

**Evaluation Manual
for the Authorisation
of Plant protection products
according to Regulation (EC) No 1107/2009**

NL part

Plant protection products

Chapter 7 Ecotoxicology; aquatic

version 2.3; March 2019

ctgb

**Board
for the Authorisation
of Plant protection products and Biocides**

Chapter 7 Ecotoxicology; aquatic

Category: Plant protection products

General introduction	3
I Aquatic and sediment dwelling organisms	3
4. NL framework	3
4.1. Introduction	3
4.2. Data requirements	3
4.3. Risk assessment.....	4
4.4. Approval.....	7
4.4.1. Criteria and trigger values	7
4.4.2. Decision making.....	8
4.5. Developments	8
II Effects on a sewage treatment plant (STP)	8
2. NL framework	8
2.1. Introduction	9
2.2. Data requirements	9
2.3. Risk assessment.....	9
2.4. Approval.....	9
2.4.1. Criteria and trigger values	9
2.4.2. Decision making.....	10
2.5. Developments	10
3. References	11

Changes in the Evaluation Manual

Evaluation manual PPP NL part Chapter 7 Ecotoxicology; Aquatic			
Version	Date	Paragraph	Changes
2.0	January 2014		
2.1	October 2016	Chapter 1.3	Further elaboration or clarification on risk assessment issues that are used by Ctgb included in the text of 1.3: <ul style="list-style-type: none"> - Expression of the endpoints from aquatic studies - Species Sensitivity Distribution: Acceptability criteria HC5
2.2	April 2017	Chapter 4.3	Change with respect to the endpoint for algae and aquatic plants, based on the decision of the Board end of 2017.
		Appendix 1	Updated
2.3	March 2019	2	Bgb link update
		All paragraphs	Link checked

GENERAL INTRODUCTION

This chapter describes the data requirements for estimation of the effects of a plant protection product and its active substance on the aquatic environment and STP, and how reference values are derived in the NL framework (§2 - §2.5).

This chapter consists of two parts: a part about effects on aquatic and sediment dwelling organisms (I), and a part about effects on sewage treatment plants (STPs) (II),

I AQUATIC AND SEDIMENT DWELLING ORGANISMS

4. NL FRAMEWORK

The NL framework (§2 - §2.5) describes the authorisation procedure for plant protection products based on existing substances, included [Commission Implementing Regulation \(EU\) No 540/2011](#) and new active substances. A new substance is a substance not authorised in any of the Member States of the EU on 25 July 1993.

The plant protection product that contains such substances may be authorised if the criteria laid down in the [Regulation \(EC\) No 1107/2009](#) are met, also taking into account the national stipulations described in the [Bgb](#) (Plant protection products and Biocides Decree). The evaluation dossiers must meet the requirements in [Commission Regulation \(EU\) No 283/2013](#) and [Commission Regulation \(EU\) No 284/2013](#) implementing Regulation (EC) No 1107/2009 (see Application Form and corresponding instructions).

A Member State may deviate from the EU evaluation on the basis of agricultural, phytosanitary and ecological, including climatological, conditions which are specific for the Netherlands.

The NL framework describes the data requirements (§2.2), evaluation methodologies (§2.3), criteria and trigger values (§2.4) for which specific rules apply in the national approval framework or when the national framework has been elaborated in more detail than the EU framework.

The NL procedure described in §2 - §2.5 of this chapter can also be used for evaluation of a substance for approval, and consequently inclusion in [Commission Implementing Regulation \(EU\) No 540/2011](#) in case no European procedure has been described.

4.1. Introduction

This chapter describes the aspects for aquatic and sediment dwelling organisms for which specific rules apply in the national approval framework .

NL-specific drift percentages, deviating from the EU evaluation methodology, are used as input for calculation of the PEC for aquatic and sediment dwelling organisms. There is a national system of drift-reducing measures as well. This serves to meet the specific NL conditions (climatological conditions; specific standard drift-reducing measures packages from the Activity Decree (expected January 2017). This is elaborated in §2.3.

This chapter is related to Chapter 6 Fate and Behaviour in the environment; behaviour in surface water, sediment and sewage treatment plant (STP) where the estimated or measured concentrations in water and sediment are determined.

4.2. Data requirements

The data requirements for chemical Plant protection products are in compliance with the

provisions in EU framework (see §1.2 of this chapter). NL-specific data requirements and further elaborations of the EU data requirements are given in the text below.

Experiments carried out after the 25th of July 1993 must have been carried out under GLP.

There may be no doubt about the identity of the tested product or the purity of the tested substance for each study.

For animal welfare reasons it is recommended to limit the vertebrate tests with formulations and also metabolites as much as possible. In some cases it is even not allowed to submit fish studies with formulations, i.e. in the case that already fish studies are available with a comparable formulation

4.3. Risk assessment

The evaluation methodologies for chemical plant crop protection products are in compliance with the provisions in EU framework (see §1.3 of the EU part).

The national evaluation is in line with the risk evaluation methodology for aquatic and sediment dwelling organisms as elaborated in the [Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters \(EFSA Journal 2013; 11\(7\):3290\)](#), with the exception of the drift percentages used for the calculation of the concentration in surface water. The used drift percentages are NL-specific, to meet the NL-specific climatological conditions and the specific standard drift-reducing measures packages from the Activity Decree (expected January 2017).

National drift figures can be applied on the basis of [article 8f](#) of the [Bgb](#) (Plant protection product and Biocides Decree).

Artikel 8f. Driftcijfers

Bij de risicobeoordeling voor waterorganismen, vogels, zoogdieren, niet-doelwitarthropoden, niet-doelwitplanten of oppervlaktewater bestemd voor de bereiding van drinkwater, hanteert het college specifieke driftcijfers. Het college stelt deze cijfers vast en maakt hen bekend op zijn website.

For the drift percentages reference is made to chapter 6: Fate and Behaviour in the environment; behaviour in surface water and sediment .

In addition, further elaboration or clarification on risk assessment issues that are used by Ctgb are included in the text below:

1. Expression of the concentration in water concerning endpoints of aquatic toxicity tests

Acute

Static tests, one active substance:

a) Concentration at the end of the test > LOQ:

- If the measured concentration during the test stays between 80 and 120% of the nominal concentrations for all measurements and all dose levels, then the endpoint

based on nominal concentrations is acceptable. If the endpoints in the test report are already based on measured concentrations this does not have to be corrected / assessed of course.

- If the concentration falls below 80% of nominal in any dose level at any time point, the endpoint based on geometric mean measured concentrations must be used.

b) Concentration at the end of the test < LOQ:

When the substance is **very unstable** (DT50 < 3 hours), the initial measured concentration may be used for calculating the endpoint of the test.

- When the substance is **unstable**, then for the time point at which the measurable residue is below the LOD/LOQ, half of the LOD/LOQ should be used for calculating the geometric mean measured concentration for the endpoint. If measurements were only done at the beginning and end of the test, and it cannot be estimated when the concentration of the substance went below the LOD/LOQ (if both the LOD and the LOQ are given, take the lowest), in principle the test must be rejected.
- For tests with formulations, which are often static tests, it is important to measure frequently to avoid the situation described above. If this has not been done, the test should in principle be rejected (unless no effects are observed at concentrations which could be measured (EC50 'greater than') or the degradation pattern at higher dose levels, can be extrapolated to the lower concentrations, so long as the measured concentration at the end of the test is not below the LOD/LOQ (see below more information)). The reason for rejecting these tests is that (1) it cannot be determined whether the criteria of 'very unstable' (DT50 <3 hours) are fulfilled, and (2) no reliable geometric mean concentrations can be calculated.
- The period over which a geometric mean concentration must be calculated depends upon the period over which effects were observed in the test. For example, if there is complete mortality within 2 hours, it is acceptable to use initial measured concentrations to set the endpoint. If there is mortality during the first two days and none afterwards, then the geometric mean concentration over the first two days must be calculated. If there are concentrations that fall below the LOD/LOQ during the course of the test and the effects are observed during the whole test period, then the geometric mean concentration must be calculated over the whole test period using a value of half of the LOD/LOQ for the concentrations which were no longer measurable.

