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Part 1:

Relevance and adequate consideration in environmental risk assessment of plant protection products and biocides

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Ecotoxicological combined effects from chemical mixtures

Part 1:
Relevance and adequate consideration in environmental risk assessment of plant protection products and biocides

by

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Abstract

The Project “Ecotoxicological combined effects from chemical mixtures“ funded by the Federal Environment Agency (UBA, FKZ 3709 65 404) deals with the possibilities of performing mixture toxicity assessment within the environmental risk assessment of the authorization of biocide and plant protection products. To this end a review on the state of scientific knowledge about the predictivity of combined effects is collated. Central in this context is the reference model of Concentration Addition which allows extrapolating combined effects for mixtures based on knowledge about the effects of the components. Building on this, options for risk regulation are developed. Their applicability is considered in the context of those data that are currently available within the authorization process for biocide and plant protection products. Deficits with respect to a – scientifically sensible – homogeneous data base can often be overcome with pragmatic decisions if additional requirements for the authorization process are not an option. Tiered schemes to specifically account for combined effects during environmental risk assessment of biocide and plant protection product authorization are suggested, accompanied with a software tool for its implementation.

Kurzbeschreibung

Das durch das Umweltbundesamt (UBA) geförderte Projekt „Ökotoxische Kombinationswirkungen von Stoffgemischen“ (FKZ 3709 65 404) befasst sich mit den Möglichkeiten der Berücksichtigung von Kombinationswirkungen in der Umweltrisikobeurteilung von Biozidprodukten und Pflanzenschutzmitteln. Hierfür wurde der Stand der wissenschaftlichen Erkenntnisse zur Prognostizierbarkeit von Mischungseffekten zusammengetragen. Als zentral ist das Modell der Konzentrationsadditivität anzusehen, das es gestattet, auf der Basis des Wissens über die Effekte der Komponenten, Extrapolationen über die zu erwartenden Kombinationseffekte der Mischungen anzustellen. Darauf aufbauend werden Optionen für die Regulation zur Beurteilung von Gemischtoxizitäten ausgelotet und deren Anwendbarkeit mit den in der Biozid- bzw. Pflanzenschutzmittelzulassung verfügbaren Informationen betrachtet. Es zeigt sich dass in Risikobetrachtungen unterschiedliche Arten von Mischungen (Wirkstoffe mit Formulierungshilfstoffen; Wirkstoffmischungen; Mischungen verschiedener Produkte bei gleichzeitiger oder sequentieller Anwendung) aus Anwendungssicht aber auch hinsichtlich möglicher Kombinationswirkungen relevant sind. Ein Mangel an – wissenschaftlich gebotener – homogener Datenlage für die Beurteilung kann, wenn auf entsprechende zusätzliche Anforderungen im Zulassungsverfahren verzichtet werden soll, durch pragmatische Lösungen überbrückt werden. Spezifische Vorschläge zur Berücksichtigung von Kombinationswirkungen in der Umweltrisikobeurteilung von Biozidprodukten und Pflanzenschutzmitteln bei unterschiedlichem Informationsstand werden ebenso vorgestellt wie ein zugehöriges edv-Programm zur Prognose der Mischungstoxizität.

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List of Abbreviations

a.s.	Active substance
AL	Acceptable level
AR	Application rate
AV	Avoidance factor
BCF	Bioconcentration factor
BEAM	Bridging Effect Assessment of Mixtures to Ecosystem Situations and Regulation (EVK1-CT1999-00012)
B-index	Behandlungsindex
BLM	Biotic ligand model
BMD	Benchmark dose (concentration)
BPD	Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998
BPR	Proposal of the European Commission COM (2009) 267
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
bw	Body weight
CA	Concentration addition (LOEWE Additivity)
CBA	Component based approach
DAA	Diacetone alcohol
DDD	Daily dietary dose
DF	Drift factor
DMT1	Divalent metal transporter 1
DR	Deviation ratio
EC	European Commission
EC50	Median effective concentration
ECx	Effect Concentration for effect level x
ECxCA	Effect concentration of a mixture calculated under the assumption of concentration addition
ECxIA	Effect concentration of a mixture calculated under the assumption of independent action
EFSA	European food safety authority
ER50	Median effect application rate
ERA	Ecotoxicological risk assessment
est	Estimated
ETE	Estimated theoretical exposure
EU	European Union
exp	Experimental
FIAM	Free ion activity model
FIR	Food intake rate
FRAC	Fungicide Resistance Action Committee
HI	Hazard index
HQ	Hazard Quotient
HRAC	Herbicide Resistance Action Committee
IA	Independent Action (BLISS Independence, Response Addition, Abbott's Formula)
IF	Interaction factor
IRAC	Insecticide Resistance Action Committee

