Frequently Asked Questions in the context of aquatic Mixture Toxicity and in relation with the use of the MixTox Tool

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- **Disclaimer**: This FAQ answers common questions on complex areas of the aquatic mixture risk assessment. Please, be aware that for these topics, no strict guidance exists in the Aquatic Guidance Document (AGD, EFSA Journal 2013; 11 (7): 3290); thus the given answers should be considered only as recommendations from the group which developed this MixTox tool (i.e. members of authorities from DE, DK, AT, NO and NL).

1. Metabolites

- Question: How metabolites formed in water could/ should be included in the aquatic mixture risk assessment?

Incorporation of metabolites in the mixture toxicity risk assessment should be done on a case-by-case basis. Many variables influence this assessment. However, some structured suggestions are provided below:

SCHEME

Example based on a mixture of 2 a.s. (parents A and B, i.e. "A" and "B" in following text) and a metabolite (from parent A, i.e. "metab.")

1- Is the metabolite clearly toxic (i.e. of equal or higher toxicity compared to the parent)?
   - Yes, go to 2
   - No: conduct a MixTox risk assessment based simply on a.s. A and B (see below, section M1)

2- Is the metabolite much more contributing to the risk* than the a.s.?
   (i.e. > 90% of risk due to PEC_{metab}/RAC_{metab} + PEC_A/RAC_A attributed to PEC_{metab}/ RAC_{metab} at the most critical/ worst-case(s) FOCUS step and scenario(s)) ?)
   - Yes: conduct a MixTox risk assessment based on the metab. and B.
   - No or unknown: go to 3

   * it is meant the risk, not the toxicity

3- Is the maximum formation rate of the metabolite within the test duration of the a.s.?
   (based on information available from water-sediment degradation tests)
   - Yes: see below, section M2
   - No or unknown: see below, go to 4

4- Is the maximum formation rate of the metabolite outside the test duration of the a.s.?
   - Yes: see below, section M3
   - No: if the toxic metabolites formed in another compartment, see below section M4

PROCEDURE

As above: Example based on a mixture of 2 a.s. (parents A and B) and a metabolite (from parent A, i.e. "metab.")

Section M1. Evidently non-toxic metabolites

No further consideration needed in the mixture toxicity risk assessment.

MixTox risk assessment:

→ Conduct a mixture toxicity risk assessment using the endpoints of the a.s. tests ("A " and "B") with the mixed Tox tool

Section M2.Toxic metabolites with maximum formation rates within the test duration.

The toxicity due to the metabolite is reflected in the test performed with the parent A

For a.s. test evaluation:

- ***The toxic metabolite is of less toxicity compared to the parent:***
  No need for special consideration to derive the endpoint

- ***The toxic metabolite is of equal or higher toxicity compared to the parent***:
- *Option1:* The test endpoint is expressed in mg parent/L.
  The test endpoint is derived considering that the toxicity in the test is only attributed to the concentration of the parent in the test (instead of the sum parent + metab, (option 2)). This approach is considered as suitable and conservative if metabolites have not been measured in the parent test (please note that however generally, especially toxic metabolites should be measured).

  Option 2: The test endpoint is expressed in mg sum parent + metab./L.
  If the metabolite is sufficiently measured in the parent test, the test endpoint can be calculated based on the average concentrations of parent and metabolite. In this case the test endpoint is more accurate but less conservative than in Option 1, because the concentration of the parent plus the metabolite will always be higher than the concentration of the parent alone.
  The calculation of the test endpoint could be done in a similar way as for a product endpoint using the information from Appendix J. of EFSA report 2019 (recurring issues meeting, EFSA Supporting publication 2019:EN-1673) (Section 4.1 Case 1: All active substances have been analytically measured). Please note, that the proportions of the actives and the metabolites as measured in the test should be regarded for the mixture toxicity assessment.

Please note that:
- In Option 1, the value of the test endpoint will be less accurate than in Option 2,
- In Option 2, the risk of mistakes in calculations are higher than in Option 1.

