for example be based on the phylogenetic relationship between the strain under evaluation and the microorganism for which the effect was observed, or on the likelihood that the observed effect is caused by metabolites which are already identified for the microorganism under evaluation.

If the observed hazardous effects are not likely caused by known metabolites, all unknown metabolites with hazardous effects shall be identified. Possible analytical methods to identify the responsible metabolites can for example be found in chapter 9 of the OECD working document on the risk assessment of secondary metabolites of microbial biocontrol agents (OECD No. 98, 2018).

When the (geno-)toxicity studies show hazardous effects, information from the chemical analysis shall be compared with quantitative structure-activity relationship (QSAR) models, read-across, or interrogation of whole genome sequencing to identify which metabolite(s) is causing the effects observed.

When the metabolite responsible for the toxic effect has been identified, a further literature search is necessary to determine whether the metabolite conveys more types of hazards and whether there are other organisms affected by it.

Step 9: Genomic analysis (for metabolites identified due to published literature)

Genomic analysis is strongly recommended and should be seen as an opportunity to clearly clarify the (potential) production and presence for all metabolites of potential concern. This step may for example be used for microorganisms for which a toxic metabolite is known to be produced by closely related

strains, however it has not been determined if the strain under evaluation is capable of producing this metabolite. Genomic analysis may be used to determine if the strain under evaluation contains the genetic information required for the production of the metabolite. However even if a strain contains the genetic information, it cannot be necessarily concluded that genes potentially involved in metabolite production are actually expressed and that the metabolite is produced, unless other omics analyses are provided by the applicant, such as metabolomics and transcriptomics data.

Question 9.1: If the gene(s) encoding the metabolite is known, does a genomic analysis show absence of the gene?

The applicant can address this question by demonstrating that the strain under evaluation does not or is not able to produce the identified metabolites, e.g. by providing genomic data to prove absence or non-functionality or lack of expression of the associated gene(s).

Genomic data provided in accordance with the guidance currently under elaboration by EFSA[13] can be used as outlined in the OECD working document[14] on the risk assessment of secondary metabolites of microbial biocontrol agents. Additional information and methods are given in chapter 9 of this OECD Working Document, which can be used to obtain data on the presence of known genes or gene clusters associated with metabolites and their expression.

Absence of the gene encoding the metabolite of potential concern would exclude the production of such a metabolite by the microbial strain under evaluation, hence no further assessment would be required for this metabolite.

Even when submitting applications for well-studied microorganisms (see Step 5), genomic analysis can be an efficient method to exclude metabolites from further assessment.

Actions resulting from Step 9:

When this genomic analysis is available, the absence of gene(s) encoding the metabolite of potential concern shall allow to conclude that no further assessment is necessary for this particular metabolite.

This result shall be included in the overview table and one can **proceed to Step 10** (where applicable) or **Step 11**.

Collecting additional data for microorganisms for which the body of knowledge is not sufficient (conditional)

Step 10: Collecting information regarding the production of hazardous metabolites / metabolites with antimicrobial activity – or less well known microorganisms

Step 10 is a **conditional** step. Whether or not this step is required, depends on the available body of knowledge for a microorganism (see Step 5). For microorganisms for which the available information is not considered sufficient, the inclusion of Step 10 allows for the collection of sufficient information in Stage 2 to be able to conclude on the set of metabolites of potential concern which are present in the MPCA-AM and which should be addressed in the assessment.

[13] EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain: https://www.efsa.europa.eu/sites/default/files/consultation/consultation/consultation_EFSA-Statement-WGS-microorganisms.pdf

[14] http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2018)33&doclanguage=en

Question 10.1: Is there any indication for (geno-)toxicity in tests with crude extracts?

Information on genotoxicity of metabolites is often more relevant than acute toxicity, because of the usually low levels of production and/or exposure, which may still lead to harmful effects. However, since microorganisms may produce a large variety of metabolites of potential concern, testing of a crude extract[15].(i.e. the chemical constituents of the MPCA-AM with cell walls removed) or a cell-free filtrate, or cells grown in dual cultures or in microbial consortia[16] could be considered, if more appropriate. In such a test, the study design needs to be carefully considered as the concentrations of each component can be expected to be low and a component with genotoxic potential is present at such low concentration it would not be detected in the test.

When performing genotoxicity studies with a crude extract it is necessary to avoid interference by constituents in the test samples (examples by OECD, 2005):

- interference from toxic components;
- provision of nutrients by lysates (e.g. histidine, which would allow growth of the auxotrophic tester strains in the Ames Salmonella assay);
- growth factors that may produce abnormal growth, growth inhibition, or DNA synthesis (e.g. erythropoietin which causes micronuclei in bone marrow via induction of abnormal cell proliferation; lectins that may stimulate DNA synthesis in *in vitro* mammalian cell tests);
- enzymatic activity that could mimic endogenous activity in the test organism (e.g., kinase or phosphokinase activity in the TK+/- or HPRT assays);
- the occurrence of potentially active constituents as bound or complexed forms (e.g. as glycosides or other conjugates, or bound to macromolecular constituents);
- intracellular molecules with nuclease or proteolytic activity from *in vitro* lysates that would not normally have access to mammalian cells in vivo.

It is important that a flexible approach is adopted with modifications of standard test protocols as necessary to adjust for the factors listed above. Selection of the methodology for further testing depends on how the results are interpreted at each stage. This is further described in the OECD 2015 report and in the Statement[17] of the EFSA Scientific Committee (2018).

Question 10.2: Are there any genes for known toxins or relevant antimicrobial agents present in the genome?

Technologies of DNA sequencing and bioinformatics enable the screening of genomic data for physiological traits encoded in a genome of interest. The so-called “whole-genome sequencing” (WGS) is an essential tool that may prove helpful in the detection of genetic determinants for hazardous metabolites and toxins, relevant antimicrobial agents as well as antimicrobial resistance. Both chromosomal and extrachromosomal genetic elements such as plasmids must be included in the whole genome sequencing. It should be noted that antimicrobial resistance genes and their encoded

[15] Testing of crude extracts of the TGAI shall be considered with care, in case it is constituted of spores, as the cells would need to grow out before they produce metabolites. It is therefore recommended to first grow the cells and then harvest them and produce the extract.

[16] Testing an extract from cells of the microorganism under evaluation grown in dual cultures or microbial consortia shall be considered with care as it could include metabolites produced by the other microorganisms and no distinction could be made eventually.

[17] See: www.oecd.org/chemicalsafety/testing/Genetic%20Toxicology%20Guidance%20Document%20Aug%2031%202015.pdf

proteins are out of scope of this guidance document on metabolites of concern. This issue is addressed in a separate guidance document[18].

WGS should be interrogated for the presence of genetic material coding for known toxins or relevant antimicrobial agents.

For this purpose, a comparison against at least two up-to-date and curated international databases (e.g. CARD[19], ARG-ANNOT[20], ResFinder[21], Norine[22], Accelrys[23]) should be performed. The outcome of the analysis should be presented as a table focusing on complete genes coding hazardous metabolites/toxins and relevant antimicrobial agents. The table should include at least the gene identification, the function of the encoded protein, the percentage of identity and the e-value.