JKI	Julius-Kühn Institute in Kleinmachnow, Germany
*K*oc	Soil organic carbon-water partitioning coefficient
*K*ow	Octanol-water partition coefficient
LC	Lethal concentration
LOEC	Lowest observed effect concentration
LR50	Median lethal application rate
MAF	Multiple application factor
MDR	Model deviation ratio
MM	Mixed model
MoA or MOA	Mode of Action
MOE	Margin of exposure
MS	Member states
MSDS	Material safety data sheets
MTI	Mixture toxicity index
MUF	Mixture uncertainty factor
MW	Molecular weight
NOAEL	No observed adverse effect concentration
NOEAEC	No observed ecological adverse effect concentration
NOEC	No observed effect concentration
NOEL	No observed effect level
PAH	Polycyclic aromatic hydrocarbons
PBO	Piperonyl butoxide
PBPK	Physiologically based pharmacokinetics
PCB	Polychlorinated biphenyls
PD	Fraction of the food type in the diet
PEC	Predicted environmental concentration
p*K*a	Negative base-10 logarithm of the acid dissociation constant
PNEC	Predicted no effect concentration
POD(I)	Point of departure (index)
PPP	Plant protection product
PPR	EFSA panel on plant protection products and their residues
PSM	Pflanzenschutzmittel
PT	Fraction of diet obtained in the treated area
QSAR	Quantitative structure activity relationship
R^2	R-square: the coefficient of determination
RI	Ratio of inhibition
RPF	Relative potency factor
SI	Synergistic interaction
SSD	Species sensitivity distributions
STU	Sum (summation) of toxic unit
TEF	Toxic equivalence factor
TER	Toxicity exposure ratio, inverse of HQ
TERmix	Toxicity exposure ratio of a mixture
TU	Toxic unit
TUS or TUs	Toxic unit summation, equivalent to STU
UBA	Umweltbundesamt, Federal Environment Agency
ULV	Ultra low volume
US	United States of America
VBA	Visual Basic

VDF	Vegetation distribution factor
WHO/IPCS	The World Health Organization / International Programme on Chemical Safety (IPCS)
WMA	Whole mixture approach

1 Executive Summary

The project **'Ecotoxic combination effects of chemical mixtures'** funded by the Federal Environment Agency (UBA, FKZ 3709 65 404) dealt with the relevance and means to account for mixture toxicity within the environmental risk assessment in the authorization process for plant protection products (PPP) and biocide products.

Since the conclusion of the EU Council of Ministers in 2009 on 'Combination effects of chemicals' (Council conclusion 17820/09) it is apparent that environmental regulations dealing with the assessment and management of risks for adverse biological effects from chemical exposure will progress toward including the consideration of potential combined effects resulting from exposure to chemical mixtures. While in principle there is sufficient evidence that mixture toxicity is an issue for regulatory risk assessment, the means on when and how to deal with this novel issue are less clear.

With the intention to generate a coherent and consistent approach for the assessment of mixture toxicity across different regulatory areas the specific project goals therefore were:

- To summarise the available knowledge on the applicability of mixture models for the prediction of ecotoxicological mixture effects and identify key aspects for their use in risk assessment approaches;
- To develop generic options for mixture assessments within environmental risk assessment schemes and identify gaps and necessary framework settings;
- To specify the types and needs of mixture considerations within the existing risk assessment frameworks for PPP and biocide product authorization;
- To develop specific implementation schemes for mixture assessment within the biocide and PPP authorization process and provide a tool for its consistent implementation.