Geometric mean concentrations are calculated according to the following formula:

$$\frac{i \times \sqrt{C_{t=0} \times C_{t=i}} + (j-i) \times \sqrt{C_{t=i} \times C_{t=j}}}{j}$$

In this formula, 0, i and j are sample time points.

For example, for a test with algae of 72 hours with sample points at day 0, 1 and 3, the formula is then:

$$\frac{24 \times \sqrt{C_{t=0} \times C_{t=24}} + 48 \times \sqrt{C_{t=24} \times C_{t=72}}}{72}$$

- If, at a higher dose level, the measurable residue has not fallen below the LOD/LOQ by the end of the test, information gleaned from the degradation pattern at that dose level can be taken into account when calculating the geometric mean measured concentration in lower doses, as follows: The degradation pattern seen in the higher exposure level may be extrapolated to lower exposure levels where concentrations

fell below LOD/LOQ. In order for this methodology to be considered, it must be clear that the degradation pattern at lower concentrations is not different from the pattern at the (higher) concentration used for extrapolation (expert-judgement).

Static tests, more than one active substance:

- In the case of a test with a formulation with more than one active substance, the degradation pattern of the substance which degrades most quickly should be used for the calculation of the endpoint of the formulation, unless this substance:
 - does not contribute significantly to the toxicity of the formulation; and/or
 - belongs to the group of 'very unstable' substances.

Semi-static and flow-through:

The procedure as described above is also valid for semi-static and flow-through tests. However, in well-performed flow-through tests the test concentrations should be well maintained (if the measured concentrations fall below 80% the test should be rejected). If, in a semi-static test, the concentrations at the end of the test medium renewal intervals are below the LOD/LOQ, the test should in principle be rejected. In such a case a flow-through test should have been performed, or at least a shorter renewal interval should have been used (e.g. in a *Lemna* study, where flow-through conditions are not feasible).

Chronic tests

In most cases, chronic tests are performed under flow-through conditions. Some exceptions to this include the 28 day water-spiked test with *Chironomus riparius* and modified exposure tests. In these cases, in principle the same criteria are valid as were outlined for acute tests. However, it is also possible to show that the exposure in the test is, at any moment, worst-case compared to the calculated exposure profile(s). If that is the case, the nominal/initial measured concentration may be used for calculating the endpoint.

For more details see appendix 1, in which a more detailed elaboration is made of what is described above. Also examples are given.

2. Species Sensitivity Distribution: Acceptability criteria HC5

If an SSD is run, the data normality must be accepted at no less than 0.05 significance level to be acceptable for use in RA (look under "goodness-of-fit" in ETX 2.0). Modelling which does not pass at least this level (i.e. only passes at 0.025 or 0.01) indicates a poor fit for the data and a less reliable outcome¹.

There are several other issues like the use of the ErC50 or EyC50/EbC50 in the risk assessment for algae and aquatic plants, the geometric approach, the use of the NOEC or NOEAEC from micro-/mesocosm studies in risk assessment and the use of the PEC_{sw-twa}. For these issues reference is made to the EU part for aquatic and sediment organisms of the Evaluation Manual.

¹ As the significance level decreases (and the critical value increases), it becomes less and less probable that the sample derives from a normal distribution.

3. Endpoint to be used in the risk assessment for algae and aquatic plants

In the Board meetings of November and December 2016, the Board has decided to follow in principle the EFSA aquatic guidance document ([Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters \(EFSA Journal 2013; 11\(7\):3290\)](#).) with respect to the use of the growth rate (E_rC_{50}) endpoint in the risk assessment for algae and aquatic plants, despite the fact that the Board was of the opinion that the yield is a better endpoint in relation to the protection goals for these taxonomic groups. Hence, it is a procedural decision. The date of entry into force of this decision is the 1st of January 2017.

However, it is acknowledged that uncertainty is introduced with respect to the protectiveness of the risk assessment for algae and aquatic plants (E_rC_{50} for algae is in general a factor of 4-5 higher than the E_yC_{50} and for aquatic plants this is about a factor of 2, while the trigger values has not changed). To reduce the uncertainty the following approach will be followed:

- If the PEC/RAC ratio based on the E_rC_{50} for algae and/or aquatic plants is just below the trigger of 1 (factor of 2 or less below the trigger: $0.5 > \text{PEC/RAC} \leq 1$), and the PEC/RAC based on E_yC_{50} is clearly higher than trigger of 1 (factor of 2 more: $\text{PEC/RAC} \geq 2$), the protectiveness of the risk assessment comes into question. In such cases drift reduction measures one class higher than would be necessary on basis of the risk assessment with the E_rC_{50} should be applied (e.g. from DRT class 50% to DRT class 75%, or from DRT class 75% to DRT class 90%). If it is not possible to go to a higher drift reduction class, because based on the E_rC_{50} the highest possible drift reduction class should be applied, the possible risk in the assessment will be highlighted and clearly described, and the decision will be left to the Board how to proceed with the application.

EFSA is busy with a corrigendum of the EFSA aquatic guidance document (2013) and one of the activities is a proper calibration with respect to the protectiveness of the E_rC_{50} endpoint for algae and aquatic plants. Depending on the outcome of the calibration the proper endpoint for algae and aquatic plants to be used in the risk assessment may change.

Combination toxicity

Combination products are formulated plant protection products that contain more than one active substance. Combinations of plant protection products of which, in accordance with the recommendations in the directions for use, the user prepares a combination in a tank (tank mix) are also considered as combination products. The issue of combined toxicity is further described in Appendix A. Also in the [Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters \(EFSA Journal 2013; 11\(7\):3290\)](#), a section is included about mixture toxicity. However, this section is still unclear on several points and Ctgb prefers the approach as described in Appendix A.

4.4. Approval

The evaluation of products on the basis of existing active substances already included in [Commission Implementing Regulation \(EU\) No 540/2011](#), or new substances, has been laid down in [Regulation \(EC\) No 1107/2009](#). Where no European methodology is agreed upon, a national methodology is applied as described in the [Bgb](#) (Plant protection product and Biocides Decree).

4.4.1. Criteria and trigger values

For the criteria and trigger values for aquatic and sediment dwelling organisms for the national authorisation reference is made to the EU part (§ 1.4.2 EU-chapter).

4.4.2. Decision making

For decision-making as regards aquatic and sediment dwelling organisms for the national authorisation reference is made to the EU framework (§ 1.4.3 EU-chapter).

4.5. Developments

Multiple stress and mixture toxicity

In many crops during the growing season more than one compound will be used. In some crops this can add up to more than 50 applications and some of these compounds will be applied together, e.g. an herbicide together with an insecticide and/or fungicide. Sometimes even two or three herbicides or two or three fungicides or two insecticides may be applied simultaneously, up to 5 or 6 compounds at the same time. When these combinations (e.g. tank mixes) are not sold as a formulation the legislative process does not take account for the potential combined effects of the use of these tank mixes. Neither does the legislative process take into account that different compounds of the same group (e.g. insecticides) or of different groups (e.g. insecticides, herbicides, fungicides) are used over time in the same growing season.

When a compound is allowed on the market this decision is sometimes based on the potential of recovery. Whether under different crop scenarios the recovery option is appropriate to use in the derivation of the RAC needs to be evaluated from an ecological point of view, since during the growing season drainage ditches may be affected multiple times by the use of plant protection products. Research on multiple stress of pesticides on aquatic communities representative for Dutch drainage ditches, and how to deal with mixture toxicity of pesticides, has already been initiated in the past (Hartgers *et al.*, 1998[1]; Deneer, 2000 [2]; De Zwart, 2005 [3]; Van Wijngaarden *et al.*, 2004 [4]; Arts *et al.*, 2006 [5]; Van den Brink *et al.*, 2002b [6] & 2009 [7]). In 2009 a literature research was started to update the knowledge on mixture toxicity (Verbruggen & Van den Brink, 2010) [8]. In addition, a working group has been installed to look into the problem of multiple stress caused by pesticides in Dutch drainage ditches. This group has analyzed some of the more realistic worst cases of pesticide use in crops (e.g. potatoes and fruit). A report is still to be expected.

