Evaluation Manual

for the Authorisation of Biopesticides according to Reg. (EC) No 1107/2009

Appendices to Part I: Micro-organisms

Appendix 1 – Roadmap for SANCO/2020/12258

on the risk assessment of metabolites produced by micro-organisms used as active substances in plant protection

Version 1.0, March 2023

Board for the Authorisation of Plant Protection Products and Biocides

ctgb

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Document history

Version	Date	Amended section(s)	General description of changes
1.0	March 2023	**-**	Initial version of Appendix 1 ('Roadmap for SANCO/2020/12258') to EM Biopesticides – Part I: Micro-organisms.

*** Disclaimer ***

With this document Ctgb intends to (i) provide support to applicants and evaluators when applying SANCO/2020/12258 in the context of respectively compiling and evaluating regulatory dossiers for the approval of microbial active substances under Regulation (EC) No 1107/2009, and ultimately to (ii) establish a rational, efficient, and predictable approach for dealing with secondary metabolites in this context (please refer to 'Purpose of this document' below, for a full description of its scope).

Having been produced informally in the course of interpreting SANCO/2020/12258 for actual use, the document strictly reflects Ctgb's practical understanding of the matter.
As such, it has no formal status within the existing regulatory framework, nor can it be considered to carry any authoritative charge in itself. The document is not legally binding.
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Glossary of terms

(A)AOEL	(Acute) acceptable operator exposure level
ADI	Acceptable daily intake
ARfD	Acute reference dose
CRS	Closely related strain
EC_{50}	Concentration at which 50 % of the test population exhibits a defined effect
EM	Evaluation manual
GD	Guidance document
GLP	Good laboratory practice
LD_{50}	Dosing at which 50 % of the test population exhibits mortality
LOQ	Limit of quantification
MPCA	Microbial pest control agent
MPCA-AM	Microbial pest control agent as manufactured
MoA	Mode of action
MoC	Metabolite of concern
MoPC	Metabolite of potential concern
MPCP	Microbial pest control product
NOAEL	No observed adverse effect level
NOEC	No observed effect concentration
NOEL	No observed effect level
NTO	Non-target organism
PEC	Predicted environmental concentration
PEC_{GW}	Predicted environmental concentration relating to groundwater
PNEC	Predicted no effect concentration
PPP	Plant protection product
RRF	Relative response factor
TTC	Threshold of toxicological concern

Definitions used in this document

For easy reference, definitions relevant to this Appendix are adopted from the main document 'Part I: Micro-organisms'.

'Claimed active metabolite' means a metabolite present in the MPCA-AM that is claimed to contribute to the plant protection action and whose quantitative presence in the final product is considered indispensible to the effect (see A.1.4.1 of EM Part I: Micro-organisms for further explanation). Claimed active metabolites are included in the specification

'Deactivated micro-organism' means a micro-organism that is no longer capable of replication or transfer of genetic material.

'Part A active substance' means a substance for which a dossier shall be submitted in accordance with Part A (of (EU) No 283/2013 and (EU) No 284/2013). This group concerns chemical substances, extracts from biological material, semiochemicals, and metabolites produced by a micro-organism (either purified or as part of a fermentate in which the micro-organism has been deactivated).

Efficient referencing to key documents

'Literature GD' refers to the Guidance of EFSA – Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA J. 2011;9(2):2092

'Metabolite GD' refers to SANCO/2020/12258, d.d. 23 October 2020

'OECD 98' refers to the OECD Working document on the risk assessment of secondary metabolites of microbial biocontrol agents, Series on Pesticides No. 98

Purpose of this document

Guidance documents within the (EU) No 1107/2009 framework are at the boundary between scientific and regulatory practice.
SANCO/2020/12258 on the risk assessment of metabolites produced by microbial active substances (henceforth: Metabolite GD) addresses a complex reality that is yet not understood to a full degree of confidence and moreover challenges standard research methodology. To keep a clear focus, the Metabolite GD sets up a logical step-wise system in which scientific considerations are reconciled with regulatory procedures. To avoid narrowing of this focus, the inevitable categorization process is largely left to the applicants, consultants, and evaluators that use the document in their routines.

During the national-level implementation of the Metabolite GD, Ctgb sensed that, despite the document's encouragement of a less rigid approach, the lack of (i) a categorization process[1] and (ii) an accompanying pathway system[2] complicates the navigation of the document. Furthermore, the degrees of freedom that the Metabolite GD offers to those experts that are comfortable with setting their own course are only then leading to productive outcomes, when all participants in the approval process share this level of comfort.

To address these difficulties, Ctgb has drafted a roadmap for the Metabolite GD that outlines a more detailed way to categorize metabolites, and includes a more clearly defined starting point for the various categories.

In its capacity as informal, supportive document, the Roadmap intends to:

- **Amend the currently available 'service package' to offer additional, more systematic support.** The addition of the Roadmap completes the support level range[3] that is available to applicants that have to perform the metabolite assessment.
- **Facilitate effective use of the Overview table.** The Metabolite GD frequently refers to an 'Overview table' whose development was intended for a later stage. A table that effectively keeps track of metabolites, their effects, and their statuses, relies on a integer system of input parameters and accompanying categories. The roadmap provides such a system and thus may be used as an instruction manual to the Overview table that is included in this Appendix as well.
- **Offer an adaptable, shared platform that reflects the actual state of experience.** As soon as there is a build-up of actual hands-on experience with the metabolite assessment according to SANCO/2020/12258, critically evaluated approaches and efficient pathways will become available. Unlike a guidance document, the Roadmap allows more flexible editing and can thus be used as an up-to-date record of recommended procedures for multiple cases.
- **Initiate harmonization of the metabolite assessment.** Though not relevant at this stage, it is conceivable that broader harmonization of the metabolite assessment is endorsed at member state level. Depending on its degree of usage, the roadmap may provide useful input in terms of tried and tested approaches (see also the point above).