It should be pointed out that cumulative exposure assessment, i.e. the multiple exposure of an organism against the same substance via different sources and/or pathways, was outside these terms of reference. The project approached the listed objectives in a consecutive manner. The available knowledge was summarised as a state of the art description through a comprehensive review of currently existing scientific evidence. Generic options for regulatory mixture assessment were subsequently reflected by consideration of the identified key issues. The latter step allowed identification of additionally necessary non scientific decisions regarding a framework for implementing mixture assessment in regulatory procedures. The practicability of mixture assessment within the biocide and PPP authorization process were then specified through a detailed consideration of the specific European regulatory frameworks, the in depth analysis of types of mixtures potentially under scrutiny, analysis of specific experimental evidence, and where possible the re-analysis of selected available regulatory documentation. The generated knowledge was subsequently used to develop specific schemes and a tool for the systematic and consistent inclusion of mixture assessments for different biocide and PPP related aspects. The schemes are elaborated in sufficient detail to accommodate situations of varying data availability and allow evidence-based mixture risk decisions.

1.1 State of the art in mixture ecotoxicity assessment

It has been demonstrated through various observational studies that organisms in their environment are typically confronted with mixtures of chemicals rather than that chemical exposure is against individual compounds. Furthermore, eco-epidemiological evidence and experimental studies show that mixture exposure may provoke combined effects and that ignoring these will underestimate resulting adverse outcomes. Moreover, it is now a wide held belief that accounting comprehensively for mixture toxicity via direct observation is possible only for few selected cases as mixture occurrence in the environment is too variable and divergent to be comprehensively investigated. Therefore, in experimental studies on mixture toxicity, models that allow calculation of expected combined effects on the basis of knowledge about the components biological activities have become an established means for assessment.

A potential basis for the assessment and thus also regulatory usable prediction of mixture toxicity in environmental organisms exists with the models of Concentration Addition (CA) and Independent Action (IA). These models allow the calculation of expected combined effects purely based on concentration-effect information for the components of a mixture of concern and their concentrations in the mixture. As they do not require any further environmental system dependent information but rely on individual compound-biology interaction properties they can be considered as generic concepts. In order to discuss the applicability of these concepts for component-based extrapolation purposes within environmental risk assessment schemes, the premises, scope for inference and uncertainty aspects were identified by means of a systematic literature review. The literature search retrieved about 800 references on these topics whereby the focus of analysing the evidence was placed on reviews of experimental studies and summary reports. Only for specific aspects original experimental studies and various other types of reports were considered as well. While the interest was in mixture toxicity evidence for pesticides and biocides with respect to ecotoxicological effects, a variety of endpoints, modelling approaches and generic studies, e.g. on validating the predictivity of extrapolation techniques were also considered. Major findings from these efforts concerning the applicability of the reference models for the assessment and prediction of combined effects using a component-based approach can be summarised as follows:

- Non-interaction between the components of a mixture of concern is widely accepted as a default assumption in the formulation of reference models for calculating the expected combined effects, while interactive effects are thought to be not predictable in a systematic fashion;
- Concentration Addition (CA) and Independent Action (IA) can be regarded as the major reference models for a component-based combined effect prediction. They have found widespread application and both reference models are thought to be suitable for distinguishable situations and each model comprises a specific set of conditions for use;
- Generalisations on the quantitative differences between predicted and observed mixture toxicity prediction are available for various fields (e.g. aquatic, terrestric bioassays, bioassays using vertebrates, invertebrates or plants). The mixture models seem to have a reasonable predictive power for various ecotoxicological effect endpoints and types of mixtures including transformation products. However, there is dispute whether this holds for mixtures including metals and the empirical evidence is still limited for biocide containing mixtures;
- The use of data for the same exposure setting, biological effect and species is considered a precondition for the application of either concept. The mode of action of

the mixture components is regarded as the governing factor for the accuracy of the predictions, although for mixtures of only a few components both concepts tend to predict quantitatively similar mixture toxicities.