II EFFECTS ON A SEWAGE TREATMENT PLANT (STP)

2. NL FRAMEWORK

The NL framework (§2 - §2.5) describes the authorisation procedure for plant protection products based on existing substances, included [Commission Implementing Regulation \(EU\) No 540/2011](#) and new active substances. A new substance is a substance not authorised in any of the Member States of the EU on 25 July 1993. The plant protection product that contains such substances may be authorised if the criteria laid down in the [Regulation \(EC\) No 1107/2009](#) are met, also taking into account the national stipulations described in the [Bgb](#) (Plant protection products and Biocides Decree) . The evaluation dossiers must meet the requirements in [Commission Regulation \(EU\) No 283/2013](#) and [Commission Regulation \(EU\) No 284/2013](#) implementing Regulation (EC) No 1107/2009 (see Application Form and corresponding instructions).

A Member State may deviate from the EU evaluation on the basis of agricultural, phytosanitary and ecological, including climatological, conditions which are specific for the Netherlands.

The NL framework describes the data requirements (§2.2), evaluation methodologies (§2.3), criteria and trigger values (§2.4) for which specific rules apply in the national approval

framework or when the national framework has been elaborated in more detail than the EU framework.

The NL procedure described in §2 - §2.5 of this chapter can also be used for evaluation of a substance for approval, and consequently inclusion in [Commission Implementing Regulation \(EU\) No 540/2011](#) in case no European procedure has been described.

2.1. Introduction

This chapter describes the data for effects on an STP for which specific rules apply in the national decision scheme or when the national decision scheme has been elaborated in more detail than the EU framework.

Methods for exposure estimation for an STP have not been laid down in EU framework. Criteria for this aspect have neither been described. This aspect has therefore been elaborated nationally (see §2.3. and 2.4.1). For the methods for exposure estimation of an STP we refer to Chapter 6 Fate and behaviour in the environment; behaviour in surface water, sediment and sewage treatment plant (STP). The national elaboration of criteria setting is described in §2.4.1.

This chapter deals with substances which, in view of the nature of their use, may reach a sewage or waste water treatment plant. This category includes plant protection products that are used in mushroom growing, chicory forcing, greenhouse cultures, and for pre-treatment of cut flowers. Use on hard surfaces (pavements) by municipalities, private organisations, companies and households may also contribute to Plant protection products reaching STPs via runoff [9].

2.2. Data requirements

The data requirements for chemical plant protection products are in compliance with the provisions in EU framework (see §1.2 of the EU part).

Experiments carried out after the 25th of July 1993 must have been carried out under GLP.

There may be no doubt about the identity of the tested product or the purity of the tested substance for each study.

2.3. Risk assessment

Methods for exposure estimation of an STP are given in Chapter 6, Fate and behaviour in the environment; behaviour in surface water, sediment and sewage treatment plant (STP). The exposure is compared with a criterion derived on the basis of the toxicity to micro-organisms in an STP.

2.4. Approval

The evaluation of products on the basis of existing active substances already included in [Commission Implementing Regulation \(EU\) No 540/2011](#), or new substances, has been laid down in [Regulation \(EC\) No 1107/2009](#). Where no European methodology is agreed upon, a national methodology is applied as described in the [Bgb](#) (Plant protection product and Biocides Decree).

2.4.1. Criteria and trigger values

The criteria and trigger values are in compliance with the European regulations, see §1.4 of the EU part of the Evaluation Manual PPP.

2.4.2. Decision making

Decisions on approval are taken in compliance with the European regulations, see §1.4 of the EU part of the Evaluation Manual PPP.

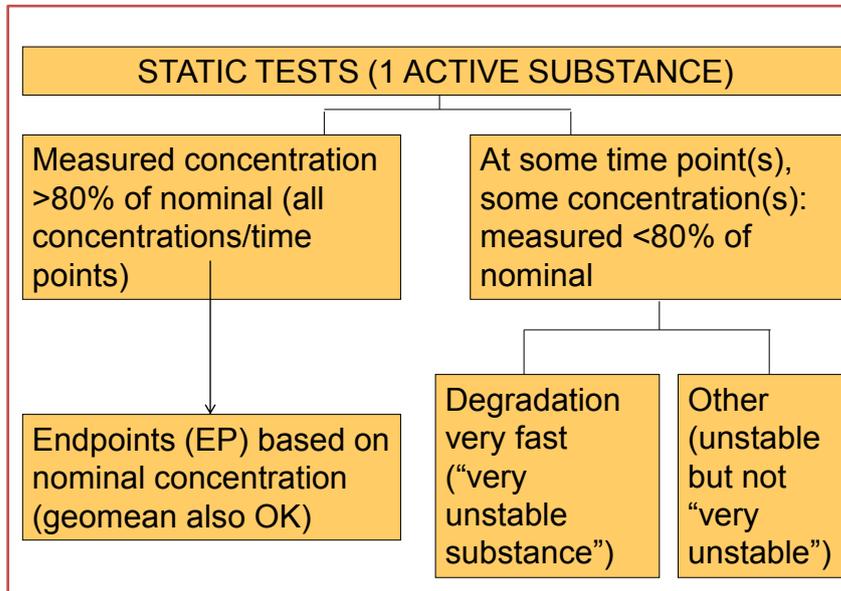
2.5. Developments

None.

3. REFERENCES

- 1 Hartgers EM, Aalderink GH, Van den Brink P., Gylstra R, Wiegman JWF, Brock TCM. 1998. Ecotoxicological threshold levels of a mixture of herbicides (atrazine, diuron and metolachlor) in freshwater microcosms. *Aquatic Ecology* 32: 135-152.
- 2 Deneer JW. 2000. Toxicity of mixtures of pesticides in aquatic systems. *Pest Manag Sci* 56:516-520.
- 3 De Zwart D. 2005. Ecological effects of pesticide use in The Netherlands: Modeled and observed effects in the field ditch. *Integr Environ Assess Manag* 1:123-134.
- 4 Van Wijngaarden RPA, Cuppen JGM, Arts GHP, Crum SHJ, Van den Hoorn MW, Van den Brink PJ, Brock TCM, 2004. Aquatic risk assessment of a realistic exposure to pesticides used in bulb crops: A microcosm study. *Environ Toxicol Chem* 23: 1479-1498.
- 5 Arts GHP, Buijse-Bogdan LL, Belgers JDM, Van Rhenen-Kersten CH, Van Wijngaarden RPA, Roessink I, Maund SJ, Van den Brink PJ, Brock TCM. 2006. Ecological impact in ditch mesocosms of simulated spray drift from a crop protection programme for potatoes. *Integr Environ Assess Manag* 2:105-125.
- 6 Van den Brink PJ, Hartgers EM, Gylstra R, Bransen F, Brock TCM. 2002b. The effects of a mixture of two insecticides on freshwater microcosms. II. Water quality, responses of zooplankton, phytoplankton and periphyton and ecological risk assessment. *Ecotoxicology* 11, 181-197.
- 7 Van den Brink PJ, Crum SJH, Gylstra R, Bransen F, Cuppen JGM, Brock TCM 2009. Effects of a herbicide-insecticide mixture in freshwater microcosms: Risk assessment and ecological effect chain. *Environmental Pollution* 157:237-249.
- 8 Verbruggen EMJ, Van den Brink PJ. 2010. Review of recent literature concerning mixture toxicity of pesticides. RIVM report 601400xxx/2010.
- ⁹⁹⁹9 FRAUNHOFER-INSTITUT FÜR UMWELTCHEMIE UND ÖKOTOXIKOLOGIE (2001). Ökotoxikologische Prüfung von Pflanzenschutzmitteln hinsichtlich ihres Potentials zur Grundwassergefährdung - Ecotoxicological testing of pesticides with respect to their potential of endangering groundwater communities, UBA-Text 76/01.

