Assessment of the toxicity of combination products for organisms

1. Introduction

According to the Uniform principles (Commission Regulation 546/2011), Member states have to take into consideration all relevant information regarding the potentially adverse effects of the plant protection product, its components or its residues when performing a risk assessment for that product. Furthermore, the practical conditions of use and, in particular, the purpose of use, the dose, the manner, frequency and timing of applications, and the nature and composition of the preparation, has to be taken into account. In the specific principles (Commission Regulation 546/2011), and in the data requirements it is stressed that the potential risk from the product should be considered, and not only the potential risk from the active substance. This means that in many cases it is not sufficient to only look at the risk of the active substance to non-target organisms. Combination toxicology is assessed for formulations containing more than one active substance, and for combinations of products (i.e. tank mixes) that are specified on the label.

Although it is stated that formulation toxicology should be taken into account, it is often not clearly described in guidances, how to take this into account. In this chapter the Dutch approach for assessing combination toxicology is given. However as long as there is no general agreement on how to perform combinations toxicology, alternative methods could also be possible.
Whenever European or zonal agreements are used, this will overrule the Dutch proposed methods described below.

In section 2 of this appendix, the risk assessment scheme for a formulated product is presented, and in section 3, the risk assessment scheme for a combination of active substances is presented.

In appendix 1 of the appendix A the theoretical background of combination toxicology is described and the results of experimental research are presented. This is mainly related to aquatic organisms, on which relatively more research has been done. Furthermore, in appendix 1 of this appendix A in which cases concentration-addition or partial addition is applicable is discussed. In section 4, considerations of the different approaches as described in sections 2 and 3 are discussed.

2. Risk assessment for formulated products with more than one active substance using formulation toxicity data

When formulation data is available, a risk assessment can be performed based on the same principles as for active substances, as described in the various ecotox chapters of this evaluation manual. In most cases, formulation data is required and available. However, estimation of exposure to the formulation is difficult for multiple applications and long-term scenarios, as information on dissipation is usually only available for the active substances. Therefore, it is assumed that the toxicity of the formulation is caused by the active substances. The endpoint for the formulation should then be recalculated to be expressed in total active substance, and the predicted exposure will also be expressed in total active substance. However, it should be noted that this is not always a worst-case assumption. See section 4 for further considerations on the risk assessment.

3. Risk assessment based on combination of active substances, or products (in case of tank mixes)

From the information in Appendix 1, it can be concluded that the level at which individual active substances contribute to the combined toxicity of a product is still not well known . The available data, mainly from aquatic organisms, also show that in the case of partial addition (see appendix 1), the extent of combination toxicity does not deviate much from what is predicted by concentration-addition. On the basis of these two considerations, the Ctgb assumes concentration-addition in the assessment of the toxicity of combinations of active substances. Based on the precautionary principle, concentration-addition is also assumed for other organisms. Two approaches can be followed: calculation of the CombiTER (see 3.1) or calculation of a mixture endpoint (see 3.2). These calculations are based on the same scientific principles.

Using a mixture endpoint in risk assessment will obtain the same TER as when calculating the risk using CombiTER calculations. In cases of substance specific refinements, the approach described in 3.1 is more useful, while in cases where it is more important to compare endpoints (because of possible formulation effects), the approach described in 3.2 is more useful.

3.1 Combi TER approach

For plant protection products the TER (Toxicity-Exposure Ratio) is used as a standard in the risk assessment (except for bees and other non-target arthropods, where HQ-values are calculated). The TER must be higher than a trigger value to comply with the standards.

For the risk assessment of products containing more than one active substance and for tank mixtures the following formula is used:

$trigger_{substance\ 1} / TER_{substance\ 1} + trigger_{substance\ 2} / TER_{substance\ 2} + trigger_{substance\ i} / TER_{substance\ i}$.

When for each substance the trigger values are equal, the combined TER value can be calculated according to:

- $TER_{combi} = 1/((1/TER_{substance\ 1})+(1/TER_{substance\ 2})+(1/TER_{substance\ 3}))$

An acceptable risk is expected when $TER_{combi} > trigger$.

In case of unequal triggers, the combined TER value can be calculated using the following formula:

- $Trigger_{combi} = trigger_{substance\ 1}/trigger_{substance\ 2}/trigger_{substance\ i}$
- $TER_{combi} = trigger_{combi} /((trigger_{substance\ 1} /TER_{substance\ 1})+(trigger_{substance\ 2} /TER_{substance\ 2})+(trigger_{substance\ i} /TER_{substance\ i}))$

An acceptable risk is expected when $TER_{combi} > trigger_{combi}$.