N.B. Although the introduction of yet another comprehensive document dedicated to metabolites produced by microbial active substances might suggest otherwise, it must be emphasized that the assessment of metabolites should in principle reflect the understanding

[1] To illustrate, questions typically relating to categorization are 'which are the relevant parameters that can be assigned to a metabolite?' and 'which values can these parameters assume?'.
[2] A pathway system should be primarily designed to help finding the most efficient route through the stepwise process for any metabolite with any given set of parameters.
[3] The support level range would now roughly vary from low support ('Regulation only'), to medium ('Regulation plus Metabolite GD'), and high ('Regulation plus Metabolite GD plus Roadmap').

that the vast majority of interactions with micro-organisms in the natural world is non-problematic – or even beneficial – and that the estimation of risk should be proportionally aligned with this notion.
As a consequence, the assessment should establish whether any secondary metabolite produced by the respective micro-organism is of concern or not *via the most efficient course through the process – ideally only following the steps that actually matter in a given case.*
When functioning as intended, the roadmap helps to pinpoint this course.

In essence, to conclude that a metabolite is of no concern one (or more) of the following points need to be substantiated:

- The metabolite is not relevant to the context of the dossier;
- The toxicological / antimicrobial effects associated with the metabolite are not observed for the MPCA under triggering conditions;
- The MPCA has no functional genetic basis for metabolite production;
- The metabolite is not produced under circumstances known to trigger its production;
- There is no group of humans / other NTOs for which there is relevant exposure.

Roadmap for SANCO/2020/12258

The assessment is broken down into four stages, each including a number of Steps. Directly below, the stages are briefly described and bookmarked:

N.B. The original terminology of the Metabolite GD has been adapted to match that of the amended Data Requirements.

STAGE 1 – DETERMINE THE TYPE OF ASSESSMENT NECESSARY FOR THE METABOLITES OF THE ACTIVE SUBSTANCE

In the first stage, it must be established whether (i) the plant protection action is (partly) based on the MoA of a specific metabolite, and (ii) whether the MPCA is capable of metabolite production at all.

Step 1 – Starting up the assessment and evaluating any claimed active metabolites in the MPCA-AM (N.B. see footnote 4)	
Action	**-** Add the Overview table (see end of this Appendix) to MA.2.8. - For any claimed active metabolite that is present in an MPCA-AM in which the micro-organism itself has been deactivated (i.e., a non-purified claimed active metabolite): state the name of the respective metabolite in column 'Metabolite identifier' (C1) of the Overview table and add a 'Y' to C2. - For any metabolite present in a non-deactivated MPCA-AM, which is claimed to contribute to the MoA (see A.1.4 'Specification of active components and their specified limits' of EM Biopesticides – Part I for background): state the name of the respective metabolite in column 'Metabolite identifier' (C1) of the Overview table and add a 'N' to C2. Put 'Y' in C3, when a contributary effect is to be expected.

[4] Amending Regulation (EU) 2022/1439 to Reg. (EU) No 283/2013, and its ensuing interpretation (see 'ANNEX I and II – Introductory notes' of EM Biopesticides – Part I) caused a modification of Step 1 as described in the Metabolite GD.
Cases in which the MoA is fully based on the effect of one or more metabolites that are furthermore purified from the MPCA-AM are already directed to the Part A route at Regulation-level. Assessment according to the Metabolite GD is not relevant for these cases. As such, they are not considered in the roadmap below.
For the other 'Part A case' with a microbial origin that is now covered by the Regulation (i.e., MPCA-AM in which the micro-organism has been deactivated and that either contains claimed active metabolites or not), the Metabolite GD may however be employed for practical reasons (refer to the note on 'Part A and B crossover' under 'ANNEX I and II – Introductory notes' of EM Biopesticides – Part I). Consequently, the process flow below allows for this possibility.

	Once additional information may be required to substantiate this effect, a provisional '?' is entered in C3. The entry is updated once adequate data will become available; 'Y' or 'N' when additional data demonstrate the respective presence or absence of involvement in the MoA. *N.B. Metabolites present in the MPCA-AM that contribute to the MPCA's efficacy ('Y' in C3) must be characterized as described under (EU) No 283/2013, Part A, 1.9. For practical purposes, these metabolites must go through Step 7 (see Step 7 for further info).*
Follow-up	- For cases in which a metabolite present in a deactivated MPCA-AM is considered to be the active substance ('Y' in C2) AND in which the applicant prefers to follow the Metabolite GD-route for 'impurity assessment': **go to Stage 2, Step 3.** - For all other cases in which metabolites have been defined during this step (thus evidencing the capacity of the MPCA to produce metabolites in the first place): **go to Stage 2, Step 3.** - For all cases in which no metabolites have yet been defined at this point: **go to Step 2.**
Step 2 – Exclusion of metabolite production	
Action	**-** If applicable, mention explicitly under MA.2.8 that the active substance is a virus that, due to its nature, does not produce any metabolites by itself and that further metabolite-assessment is therefore unnecessary. *N.B. For bacteriophages however, metabolites need to be covered by the assessment. In this case, any metabolites will however not be produced by the MPCA but by the 'production host'. Though such a context is formally outside the scope of the Metabolite GD, the GD is by default considered the most appropriate piece of Regulatory framework.*
Follow-up	- For viruses: **abort the metabolite-assessment (and start an alternative procedure for bacteriophages, if applicable).** - For MPCAs that are not viruses: **go to Stage 2, Step 3.**

STAGE 2 – COLLECTING A BASIC SET OF INFORMATION ON METABOLITES

In Stage 2, the applicant assembles a comprehensive body of relevant literature and other data on (hazardous / antimicrobial) metabolites produced by the MPCA and CRS, that ultimately helps to reach a conclusion on any MoPCs that may be produced by the MPCA in specific.