List of toxins of concern such as mycotoxins, phytotoxins, relevant antimicrobial agents and their producing strains can for example be found in the OECD working document on the risk assessment of secondary metabolites of microbial biocontrol agents.

Actions resulting from Step 10:

Based on the results of the tests carried out with crude extracts under the strict conditions described above, and on the screening of genomic data (WGS), the identified metabolite(s) responsible for the hazardous or antimicrobial effects shall be reported in the overview table.

The assessment can **proceed to Step 11**.

Based on the analysis of the genomic data available and by comparison with the relevant databases any 'hit' for a gene coding for hazardous metabolites or referenced toxins for the given genus shall allow the applicant and the risk assessor to identify a toxin or a hazardous metabolite and report it in the overview table. The assessment can **proceed to Step 11**.

If none of the tests referred under this section are 'positive' for the known toxins or antimicrobials produced by the species to which the strain under evaluation belongs, no further assessment is necessary.

Outcome of Stage 2: metabolites of potential concern

Step 11: Metabolites of potential concern based on literature and experimental data for active substance.

The metabolites included at this stage in the overview table are the metabolites of potential concern. They have been included in the overview table based on the responses in Stage 2.

If no metabolites of potential concern have been identified (i.e., Steps 3 – 7 all green (see decision tree in annex II) or genomic analysis shows absence of gene(s) for metabolite(s) identified in the published literature), no further assessment is necessary. In this case the body of knowledge submitted in the dossier is considered sufficiently robust and exhaustive, with no indication on production of

[18] SANTE/2020/12260
[19] https://card.mcmaster.ca/
[20] http://en.mediterranee-infection.com/article.php?laref=283%26titre=arg-annot
[21] https://cge.cbs.dtu.dk/services/ResFinder/
[22] http://bioinfo.lifl.fr/norine/
[23] http://accelrys.com/products/databases/database-access/index.html

metabolites of potential concern for the investigated strain and closely related microorganisms.

The results of the additional literature search will be included in the overview table.

Stage 3: Determine which metabolites are of concern

This stage will address the risk arising from hazards identified in the previous stage.

The expected deliverable would consist in an updated overview table, in which the metabolites of potential concern would be categorised either as being of no concern or as being of concern for the risk assessment.

To determine if a metabolite is of actual concern (i.e., a 'relevant metabolite' according to the data requirements), the exposure to the metabolite needs to be determined. As exposure depends both on the metabolites present in the MPCA-AM and on *in situ* production, it may be challenging to determine the actual level of exposure.

Step 12: Determine if a metabolite of potential concern is actually produced by the strain

Question 12.1: Can it be adequately justified that the strain does not produce the metabolite (e.g. using WGS data)?

In the previous steps, the use of genomic data may have helped to prove absence, non-functionality, or lack of expression of the genes associated to the metabolite, hence evidencing that the strain does not produce a metabolite of potential concern. However, if other strains of the same species are known to be producing a toxic metabolite under specific conditions, one could test this strain under evaluation under the same specific conditions and determine if the metabolite is actually produced.

Actions resulting from Step 12:

When it can be evidenced that the strain under evaluation is not producing the metabolite of concern under the conditions known to be triggering this production by other strains of the same species, it can be concluded that no further assessment is necessary.

Should that not be the case the assessment shall **proceed to Step 13**.

Step 13: Determine exposure routes of the metabolite

Exposure to metabolites of potential concern can come from two sources: the microbial PPP and metabolites produced by the microorganism after application (*in situ* production). Each of these sources needs to be addressed. The exposure route for humans or the relevant non-target organism to the metabolite of potential concern should be determined and described. Based on this exposure route, it can be concluded whether or not there is an actual risk linked to the metabolite or if it would be of no concern; the relevant environmental compartments and/or the edible parts of crops can also be identified by this qualitative exposure assessment.

The data requirements for microorganisms state in section 6 "Residues in or on treated products, food and feed", Introduction point (iii) that "*for the evaluation of risk arising from residues, experimental data on levels of exposure to the residue may not be required where it can be justified that the micro-organism and its metabolites are not hazardous to humans in the concentrations that could occur as a result of authorised use. This justification can be based on open literature, on practical experience and on information submitted.*" It is emphasised that if applicants do not generate specific residues trials when residues of metabolites of concern could potentially be found in edible crops then the case for not doing so must be robustly clarified. In addition, information on the magnitude of expected residues shall be provided together with a sufficiently validated method of analysis with a reported LOQ.

Question 13.1: Can it be adequately justified that in situ production of the metabolite is not relevant for the risk assessment?

In situ production of metabolites refers to the production of metabolites by the microorganisms after application. *In situ* production may be relevant only for hazardous metabolites; *in situ* production of antimicrobial metabolites is not considered as a foreseeable risk to human and animal health.

The *in situ* production of many metabolites of potential concern is in general not expected to be relevant, because their production is limited due to energy/resources constraints and therefore triggered only under certain conditions and rapidly decreasing under environmental and agricultural conditions. Furthermore, metabolites produced *in situ* normally break down rapidly under these conditions Therefore, at least for consumers the expected exposure should in general be very low. However, this conclusion about the *in situ* production of the specific metabolite should be adequately justified.

The relevance of *in situ* production of a certain metabolite should be assessed for each group of non-target organisms for which a hazard is identified due to the metabolite. As a result of this approach, the environmental compartments can be pinpointed by which non-target organisms may be exposed to the metabolite due to *in situ* production. Information on the proposed use, the ecology of the microorganism including the environmental conditions which trigger the production of the metabolite, and the properties of the metabolite may be used. Several examples are provided to illustrate the process:

- If a hazard has been identified due to toxicity to birds and the microorganism is applied in protected crops, no exposure to birds is assumed and *in situ* exposure can be adequately justified to be not relevant for birds.
- If a hazard has been identified due to toxicity to aquatic organisms and the microorganism is applied as a foliar spray in field crops, exposure of aquatic organisms to the microorganism will occur. The relevant environmental compartment in which *in situ* production can lead to exposure of aquatic organisms is the aquatic system itself. Information on the natural habitat, the ecology of the microorganisms and on the prerequisites for the production of the metabolite may be used to assess the relevance of *in situ* production. When the microorganism is for example known to only produce the metabolite during direct interaction with its insect host and this host is strictly terrestrial, the *in situ* production in the aquatic system can be justified to be not relevant for the assessment.
- When a hazard has been identified for humans and the microorganism is used in edible crops, consumers and workers[24] may be exposed. With regard to consumer exposure, the edible parts may contain *in situ* produced metabolites either due to dispersal of the microorganism to the edible parts where the metabolite may be produced (either in- or outside of the plant), or by diffusion of the metabolite upon *in situ* production in another part of the plant. Available information may be used on the capacity of the microorganism to grow endophytically or in the phyllosphere and on the environmental conditions which trigger metabolite production to justify that the microorganism does not grow on edible parts and/or that metabolites are not expected to be present on edible parts. Moreover, available information about the

[24] Also relevant for workers, when applied on non edible crops

persistence of the metabolite and toxicological reference values should be used to assess the relevance of the *in situ* production of the metabolite.