MixTox risk assessment:

➔ Conduct a mixture toxicity risk assessment based on e.g. A (forming a metabolite) and B (has no relevant metabolite). Use the mixture tox tool to enter either the endpoint of A

expressed in mg sum parent A/L (Option 1) or the endpoint of A in mg sum A + metab./ L (Option 2) in combination with the endpoint of B expressed in mg B /L.

➔ In terms of risk calculations, the PEC values used for A should be the sum of PEC A (that dissipates over the time) plus PEC metabolite (that forms over the time).

- For Option 1, use PECmax of substance A only, since only the a.s. is considered for EP derivation;
- For Option 2: use PECmax for A and PECmax for metabolite (for refinements, use PECmax of FOCUS higher steps).

Section M3. Toxic Metabolites with maximum formation rates outside the test duration*

MixTox risk assessment: →Conduct a mixture toxicity risk assessment based on calculated toxicity using a.s. and metabolite data.

* outside the test duration, means e.g. after 96 h. if species most at risk for the acute risk assessment is the fish

Section M4. For toxic metabolites formed in another compartment

This is to address the case of e.g. soil metabolite entering waterbodies via run-offs. Currently, such metabolites are only considered in a separate assessment, such as a single substance (i.e peaks of exposure considered as toxicology independent). Generally such metabolites are not covered and currently, no mixtox approach is available for this case; it will be the subject to future development.

Remarks and exceptions:

- In case a metabolite is more toxic than the parent and the test with a.s./PPP was performed under flow-through conditions, then the endpoint of the test with the a.s./ PPP would be less conservative (unusual situation). In this case, conduct a mixture toxicity risk assessment based on calculated toxicity using active substance and metabolite data as in 3. Alternatively, if available, the EP derived from a test performed with the a.s./ PPP in a static design might be preferred.

- The above considerations are not correct, if e.g. a surfactant in the formulation PPP would change the formation rate of the metabolite. However, this is not expected and difficult to demonstrate, unless it leads to synergy.

2. Using different FOCUS steps, in particular FOCUS Step 4

- Question: How to proceed, if data from a higher FOCUS Step (typically FOCUS Step 4) data are only available for some active substances?

To obtain exact results for the mixture toxicity calculation and mixture risk assessment, the risk calculations should be performed using the PECsw from the same FOCUS Step (1,2,3, or 4) for all a.s.

To ensure an accurate assessment, missing data from a higher FOCUS Step should be requested, even if the higher FOCUS Step is not necessary to demonstrate acceptable risk for a particular a.s. Although assessing FOCUS Step 4 for all a.s. in all FOCUS scenarios, even if not needed, may become a time-consuming effort, the FOCUS Step 4 data should be provided for all a.s. and all FOCUS scenarios unless it can be clearly demonstrated that a particular FOCUS scenario should be considered as worst-case scenario. If mixing different FOCUS Steps is reasonable depends on the

assessment step considered (cf. below); it is strongly advised to include FOCUS Step 4 calculations instead of mixing different FOCUS Steps.

Remarks:

If mixing different FOCUS Steps is possible depends on the respective assessment step:

- for assessment Steps 3 and 4 (product mixture assessment), the ratio in Step 3 needs to be accurate for deriving a correct conclusion (i.e. precise PEC input data). Mixing different FOCUS Steps is not possible.

- For assessment Step 5 and 6 (driver assessment) currently FOCUS Step 4 cannot be calculated and it is also not possible to mix different FOCUS Steps. Mixing different FOCUS Steps is only possible for the assessment Step 8 (as it can handle also "fictional" exposure scenarios), for which it is assumed that using data from earlier FOCUS Step will lead to higher $PEC_{sw,max}$ for those a.s. and, thus, it will give a conservative estimate for the mixture risk assessment. However, this approach should only be taken in exceptional circumstances.