Steps 3 to 5 are mandatory, 6 - 8 and 10 are triggered by the outcome of Step 5, and Step 9 varies in degree of advisability, depending on the context.

Step 3 – Collecting literature data on metabolite production of MPCA and/or CRS	
Action	**-** Perform a Literature GD-compliant search on metabolites produced by the MPCA and CRS. Follow subquestions 3.1 – 3.6 and update the Overview table in the process. The resulting data can be organized into four categories[5]: (Cat. A) metabolites produced by MPCA and/or CRS, for which toxic / antimicrobial effects have been observed; (Cat. B) toxic / antimicrobial effects observed for MPCA and/or CRS; (Cat. C) MPCA/CRS-produced metabolites for which no toxicological data are available; (Cat. D) metabolites produced by MPCA and/or CRS for which a null-effect has been observed, and null-effects observed in studies with the MPCA. - Update the Overview table according to the categorization: 3.1 and 3.2 (Cat. A) – the name of any MPCA -or CRS-produced metabolite for which toxic / antimicrobial effects have been observed is added to C1. In C4, the nature of the observed effect is entered along with the test species and the strain (MPCA or CRS) with which the test has been conducted. 3.3 and 3.4 (Cat. B) – all toxic / antimicrobial effects observed for the MPCA and/or CRS are added to C4, along with the name of the strain. For Cat. B, no metabolite has been identified. As such, C4 will be filled in for a table row in which C1 has been left blank. 3.5 (Cat. C) – metabolites, described for the MPCA and/or CRS, for which no toxicological data are available are marked with a '?' in C4 on the position where otherwise an effect would have been entered. 3.6 (Cat. D) – when studies are available that show null-effects for the identified metabolite, 'null' is entered in C4 along with the name of the test species and the tested strain. For studies performed with the MPCA that show null-effects without identifying any metabolites, 'null' is entered into C4 along with the test species and strain. On that table row, C1 is blank.
Follow-up	- For all metabolites in Cat. A: **go to Step 5.** - For all effects in Cat. B: **go to Step 5.** - For all Cat. C en Cat. D identified metabolites: **go to Step 4.** - For all null-effects in Cat. D: **go to Step 5.**

[5] Note that this categorization results in a slight adaptation of Steps 3 and 4 of the Metabolite GD that intends to avoid unnecessary repetition of actions for Cat. A and B in Step 4.

Step 4 – Screening the literature for any indications of toxic / antimicrobial effects related to Cat. C and Cat. D metabolites	
Action	**-** Perform an additional, Literature GD-compliant search for all Cat. C and Cat. D identified metabolites that focuses on (i) the conditions under which the MPCA and/or CRS produces the metabolite, (ii) the expected quantities produced and the sensitivity of the analytical method used to quantify the metabolite, (iii) the mechanism by which the MPCA and/or CRS regulates production of the metabolite, and (iv) the influence of the metabolite on the MoA. - Update the Overview table based on the results of the additional search, if possible according to the procedure described under Step 3, 'Action'. If the additional literature data involve toxicity studies performed with analytical standards of the metabolite, no strain is defined under the study entry in C4. - Enter a 'N' on the metabolite-position of C5, but only when at this stage sufficient data are available to conclude that a metabolite is not relevant in the context of the dossier.
Follow-up	- In all cases: **go to Step 5.**
Step 5 – Checking whether the literature search is compliant with the Literature GD and whether the collected data allow drawing a conclusion on the relevance of any of the hazardous metabolites and effects defined for the MPCA and/or CRS	
Action	**-** Verify whether the literature searches comply with the Literature GD's criteria. - Prepare reasoned statements that convincingly substantiate that the collected data suffice to draw a conclusion on the relevance of each of the metabolites and effects identified for the MPCA and/or CRS.
Follow-up	- Whenever the literature search was found to be incompliant with the Literature GD: **go back to Step 3.** - When the search is Literature GD-compliant and the collected body of data is sufficiently solid[6] to support the conclusion that[7]… • …there are no toxic / antimicrobial effects associated with the metabolite concerned (only study entries included in C4 that report 'null' effects –possibly accompanied with studies reporting '?'-effects – for that metabolite): **go to Step 11 for the respective metabolite.** • …toxic / antimicrobial effects have been observed for the metabolite concerned (in C4, at least one study entry is included that reports a toxic / antimicrobial effect for that metabolite): **go to Step 7, Step 9 (optional), or Step 11 – whichever route is most efficient to establish the relevance of the respective metabolite.** • …toxic / antimicrobial effects have been defined for the MPCA and/or CRS that could not be associated with a metabolite (i.e., non-associated effects; C4 includes at least one study entry that reports a toxic / antimicrobial effect, but C1 is blank for that table row): **go to Step 6 for the respective effect.** • …the metabolite concerned is not relevant for the context of the dossier (C5 includes a 'N' on the metabolite position): **go to Step 11 for the respective metabolite.**

[6] Deciding on what is considered 'sufficiently solid' (and what is not) represents a process that is subject to careful and ongoing refinement. At this stage, Ctgb has not yet defined a systematic approach that goes beyond the Literature GD – which does not suggest that Ctgb would *a priori* disconsider 'non-compliant' generalist knowledge that could nonetheless add to the weight of evidence approach.