All justifications on the relevance of *in situ* production should be substantiated with reliable studies or literature references included in the dossier. Please note that when based on available information it cannot be concluded that *in situ* production is not relevant for the risk assessment, this does not mean that *in situ* production is necessarily relevant. However, as the relevance of *in situ* production cannot be excluded at this stage, more information on the exposure and toxicity of the metabolite is required to perform a risk assessment (see Step 14 and Stage 4).

Question 13.2: Is the metabolite present in the product?

Metabolites of potential concern present in the microbial PPP can be measured and quantified, where technically possible. A chemical analysis of the presence of metabolites in material taken from an appropriate and justified point in the production process should be performed.

Actions resulting from Step 13:

When it can be duly justified that the *in situ* production of the metabolite is not relevant for the risk assessment and when it has been proven by analytical search that the metabolite is not present in the plant protection product, it can be concluded that no further assessment is necessary for this metabolite.

Should that not be the case for one or the other assessment under this step, the assessment shall further **proceed to Step 14**.

Step 14: Determine if a metabolite of potential concern is of actual concern for the risk assessment

The presence of detectable amounts of relevant antimicrobial agents in the formulated product is considered to pose an unacceptable risk to human and animal health, unless it is demonstrated that the expected concentrations in environmental compartments are below the LOQ under realistic conditions of use.

Question 14.1: Does a qualitative assessment demonstrate that the exposure of humans or the relevant non-target organisms to the metabolite is of no concern?

A qualitative or semi-quantitative assessment should be based on information regarding the exposure to the metabolite as well as its toxicological properties. Both the exposure resulting from the presence of the metabolite in the product and from *in situ* production should be addressed.

In certain cases it may be concluded that exposure is only theoretical. This should be fully justified, e.g. by considering the biology of the microorganism and its requirements (nutrients, energy, translocation, presence of the host and/or the plant pest) and the properties of the metabolite (e.g., persistence). In addition, the natural exposure level to the metabolite or the exposure to the microorganism producing the metabolite (e.g., the microbial species to which the strain under application belongs) may be taken into account.

Qualitative assessment for metabolites in the product when the *in situ* production is not relevant

Based on the concentration present in the microbial PPP (where relevant, calculated from the analysis of the MPCA-AM), exposure to metabolites of concern for operators, workers, bystanders, and residents needs to be determined using the appropriate model[25].

The exposure to the metabolite for operators, workers, bystanders, and residents needs to be determined in accordance with the applicable guidance documents for chemical substances.

To address hazards arising from human dietary exposure, a worst-case theoretical estimate of the residues can be made by assuming that the entire amount of the metabolite of concern in the product applied will end up on the edible parts. With data on crop yields[26] a theoretical estimate of the residues can be calculated, by taking the lowest mean crop yield for the EU in the last five years (a low level of crop yield from a possible range should be used to give a worst case estimate of the residue, since the aim should be to assess the highest likely residues that could arise following the intended use). Together with the application rate (CFU/kg/ha) and the metabolite concentration (in mg/ha), the maximal residue of the metabolite in microgram/kg crop can be calculated. With this worst case approach dietary uptake from a given crop can be compared with the TTC value[27].

The expected consumer exposure to these residues can be estimated using EFSA Pesticide Residue Intake Model (PRIMo) and this can then be compared with health-based reference values (Acceptable Daily Intake (ADI), Acute Reference Dose (ARfD), when available – see step 17), natural exposure levels or considered in relation to the Threshold of Toxicological Concern (TTC) when no reference values are available (see specific EFSA opinion[28]).

To determine Predicted Environmental Concentrations (PEC) in relevant environmental compartments for relevant non-target organisms (i.e., non-target organisms for which a hazard is identified), the pesticide fate models developed for chemical active substances can be used (see FOCUS DG SANTE[29]). When physical-chemical parameters needed as input for these models are not available, conservative default values should be used as prescribed by the respective guidance documents.

All assumptions made for the calculations must be reported and justified.

Qualitative assessment for metabolites produced *in situ*

For the assessment of the *in situ* production of metabolites the EFSA Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products does not provide any guidance.

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[25] EFSA (European Food Safety Authority), 2014. Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products. EFSA Journal 2014;12(10):3874, 55 pp., (doi:10.2903/j.efsa.2014.3874)

[26] Can be found at EUROSTAT

[27] TTC is based on the assumption that all the routes of exposure are identified and quantified and that the exposure can be clearly estimated whereas the structure of the metabolite has to be identified and some toxicological information shall be available to determine the Cramer class. For further information the applicant is invited to discuss the usefulness of this approach with the rapporteur Member States and EFSA.

[28] Guidance on the establishment of the residue definition for dietary risk assessment. EFSA Journal 2016;14(12):4549, 129 pp. (doi: 10.2093/j.efsa.2016.4549)

[29] https://esdac.jrc.ec.europa.eu/projects/focus-dg-sante

In general, when a concern exists for concentration of the metabolite expected to be present due to the *in situ* production and it is assumed that these concentrations are above the expected natural background level, the performance of field trials to measure the concentration of the metabolite of potential concern shall be carefully checked. Moreover, the decrease of the respective metabolite levels over time can be addressed in these experiments.

When exposure cannot be excluded, an alternative approach may be to use the concentration of metabolite formed under production promoting conditions which can be used as a maximum for metabolite production. Examples of such conditions favourable for metabolite production may be laboratory conditions which can be justified to be conditions which maximize the formation of the metabolite (e.g., nutrient-rich medium), or during interaction of the microorganisms with the target or host organism. Information on population dynamics of the microorganism in the relevant environmental compartment can then be used to infer information on the maximal metabolite production in the relevant environmental compartment upon application and to which the relevant non-target organisms (i.e., the organism for which the hazard is identified) might be exposed.

Natural exposure levels

Available information on concentrations under natural (untreated) conditions and upon application can be compared to assess if the estimated exposure due to the use of the product is significantly higher than natural exposure situation. Natural exposure level refers to the level of the metabolite present in the environment relevant for the respective environmental compartment taking into account in what way exposure levels have been altered (e.g. agriculture). When the microorganism is ubiquitous in the environment, a certain background level of metabolites of concern may be present. The metabolite(s) of concern may also be produced by other microorganisms naturally present on food crops. Such data (including appropriate investigations with validated analytical methods) can be used to provide the justification why the metabolite is considered not to be of concern for the risk assessment[30] (see section 6 (iii) of the data requirements for microorganisms). It should be noted that arguments relating to natural exposure should be used and considered carefully. For example, whilst the microorganism producing metabolites of concern may occur in the terrestrial environment, it may not occur in the aquatic environment. However, due to the use of the plant protection product other environmental compartments may be exposed and this may result in further information being required.