- In most cases CA predicts the higher mixture toxicity compared to IA, which is why CA would seem a reasonable worst case model for a non interactive combined effect prediction. The determinants of the differences between the combined effect predictions from the models are known and can be simulated or quantified for specific situations;
- Limited knowledge is available concerning mixtures of formulation additives and active substances in biocide and PPP and the combined effects from mixtures in higher tier studies (i.e. semi-field and field studies as e.g. aquatic micro- or mesocosms and earthworm field studies). Indicators for synergism (i.e. combined effect are larger than expected for non interactive combined effects) are currently lacking;
- While mixture effects have been observed at low level effects of the individual components the way for their assessment is disputed;
- The question of whether a combined effect is likely or of relevance for a given mixture risk assessment can only be answered by explicit theoretical or experimental consideration for that mixture in the assessment process.

From the primary literature survey the project deduced the need to add more in depth considerations to gain more detailed knowledge of the following issues:

- Evidence on the suitability of the reference models of CA and IA for biocide mixtures. A reanalysis of published mixture studies including biocides was performed. It was concluded that this further systematic study was needed, as there are inconsistent reports on possible synergistic interactions in this field of application;
- Evidence for combined effects of active substances and formulation additives. For this, regulatory data for 15 selected plant protection products were re-analysed applying mixture considerations and available literature was evaluated;
- Evidence for the existence of indicators of synergistic effects and low dose combination effects. To achieve this, the progress achieved by toxicogenomic methods to detect low dose combined effects or to provide possible indicators for synergism was analysed by performing a systematic literature review.

1.2 Options for regulatory mixture ecotoxicity assessment

When it comes to the question of how the joint toxicity of ingredients of biocide or plant protection products (PPP) to non-target organisms in the environment may be considered in an environmental risk assessment three generic options may be differentiated:

(i) the whole mixture approach (WMA), i.e. direct experimental testing of the mixture of concern, just like a single substance,

(ii) the component-based approach (CBA), i.e. calculating the expectable joint toxicity from toxicity data for individual mixture components by applying corresponding models, in particular those based on the reference models of Concentration Addition (CA), Independent Action (IA), and so-called mixed model (MM), and

(iii) the assessment factor approach, i.e. safeguarding against mixture effects by means of a special factor, similar to the use of other uncertainty or extrapolation factors in chemicals risk assessment.

The whole mixture approach is the only reliable way to account for joint effects that result from synergistic or antagonistic interactions of components and that are hence unpredictable by CA or IA, but it is strongly limited by technical, economical and ethical constraints. Component-based approaches may be hampered by limited knowledge about all relevant mixture components and missing data on their individual toxicities, and the optimal choice between different component-based approaches may be constricted by unfulfillable data requirements or insufficient knowledge about modes of action (MoA) responsible for the toxicity as observed in non-target organism. Assessment factors may be used (i) to account for unknown mixture components or components with unknown toxicity, and (ii) for safeguarding against synergistic interactions, but scientifically justifiable quantitative values are difficult to define.

The reflections about the requirements for any of the above outlined options, such as e.g. data availability, demonstrate that for generating coherent approaches within a regulatory field or even more so for harmonised approaches across different regulatory arenas, there is a need for additional settings. These cannot be derived from either data or scientific knowledge alone. Rather additional framework definitions with respect to the level of acceptable pragmatism, the requirements for using available or calling for additional data, the acceptable risk outcomes and the accepted uncertainty are required. First common principles for devising a transparent and coherent framework of mixture assessment regulation were formulated.

The suggested principles include:

- Environmental safety requirements for mixtures should neither be higher nor lower than the corresponding requirements for single substances;
- As far as possible, assessments of environmental risks for mixture effects should be implemented without additional experimental testing of whole mixtures, both for ethical and economic reasons;
- CA is considered to provide a reasonable default assumption for the joint eco-toxicity of PPP and biocidal product ingredients, provided that there are reasons to assume that all relevant mixture components are included in the calculation;
- As input data, the original scientific concept of CA requires effect concentrations (or doses) that refer to the same biological effect in the same species under identical test conditions. For regulatory use, however, pragmatic simplifications and assumptions are unavoidable. This may refer to the merging of data for different test conditions, endpoints and species and to the use of NOEC values as a surrogate for quantitative estimates of low effect concentrations. In any case, the potential additional errors that may be introduced by such deviations from the original concept should be made transparent. Where possible they should be removed in a stepwise manner;
- IA and mixed models (MM) are much more data demanding and bear a higher risk of underestimating the actual mixture toxicity than CA. Therefore, the use of IA and MM should be restricted to situations where knowledge about MoAs and dose response relationships of mixture components supports the proper usage of these models;
- Depending on the specific assessment situation, CBA-based mixture toxicity estimates may be complemented by specific assessment factors for potential synergistic interactions.