In this formula, ‘triggers’ are the trigger values as mentioned in the corresponding chapter of the Evaluation Manual.

For bees and non-target arthropods HQ-values are calculated in the assessment.
These values may be summed up for the different active substances and related to the trigger (for bees the trigger is 50 and for non-target arthropods the trigger is 2 in the first tier assessment and 1 in the case of extended laboratory tests). If the summed HQ-value is lower than the trigger value, the risk is acceptable. If this is not the case the product is not permissible, unless an adequate risk assessment shows that there are no unacceptable effects under field conditions after application of the product according to the proposed GAP.

Additional remarks:

- The ratio between the concentrations of the active substances in a product will change after application, because the active substances will behave differently in the environment after application, dependent on the characteristics of the substances themselves, and the environment (half-life and sorption will differ for each active). See also section 4.
- For plant protection products, acute and chronic data and/or assessments should not be combined. However, TERs or mixture endpoints from different tiers of the assessment may be combined (e.g. an acute HC5 for active substance 1 and an acute LC50 for active substance 2), as long as it is corrected for the trigger value.
- If the difference in toxicity of the active substances for the different species is large and the calculated PEC-values are in the same order of magnitude, it is not necessary to estimate the potential combined effect. In that case, the risk assessment may be based upon the most toxic active substance.

3.2 Mixture endpoint

In the most recent guidance documents for ecotoxicological risk assessment (birds and mammals, aquatic organisms), the concentration-addition approach is used to take mixture toxicology into account. Although the scientific knowledge behind this approach is the same as given above, the aim of the calculation is to come to an combination endpoint, rather than a combination TER. The first step is to calculate the fraction of each active substance in the mixture. This will give the ratio between the different actives in the mixture and to the sum of these ratios should be 1.
The LD50 mixture can be calculated as:

endpoint mixture = $(1/((\text{fraction}_1/\text{endpoint}_1)+ (\text{fraction}_2/\text{endpoint}_2) +(\text{fraction }_i/\text{endpoint}_i))$

4. Formulation data versus combination toxicity – general considerations

For the aspects for which the risk assessment is based on product data, combination toxicity calculations are less relevant and are considered to be less certain than data based on formulations. This applies to the risk assessment for non-target arthropods and non-target plants. For soil micro-organisms, formulation data is also considered to be more relevant, as the risk assessment is not suitable for combination addition calculations.

Formulation data on reproductive effects for birds, mammals, fish and aquatic invertebrates are usually not available, nor required according to the data requirements. In the current aquatic guidance document Aquatic guidance (EFSA 2013) chronic data with the formulation is only required when acute toxicity endpoint of the is a factor of ten or more toxic than would be expected based on the active substances, in order to prevent unnecessary testing. However it should be taken into account that exposure to the formulation/combination of the actives could also trigger reproductive effects. Therefore, a combitox risk assessment must also be performed for the reproductive risk assessment, using combination toxicology calculations as described in section 3.1. Only when it can be excluded that combined effects may occur, because the effects seen in the organisms are clearly not related, combination toxicology may be disregarded for reproductive effects.

For all other cases, values that relate the lowest TERs should be used to calculate potential combined toxicity. In those cases where the formulation is more toxic than the calculated potential combined toxicity, however, the determination of the relevant exposure might be problematic. This is especially relevant for the aquatic risk assessment, for the following reasons:
The PECsw should reflect the ratio of the active substances (a.s.) as present in the formulation. This is the case only for an initial PECsw after a single application based on drift only, due to different fate and behaviour of the individual a.s. However, using such a PECsw can underestimate the exposure, since the additional exposure to both a.s. via possible run-off and drainage, and from possible multiple applications, is not taken into account. Therefore, in these cases the use of a ‘formulation PECsw’, based on drift and a single

application only, is not acceptable. However, a standard PECsw (i.e. taking into account multiple application and different exposure routes) based on the sum of the a.s. is also not correct for comparison with a formulation toxicity endpoint (expressed in a.s.), as described above. Currently, there is no clear guidance on how to solve this problem. As a first step, the formulation toxicity endpoint and calculated combination toxicity endpoint from the a.s. data (see 3.2) might be compared (take care that the units should be the same, either expressed in total a.s. or in formulation).