Toxicological endpoints

The relevant toxicological threshold to which the expected exposure is compared, depends on the non-target organism for which the hazard is identified. Reference values available from food or feed safety evaluations shall be considered if available and relevant for the non-target organism for which the risk assessment is performed. Bridging and/or read across within chemical groups shall be adequately justified. Comparison of exposure to the metabolites of concern in relation to the Threshold of Toxicological Concern (TTC) values may be conducted, if feasible. Guidance on the applicability of the TTC concept can be found in publications by EFSA[31].

[30] Such approach cannot be used if newly produced studies show e.g. genotoxicity, as, for instance, not all naturally occurring metabolites are non-toxic or have no impact on human health.

[31] EFSA (2012) Scientific Opinion on Exploring options for providing advice about possible human health risks based on the concept of Threshold of Toxicological Concern (TTC); EFSA Journal 2012, 10 (7):2750
+ EFSA (2016) Review of the Threshold of Toxicological Concern (TTC) approach and development of new TTC decision tree.

Actions resulting from Step 14:

When it can be duly established via a qualitative assessment or semi-quantitative assessment that exposure of humans or relevant non-target organisms to the metabolite is of no concern, it can be concluded that no further assessment is necessary for this metabolite.

Should that not be the case for one or the other assessment under this step, the assessment shall further **proceed to Step 15** as the metabolite can be considered of concern and reported as such in the overview table.

Outcome of Stage 3: metabolites of concern

Step 15: Metabolites of concern.

All potential metabolites of concern identified in previous stages will be reported in the updated overview table, including the relevance of their respective *in situ* production (question 13.1) and the confirmation of their presence in the microbial PPP (question 13.2), qualifying the latter as of concern for the risk assessment to proceed under Stage 4.

Stage 4: Risk assessment for metabolites of concern (i.e., relevant metabolites)

The risk assessment is necessary for all metabolites for which:

- The exposure assessment could not show the non-relevance for humans and non-target organisms.
- The toxicological effects described are considered relevant for the proposed uses.

All identified types of toxicity (e.g., acute toxicity, genotoxicity) for humans and relevant non-target organisms (i.e., for which the hazard was identified) need to be addressed in the risk assessment.

For the risk assessment the relevant standard approaches outlined in the regulations and guidance documents for microorganisms and for chemical active substances should in principle be followed. Where these approaches are not appropriate or technically feasible, the information provided below could be considered.

Exposure assessment

The exposure route for the relevant non-target organism (i.e., for which the hazard is identified) to the metabolite should be determined and described (see Step 13). Based on the exposure route, the relevant environmental compartments and/or the edible parts of crops can be identified.

> Example 1: Supposed that a hazard to bees has been identified for a given metabolite of concern. The metabolite is not detected in the product, but it cannot be adequately justified that *in situ* production is not relevant. A qualitative risk assessment does not demonstrate that the exposure of bees to this metabolite is of no concern. The product is applied to flowering crops. To quantify exposure of bees to the metabolite, the concentration of the metabolites on flowers shall be determined and the risk assessment to bees be further carried out.
>
> Example 2: For a metabolite a hazard to humans is identified, namely acute oral toxicity. The metabolite is present in the product, and it cannot be adequately justified that *in situ* production is not relevant. Therefore, a qualitative risk assessment does not demonstrate that the exposure of humans to this metabolite is of no concern. The product is applied as a foliar spray on tomatoes. For example[32], to quantify the oral exposure of humans, the concentration of the metabolite on tomatoes shall be determined and the risk assessment to humans be further carried out. Toxicological reference values (AOEL, AAOEL ADI, ARfD) would be needed for the metabolite[33].

Step 16: Determine exposure

Regarding risks to human health resulting from dietary exposure, the data requirements state that "*if relevant quantities of the micro-organism or of produced metabolites, especially toxins, have been found to be persistent […] full experimental residue data as provided for in Section 6 of Part A (chemical data requirements) is required, if concentrations of the micro-organism and/or its toxins in or on the treated foodstuffs or feedingstuffs are expected to occur in concentration higher than under natural*

[32] Other exposure routes (dermal, inhalation) may also be assessed.

[33] See step 17

conditions or in a different phenotypic state".

This means that if a qualitative assessment (see question 14.1) does not demonstrate that exposure of humans to the metabolite is of no concern, and when the metabolite is persistent and occurring in concentrations higher than under natural conditions, the full experimental residue data shall be required as provided in Part A of the data requirements, to enable Maximum Residue Levels to be determined (see question 16.2).

Question 16.1: For exposure resulting from a metabolite present in the product, perform a quantitative exposure assessment in accordance with current EFSA guidance for fate modeling and/or operators/workers/bystanders/residents, as appropriate.

When *in situ* production is not relevant for the risk assessment but exposure to a metabolite of potential concern present in the microbial PPP cannot be excluded, the exposure to the metabolite for operators, workers, bystanders, and residents needs to be determined in accordance with the applicable guidance documents for chemical substances[34,35].

To determine the predicted environmental concentrations of metabolites, the environmental fate and behaviour modelling approach as for chemical active substances can be used. When information on the physical-chemical parameters needed as input for these models is not available, conservative default values should be used.

Question 16.2: For those metabolites for which it has not been adequately justified that in situ exposure is not relevant for the risk assessment, determine exposure by measuring concentrations of the metabolite in the relevant environmental compartment (e.g. edible parts).

The EFSA Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products does not provide any guidance on worker exposure to substances produced *in situ*.

When *in situ* production of a metabolite is relevant for the risk assessment and a qualitative assessment does not demonstrate that exposure to the metabolite is of no concern, exposure to the metabolite of the relevant non-target organism (i.e., for which the hazard was identified) should be determined by determining the concentrations of the metabolite on edible parts and/or relevant environmental compartments.

To determine concentrations of metabolites, chemical analyses can be used of edible parts and/or relevant environmental compartments upon application of the product in accordance with the representative good agricultural practice, under normal conditions of use.

By determining the concentration of metabolites, both the exposure resulting from the presence of the metabolite in the product and from *in situ* production are addressed. The concentrations of the metabolite in edible parts and/or relevant environmental compartments may for example be determined as part of efficacy trials performed with the representative formulation.

[34] EFSA (European Food Safety Authority), 2014. Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products. EFSA Journal 2014;12(10):3874, 55 pp., doi:10.2903/j.efsa.2014.3874

[35] EFSA Guidance on dermal absorption, June 2017 https://www.efsa.europa.eu/en/efsajournal/pub/4873

An alternative approach which can be used to demonstrate absence of the metabolite in the relevant environmental compartment, is to analyse the population density of the microorganism in this environmental compartment.

Note that this approach can only be used when the edible parts are not present when the microorganism is applied and it can be demonstrated that the microorganism is not present inside the plant.

An example is addressing oral toxicity of a metabolite by demonstrating the absence of the microorganism on harvested wheat grains after use of a microorganism as seed treatment. However, when the relevant environmental compartment (e.g., the edible part) is exposed to the microorganism during application, the absence of the microorganism from the relevant environmental compartment does not necessarily exclude the presence of the metabolite, as the metabolite could persist longer than the microorganism.