[7] Note that the differentiation provided here allows a more efficient process flow than Step 5 in the Metabolite GD, which offers a less specific definition of routes at this stage.

	- When the search is Literature GD-compliant, but the collected body of data provides insufficient support to conclude on the relevance of any metabolite / effect identified for the MPCA and/or CRS: **go to Step 10.**
Step 6 – Evaluating (eco)toxicological studies with the MPCA to establish a relation between observed non-associated toxic / antimicrobial effects and the presence of a metabolite	
Action	**-** Evaluate the (acute) (eco)toxicological studies that have been performed with the MPCA in support of the dossier. Whenever non-infectivity/pathogenicity-related effects are observed in the control groups that have been exposed to material in which the MPCA has been deactivated, MoPCs may be present. - When the test results confirm or indicate that observed toxic / antimicrobial effects are caused by a metabolite… …and the effect is the same as the non-associated one that has already been reported in C4 (C1 is blank on this row): a new study entry is added in C4 on the same row, mentioning the effect, test species, and the name of the MPCA. Finally, a 'Y' is added on the effect-position of C5. …and the observed effect has not yet been reported in the Overview table: add a study entry in C4 on a new row with a blank C1 and state the nature of the effect, test species, and the name of the MPCA. Also in this case, a 'Y' is entered on the effect-position of C5. *N.B. Try to already relate the effects observed in the (eco)tox-tests to metabolites identified in previous steps for which (i) no effect is yet known ('?' in C4), or (ii) the same effects have been observed (check C4).* *Establishing an initial relation in case of (i) may be done based on a comparative assessment of the metabolite's structure with that of molecules known to cause the observed effect. Note that this exploration is preliminary and is exclusively intended to facilitate a focused analysis under Step 7. The Overview table is not yet updated to reflect the findings.* - When the test results show a null-effect, a study entry is added in C4 on a new row with a blank C1, which reports a 'null' effect, along with test species, and the name of the MPCA.
Follow-up	- For the cases supported by sufficient information to allow an effective analytical screening: **go to Step 7.** - For observed effects that cannot yet be related to a metabolite: **go to Step 8.**
Step 7 – Identification of metabolites in the MPCA-AM/MPCP*, using appropriate analytical methods * Preferably, the MPCA-AM is screened, as metabolite-levels in the MPCP are generally lower due to dilution with co-formulants. Also, co-formulants may provide (additional) interference. In conclusdion, testing with the MPCP should be limited to the cases where the MPCA-AM is a non-isolated intermediate.	
Action	**-** Select a suitable analytical technique for each of the metabolites that are directed here via Step 5. Add a 'Y' or 'N' to C8 for the respective metabolites, reflecting whether the metabolite has been detected or not, along with the nature of the test material (MPCA-AM or MPCP). Enter a 'Y' on the metabolite-position of C5, when the metabolite has been detected in the material. **-** Select a suitable analytical technique for the suspected metabolites directed here via Step 6 and update the Overview table according to the screening results to… …finalize the preliminary relationships between effects observed in the (eco)tox-tests performed under Step 6 and candidate-metabolites, once the metabolite has

	been detected in this step. Enter the name of the detected metabolite in C1 (of the row in which C4 reports the effect observed in the study), and add 'Y' on the metabolite-position of C5. Finally, a 'Y' is reported in corresponding C8 under the appropriate material (MPCA-AM or MPCP). …include any unforeseen substances identified in the 'semi-specific' analytical screening, that are both non-trivial and confirmed not to be process-impurities (see A.1.5, 'The essential process checkup; Potential sources of relevant impurities' of EM Biopesticides – Part I). Enter the name in C1, fill in a 'Y' on the metabolite-position of C5, and add a 'Y' to C8 under the appropriate material (MPCA-AM or MPCP). When these new metabolites can reasonably be related to a non-associated effect, add the new C1-, C5-, and C8-data to the row previously created for the non-associated effect. If insufficient data are available to establish a relation, additional data are needed (see '*Follow-up*'). *Note that non-associated effects, for which the analytical screening does not identify a causative metabolite, remain unchanged in the Overview table.* *N.B. Although not required at this stage, it may be advantageous to perform the screening in the context of a 5-BA, especially when there are strong indications that the concerning metabolite will be classified as MoC. In this case, the derived max. content (average + 3xSD) will be entered in C8 under the relevant test material.* *Active metabolites (for which a 'Y' has been entered in C3) need to be included in a 5-BA at any rate, to establish a specification range. For these metabolites, both a min. (average - 3xSD) and a max. (average + 3xSD) are reported in C8 under the relevant test material.*
Follow-up	- For detected metabolites that could be successfully associated with an observed toxic / antimicrobial effect: **go to Step 11.** - For metabolites associated with an observed toxic / antimicrobial effect, that were not detected in the analytical screening, but whose production by the MPCA can nonetheless not be excluded: **go to Step 11 (possibly after a strongly recommended, optional WGS-screening via Step 9*).** - For observed toxic / antimicrobial effects that remain non-associated to a causative metabolite after the analytical screening: **go to Step 8.** - For newly detected metabolites for which insufficient data are available to justify linking with a yet non-associated effect: **start at Step 4 to collect literature data for these new metabolites.** * The route via Step 9 is recommended as a more efficient approach than the one that may involve initial misclassification of the metabolite as MoPC.
Step 8 – Evaluation of the relevance of observed toxic / antimicrobial effects to the MPCA[8]	
Action	**-** Check whether a non-associated effect recorded for a CRS may also be relevant for the MPCA by analyzing the phylogenetic relationship between that particular CRS and the MPCA. Enter a 'Y' or 'N' on the effect-position of C5 on the corresponding row of the Overview table, dependent of whether the analysis resp. confirms or refutes the effect's relevance for the MPCA. - Assess whether any identified metabolites on the finalized list may be associated with a non-associated effect for which non-relevance for the MPCA has not (yet) been established (effect-position of C5 reports a 'Y' or is blank). Update the Overview table in case of a confirmed match between metabolite and (previously) non-associated effect, by merging the data on the row relating to the