4. **APPENDIX 1: DERIVATION OF ENDPOINTS FROM STUDIES WITH AQUATIC ORGANISMS (BASED ON EXPERT VIEW MATHIEU PLUIJMEN (CHARLES RIVER LABORATORIES))**

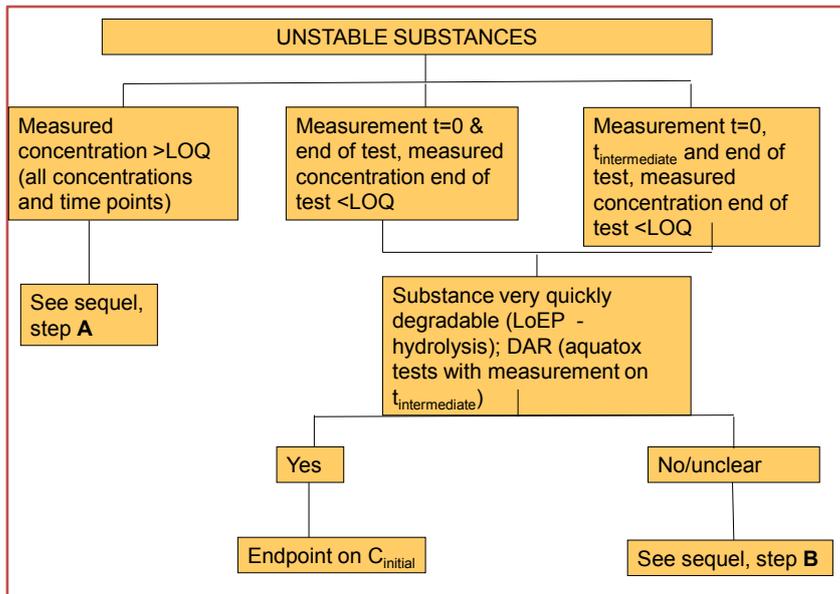


1

VERY UNSTABLE SUBSTANCES, suggested criterion:

- First possible measurement in aquatox tests typically @ 24 hours
- Whether substance still can be measured reasonably after 24 h depends on:
 - Initial (nominal) concentration
 - LOQ (often arbitrarily established, for the purpose of the study).
 - Degradation time (DT_{50} 2, 3, 3.6 and 4 h: % left after 24 h 0.02%, 0.4%, 1.0% en 1.6%).
- Criterion $DT_{50} = 3$ hours seems reasonable, because:
 - Complete dissipation (0.002% left) after 2 days (minimal interval between applications is 5 days)
 - Substance often not measurable in test after 24 h at low nominal concentrations (<1 mg/L)

4



5

Step A:

UNSTABLE, MEASURED CONCENTRATION >LOQ (all concentrations and time points)

Calculate geomean concentration for each test concentration

- i. Measurement only on t_0 (C_0) and t_{end} (C_{end}) $\rightarrow C_{geomean} = (C_0 * C_{end})^{0.5}$
- ii. Measurement t_0 (C_0), $t_{intermediate}$ (C_i) and t_{end} (C_{end}) \rightarrow calculate weighted geomean:
 - $((t_{int}-t_0)*geomean(C_{int},C_0) + (t_{end}-t_{int}) * geomean(C_{end},C_{int})) / (t_{end}-t_0)$
- iii. In case of 100% mortality/immobility **before** end of test:
 - 100% effect within 2 hours \rightarrow use $C_{initial}$
 - 100% effect later than 2 hours and measurement on timepoint t_x directly after timepoint with 100% effect (earlier than end of test) \rightarrow base $C_{geomean}$ on period t_0-t_x .

6

Example Step A(i): UNSTABLE, MEASURED CONCENTRATION >LOQ (all concentrations and time points)

Measurement @ t_0 & t_{end} $\rightarrow C_{geomean} = (C_0 * C_{end})^{0.5}$

	measured conc	measured conc	measured conc
nominal conc	t=0	t=48	geomean, t=0-48
(mg/L)	(mg/L)	(mg/L)	(mg/L)
2.0	1.8	0.22	0.63
4.0	3.4	0.48	1.3
8.0	7.1	0.77	2.3
16	16.2	1.8	5.4
32	28.9	3.0	9.3
64	62.9	10.9	26

7

Example Step A(ii): UNSTABLE, MEASURED CONCENTRATION >LOQ (all concentrations and time points)

Measurement @ t_0 (C_0), $t_{intermediate}$ (C_i) & t_{end} (C_{end}) \rightarrow calculate weighted geomean

nominal conc	measured conc	measured conc	measured conc	measured conc	measured conc	measured conc
	t=0	t=24	t=96	geomean, t=0-24	geomean, t=24-96	geomean, t=0-96
(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
2.0	1.8	0.90	0.22	1.27	0.44	0.65
4.0	3.4	1.80	0.48	2.47	0.93	1.3
8.0	7.1	3.24	0.77	4.80	1.58	2.4
16	16.2	7.50	1.8	11.0	3.67	5.5
32	28.9	15.1	3.0	20.9	6.73	10
64	62.9	28.8	10.9	42.6	17.7	24
			Weighting factor	24/96	72/96	

8

**Example Step A(iii): UNSTABLE, MEASURED CONCENTRATION >LOQ
(all concentrations and time points)**

100% effect > 2 h & measurement @ t_x directly after timepoint with 100% effect
(earlier than end of test) → base C_{geomean} on period t_0-t_x

nominal conc (mg/L)	% immo- bility t=6	% immo- bility t=24	% immo- bility t=48	measured conc (mg/L) t=0	measured conc (mg/L) t=6	measured conc (mg/L) t=24	measured conc (mg/L) t=48	measured conc (mg/L) geomean	geomean calculated over t
2.0	n.a.	10.0	15.0	1.8	n.a.	0.48	0.22	0.57	0-24-48
4.0	n.a.	15.0	25.0	3.4	n.a.	1.11	0.48	1.2	0-24-48
8.0	n.a.	35.0	50.0	7.1	n.a.	1.32	0.77	1.9	0-24-48
16	n.a.	60	75	16.2	n.a.	3.94	1.8	4.9	0-24-48
32	n.a.	100	100	28.9	n.a.	7.99	n.a.*	15	0-24
64	100	100	100	62.9	24.2	n.a.	n.a.	39	0-6

* If measured: not to be taken into account.

9

Step B-1:**UNSTABLE (but not very unstable), MEASUREMENT T=0 & END,
MEASURED @ END <LOQ IN ALL CONCENTRATIONS**

Test not acceptable, since it is unknown when concentrations were <LOQ, calculation of a reliable geomean not possible.

Options for new test:

- Measurements at intermediate timepoints (minimal after 24 hours);
- Lower LOQ;
- Provide evidence that the substance meets criterion “very unstable” under test conditions (in presence of test organisms).
 - E.g. in case of algal test measurement of concentration after 0, 4, 8 en 24 h.
- If substance def not “very unstable”: flow-through test or semi-static test with intervals as short as possible.
 - Algae: on basis of measuring concentrations after 0, 4, 8 en 24 hours, determination of DT_{50} ; calculation of $C_{TWA,72 \text{ uur}}$ with that DT_{50} (see slide 13).

10

Example Step B-1: UNSTABLE, MEASUREMENT @ T=0 & END, MEASURED CONCENTRATION END <LOQ IN ALL CONCENTRATIONS

nominal conc (mg/L)	measured conc t=0 (mg/L)	measured conc t=48 (mg/L)
2.0	1.8	<LOQ
4.0	3.4	<LOQ
8.0	7.1	<LOQ
16	16.2	<LOQ
32	28.9	<LOQ
64	62.9	<LOQ

LOQ 0.1 mg/L; processed data

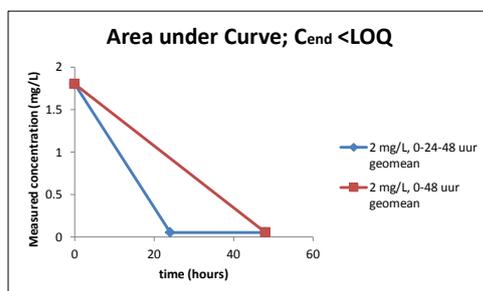
nominal conc (mg/L)	measured conc t=0 (mg/L)	measured conc t=24 (mg/L)	measured conc t=48 (mg/L)	geomean measured conc t=0-48 (mg/L)	geomean measured conc t=0-24-48 (mg/L)
2.0	1.8	0.05	0.05	0.30	0.17
4.0	3.4	0.05	0.05	0.41	0.20
8.0	7.1	0.05	0.05	0.60	0.26
16	16.2	0.05	0.05	0.90	0.34
32	28.9	0.05	0.05	1.2	0.42
64	62.9	0.05	0.05	1.8	0.54

11

Example Step B-1 (next): UNSTABLE, MEASUREMENT T=0 & END, MEASURED CONCENTRATION END <LOQ IN ALL CONCENTRATIONS

Geomean concentration \approx proportional to Area Under concentration-time Curve (AUC)