If the approach described above does not adequately demonstrate a safe use regarding human health, residue trials as provided in Part A of the data requirements may be required.

Toxicity assessment

Step 17.a: Determining reference values (for human toxicology) for metabolites of concern from literature or TTC

Question 17.a.1: Can reference values for the metabolite of concern be determined based on available information/data?

For concerns related to the safety of humans

Toxicity data may be available. However, in order to derive health-based reference values the typical acute studies (oral, dermal, inhalation toxicity studies, primary irritation and dermal sensitisation studies) are often inappropriate for deriving reference values, such as (A)AOEL, ADI or ARfD, although acute studies may be useful to inform on whether acute reference doses are required i.e. AAOEL and ARfD. Higher tier toxicity tests, in particular toxicity studies with repeated administration, may be helpful to identify a NOAEL or a LOAEL which can be used to derive the reference values. In addition, genotoxicity studies are also relevant when considering if reference values can be set, since reference values cannot normally be set if there is a concern for non-threshold genotoxicity.

If toxicity studies relevant to the hazard(s) identified are available and suitable to derive reference values for a quantitative risk assessment, go to step 19.

If reference values for the metabolite of concern are already available, go to step 20.

Question 17.a.2: Is the Threshold of Toxicological Concern (TTC) approach appropriate for the metabolite of concern?

For concerns related to the safety of humans

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 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 established at 0.15 µg/person/day or 2.5 ng/kg bw/day. For substances that have the potential to be DNA-reactive mutagens and/or carcinogens based on the weight of evidence, the relevant TTC value is lower: 0.0025 µg/kg bw/d (2.5 ng/kg bw/d)[36]. However, the non-exceedance of this TTC value does not automatically allow to conclude on the acceptability of such substances: an expert judgement shall be provided in this case.

Substances qualifying for Cramer classes I, II and III, would be evaluated using TTC values of 30, 9.0 and 1.5 µg/kg bw per day, respectively (EFSA Scientific Committee[37], 2012).

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

[36] According to the Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA, 2019).

[37] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2012.2750

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 metabolites produced by microorganisms in food and feed. If possible, concentrations of metabolites of concern can be compared with tolerable quantities in food/feed.

If the TTC approach is not appropriate, go to step 18.

If the TTC approach is appropriate, go to step 20.

Step 17.b: Determining toxicity endpoints (for ecotoxicology) for metabolites of concern from literature/available data

Question 17.b: Can toxicity endpoints for the relevant group of non-target organisms be determined for the metabolite of concern based on available literature/data?

For concerns related to the safety of non-target organisms other than humans

In contrast to concerns related to human safety, for other non-target organisms, the most reliable information for ecotoxicology may be derived from higher-tier studies such as field studies.

Although, sometimes it is quite difficult to determine the cause of effects, as mentioned earlier, the studies should enable a clear distinction between pathogenic and toxic effects by using appropriate control groups. According to the test guidelines, a control group of non-target organisms treated with the inactivated microorganism (considering also the thermos-stability of toxins) may prove useful to evaluate (eco-)toxic properties of the metabolite(s). Sterile filtrates could also be included as additional control group.

The identification of responsible "bioactive" compounds would be very useful in case high amounts of metabolites are expected in the test solution. However, this information is generally absent in the eco-toxicological studies with the MPCA-AM. Therefore, crude extract testing may provide useful information in the hazard assessment and in the context of mixture toxicity. It should be noted however that this type of exposure represents a worst-case situation, as under field conditions, metabolite production is highly regulated in time and space.

An illustrative example of the usefulness of appropriate control groups in eco-toxicity assessment:

Assuming that the crude extract control (one single concentration) is showing 40% mortality to fish and that the metabolite of concern has been identified and quantified in the crude extract, then the risk can be calculated by using this single concentration causing 40% effect and the PEC. If the risks are considered acceptable, no further assessment needed. If the risks are not acceptable, the applicant shall conduct an appropriate dose-response study with the crude extract to determine the exact LC50/EC50. This would mean, ideally in every effect study that is provided, a crude extract control and an inactivated control should be included.

Shall the question under step 17b be answered positively the applicant and risk assessors will be able to conduct a quantitative risk assessment and **proceed to step 19** to set a suitable eco-toxicity endpoint.

In case the question under step 17b is answered negatively the applicant will have to perform the appropriate toxicity tests with the metabolite of concern for the hazards identified in stage 2 and then **proceed to Step 18**.

Step 18: (Eco-)Toxicity testing

Question 18.1: Toxicity testing strategy for the metabolite of concern to follow-up on the hazards identified in stage 2.

For concerns related to the safety of humans

For metabolites of concern that reach this step due to concerns for human health, all hazards indicated in stage 2 (e.g. through literature search, QSAR where relevant) should trigger the performing of the appropriate tests which may include genotoxicity[38], repeated-dose toxicity or specific toxicity tests (e.g. DART developmental and reproductive toxicity, neurotoxicity tests), and/or eco-toxicity tests. The tests choice should be determined on a case-by-case basis and as a function of observed or anticipated hazards.
It is appropriate to propose to conduct studies according to the genotoxicity testing guideline in order to address identified hazards indicated in the open scientific literature. Taking into account the versatile and open-end nature of existing published studies, the most appropriate assay will have to be conducted by the applicant.
In case of a versatile/non-specific outcome of existing open scientific literature, a well-designed oral subacute toxicity study[39], focusing on the most relevant identified adverse findings, should be conducted in order to generate a Point of Departure for the establishment of reference values.

The conduct of such studies will however only be possible if it would be feasible to extract/synthetise sufficient quantities of substance to be tested.

Genotoxicity characterization, where relevant

With regard to genotoxicity, if technically possible, for compounds suspected to be of genotoxic concern from the scientific literature and following (Q)SAR prediction and read-across, and when their exposure is exceeding 0.0025[40] µg/kg bw per day, it seems appropriate to conduct the testing battery[41] which should include as a minimum two *in vitro* tests, covering all three genetic endpoints, i.e. gene mutations, structural and numerical chromosomal aberrations. A list of possible assays currently available could be for instance:

- A bacterial gene mutation assay ('Ames test'),
- A gene-mutation assay in mammalian cells (preferably a mouse lymphoma assay),
- An *in-vitro* micronucleus assay (with specific probing to detect aneugenicity).

In case of a positive response in any *in-vitro* assay, the appropriate follow-up testing should be determined, including *in-vivo* testing, taking into account the genotoxic endpoint(s) identified as positive *in vitro*. The opinions of the EFSA Scientific Committee on genotoxicity testing (2011, 2017 and any subsequent updates [42]) should be taken into account.