Where CBA-based assessments point to an unacceptable risk, experimental testing of the mixture may be considered as an ultimate option for clarification.

1.3 Relevance of mixture risk assessment in biocide and PPP product authorization

Prior to detailing possible mixture considerations within the specific risk assessment schemes for biocides and plant protection products the following questions were investigated:

(i) What types of mixtures would have to be addressed in a future risk assessment amended for mixture considerations?

(ii) How does the current EU regulatory framework account for the assessment of mixture toxicity?

(iii) Would a mixture assessment be plausible with the available reference models and would it be possible on the basis of the information typically available in the risk assessment process?

(iv) Would the consideration of mixture toxicity have great impact on the authorization of plant protection products (PPPs)?

Biocides

Biocides are involved in at least three fundamentally different mixture types.

a) A biocidal product, containing a mixture of several active substances;

b) A biocidal product, containing a mixture of one (or more) active substance(s), mixed with additional biologically active substances or substances that alter the biological activity of active substances by interfering with its uptake or biotransformation;

c) A biocide co-occurring with other biocides, other anthropogenic chemicals or with natural compounds in a surface water body, ground water, soil and sediments.

Mixtures of the last type were not considered, as they are outside the current scope of the Biocidal Product Directive, and are potentially subject for the Water Framework Directive or similar media-oriented regulations.

As data and first-hand documentation from the authorization of biocide products (mixtures of either several active substances, or mixtures of active substances with other biologically active substances) were not available for a review and analysis of empirical evidence, a generic approach for biocide assessment was developed by the project. This included

- a brief overview of the existing and foreseeable regulatory demands for biocide mixture assessment, and
- a review of published studies on mixtures involving biocides. The scientific state of the art was compiled and critically reviewed, focusing on wood preservatives and antifouling agents, i.e. two product groups with high potential environmental impact.

Regulatory demands

Mixtures and substances are newly defined in Regulation (EU) No 528/2012[1] in concordance with article 3 of Regulation (EC) No 1907/2006 (REACH). Article 3(1)a of Regulation (EU) No 528/2012 then acknowledges the fact that biocidal products might contain mixtures of active substances. And finally, in Annex VI, "Common principles for the evaluation of dossiers for biocidal products", points 53 and 54 it is stated that *"In each of the areas where risk assessments have been carried out, the evaluating body shall combine the results for the active substance together with the results for any substance of concern to produce an overall assessment for the biocidal product itself. This shall also take account of any cumulative or synergistic effects. For biocidal product containing more than one active substance, any adverse effects shall also be considered together to produce an overall assessment for the biocidal product itself."*

A substance of concern is defined in this context as "*any substance, other than the active substance, which has an inherent capacity to cause an adverse effect, immediately or in the more distant future, on humans, in particular vulnerable groups, animals or the environment and is present or is produced in a biocidal product in sufficient concentration to present risks of such an effect*" (Art 3(1)f of Regulation (EU) No 528/2012).

Article 8.3 ("Evaluation of applications") of Regulation (EU) No 528/2012 reads as follows: *"Where the evaluating competent authority considers that there are concerns for human health, animal health or the environment as a result of the cumulative effects from the use of biocidal products containing the same or different active substances, it shall document its concerns [...] and include this as part of its conclusions."* The new regulation of the European parliament and of the Council entered into force on 27 June 2012 and will replace and repeal the current directive on biocides (98/8/EC) and will enter into operation on 1 September 2013.

Recent regulatory developments in the area of biocides thus follow and even go beyond previous developments in the area of PPP risk assessment. Pragmatically useful, scientifically sound and sufficiently protective approaches for the assessment of mixture effects of biocides hence need to be developed.