Graphic presentation of the above-mentioned situation:



12

Example Step B-1: MEASUREMENT T=0 & END, MEASURED CONCENTRATION END <LOQ IN ALL CONCENTRATIONS, follow-up research for algae

- Flow-through or semi-static test not possible for algae.
- Proposal algae: on basis of measurement concentration after 0, 4, 8 en 24 hours, determination of DT_{50} , calculation of $C_{TWA,72\text{ uur}}$ with that DT_{50} .
- $C_{TWA,72\text{ h}} = C_0 * (1 - \exp(-k*72)) / (k*72)$, in which $k = \ln(2)/DT_{50}$
- Quick calculation in Excel possible:

nominal conc (mg/L)	measured conc t=0 (mg/L)	measured conc t=4 hr (mg/L)	measured conc t=8 hr (mg/L)	measured conc t=24 hr (mg/L)
2.0	1.8	0.36	<LOQ	<LOQ
4.0	3.4	0.68	0.15	<LOQ
8.0	7.1	1.42	0.24	<LOQ
16	16.2	4.86	1.55	0.14
32	28.9	11.6	4.70	0.81
64	62.9	37.7	20.6	6.90

13

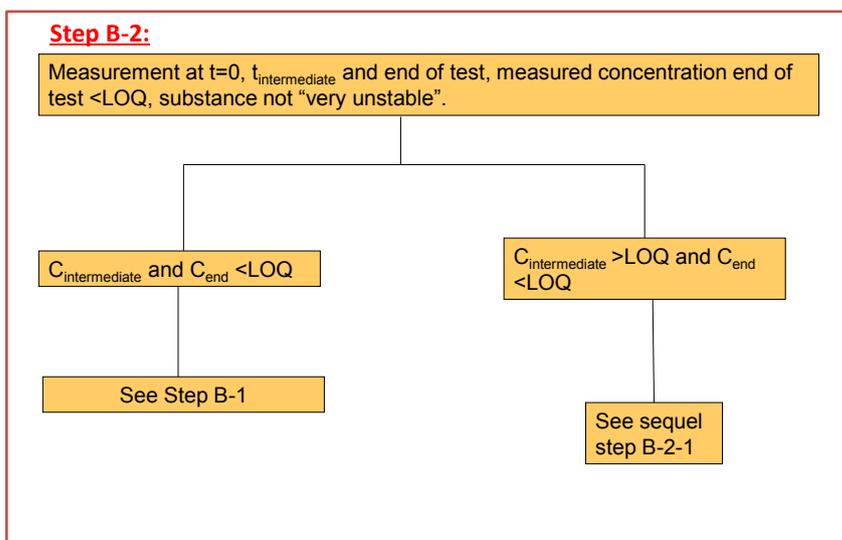
Example Step B-1: MEASUREMENT T=0 & END, MEASURED CONCENTRATION END <LOQ IN ALL CONCENTRATIONS, follow-up research for algae

LOQ 0.1 mg/L; processed data

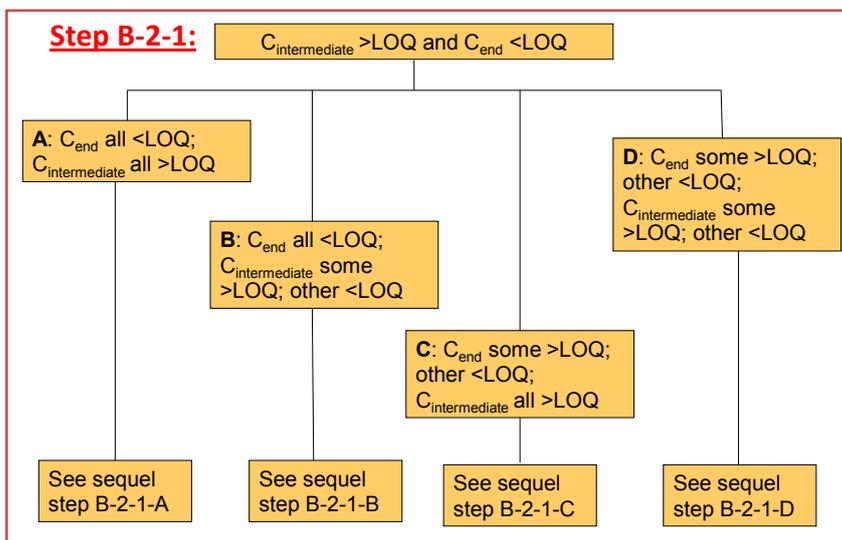
nominal conc (mg/L)	measured conc t=0 (mg/L)	measured conc t=4 hr (mg/L)	measured conc t=8 hr (mg/L)	measured conc t=24 hr (mg/L)	DT_{50} (hr)	R^2	C_{TWA} 0-72 hr (mg/L)
2.0	1.8	0.36	0.05	-*	1.5	0.9966	0.020
4.0	3.4	0.68	0.15	-**	1.8	0.9997	0.039
8.0	7.1	1.42	0.24	-**	1.6	0.9992	0.080
16	16.2	4.86	1.55	0.14	3.7	0.9689	0.21
32	28.9	11.6	4.70	0.81	4.9	0.9635	0.37
64	62.9	37.7	20.6	6.90	7.8	0.9658	0.84

* Omitted, only take into account first point <LOQ (t=8 hr).
** Omitted because of bad regression line if taken into account as 0.05.

14



15



16

Step B-2-1-A:

C_{end} all <LOQ, $C_{intermediate}$ all >LOQ

Quick check: is $C_{end} = 0.5 * LOQ$ reasonable?

- A. $C_{end} = 0.5 * LOQ$ **reasonable** i.e. **worst case** → calculate geomean concentration on basis of C_{end} with $C_{end} = 0.5 * LOQ$ (methodology: see Step A-ii en A-iii).
- B. $C_{end} = 0.5 * LOQ$ **over-estimation** → geomean over-estimated.
 - If $C_{end} = 0.5 * LOQ$ over-estimation **and** effects over whole test period:
 - » Estimate for each concentration C_{end} on basis of DT_{50} or % degradation over period $t_0 - t_{intermediate}$
 - » Calculate geomean over whole test period (see Step A-ii, use in formula C_{end} from previous step over period $t_{end} - t_{intermediate}$)
 - If $C_{end} = 0.5 * LOQ$ overestimation **and** effects **not** over whole test period:
 - » Calculate on basis of DT_{50} or % degradation, estimated over period $t_0 - t_{intermediate}$, de $C_{intermediate}$ on t_x ; t_x is the earliest time point at which 100% effect was observed.
 - » Calculate geomean over whole *relevant* test period (see Step A-ii, use in formula $C_{intermediate}$ on t_x from previous step)

17

Example Step B-2-1-A: C_{end} all <LOQ, $C_{intermediate}$ all >LOQ

Is $C_{end} = 0.5 * LOQ$ OK? LOQ = 0.1 mg/L

nominal conc (mg/L)	measured conc (mg/L) t=0	measured conc (mg/L) 48	measured conc (mg/L) 96		estimated conc* (mg/L) C0/C48	0.5*LOQ	used conc (mg/L)	geomean conc (mg/L) 0-96	geomean conc (mg/L) 0-96 on basis of 0.5*LOQ
2.0	1.8	0.11	<LOQ	16	0.005	no	0.005	0.10	0.21
4.0	3.4	0.15	<LOQ	23	0.007	no	0.007	0.16	0.29
8.0	7.1	0.34	<LOQ	21	0.017	no	0.017	0.34	0.49
16	16.2	0.84	<LOQ	19	0.041	no	0.041	0.82	0.88
32	28.9	1.35	<LOQ	21	0.067	yes (worst c)	0.05	1.2	1.2
64	62.9	2.97	<LOQ	21	0.15	yes (worst c)	0.05	2.1	2.1
mean factor				20					

* Estimated on basis of mean factor

Uncertainty: is degradation time in period 48-96 h the same as in period 0-48 h? Constant degradation during 0-48 h at 2-64 mg/L supports the assumption - other considerations:

- What is degradation pattern in other studies (with other organisms or studies in DAR with the same organism)?