[8] To make the process flow more efficient and logical, and to avoid repetition, Steps 7 and 8 have been fitted with a more clearly defined set of actions. The initial purpose of the Steps is still secured within the structure.

	metabolite (C1 and possibly C8) with those on the row pertaining to the respective effect (C4 and possibly C5).
Follow-up	- For non-associated effects that are potentially relevant for the MPCA (effect-position of C5 reports a 'Y' or is blank, and C1 is empty): **go to Step 10 for a broad, non-specific WGS-analysis.** - For non-associated effects that are not relevant for the MPCA (effect-position of C5 reports a 'N', and C1 is empty): **go to Step 11.** - For a successfully established relationship between a non-associated effect and an identified metabolite: **go to Step 11 (possibly after a strongly recommended, optional WGS-verification via Step 9*).** * WGS-verification is strongly recommended when the metabolite has at this stage not yet been confirmed as potentially relevant for the MPCA itself (no 'Y' on the metabolite-position of C5). When this relevance has been confirmed, WGS-verification is, to a higher degree, considered 'nice to have'.
Step 9 – Analyzing genomic data for the absence or lack of expression of genes known to encode identified metabolites (OPTIONAL STEP)	
Action	**-** Use the available WGS-data to establish whether the MPCA has functional* genes that encode hazardous metabolites that have yet only been identified for CRS** - Enter 'Y' or 'N' into C6 of the Overview table when the respective presence or absence of a functional genetic basis for metabolite production in the MPCA has been evidenced. *Note that when the required genetic basis is (largely) present ('Y' in C6), it cannot yet be unambiguously concluded that the MPCA actually produces the concerning metabolite, whereas absence of such basis ('N' in C6) is sufficiently conclusive to eliminate the concerning metabolite from further assessment.* * Genes that contain all mechanistic elements necessary for complete transcription / translation, e.g., a functional promotor, a lack of any critical mutations, etc. ** For hazardous metabolites whose relevance for the MPCA has already been confirmed, this step may serve for purposes of verification.
Follow-up	- In all cases: **go to Step 11.**
Step 10 – Broad genotox -and WGS-screening for micro-organisms that lack a sufficiently solid knowledge base on metabolite production (CONDITIONAL STEP TRIGGERED BY A LACK OF DATA)	
Action	For cases that are directed here via Step 5, (i) genotox-studies, and (ii) broad, non-specific WGS-analyses are carried out. For cases that end up here via Step 8 (non-associated toxic / antimicrobial effects that are potentially relevant for the MPCA), only (ii) needs to be performed. - For (i): Perform a genotox-assay with MPCA-AM extract or filtrate and check whether the results allow drawing a conclusion on the MPCA's ability to cause genotoxic effects through metabolite production. Update the Overview table based on the outcomes. In case of a positive result, 'GEN' is entered as effect in C4 on a new row in the table, along with the test species and name of the MPCA. Fill in a 'Y' on the effect-position in C5 on the same row. In case of a negative result, 'null' is entered as effect in C4 on a new row, along with the test species and MPCA name[9]. **-** For (ii): Perform a comparative assessment of WGS-data against international

[9] Aside from stating a number of caveats that need to be considered when performing genotoxicity studies, the Metabolite GD assigns substantial weight to these particular studies for MPCAs that lack a firm knowledge base on metabolites. In that sense, it is however important to emphasize that genotoxicity studies are not specifically designed to deal with complex matrices such as crude microbial extracts. Results must be used with caution.

	reference data bases and update the Overview table based on the outcome by creating a new table row for each new metabolite and adding the names in C1. Put '(MIA)' after the name, whenever the metabolite meets the criteria for a medically important antimicrobial. Add 'Y' to C6 for all metabolites found. For 'Step 5-cases' only: **-** Check whether a relationship can be confirmed between metabolites identified in the WGS-screening and effects observed in the genotox-tests. In case of a match, the respective metabolite-row (entry in C1) is merged with the corresponding effect-row (entries in C4 and C5). For 'Step 8-cases' only: **-** Check whether the WGS-screening has produced any new metabolites that may be related to non-associated effects potentially relevant for the MPCA. In case of a match, the name of the new metabolite is entered in C1 of the row corresponding to the effect (entries in C4 and C5). Fill in a 'Y' on the metabolite-position of C5, and put a 'Y' in C6.
Follow-up	- For 'Step 5-cases' where no effects have been observed in the genotox-tests: **go to Step 11.** - For 'Step 5-cases' where metabolites have been identified in the WGS-screening that cannot be linked to any effect that may have been observed in the genotox-tests: **start at Step 4 to start collecting specific contextual data.** - For 'Step 5-cases' where metabolites have been identified in the WGS-screening that can be associated with an effect observed in the genotox-tests: **go to Step 11.** - For 'Step 8-cases' where metabolites have been successfully linked to corresponding effects: **go to Step 11.** - For 'Step 8-cases' where metabolites have been identified that cannot be linked to any effect reported in the Overview table: **start at Step 4.** - For 'Step 8-cases' where non-associated effects that are potentially relevant for the MPCA can still not be attributed to a metabolite: **go to Step 11.**
Step 11 – Making an overview of MoPCs based on the preceding steps	
Action	**-** Draw up a conclusion by adding a 'Y' to C7 for identified metabolites for which… …in C4 a toxic / antimicrobial effect has been defined that has been observed for the MPCA; …in C4 a toxic / antimicrobial effect has been defined that has only been observed for CRSs, while in C5 is noted that the metabolite and/or the effect are also potentially relevant for the MPCA; …in C4 a toxic / antimicrobial effect has been defined that has only been observed for CRSs while C6 reports that the MPCA has a functional genetic basis for production of the metabolite; …in C4 a toxic / antimicrobial effect has been defined that has only been observed for CRSs while C8 shows that the metabolite has also been detected in the MPCA-AM/MPCP. *Exception: for in situ-produced antimicrobial metabolites ('AM' as the only effect in C4 and 'N' in C8) no further assessment is required as these metabolites do not constitute a foreseeable risk. Enter an 'N' in C7 for these specific cases.* - Enter a '?' in C7 for identified metabolites for which in C4 a toxic / antimicrobial effect has been defined that has only been observed for CRSs, but that has not yet been unambiguously related to the MPCA. In that case, C5 is blank, C6 does not report anything conclusive ('Y' or nothing), and C8 contains a 'N' or is empty.