Evidence for synergism with biocide mixtures

From the initial systematic literature review it was concluded that a further more detailed analysis was needed, as there are inconsistent reports on possible synergistic interactions in this field of application. A literature search using Scopus retrieved 961 peer reviewed studies on the joint toxicity of biocides in general and on the combination ecotoxicology of antifouling agents (11 biocides) and wood preservatives (25 biocides) in particular. 89 of these publications were finally selected for an in-depth review. The most commonly analysed mixtures contained copper, tributyltin and/or irgarol. Those were usually combined in binary mixtures with either other biocides, or with common environmental pollutants such as PAHs, PCBs or heavy metals. The ecotoxicology of individual as well as mixed biocides has been investigated using the standard battery of mainly limnic assays (algal growth inhibition assay, bacterial respiration and/or growth, short-term toxicity to fish, short-term and long-term toxicity to daphnids, etc.). Studies on multi-component mixtures are severely underrepresented. There is also a clear lack of studies on the terrestrial effects of biocide mixtures (important for mixtures containing biocides from product group 21 (wood

[1] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products.

preservatives)) and on the impact of biocide mixtures in the marine environment (important for mixtures containing biocides from product group 21 (antifoulants)).

Data for 382 different mixtures were re-analysed in more detail (336 two-compound mixtures, 32 three-compound mixtures 7 four-compound mixtures, 1 six-compound mixtures, and six 12-compound mixtures) which included at least one biocide. The major findings of this exercise can be summarised as follows:

- Concentration Addition is the most widely used reference concept for assessing experimental data on the joint toxicity of biocides, followed by Independent Action (Response Addition) and Effect Summation;
- The empirical knowledge on the ecotoxicity of biocide mixtures is still limited. Only 50 % of the active substances that are currently in the EU review programme have been subject to mixture studies. In particular, only a single study has been performed to our knowledge that used realistic biocide mixtures (in terms of mixture ratio, composition and concentration);
- Several cases of strong synergistic or antagonistic mixture toxicities have been described in the literature. However, these can be traced back to only a very few publications; after exclusion of these mixtures with strong deviations from non interaction predictions, the ratio of predicted to observed mixture EC50 of the 89 analysed binary mixtures was 1.02, for the 3-compound mixtures it was 1.12. The data hence indicate that CA performs equally for mixtures of antifoulants as will be discussed for pesticide mixtures under the above made restrictions;
- With very few exceptions the compounds in the investigated mixtures had dissimilar or unknown modes and mechanisms of action in the test organisms and belong to different chemical classes;
- The documentation of the mixture studies in the analysed publications varies dramatically. In the majority of cases a consistent, quantitative assessment and comparison of the results from different publications is hampered by limitations in either the study design or its documentation, or by use of different approaches to quantify deviations between predicted and observed mixture toxicities. This concerns for example situations in which the results are only presented visually (as isoboles), or where single substance toxicity data or mixture toxicity data are not provided. In particular, studies that used Independent Action often assessed the predictive power of this concept by comparing predicted with observed mixture effects at an *a priori* defined concentration. This makes a comparison across studies impossible. Improved study designs and data documentation is therefore urgently needed in order to allow firmer conclusions for risk assessment.

Plant Protection Products

Plant protection products (PPPs) are involved in several types of mixtures resulting from PPP application on the same agricultural crop.

a) all PPPs, as they consist of one (or more) active substance(s) together with formulation additives that may be biologically active themselves or alter the biological activity of the active substance(s) by interfering with its uptake or biotransformation;

b) PPPs, containing a mixture of active substances, so-called combination products;

c) PPPs that are mixed prior to application (Tank mixtures);

d) PPPs that are used in serial applications (Spray calendars).

Here we considered mixtures of active substances applied together, namely combination products (i.e., products containing more than one active substance) and tank mixtures, as well as mono-formulations (i.e., products consisting of one active substance and its formulation additives), the latter, in order to evaluate the influence of formulation additives on the ecotoxicity of active substances. Exposure modelling was beyond the scope of the present study, which precluded a more than principal consideration of serial applications. Likewise, the options for an exposure refinement that are foreseen in the risk assessment process (if a risk cannot be excluded otherwise) were not considered here.