When the endpoint of the formulation is a factor of 5 or more lower than the calculated endpoint determined as described in section 3.2 using active substance data, then the risk assessment should be performed using formulation data. However, as explained above, a 'formulation PECsw' based on drift and a single application only is not acceptable when additional exposure to both a.s. via possible run-off and drainage and/or from multiple applications is possible. In this case, an adequate PECsw could be a PECsw based on total a.s., with a correction factor based on the difference in toxicity between formulation and calculated combination toxicity. However, this must be decided on case by case basis, and should be discussed with the Ctgb in the pre-submission phase.

In addition, for exposure to soil organisms the PECs based on the total a.s. can be an underestimation in cases of slow dissipating active substances, or fast dissipating active substances if the formulation makes those active substances more stable. In those cases exposure without dissipation will be considered as a worst-case.

When the formulation is not considered to be more toxic (by a factor of 5) than expected based on combined toxicity estimations, the toxicity endpoints and PECsw can be expressed in total a.s..

The factor of 5 is based on section 10.3.11 from 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).

Appendix 1 Background information to the concept of combination toxicology:

1. Theoretical background, toxicity of chemical mixtures

If the toxicity is based on studies of the individual active substances, the joint toxicity can be estimated on the basis of four types of interactions (according to Könemann, 1981):

1. the substances attenuate each other's toxic effects (antagonism)
2. the effects of the substances are completely independent of each other (non-additive)
3. the effect of one substance contributes to the effect of one or more of the other substances. This contribution may be complete (concentration addition) or partial (partial addition)
4. the effect of one substance enhances the toxic effect of the other substances in such a way that the combined effect is greater than the sum of the individual effects (potentiation, supra-addition, synergism).

On theoretical grounds, it is assumed that the mixture toxicity of active substances with the same or very similar mechanism of action (for example, a mixture of two organophosphate ester insecticides or an organophosphate ester and a carbamate) is established by concentration addition.

The following section addresses research on the toxicity of combinations of toxic substances for *aquatic organisms* (a considerable amount of research has been done on this topic).

1.1 Toxic unit model

For aquatic organisms, the toxicity (M) of a mixture of substances is determined by expressing the concentration of the individual substances (C_i) as a fraction of an effect parameter, usually the LC_{50} or $EC_{50.}$ In that case, the mixture toxicity M is $\sum C_i / L(E)C50_i$. This is expressed in toxic units (TU).

In the case of concentration addition, 50% effect occurs if M=1.0 TU, regardless of how many substances are in the mixture. In the case of partial addition, 50% effect will be found if $1.0 < M < n$, where n is the number of substances in the mixture. The exact value of M can be calculated if the dose-response relationships of the individual substances are known. In the case of non-addition, M = n at 50% effect. In the case of antagonism and potentiation, M takes on values of >1.0 to <1.0, respectively.

In the section below, concentration addition and partial addition are addressed as an underlying explanation of the toxicity of mixtures. In both cases, it is assumed that one substance does not affect the biological activity of the other (unlike for antagonism and potentiation).

2. Results of experimental research

2.1 Mixtures of non-plant protection products

Könemann (1981) showed that the toxicity of mixtures (from 3 to 50 substances with an assumed narcotic effect, the 'minimum toxicity') can be successfully described by the concentration addition model. The toxicity of these mixtures could be predicted by adding the fractions of the LC50 of the individual substances in the mixture. Expressed as an equation: $M = \sum C_i / LC50_i$. M is expressed in toxic units (TU). At an M value of 0.9 to 2.5 TU (geometric mean 1.3 TU), in sub-acute toxicity tests (14 d), Könemann reported 50% mortality in fish exposed to mixtures of substances.

Hermens et al. (1984a) showed for substances with an assumed narcotic effect that a combined effect also occurs at sublethal levels. The M values for immobilisation of *Daphnia magna* (48 hours) were 1.0 to 1.2 TU (mixtures of 10 to 50 substances).

In mixtures of 5, 10 and 25 substances, reproductive inhibition occurred at M values of 1.5 to 2.0 TU (test duration approximately 16 d). It appears that the combination effect is somewhat smaller than with the toxicological endpoint of mortality: in the same mixtures, 50% mortality was observed with M values of 0.9 to 1.5.

In more recent studies with organic micro-contaminants (excluding the above-mentioned studies) and metals (Hendriks, 1995), a 50% effect occurred frequently with aquatic animals at M values between 0.5 to 2 TU (the toxicological endpoint was not specified.).