-Note that an evident prerequisite for mixing different FOCUS Steps directly in assessment Step 8 is that synergism is excluded (check MDR calculation in Step 2; in case of a synergism and calculated mixture toxicity include the MDR into the AF/ETR-trigger as suggested in the AGD 10.3.4). Even if a slight synergism occurs, care should be taken and FOCUS Step 4 data should be requested.

- To achieve mixing different FOCUS Steps technically in the tool, enter e.g. into the FOCUS Step input tables (sheet input PEC) the PEC values corresponding to FOCUS Step 2 or 3 for those a.s. which are missing FOCUS Step 4 values (i.e. these are "fictional" but conservative values). The tool does not automatically go back to an earlier FOCUS Step. Of course, for all substances which have FOCUS Step 4 values, use those.

- Lastly, this procedure requires that only the $PEC_{sw,max}$ values are used; any consideration of FOCUS profiles and PECtwa of the active substances should be excluded.

3. Chronic mixed Toxicity

- Question: How should the chronic mixture toxicity be included in the assessment?

Background information:

How and whether to conduct a chronic mixture toxicity assessment is currently not harmonized between zones and member states.. Only limited specific information for chronic mixture toxicity is provided in the AGD, but the approach developed for acute mixture toxicity was meant to be applied also for chronic (see e.g. section 10.3.4. about MDR and step 1 scheme).

In order to facilitate a chronic assessment, please see the following suggestions:

- In general, the chronic mixture toxicity assessment can follow the acute approach from the AGD (section 10.3.11). When using a calculated mixture toxicity approach (which is likely as often chronic product data are not available) going directly to the RQ_{mix} (Step 8b) is preferred (after synergism is discussed, i.e. Step 7). This is also due to technical reasons, as the ETR-triggers are hard coded in this tool while the AF for the RQ_{mix} can be adapted (which is necessary for a correct chronic toxicity assessment).

Measured mixture toxicity:

- If a chronic formulation test is available, the standard steps of the risk assessment scheme (AGD 10.3.11) should be followed.

- A formulation test (chronic) should be delivered if it is not possible to extrapolate the mixture toxicity from data of the a.s. For instance, if a PPP is more acutely toxic than the a.s. by a factor 10, a product test is mandatory (AGD 10.3.2; unless demonstrated that exposure will not occur).

Calculated mixture toxicity:

- In accordance with the B&M GD, EC_{10} values should be preferably used for the mixture toxicity calculations (if they are available) because the NOEC strongly depends on dose-spacing and could already reflect a certain effect level – besides exhibiting several other drawbacks.

- In long-term/chronic toxicity tests, typically more diverse biological endpoints are tested than in the acute assessment. Thus, a two-step approach is reasonable.

- In a first step, a conservative approach can be taken (i.e. make worst-case assumptions) by combining different (lowest) endpoints, e.g. egg production for substance A and body weight for substance B.
- In a second step, a less conservative approach can be taken as a refinement step, that is combining comparable endpoints, e.g. referring to the example above, combine only egg productions or only body weight effects. However, if for substance A egg production is relevant and for substance B egg hatching, a discussion may be indicated why these endpoints should not be considered jointly.

4. Simplified approach

- Question: What to do with the simplified mix-tox assessment mentioned in the guidance?

The simplified approach is included in the guidance (10.3.7) and can, thus, be taken if conducted correctly. It was not included in the tool in order to not further increase its complexity, but it is a valid approach.
Note that if the check on synergism indicates that synergism occurs, the simplified approach is not valid.
Also, the individual ETR should be below their respective trigger values divided by the number of a.s. in the product..
Note also that the driver is often based on the product composition (as stated in the guidance) but this is scientifically not correct (as clarified in an email exchange with EFSA). The tool (assessment Step 5- Driver assessment) can be used to check if this is relevant in the specific case (i.e. if the results of Equation 14 are different in case the proportions are based on the composition of the product instead of the composition at the PECmix, the latter of which may lead to different results for the FOCUS scenarios under assessment).