	- Enter a 'N' in C7 for all other cases. *N.B. This includes all effects that are still non-associated at this stage (i.e., they have not yet been related to a causative metabolite). These cases have either been directed here via Step 8 or 10.* *For 'Step 8-cases', the conclusion is supported by a confirmed non-relevance for the MPCA.* *For 'Step 10-cases', the conclusion is more pragmatic; the metabolite assessment is aborted under the assumption that the observed effects are not caused by a metabolite. After all, the search for a metabolite-effect relationship has at this stage received the maximally justifiable amount of attention, without a positive result. Any remaining uncertainties related to this may still be addressed in the respective risk assessment.*
Follow-up	- For MoPCs verified for the MPCA ('Y' in C7): **go to Stage 3, Step 13.** - For MoPCs verified for the MPCA that have skipped Step 7 ('Y' in C7, no input in C8): **go to Step 7 before proceeding, as Step 13 requires analytical screening data.** - For MoPCs that have not yet been verified for the MPCA ('?' in C7): **go to Stage 3, Step 12.** - For 'metabolites of no concern' ('N' in C7): **abort the metabolite assessment.**

STAGE 3 – DETERMINE WHICH METABOLITES ARE OF CONCERN

In the third stage, MoPCs defined after Stage 2 will be either classified as MoC or as 'metabolite of no-concern'. During this translational stage from hazard to risk, the exposure to the MoPCs must be assessed.

Step 12 – Determining whether the MPCA is capable of producing the MoPC under circumstances that are known to trigger its production in CRSs	
Action	**-** Check whether the MPCA is capable of metabolite production under circumstances that are known to trigger production in CRSs. *Note that the experiments[10] and chemical analyses suitable for this step differ from those carried out in Stage 2, as they are specifically designed to trigger the MPCA to produce metabolites. For chemical analyses with induced metabolite production, useful information may be found in OECD 98.* - Update, depending on the nature of the data, C4 (in case of (eco)tox-tests performed with the MPCA), C6 (in case of WGS-screening of the MPCA) and/or C8 (in case of analytical screening of the MPCA or derivatives; record the test outcome on the 'induced-position'). Align the content of C5 with the results ('Y' of 'N' on the metabolite-position and/or effect-position, if the metabolite / effect was found to be relevant for the MPCA or non-relevant, respectively.
Follow-up	- In all cases: **go to Step 11 to update the MoPC-overview.**
Step 13 – Establishing whether there is potentially relevant exposure to MoPCs within the proposed context of use	
Action	**-** Define the critical determinants of exposure of humans and other NTOs to the MoPC: (i) information on PPP-use; (ii) ecology of the MPCA and circumstances that trigger MoPC production; (iii) relevant MoPC properties (e.g., degradation); (iv) presence of the MoPC in the MPCP (see C8). **-** Substantiate that exposure of operators, workers, bystanders, residents, and consumers to the respective MoPC is non-relevant. For MoPCs produced *in situ* ('*in situ*-MoPCs'; 'N' in C8), contextual determinants (i), (ii), and (iii) should be considered in the argumentation. For MoPCs present in the MPCP ('MPCP-MoPCs'; 'Y' or input value in C8), mainly determinants (iii) and (iv) are relevant to consider. When non-relevance of the MoPC to the mentioned exposure groups cannot be sufficiently evidenced, outcomes of residue-trials may be used to strengthen the case. All exposure groups (operators, workers, etc.) that may be subject to relevant exposure upon proposed use of the product are entered in C9. In case there is no relevant exposure of any of the groups, '-' is entered in the 'TOX-position' for the respective MoPC. **-** Use contextual determinants (i), (ii), (iii), and (iv) (dependent on whether the metabolite is an *in situ*-MoPC or an MPCP-MoPC; see above) to assess via which

[10] Standard (eco)tox tests do not cover the stated test purpose and there are currently no specific test protocols available. The tests indicated here are yet roughly conceptualized as small scale fermentation experiments where certain combinations of environmental parameters are optimized to trigger production of the metabolite. Such studies are evaluated on a fit for purpose basis.