2.2 Mixtures of plant protection products and non-plant protection products

In experiments with mixtures of 8 substances with different mechanisms of action, Hermens and Leeuwangh (1982) showed that 50% mortality in *Poecilia reticulata* (guppy) occurred at M values between 1.1 and 1.7 TU. In a mixture of 24 substances, the M value was 2.3 TU (Table 5). In experiments with *D. magna* with mixtures of 14 compounds, including 6 active substances with a variety of mechanisms of action, Hermens et al. (1984b) showed that 50% mortality occurred at an M value of 1.2 TU (Table 5). In another test, 50% reproductive inhibition occurred at M=2.6 TU (Table 7).
In an internal report from RITOX (van Lokven et al., 1993?), toxicity experiments were described with mixtures of 5, 6 and 10 substances, including 3 or 4 active substances. The endpoints were acute fish mortality (*P. reticulata, Brachydanio rerio*), immobilisation of the water flea (*D. magna*) and subacute mortality in the Early Life Stage (ELS) study with fish. Acute fish mortality occurred at M values of 1.7 to 2.5 TU. Immobilisation of *D. magna* occurred at M values of 0.9 and 4.0 TU. The M value in the ELS study (*B. rerio*) was 5.5 TU.

2.3 Binary mixtures of plant protection products

Deneer (2000) reviewed the results of studies involving mixtures of active substances that were conducted between 1972 and 1998. This review included 26 studies that investigated the toxicity of 202 mixtures on fish, crustaceans, insects, shellfish and algae.

Virtually all studies concerned mixtures of two active substances. The aim of his review was to determine the extent to which the results of the predominantly acute toxicity experiments could be described using the concept of concentration addition (CA: the substances in the mixture have the same mechanism of action and do not influence each other's activity). The criterion was that 50% effect (usually mortality) occurred at an exposure between 0.5 and 2.0 TU. Although CA theoretically occurs at an exposure to 1.0 TU, the criterion was expanded to take account of the known experimental variation in the measurement results (a factor of plus or minus 2). Deneer (2000) concluded that for more than 90% of the 202 mixtures, the observed toxicity could be described by concentration addition within the above-mentioned factor of 2 (i.e. at an M value between 0.5 and 2.0 TU).

However, if the hypothesis that the substances in the binary mixtures have a different effect (partial addition, PA) had been tested, then the conclusion would have been that partial addition also describes the observed toxicity. With partial addition, 50% mortality theoretically occurs at an M value between 1.0 and 2.0 TU (the exact value can be calculated if the dose-response relationships of the individual substances are accurately known). Based on the experimental reproducibility, partial addition describes the observed effects equally as well as concentration addition.

3. Concentration addition or partial addition?

3.1 Acute toxicity

The question of whether the acute toxicity of binary mixtures is the result of concentration addition or partial addition cannot be answered because the experimentally measured M value is usually between 0.5 and 2.0 TU.

The situation is different if the mixture contains 3 or more active substances. For CA, the theoretical M value remains 1.0. The M value for PA lies between 1.0 and n (n=the number of substances in the mixture). Table 5 shows the measured M values in acute and subacute toxicity tests with mixtures. The results are difficult to interpret because, with the exception of the binary mixtures, the studies involved mixtures in which non-plant protection products were also present.

Table 5. M values (in TU) in acute and subacute toxicity tests with mixtures of plant protection products. Toxicological endpoint: mortality.

taxon / source	M (in TU)	test duration	remarks
vis			
Deneer (2000)	0.7	48 to 96 u	geometric mean; binary mixtures; 12 tests
Hermens en Leeuwangh (1982)	1.3	14 d	geometric mean; 3 tests; 3 active substances in mixture of 8 substances
Hermens en Leeuwangh (1982)	1.3	14 d	4 active substances in mixture of 8 substances
Hermens en Leeuwangh (1982)	1.7	14 d	6 active substances in mixture of 8 substances
Hermens en Leeuwangh (1982)	2.3	14 d	11 active substances in mixture of 24 substances
van Lokven et al. (1993?)	1.7	96 h	3 active substances in mixture of 6 substances
van Lokven et al. (1993?)	2.0	96 h	4 active substances in mixture of 10 substances
van Lokven et al. (1993?)	2.5	96 h	4 active substances in mixture of 5 substances
van Lokven et al. (1993?)	5.5	14 d	ELS study; 4 active substances in mixture of 5 substances
crustaceans			
Deneer (2000)	1.1	48 u to 28 d	geometric mean; binary mixtures; 10 tests
Hermens et al. (1984b)	1.2	48 h	6 active substances in mixture of 14 substances
insects			
Deneer (2000)	0.7	96 h	geometric mean; binary mixtures; 8 tests
Deneer (2000)	0.8	96 h	mean; mixture of 3 active substances; 2 tests

The M value for subacute toxicity in the ELS study with fish was 5.5 TU. This appears to be non-additive because the mixture consisted of 5 substances. This study will be excluded from further consideration.