18

Step B-2-1-B:**C_{end} all <LOQ, C_{intermediate} some >LOQ and other <LOQ**Concentrations with C_{intermediate} >LOQ and C_{end} <LOQ: see **Step B-2-1-A**.Concentrations with C_{intermediate} <LOQ and C_{end} <LOQ:

1. Eliminate first concentrations not relevant for derivation EP.

A. 1-2 relevant concentrations left with C_{intermediate} <LOQ and C_{end} <LOQ:» Degradation at all C_{intermediar} >LOQ consistent?

- **Yes** estimate C_{intermediate} for concentrations with C_{intermediate} <LOQ on basis of % degradation or estimated DT50 at C_{intermediate} >LOQ.
 - **Condition: differences between measured and estimated (nominal) concentrations small (<1 order size), because degradation/adsorption may be concentration-dependent.**
 - Calculate geomean concentration according to **Step B-2-1-A**.
- **No** no reliable estimation of C_{intermediate} and C_{end}, test not acceptable (**Step B-1**).

19

Step B-2-1-B (next):B. >2 relevant concentrations left with C_{intermediate} <LOQ and C_{end} <LOQ:

- » In general not acceptable (too much uncertainties with extrapolation)
- » Sometimes acceptable on basis of expert judgment; considerations:
 - Much concentrations with C_{intermediar} >LOQ?
 - Is there a large concentration range with C_{intermediate} >LOQ, and is the degradation trend consistent?
 - Are the differences between measured and concentrations to be estimated small (<1 order size)?
 - Is the endpoint for the formulation (much) higher than the one calculated on basis of combitox?
 - Is it a study with vertebrates or with invertebrates?
 - Is it evident that the tested species belong to the least sensitive taxonomic group?

20

Example Step B-2-1-B: C_{end} all <LOQ, $C_{intermediate}$ some >LOQ and others <LOQ

Degradation pattern consistent; LOQ 0.1 mg/L

nominal Conc (mg/L)	measured conc (mg/L) t=0	measured conc (mg/L) 48	measured conc (mg/L) 96	% mortality 96	relevant for LC50?		estimated conc (mg/L)* 48	0.5*LOQ OK?	estimated conc (mg/L)* 96	0.5*LOQ OK?	used conc (mg/L) 48	used conc (mg/L) 96	geomean conc (mg/L) 0-96
0.10	0.12	<LOQ	<LOQ	0	No	-	-	-	-	-	-	-	-
0.20	0.17	<LOQ	<LOQ	0	Yes	-	0.058	Yes	0.020	no	0.050	0.020	0.055
0.40	0.43	0.15	<LOQ	15	Yes	2.9	n.a.	n.a.	0.051	yes (worst c)	0.15	0.050	0.15
0.75	0.75	0.24	<LOQ	40	Yes	3.1	n.a.	n.a.	0.081	yes (worst c)	0.24	0.050	0.21
1.0	0.91	0.32	<LOQ	75	yes	2.8	n.a.	n.a.	0.109	yes (worst c)	0.32	0.050	0.24
mean						2.9							

Estimated on basis of mean factor

Uncertainty: is the degradation between 48-96 uur the same as between 0-48 uur? Considerations:

- Aquaria regularly cleaned? Excess food removed?
- Information from other studies.
- Sensitive species?

21

Step B-2-1-C:

C_{end} some >LOQ and others <LOQ, $C_{intermediate}$ all >LOQ

1. Concentrations with C_{end} and $C_{intermediate}$ >LOQ: calculate geomean, see **Step A-ii** and **A-iii**.
2. Concentrations with $C_{intermediate}$ >LOQ and C_{end} <LOQ: see **Step B-2-1-A**.

22

Example Step B-2-1-C: C_{end} some >LOQ and others <LOQ, $C_{intermediate}$ all >LOQ

Degradation pattern consistent; LOQ 0.1 mg/L

nominal conc (mg/L)	measured conc (mg/L) t=0	measured conc (mg/L) 48	measured conc (mg/L) 96	% mortality for 96	relevant for LC50?	C0/C48	C48/C96	estimated conc (mg/L)* 96	0.5*LOQ OK?	used conc (mg/L) 96	geomean conc (mg/L) 0-96
0.40	0.38	0.10	<LOQ	5	Yes	3.8	-	???	no	-	-
0.80	0.84	0.26	<LOQ	15	Yes	3.2	-	???	no	-	-
1.6	1.40	0.59	<LOQ	25	Yes	2.4	-	0.13	yes	0.050	0.35
3.2	3.30	1.30	0.29	40	Yes	2.5	4.5	n.a.	n.a.	0.290	1.1
6.4	6.70	2.90	0.68	95	yes	2.3	4.3	n.a.	n.a.	0.680	2.4
Mean						2.4#	4.4				

* Estimated on basis of mean factor
Mean of 3 highest concentrations

- **1.6 mg/L:** degradation 48-96 hr to be predicted from 3.2-6.4 mg/L → estimation of C96 is OK.
- **0.4-0.8 mg/L:** degradation 0-48 hr deviates from 1.6-6.4 mg/L → estimation of C96 on basis of degradation 48-96 hr at 3.2-6.4 mg/L not acceptable.

23

Example Step B-2-1-C: C_{end} some >LOQ and others <LOQ, $C_{intermediate}$ all >LOQ

Degradation pattern not consistent; LOQ 0.1 mg/L

nominal conc (mg/L)	measured conc (mg/L) t=0	measured conc (mg/L) 48	measured conc (mg/L) 96	% mortality for 96	relevant for LC50?	C0/C48	C48/C96	estimated conc (mg/L)* 96	0.5*LOQ OK?	used conc (mg/L) 96	geomean conc (mg/L) 0-96
0.40	0.39	0.10	<LOQ	5	yes	3.9	-	???	No	-	-
0.80	0.84	0.24	<LOQ	15	yes	3.5	-	???	no	-	-
1.6	1.40	0.45	0.14	25	yes	3.1	3.2	n.a.	n.a.	0.14	0.45
3.2	3.30	1.30	0.29	40	yes	2.5	4.5	n.a.	n.a.	0.29	1.1
6.4	6.70	2.90	0.71	95	yes	2.3	4.1	n.a.	n.a.	0.71	2.4
mean						nvt	nvt				

- **0.4-0.8 mg/L:**
 - Degradation 0-48 hr deviates from 1.6-6.4 mg/L and irregular degradation trend at 1.6-6.4 mg/L during 48-96 hr → estimation C96 on basis of degradation at 1.6-6.4 mg/L not acceptable.
 - In case of vertebrates possibly C96 0.02 mg/L and 0.01 mg/L may be used for 0.80 and 0.40 mg/L nominal (reasonable and conservative assumption).

24

Step B-2-1-D:

C_{end} some >LOQ and others <LOQ, C_{intermediate} some >LOQ and others <LOQ

1. Concentrations with C_{end} and C_{intermediate} >LOQ: calculate geomean, see **Step A-ii** and **A-iii**.
2. Concentrations with C_{intermediate} >LOQ and C_{end} <LOQ: see **Step B-2-1-A**.
3. Concentrations with C_{intermediate} <LOQ and C_{end} <LOQ: see **Step B-2-1-B**.
4. Check if a sufficient number of concentrations are left for EP calculation.

25

Example Step B-2-1-D: C_{end} some >LOQ and others <LOQ, C_{intermediate} some >LOQ and others <LOQ

Degradation pattern consistent; LOQ 0.1 mg/L

nominal conc (mg/L)	measured conc (mg/L) t=0	measured conc (mg/L) 48	measured conc (mg/L) 96	% Mortality	Relevant for LC ₅₀ ?	C0/ C48	C48/ C96	estimated conc (mg/L)* 48	estimated conc (mg/L)* 96	0.5* LOQ	used conc (mg/L) 48	used conc (mg/L) 96	geomean conc (mg/L) 0-96
0.05	0.05	<LOQ	<LOQ	0	no	-	-	-	-	-	-	-	-
0.12	0.14	<LOQ	<LOQ	0	Yes	-	-	0.074	0.036	Yes	0.05	0.036	0.063
0.26	0.24	0.142	<LOQ	15	Yes	1.7	-	n.a.	0.069	yes	0.142	0.05	0.12
0.61	0.69	0.344	0.18	40	Yes	2.0	1.9	n.a.	n.a.	n.a.	0.344	0.18	0.35
1.4	1.3	0.65	0.35	75	Yes	1.9	2.1	n.a.	n.a.	n.a.	0.65	0.35	0.66
3.2	3.1	1.7	0.80	100	Yes	1.8	2.1	n.a.	n.a.	n.a.	1.7	0.80	1.6
7.4	7.6	3.7	1.7	100	no	2.1	2.1	-	-	-	-	-	-
mean						1.9	2.1						

0.12-0.26 mg/L: extrapolation reasonable on basis of consistent degradation pattern in time and at all higher concentrations.