[38] Similarly to the approach for impurities, a gene-mutation assay could be considered
[39] Or depending on the expert judgment a subchronic toxicity study (90 days)
[40] TTC-value relevant to such substances
[41] As recommended by the EFSA Scientific Committee, 2011: Kirkland et al., 2014 a,b
[42] https://www.efsa.europa.eu/en/efsajournal/pub/2379

For all metabolites for which genotoxic properties cannot be excluded after testing, risk assessors and risk managers need to take further action case-by-case to exclude any unacceptable risk for consumers in line with the approach applied to chemical active substances.

Ecotoxicity characterization

For metabolites of concern that reach this step due to concerns related to the safety of non-target organisms other than humans, GLP standard tests according to internationally agreed test guidelines with the relevant non-target group (i.e. for which a hazard was identified) and the respective metabolite of concern shall be conducted.

Higher-Tier studies with the MPCA-AM or with the representative formulation may also be performed to address the risks of metabolites of concern. If higher tier studies are performed under realistic field conditions, it must be ensured that the test item is applied at levels equal to actual or expected field exposure levels. Higher tier studies may also comprise feeding studies with infected hosts.

An example for entomopathogenic fungi is given below:

> *Insectivorous birds (and other terrestrial vertebrates) may consume mycosed insects and thus be exposed to fungal metabolites. Since the exposure to fungal metabolites is expected to be minimal or negligible following oral administration of the MPCA-AM in oral toxicity/pathogenicity studies due to the lacking metabolic activity of the microorganism in the absence of susceptible hosts, it can be assumed that a feeding study with infected host organisms is more appropriate to address the risks of in situ produced metabolites considering a realistic worst-case scenario. For further information on the risks of entomopathogens to insectivorous mammals and birds, applicants are referred to chapter 6.4 of the OECD working document on the risk assessment of secondary metabolites of microbial biocontrol agents (OECD, 2018).*

Since no test guidelines at international level may be available for the higher studies mentioned above, adapted testing protocols should be discussed in a pre-submission meeting.

Actions resulting from Step 18:

All results of conducted toxicity and ecotoxicity studies on the metabolite(s) for the metabolites of concern shall be reported in the updated overview table.

Proceed to Step 19.

Step 19: Setting of human toxicological end-points (NOAEL/NOEC) endpoints/reference values and ecotoxicological endpoints/reference values

Question 19.1: Set NOAEL/NOEC and reference values for the relevant groups (AOEL/ARfD/ADI/AAOEL for human health, as appropriate; NOEC/EC_{50} for non-target organisms). Alternatively (especially for genotoxic substances), TTC can be used if appropriate.

The establishment of reference values (AOEL/ARfD/ADI/AAOEL) will be necessary in case existing reliable toxicity studies from the open scientific literature or guideline GLP-studies are available, and a valid NOAEL/NOAEC can be identified. The appropriate safety factors necessary to establish such reference values will be applied, as for synthetic substances.

In the absence of any valid reference dose, the TTC approach may be considered.

Similarly, the establishment of reference values (NOEL/LD_{50}, NOEC/EC_{50}) will be necessary in case existing reliable ecotoxicity studies are available and a valid PNEC for the relevant non-target organisms can be identified.

Actions resulting from Step 19:

Proceed to Step 20.

Quantitative risk assessment

Step 20.a: Human Toxicological Risk Assessment

Question 20.a.1: Compare exposure to reference values as appropriate. Does the exposure exceed the reference value for any exposure group?

Step 20.b: Ecotoxicological risk assessment

Question 20.b.1: Compare exposure to ecotoxicological endpoint as appropriate. Does the exposure exceed the reference value for any exposure group?

For a metabolite of concern the standard triggers (like for a “conventional” chemical substance) should be applied.

Actions resulting from Step 20:

If the respective trigger value (as for a “conventional” chemical substance) is not breached for the assessed exposure group/organism, the risk identified for this metabolite is considered as acceptable.

In contrary, if the respective trigger value (as for a “conventional” chemical substance) is breached, the risk identified for this metabolite is considered as unacceptable.

Annex I: Decision scheme

Stage 1: Determine the assessment type.

Step 1: Role of the metabolite in the mode of action.			
1.1	Is the principal mode of action of the active substance based on the presence of metabolites in the formulated plant protection product?	no	yes
action if green (no): proceed to step 2.			
action if white: the metabolite is equivalent to a chemical active substance and a dossier is required in accordance with Part A of Reg 283/2013.			

Step 2: Exclusion of metabolite production.			
2.1	Is the microorganism a virus?	yes	no
action if green: no further assessment is needed.			
Action if white: proceed to Stage 2 (assessment of metabolites as included in Part B of Reg 283/2013).			

Stage 2: Collecting basic set of information on metabolites

The aim of Stage 2 is to identify from different sources and/or data sets the metabolites which may be produced by the strain of the microorganisms and to collect information and/or data on the hazard of this/these metabolites (i.e., toxicity or antimicrobial activity).

In Stage 2, Steps 3 – 8 are mandatory. Step 9 is an optional step which can be used to exclude metabolites of potential concern from further assessment. Step 10 is a conditional step, which is required for less well-described microorganisms.

Expected deliverable: Overview table consisting of a list of all identified metabolites and their toxic or antimicrobial effects (i.e., the metabolites of potential concern; see appendix). For hazardous effects, the organisms for which this hazardous effect was observed shall be stated. The information generated in Stage 2 shall be summarised by using the template for the overview table (see Annex III).

Literature and experimental data for the microorganism

Step 3: Literature data from literature search performed for the microorganism. Collecting information regarding the production of toxic metabolites/ metabolites with antimicrobial activity.			
3.1	Are metabolites identified for the strain under evaluation in the published literature?	no	yes
3.2	Are metabolites identified for closely related strains/species in the published literature?	no	yes
3.3	Are hazardous effects identified for the strain under evaluation in the published literature?	no	yes
3.4	Are hazardous effects identified for closely related strains/species in the published literature?	no	yes
3.5	Are there any metabolites described to be produced by the strain under assessment, or closely related strains, without toxicological information available?	no	yes
3.6.	Are there any studies reporting no effect observed for the strain under evaluation or metabolite of potential concern?	yes	no
Action if any white: List all identified metabolites and hazardous effects of both identified and unknown metabolites resulting from questions 3.1 – 3.5 in the overview table and indicate whether this metabolite or hazardous effect was identified for the strain under evaluation or for closely related strains.			

Step 4: Literature data for the metabolites identified in Step 3. As part of this step, an additional literature search shall be performed for each identified metabolite as listed in the overview table.			
4.1	For each metabolite, is there any indication of hazardous effect(s) in the published literature?	no	yes
Include the results of Step 4 in the overview table.			

Step 5: Literature data. Is the microorganism well studied?			
5.1	Is there enough published literature to assume that a literature search would provide sufficient information on metabolite production?	yes	no
Action if white: In addition to the questions in Step 6 – 8, also address the questions in Step 10, where relevant.			

Step 6: experimental (eco)toxicology data with microorganism			
6.1	Are there any hazardous effects in the toxicity or eco-toxicity studies that indicate that the strain under evaluation produces metabolite(s) of concern?	no	yes
6.2	Is the identity known of the metabolite which is responsible for the effects observed in the toxicity or eco-toxicity studies?	yes/not relevant	no
Action if any white: List all identified metabolites and hazardous effects by unknown metabolites resulting from questions 6.1 and 6.2 in the overview table and proceed to Step 7.			