	environmental compartments relevant exposure may occur. **-** Determine, based on this information, which NTO-groups may suffer relevant exposure. Add these groups to C9 on the row relating to the respective MoPC. In case there is no relevant exposure of any of the NTOs, '-' is entered in the 'ECOTOX-position' for the respective MoPC.
Follow-up	- For MoPCs to which there is no relevant exposure upon proposed use of the product ('-/-' in K9): **go to Step 15.** - For all other cases: **go to Step 14.**
Step 14 – Performing a qualitative and/or semi-quantitative[11] assessment to determine whether the indications of relevant exposure found in Step 13 can be corroborated	
Action	**-** Refine the preliminary assessment performed in Step 13… …for operators, workers, bystanders and residents that are potentially exposed to MPCP-MoPCs as a result of MPCP-use, by following the relevant guidance applying for chemical substances. In case of dietary exposure of consumers, a theoretical worst-case estimate of residue-intake is made, based on lowest mean crop yields and the highest application rate of the MoPC. The worst case intake is compared to the TTC. If possible, consumer exposure will be estimated using EFSA's PRIMo, and the outcome will be related to the ADI or ARfD (whenever available – see Step 17). In case exposure via groundwater is relevant, a PEC_{GW} needs to be established. Enter for each relevant exposure group (see C9) a corresponding critical reference value (in C11) and exposure level / intake (in C12) – maintain the same order as in C9. When the exposure level in C12 exceeds the corresponding reference value in C11, the linked input in C9 is maintained. If the value in C12 is lower, the respective input group in C9 is replaced by '-'. …for humans potentially exposed to '*in situ*-MoPCs', by checking whether MPCP-use causes a sustained exceedance of the MoPC's expected natural background level or its natural occurrence[12]. When exposure cannot be excluded, worst-case MoPC-production by the MPCA can be induced in the lab (see analytical screening under Step 12). Amended with information on population dynamics of the MPCA in the relevant compartment, the max. *in situ* MoPC-production in the respective compartment can be estimated. Update C11 and C12 as described above for MPCP-MoPCs. When *in situ* exposure (C12) exceeds the corresponding reference value (C11), the respective input in C9 remains unaltered. Otherwise, the group in C9 is replaced by '-'. …for NTOs potentially exposed to 'MPCP-MoPCs' and '*in situ*-MoPCs' (see exposure group-input in C9), by calculating PECs for all relevant compartments, following (i) standard FOCUS-modelling and decision trees for MPCP-MoPCs and (ii) a weight-of-evidence approach for *in situ*-MoPCs. Add the results to C12 on a position that corresponds with that of the respective groups in C9. Derive an estimate of the natural background level for all compartments in which

[11] As most of the qualitative assessment has already been performed during Step 13, the semi-quantitative approach already takes a more pronounced position here than it does in Step 14 in the Metabolite GD. This step is however essentially different from Step 16, although there is an apparent overlap. The semi-quantitative assessment proposed here should be fit for its purpose to distinguish between MoCs, and those that are not of concern. As such, for Step 14 it suffices to focus on the most critical exposure scenarios only, whereas at Step 16, all scenarios should be considered.

[12] Whether natural background or natural occurrence must be considered depends on the applicable regulatory framework for the Data Requirements (i.e., pre -or post the amendment as per (EU) 2022/1439).

	NTOs may suffer relevant exposure to the respective MoPC after MPCP-use. For this purpose, literature data, worst-case assumptions based on lab-induced MoPC-production, and data on *in situ* population dynamics of the MPCA may be used. Enter the relevant and available information that refers to background levels as reference values at their appropriate (ECOTOX-) positions in C11. Whenever the PECs (C12) exceed the natural background (C11), exposure is considered relevant. In that case, the corresponding input in C9 remains as is. If not, the respective NTO exposure group in C9 is replaced by '-'.
Follow-up	- For all cases: **go to Step 15.**
Step 15 – Making an overview of MoCs based on the preceding qualitative and/or semi-quantitative assessment	
Action	**-** Draw up a conclusion by adding a 'Y' to C10 for MoPCs for which in C9 one or more human and/or NTO exposure groups have been defined. For MoPCs for which relevant exposure of humans and/or NTOs can be excluded after the Stage 3 assessment (only '-' in C9), 'N' is entered in C10.
Follow-up	- For all established MoCs ('Y' in C10): **go to Stage 4, Step 16.** - For all MoPCs to which no concern-status has been assigned ('N' in C10): **abort the metabolite assessment.**

STAGE 4 – RISK ASSESSMENT FOR METABOLITES OF CONCERN

Stage 4 focuses on the risk assessment of all metabolites for which non-relevant exposure could not be established, and for which attributed toxicological effects are considered relevant for the proposed uses.

All toxicological endpoints need to be covered – in principle according to the relevant systematic that applies for chemical active substances, unless this approach is inappropriate or technically unfeasible.

Step 16 – Determining the exposure to MoCs according to standard procedure	
Action	**-** Assess the exposure of operators, workers, bystanders, en residents / consumers to 'MPCP-MoCs' according to Part A of (EU) No 283/2013 and relevant guidelines. Put the resulting exposure, expressed in appropriate terms, on the corresponding position in C12 (replacing any previously entered value if necessary). - Assess concentrations of *in situ*-produced MoCs on edible parts of crops according to Part A of (EU) No 283/2013. The resulting levels are expressed in appropriate terms and entered in C12 on the position corresponding with that in C9 and C11, replacing the existing input if necessary. Alternatively, and *only* for those cases where (i) there has been no direct contact between the MPCA and the edible parts and (ii) the MPCA cannot be present inside of the plant, the population density of the MPCA on the edible parts of treated plants can be analyzed. Only in case of negative results, dietary exposure to the metabolite can be considered non-relevant, and thus, the corresponding exposure group in C9 can be replaced by '-'. - Derive PECs for 'MPCP-MoCs' in the relevant compartments according to Part A van (EU) No 283/2013 – if needed, based on conservative standard values for any required substance properties. Enter the PECs in C12 on the position corresponding with that in C9 and C11, in case this had not yet been done earlier during Step 14. - Assess '*in situ*-MoCs' according to a context-sensitive, expert judgement-based approach that considers e.g., residue trial results, measurements in relevant compartments, or (monitoring-)data regarding population densities of the MPCA in the field. As a principle, data must be unambiguous and must be technically feasible to produce.
Follow-up	- For all cases: **go to Step 17.**
Step 17 – Determining reference values (human toxicology) and ecotoxicological endpoints for MoCs	
Action	**-** Add the critical reference value – if available – to the appropriate position in C11 (corresponding with those in C9 and C12) insofar this had not yet been done. Assess for each case whether the TTC-approach may be used. Enter a '-' in case no reference value is available and the TTC-approach is not appropriate. **-** Assess whether endpoints may be derived for NTOs exposed to the MoC based on the literature or on the ecotox-studies performed during Step 6. The ecotoxicological endpoint is entered in C11, on the position corresponding with C9 and C12. Add a '-' whenever no appropriate endpoint is available for the respective exposure group.
Follow-up	- In case no appropriate reference -or TTC-values, or ecotoxicological endpoints are available at this stage, nor any relevant toxicity studies from which reference values / endpoints may be derived: **go to Step 18.**