26

**Example Step B-2-1-D: C_{end} some >LOQ and others <LOQ,
 $C_{intermediate}$ some >LOQ and others <LOQ**

Degradation pattern not consistent; LOQ 0.1 mg/L

nominal conc (mg/L)	measured conc (mg/L) t=0	measured conc (mg/L) 48	measured conc (mg/L) 96	% mortality LC ₅₀ ?	relevant for LC ₅₀ ?	CO/C48	C48/C96	estimated conc (mg/L) 48	estimated conc (mg/L) 96	0.5* LOQ OK?	used conc (mg/L) 48	used conc (mg/L) 96	geomean conc (mg/L) 0-96
0.05	0.05	<LOQ	<LOQ	0	No	-	-	-	-	-	-	-	-
0.12	0.14	<LOQ	<LOQ	0	Yes	-	-	???	???	no	-	-	-
0.26	0.24	0.115	<LOQ	15	Yes	2.1	-	n.a.	0.055*	yes	n.a.	0.05	0.11
0.61	0.69	0.45	0.29	40	Yes	1.5	1.6	n.a.	n.a.	n.a.	0.45	0.29	0.45
1.4	1.3	0.99	0.35	75	Yes	1.3	1.2	n.a.	n.a.	n.a.	0.99	0.35	0.76
3.2	3.1	2.8	2.40	100	Yes	1.1	1.2	n.a.	n.a.	n.a.	2.8	2.40	2.7
7.4	7.6	6.7	5.6	100	no	1.1	1.2	-	-	-	-	-	-

* Estimated as C96 = C48/(CO/C48)

0.26 mg/L: estimation C96 acceptable since the degradation time at all higher concentrations in the period 48-96 uur was comparable with that in the period 0-48 uur.

0.12 mg/L: estimation C48 and C96 not reliable because of the trend in the degradation pattern, extrapolation not acceptable.

27

STATIC TESTS (2 ACTIVE SUBSTANCES A & B)

- Typically concerns a test with a formulation.
- Options for each substance A and B:
 - Stable or unstable.
 - Toxic or non-toxic.
 - Conclusion: 16 combinations possible in test.
- Use the degradation pattern of the substance which degrades most quickly for the calculation of the EP of the formulation, unless this substance:
 1. does not contribute significantly to the toxicity of the formulation; and/or
 2. belongs to the group of “very unstable” substances.

28

- INTERMEZZO: what is “not significant”?
- Exercise: formulation with substance A and B:
 - Enpoint A is 1 mg/L , endpoint B varies from 2-1000 mg/L
 - % A and B in formulation together = 100%, %A varies from 0-100%.
- Proposal for “Contribution B not significant”:
 - i. Difference combitox and tox(A) max 5% of the difference between tox(A) en tox(B)
 - Tox(A) 1 mg/L, tox(B) 11 mg/L → 5% of (tox(A)-tox(B)) = 5% of 10 mg/L = 0.5 mg/L → Combitox max 1.5 mg/L
 - and
 - ii. Max. combitox = 2*tox(A)
 - Tox(A) = 1 mg/L → Max combitox = 2 mg/L
- Rationale:
 - Factor 2 difference can also observed in the results of two the same tests with the same organism
 - 5% is arbitrarily chosen, but is quite conservative (see following slide).

29

A	A	B	B	Combitox												
%	EP mg/L	%	EP mg/L	x EP mg/L												
0	1	100	1000	1000	100	100	50	50	20	20	10	10	5	5	2	2
5	1	95	1000	20	100	17	50	14	20	10	10	7	5	4.2	2	1.9
10	1	90	1000	9.9	100	9.2	50	8.5	20	6.9	10	5.3	5	3.6	2	1.8
15	1	85	1000	6.6	100	6.3	50	6.0	20	5.2	10	4.3	5	3.1	2	1.7
20	1	80	1000	5.0	100	4.8	50	4.6	20	4.2	10	3.6	5	2.8	2	1.7
25	1	75	1000	4.0	100	3.9	50	3.8	20	3.5	10	3.1	5	2.5	2	1.6
30	1	70	1000	3.3	100	3.3	50	3.2	20	3.0	10	2.7	5	2.3	2	1.5
35	1	65	1000	2.9	100	2.8	50	2.8	20	2.6	10	2.4	5	2.1	2	1.5
40	1	60	1000	2.5	100	2.5	50	2.4	20	2.3	10	2.2	5	1.9	2	1.4
45	1	55	1000	2.2	100	2.2	50	2.2	20	2.1	10	2.0	5	1.8	2	1.4
50	1	50	1000	2.0	100	2.0	50	2.0	20	1.9	10	1.8	5	1.7	2	1.3
55	1	45	1000	1.8	100	1.8	50	1.8	20	1.7	10	1.7	5	1.6	2	1.3
60	1	40	1000	1.7	100	1.7	50	1.6	20	1.6	10	1.6	5	1.5	2	1.3
65	1	35	1000	1.5	100	1.5	50	1.5	20	1.5	10	1.5	5	1.4	2	1.2
70	1	30	1000	1.4	100	1.4	50	1.4	20	1.4	10	1.4	5	1.3	2	1.2
75	1	25	1000	1.3	100	1.3	50	1.3	20	1.3	10	1.3	5	1.3	2	1.14
80	1	20	1000	1.2	100	1.2	50	1.2	20	1.2	10	1.2	5	1.2	2	1.11
85	1	15	1000	1.2	100	1.2	50	1.2	20	1.2	10	1.2	5	1.1	2	1.08
90	1	10	1000	1.1	100	1.1	50	1.1	20	1.1	10	1.1	5	1.1	2	1.05
95	1	5	1000	1.1	100	1.1	50	1.1	20	1.0	10	1.0	5	1.0	2	1.0
100	1	0	1000	1.0	100	1.0	50	1.0	20	1.0	10	1.0	5	1.0	2	1.0
Max (i)				51		6		3		2.0		1.5		1.20		1.05

Max(i) = maximum allowed combitox on basis of criterium (i)

Red: according to EFSA GD (2014, 10.3.7) the combitox is caused by A, if the content of A is outside (above) this range

Yellow: Combitox meets both criteria: Contribution B not significant

30

Proposal:

Contribution of B is not significant when:

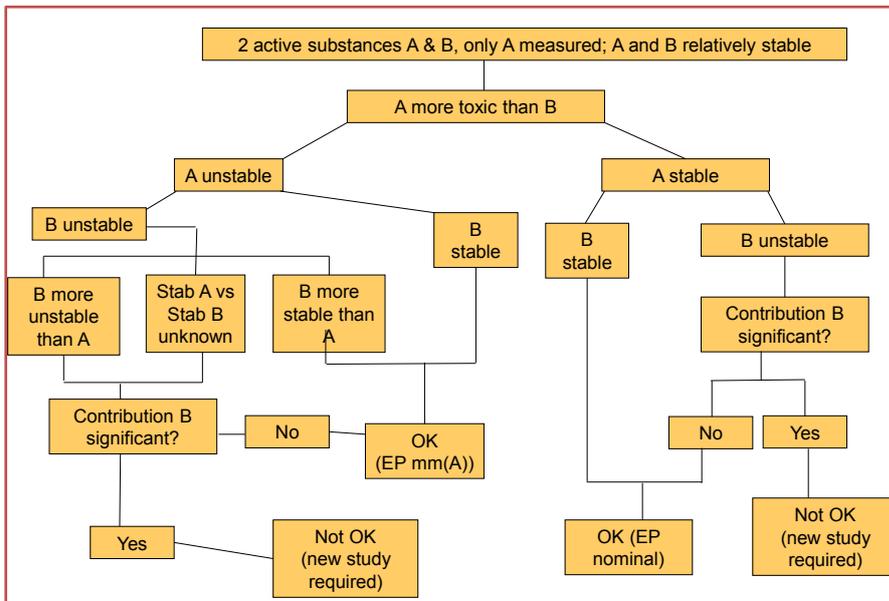
Endpoint (B) >20X higher than Endpoint (A)	and %B <%A
Endpoint (B) 10-20X higher than Endpoint (A)	and %B <50% of A
Endpoint (B) 5-10X higher than Endpoint (A)	and %B <25% of A
Endpoint (B) 2-5X higher than Endpoint (A)	and %B <10% of A