The geometric mean of all individual measurement values for fish is 0.9 TU. The geometric means of all individual measurement values for the crustaceans and insects are 1.1 and 0.7, respectively. In the data sets it is notable that the data show little variation particularly for the crustaceans.

Conclusion: the calculation of acute and subacute toxicity of mixtures, with mortality as the toxicological endpoint, can be based on the principle of concentration addition.

Table 6. M values (in TU) in acute toxicity tests with mixtures of plant protection products. Toxicological endpoint: immobility.

taxon / source	M (in TU)	test duration	remarks
crustaceans			
van Lokven et al., (1993)	0.9	48 h	3 active substances in mixture of 6 substances
van Lokven et al., (1993)	4.0	48 h	4 active substances in mixture of 10 substances

The results of the two acute toxicity tests with immobility as the toxicological endpoint differ greatly. Based partly on the observations with non-plant protection products (see above) it is assumed that the combination effect with immobility is the result of concentration addition.

Conclusion: the calculation of the acute toxicity of mixtures on crustaceans, with immobilisation as the toxicological endpoint, can be based on the principle of concentration addition.

3.2 Chronic and semi-chronic toxicity

Little is known about the chronic and semi-chronic toxicity of mixtures of active substances. Data on *algae* involve only binary mixtures (Table 7). The geometric mean of all M values for algae is 1.0 TU.

Conclusion: due to insufficient data, calculations will be based for the time being on concentration addition.

Table 7. M values (in TU) in chronic and semi-chronic toxicity tests with mixtures of plant protection products. Toxicological endpoint: growth inhibition (algae) or reproductive inhibition (crustaceans).

taxon / source	M (in TU)	test duration	remarks
algae			growth inhibition;
Faust et al., 1991	1.0	24 h	geometric mean; 29 binary mixtures
Faust et al., 1994	1.0	24 h	geometric mean; 38 binary mixtures
crustaceans			
Hermens et al., 1984b	2.6		6 active substances in mixture of 14 substances EC50 (approximately 16 d.); reproductive inhibition

For *crustaceans* only a single experiment has been done on reproductive inhibition with a mixture of 14 substances, including 6 active substances. The level of combination toxicity in this reproduction experiment is lower than that in an experiment with mortality as a parameter (compare with $M_{reproduction}$=2.6 TU (Table 7); $M_{mortality}$=1.2 TU (Table 5)).
For mixtures of 5, 10 and 25 non-reactive, non-plant protection products, Hermens et al. (1984a) showed that reproduction inhibition with crustaceans was slightly lower than concentration addition ($M_{geometric\ mean}$= 1.8). Hermens et al. (1984b) assumed that the contribution of individual substances when engaging a specific receptor is smaller than when engaging receptors that cause acute mortality. Conclusion: reproductive inhibition is based on partial addition. The degree of addition is slightly lower than concentration addition.
Basic assumption during the calculation of chronic and semi-chronic toxicity of mixtures for algae and crustaceans: based on concentration addition.

This proposal is prompted by the precautionary principle and the fact that the observed mixture toxicity deviates little from concentration addition.

Nothing is known about the mixture toxicity on aquatic vertebrates and invertebrates for other toxicological endpoints besides the above-mentioned sublethal endpoints. *Given the above, in the assessment of chronic and semi-chronic mixture toxicity for aquatic organisms, concentration addition is assumed.*

3.3 In summary

Although the effects of mixtures of active substances in plant protection products have been studied on a very limited scale and not for all relevant species and toxicological endpoints, it can be expected that active substances in a combination product jointly contribute to the toxicity of that product.
The degree with which the active substances contribute to toxicity is poorly known. The available data indicate that the degree of combination toxicity with partial addition does not deviate greatly from concentration addition. In accordance with these two considerations, the assessment of the toxicity data of combination products is based on concentration addition.

4. References

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