	- In case there are no appropriate reference -or TTC-values, or ecotoxicological endpoints at this stage, but there are relevant toxicity studies from which reference values / endpoints may be derived: **go to Step 19.** - In case appropriate reference -or TTC-values, or ecotoxicological endpoints (whichever are relevant) are available for the MoC: **go to Step 20.**
Step 18 – Performing specific (eco)tox-tests to produce reference values / endpoints	
Action	**-** Carry out GLP-compliant toxicological studies, the choice of which is instructed on a case-by-case basis by any relevant toxic / antimicrobial effects reported in the literature (see Stage 2). If there is no unambiguous and sufficiently specific hazard-indication available in the literature, an oral subacute toxicity study is recommended, provided that sufficient quantities of test substance are available. - For MoCs with a suspected genotoxic activity whose exposure exceeds the TTC for potential DNA-reactive mutagens and / or carcinogens, it is appropriate to conduct the test battery in accordance with EFSA's Scientific Opinion (see EFSA J. 2011;9(9):2379 and subsequent updates). Verify whether the data allow the use of a TTC value indicated for other classes of substances, and include the new studies in the Overview table by adding entries in C4 (including observed effect, test species and MPCA name) on the row relating to the respective MoC. **-** Perform GLP-compliant studies according to relevant international guidelines for all relevant cases for which no ecotoxicological endpoints are yet available. If necessary, representative, higher-tier studies are to be conducted. Record the new studies in C4 of the respective MoC, along with observed effect, test species, and MPCA name.
Follow-up	- For all cases: **go to Step 19.**
Step 19 – Establishing reference values and (safe) endpoints (human toxicology – ARfD/ADI/(A)AOEL/NOAEL/NOEC; ecotoxicology – NOEL/NOEC/LD_{50}/EC_{50}/PNEC) based on relevant (eco)toxicity studies	
Action	**-** Derive appropriate reference values and endpoints from the (eco)toxicity studies that are available at this stage. Enter the resulting values in C11 on the positions corresponding with those in C9 and C12.
Follow-up	- For all cases: **go to Step 20.**
Step 20 – Comparing exposure to corresponding reference values / endpoints	
Action	**-** Check whether exposure exceeds the reference -or TTC-values, or endpoints, to identify any unacceptable risk. For the ecotoxicological risk assessment of MoCs, the same standard triggers are employed as for Part A active substances. - Enter for all relevant exposure groups a 'Y' or 'N' in C13 on the position corresponding with that in C9, C11, and C12, depending on whether resp. an unacceptable or acceptable risk has been established. *Note that it is assumed here that reference values / endpoints have been expressed in a way that allows them to be compared directly, and that they relate to the same exposure group, that furthermore corresponds with the one defined in C9.*
Follow-up	**Conclude the metabolite assessment. Define any appropriate action that may be triggered by the conclusion in C13 (see note 6 to the table).**

Overview table in support of the metabolite assessment

	Stage 1		Stage 2					Stage 3				Stage 4
Metabolite identifier[1)]	**Active substance (Y/N)**	**Secondary contributor to MoA (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)[6)]**
			Toxic / antimicrobial effect observed, test species, and strain[2)]	**Potential relevance for MPCA[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. MPCP: 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..
EXAMPLE: Beauvericin **(illustrative purposes only)**	N	N	Sushi, J (2012): CYT / human cells / *B. bassiana* EXAMP1	Y / Y	Y	Y	MPCP: 3.2 mg/L Induced: Y	OP; WO; BY / -	Y	1.5 µg/d; 1.5 µg/d; 1.5 µg/d / -	1.8 µg/d; 0.8 µg/d; 0.5 µg/d / -	Y; N; N / -
The row below presents the SANCO/2020/12258 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**
PLEASE DELETE THE YELLOW HIGHLIGHTED ROWS AFTER PASTING THIS TABLE INTO THE DAR/RAR – The row below contains column numbers that are helpful when following the stepwise procedure in this Appendix												
C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**

[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 MPCA 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 MPCA 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 instead.

[4)] This column states whether or not a metabolite has been detected in the MPCA-AM or MPCP (both Step 7), or after induction of the MPCA (Step 12; Y or N for the relevant slot). 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 MPCP (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 '-'.

[6)] In general, the result in this column triggers another action, like the establishment of a threshold concentration for inclusion in the Implementing Regulation or of a Residue Definition, or the definition of a specific restriction. For each established unacceptable risk, resulting action(s) must be defined.