31

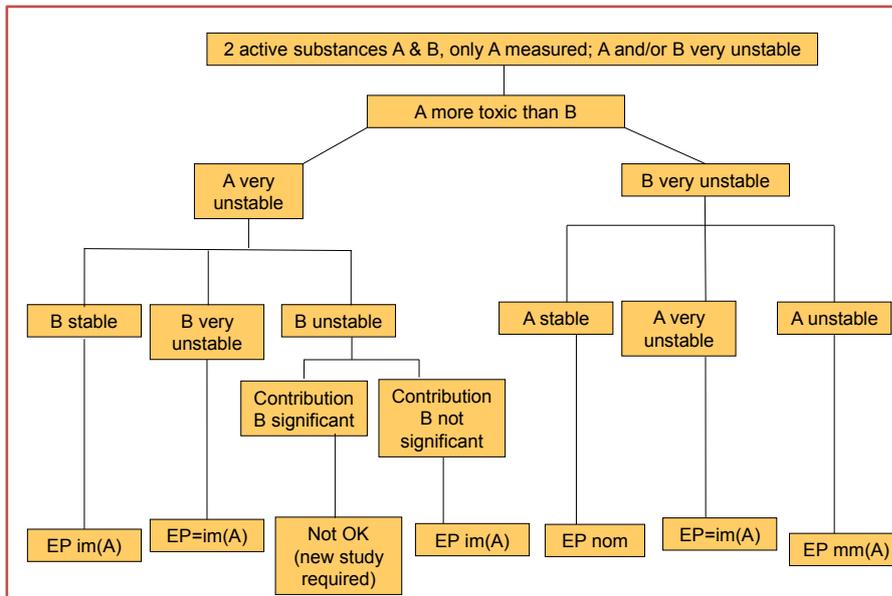
STATIC TESTS (2 ACTIVE SUBSTANCES A & B)

- In principle, both substances should be measured.
- If that is not the case, for the not measured substance information about stability is required.
- Possible sources of stability information:
 - Fate:
 - LoEP/DAR (DT₅₀ hydrolysis pH7, DT₅₀ in water-sediment systems)
 - Ecotox:
 - dRR (studies with the same formulation/matrix, but other organisms)
 - LoEP/DAR (studies with active substance and possibly other formulation(s))

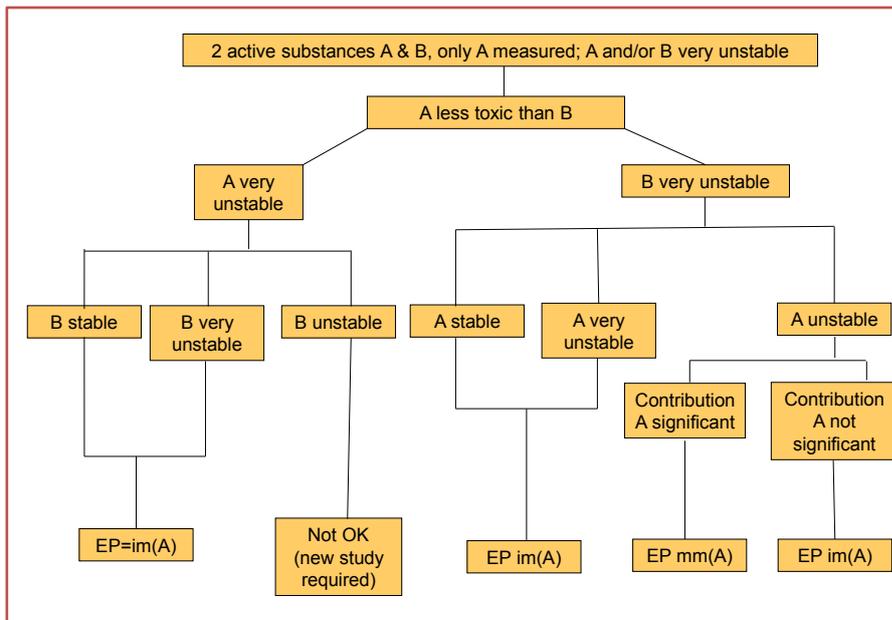
32



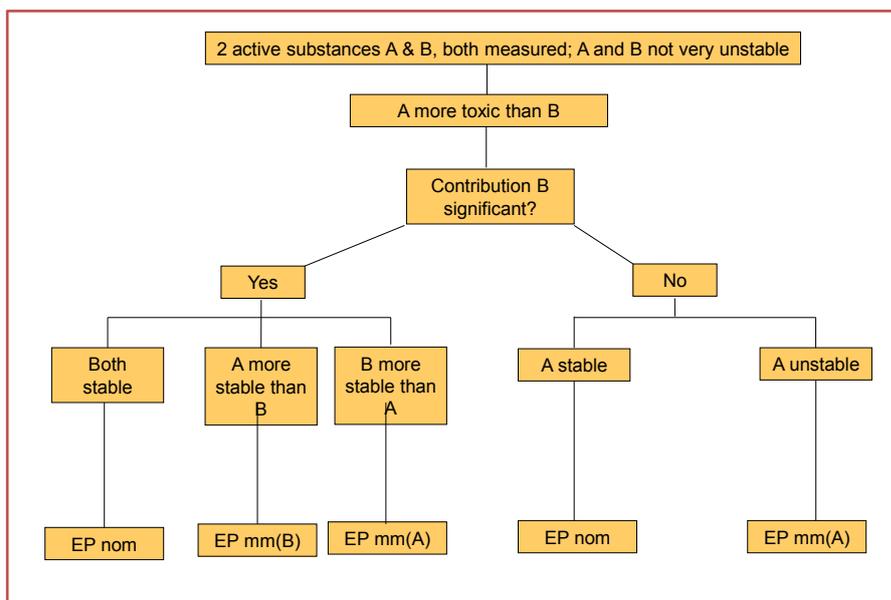
33



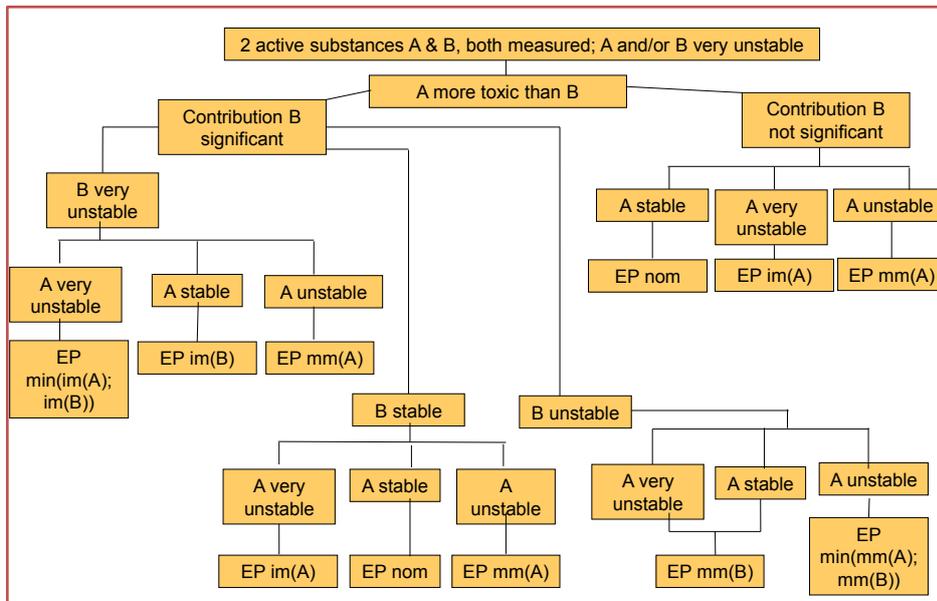
34



35



36



37