Step 7: experimental data – chemical analyses			
7.1	Is analytical chemistry data available which indicates that the strain under evaluation produces metabolites of potential concern?	no	yes
Action if white: List all identified metabolites resulting from question 7.1 in the overview table and proceed to Step 8.			

Step 8: Identification of unidentified metabolites with hazardous effects
Action: All unknown metabolites with hazardous effects as determined in Steps 3 and 6 shall be identified. A literature search for each identified metabolite should be performed and the overview table updated to include the results.

Step 9: Genomic analysis (for metabolites identified due to published literature)			
9.1	If the gene(s) encoding the metabolite is known, does a genomic analysis show absence of the gene?	yes	no/not applicable
Action if green: Include the result in the overview table; no further assessment is needed for this particular metabolite			
Action if white: Include the result in the overview table and proceed to Step 10 (where applicable) or Step 11.			

Collecting additional data for microorganisms for which the body of knowledge is not sufficient (conditional)

Step 10: Collecting information regarding the production of toxic metabolites / metabolites with antibiotic activity – less well known microorganisms			
10.1	Is there any indication for (geno-)toxicity in tests with crude extracts?	no	yes
10.2	Are there any genes for known toxins or relevant antimicrobial agents present in the genome?	no	yes
Action if all green: no additional assessment is necessary (proceed based on the results of Stage 2).			
Action if 10.1 is white: identify the metabolites responsible for the observed effects (also see Step 8). Action if any white: Include each identified metabolite in the list overview table and proceed to Step 11.			

Outcome of Stage 2: metabolites of potential concern

Step 11: Metabolites of potential concern based on literature and experimental data for active substance.
The metabolites included at this stage in the overview table are the metabolites of potential concern. They have been included in the overview table based on the responses in Stage 2 (any white for Steps 3 – 7 and Step 10 (conditional). When absence of genetic information was demonstrated for a metabolite in Step 9, this should be indicated in the overview table; this metabolite is not considered to be of potential concern). Perform an additional literature search for all metabolites of potential concern. Search terms (e.g. CAS numbers, excluding species name). Issues to address according to (current) DR: • Conditions under which the microorganism produces the metabolite must be described • Any available information on the mechanism by which the microorganism regulates the production of the metabolite shall be provided Any available information on the influence of the produced metabolites on the microorganism's mode of action shall be provided. Present information in dossier and update the overview table.
if all green (Steps 3 – 7 all green, or genomic analysis shows absence of gene(s) for metabolite(s) identified due to published literature): no further assessment is necessary.

Stage 3: Determine which metabolites are of concern

The aim of Stages 3 and 4 is to address the risk arising from hazards identified in Stage 2.

The expected deliverable: updated overview table, in which the metabolites of potential concern are categorised either as being of no concern or as being of concern for the risk assessment.

Step 12: Determine if a metabolite of potential concern is produced by the strain			
12.1	Can it be adequately justified that the strain does not produce the metabolite (e.g. using WGS data)?	yes	no
action if green: no additional assessment is necessary for this metabolite			
action if white: proceed to Step 13.			

Step 13: Determine exposure routes of the metabolite			
13.1	Can it be adequately justified that *in situ* production of the metabolite is not relevant for the risk assessment?	yes	no
13.2	Is the metabolite present in the product?	no	yes
if all green: no further assessment is needed for this metabolite.			
if one or more white: proceed to Step 14.			

Step 14: Determine if a metabolite of potential concern is of actual concern for the risk assessment			
14.1	Does a qualitative assessment demonstrate that the exposure of humans or the relevant NTO to the metabolite is in the range of background levels of exposure of no concern?	yes	no
action if green: no further assessment is needed for this metabolite			
action if white: categorize the metabolite as being of concern in the overview table.			

Outcome of Stage 3: metabolites of concern

Step 15: Metabolites of concern.
For all metabolites of concern, indicate if *in situ* production is relevant for the risk assessment (question 13.1) and if the metabolite is present in the product (question 13.2). Proceed to Stage 4.

Stage 4: Risk assessment for metabolites of concern (i.e., relevant metabolites)

Exposure assessment

Step 16: Determine exposure	
16.1	For exposure resulting from a metabolite present in the product, perform a quantitative exposure assessment in accordance with current EFSA guidance for fate modeling and/or operators/workers/bystanders/residents, as appropriate.
16.2	For those metabolites for which it has not been adequately justified that *in situ* production is not relevant for the risk assessment, determine exposure by measuring concentrations of the metabolite in the relevant environmental compartment (e.g., edible parts which would trigger a need for residue trials as provided in Part A of the data requirements).
Proceed to Step 17.	

Toxicity assessment

Step 17a: Determining reference values from literature or TTC			
17.a.1	Can reference values for the metabolite of concern be determined based on available information/data?	yes	no
17.a.2	Is the Threshold of Toxicological Concern (TTC) approach appropriate for the metabolite of concern?	yes	no
action if green: Proceed to question number 19.			
action any if white: Proceed to Step 18.			

Step 17b: Determining toxicity endpoints (for ecotoxicology) for metabolites of concern from literature/available data			
17.b.	Can toxicity endpoints for the relevant group of non-target organisms be determined for the metabolite of concern based on available literature/data?	yes	no
action if green: Proceed to question number 19.			
action any if white: Proceed to Step 18.			

Step 18: (Eco-)Toxicity testing	
18.1	Perform the appropriate (eco-)toxicity tests with the metabolite of concern to follow-up on the hazards identified in stage 2.
Proceed to Step 19.	

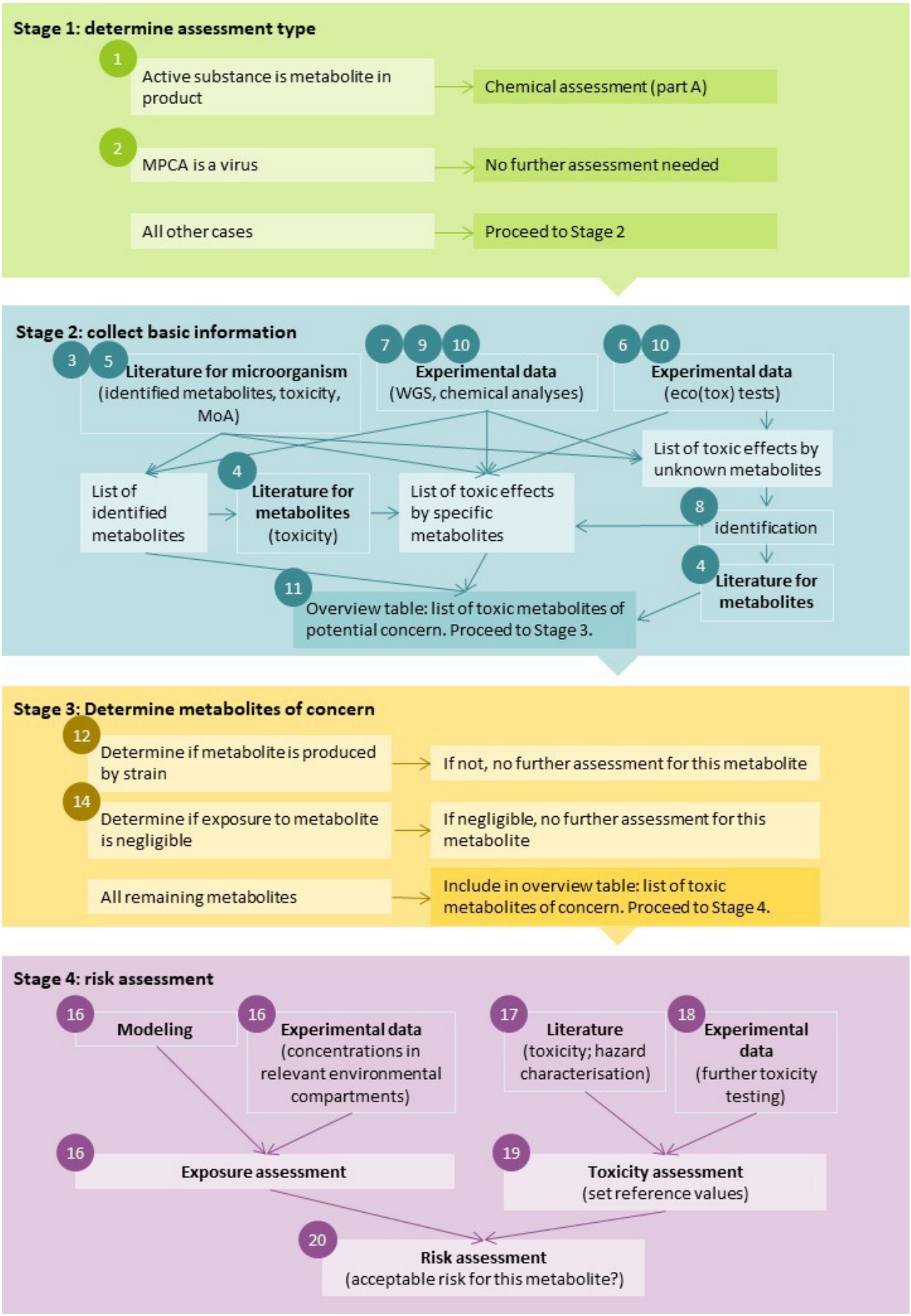
Step 19: Setting of NOAEL/NOEC and reference values	
19.1	Set NOAEL/NOEC and reference values for the relevant groups (AOEL/ARfD/ADI/AAOEL for human health, as appropriate; NOEC/EC_{50} for non-target organisms). Alternatively (especially for genotoxic substances), TTC can be used if appropriate.
action: Proceed to Step 20.	

Quantitative risk assessment

Step 20a: Human Toxicological Risk assessment			
20.a.1	Compare exposure to reference values as appropriate. Is the respective trigger value (as for a "conventional" chemical substance) breached for the assessed exposure group/organism?	no	yes
If green: acceptable risk identified for this metabolite. Update the overview table.			
If white: unacceptable risk identified for this metabolite. Update the overview table.			

Step 20b: Ecotoxicological Risk assessment			
20.b.1	Compare exposure to ecotoxicological endpoint as appropriate. Is the respective trigger value (as for a "conventional" chemical substance) breached for the assessed exposure group/organism?	no	yes
If green: acceptable risk identified for this metabolite. Update the overview table.			
If white: unacceptable risk identified for this metabolite. Update the overview table.			

Annex II: Decision tree



Annex III: Overview table

	Stage 1		Stage 2					Stage 3				Stage 4
Metabolite identifier[1)]	**Active substance (Y/N)**	**Claimed active metabolite (Y/N/?)**	**Verification of MoPC-status**				**Outcome chemical analysis[4)]**	**Relevant exposed group[5)]**	**MoC (Y/N)**	**Ref. values (TOX) and endpoints (ECOTOX)**	**Exposure level**	**Unacceptable risk (Y/N)**
			Toxic / antimicrobial effect observed, test species, and strain[2)]	**Potential relevance for micro-organism [3)]**	**WGS-evidenced (Y/N)**	**MoPC (Y/N/?)**						
Name, CAS, and/or IUPAC	Y/N	Y/N/?	Study 1: Effect / test species / strain Study 2, etc…	Metabolite / Effect	Y/N	Y/N/?	MPCA-AM: Y/N or max. PPP: Y/N or max. Induced: Y/N	TOX; TOX.. / ECOTOX; ECOTOX..	Y/N	TOX; TOX.. / ECOTOX; ECOTOX..	TOX; TOX.. / ECOTOX; ECOTOX..	TOX; TOX.. / ECOTOX; ECOTOX..
The row below presents the step-numbers associated with the respective table column												
1, 3, 7, 10	**1**	**1**	**3, 4, 6, 10, 12, 18**	**4, 6, 8, 10, 12**	**9, 10, 12**	**11**	**7, 12**	**13, 14**	**15**	**14, 17, 19**	**14, 16**	**20**

[1)] Typically the name that is unambiguously used throughout the dossier to refer to the metabolite.

[2)] For each relevant study (author and year are entered on the 'Study x'-position) the nature of the observed toxic / antimicrobial effect (? = data unavailable; null = no effect observed; ACU = acute toxicity; CYT = cytotoxicity; MUT = mutagenicity; GEN = genotoxicity; CAR = carcinogenicity; REP = reprotoxicity; NEU = neurotoxicity; AM = antimicrobial activity), the test species (or at least a detailed description of the exposed organism / material), and the name of the strain for which the effect has been observed (could be the micro-organism itself, a closely related strain, or both) is stated.

[3)] In this column, the potential relevance of an identified metabolite and observed effect is made explicit for the micro-organism in particular. If the potential relevance is confirmed for the metabolite or the effect, 'Y' is entered on the respective position in the cell. In case non-relevance is established, an 'N' is added.

[4)] This column states whether or not a metabolite has been detected in the MPCA-AM or PPP. Whenever relevant for the assessment, the 5-BA-established max. content (max.; average + 3xSD) for a metabolite is entered for the MPCA-AM (if available) and the PPP (either measured or derived).

[5)] The following codes may be used to refer to any relevant exposed group. For TOX: OP (operators), WO (workers), BY (bystanders), RE (residents), and CO (consumers). For ECOTOX: MAM (mammals), BRD (birds), REP (reptiles), AMP (amphibians), FSH (fish), AQI (aquatic invertebrates), ALG (algae), AQM (aquatic macrophytes), BEE (bees), ART (non-target arthropods other than bees), MMO (non-target meso- and macro-organisms in soil), and PLA (non-target terrestrial plants). When proposed use does not lead to exposure of any of these groups, add '-'.

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