One identified key aspect in the issue of considering mixture toxicity in the risk assessment was the need to unambiguously define to which composition of a mixture the current risk assessment scheme would relate. The two possible mixture types (mixture composition as applied in agriculture or mixture composition as expected in the environment) are both used to a different extent in the various risk assessment areas (birds & mammals, aquatic organisms and terrestrial organisms) and refinement steps. A matrix was devised to help structure the analysis and facilitate understanding of the implementation of mixture toxicity into the current risk assessment scheme.

Regulatory demands

The analysis of the legal background described and took into account current European and national directives, regulations and supporting guidance and opinion documents. Summarizing, the regulation EC No 1107/2009 concerning the placing of PPP on the market, which repealed Directive 91/141/EEC, explicitly requires with regard to human health to take "*into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available*". While there is no such explicit statement for the environmental risk assessment, both the Regulation and the former Directive require that PPP's shall be assessed "*in the light of current scientific and technical knowledge*" and that "*Member States shall ensure that use of plant protection products does not have any long-term repercussions for the abundance and diversity of non-target species*". Implicitly, this requirement calls for taking into account in the risk assessment "[...] *multiple stress by the use of multiple plant protection products, being applied at the same time (e.g. tank mixtures) or in sequence* [...]" according to an opinion of the EFSA Panel on Plant Protection Products and their Residues (PPR 2010). Moreover, Article 29 of the regulation EC No 1107/2009 requests with regard to the authorization of PPP that "*Following these principles* [Uniform principles for evaluation and authorisation of a PPP], *interaction between the active substance, safeners, synergists and co-formulants shall be taken into account in the evaluation of plant protection products*." It can thus be argued, therefore, that in analogy to the authorization of biocides the biological and regulatory relevance of combination effects of PPP reflects the current scientific knowledge and that such effects should be considered during the authorization process, while technical knowledge on how to adequately take these effects into account needs further development and guidance.

An overview on currently applied or proposed approaches on how to consider mixture toxicity in the environmental risk assessment revealed that several Member State authorities are very active in this area, but that guidance so far released is often ambiguous, incomplete and not consistent across Member States.

Mixture assessment based on data typically available in the current product authorization process

First, an analysis of the current implementation specifically on the national level (Germany) was conducted, which was based on a survey of risk assessment reports for 15 combination products that were selected by the Federal Environment Agency, Germany (UBA). This analysis aimed to provide an overview on the typical data availability for the various risk assessment areas in terms of usability for a mixture assessment. The analysis further aimed to identify aspects within the current risk assessment scheme potentially complicating mixture toxicity considerations to help developing specific implementation schemes. By applying existing or newly developed approaches of considering mixture toxicity for selected combination products, the last step of the analysis illustrates potential future consequences for the authorization of PPP.

As a result, it was found that available ecotoxicological data of individual active substances are mostly sufficiently homogeneous and complete for the legally required standard test endpoints such as acute toxicity to birds & mammals, acute toxicity to *Daphnia* and fish, and algal growth inhibition to undertake a consideration of mixture toxicity by CA. For chronic data there are usually only 'no observed effect concentrations or levels' (NOEC/NOEL) available, which do not comply with the strict scientific assumptions for usage in the model of Concentration Addition. Furthermore, data beyond the standard data requirements such as chronic toxicity or higher-tier studies (i.e. semi-field and field studies as e.g. aquatic micro- or mesocosms and earthworm field studies) were more frequently used in the risk assessment, but often only available for one of the active substances in a combination product. This prevents application of mixture toxicity consideration unless data from different levels of biological organisation and complexity are merged. In consequence, considerably dissimilar endpoints, e.g. the NOEAEC (no observed ecological adverse effect concentration) of a mesocosm study and the EC50 of algal growth inhibition test would be combined to derive a mixture toxicity estimate; an approach that can be regarded as scientifically questionable. The refinement options of exposure estimates in the risk assessment further adds complexity to considering mixture toxicity. Implementing mixture toxicity considerations, based on the approaches applied so far (and for the restricted number of examples analysed so far), did not result in a more frequent need for a refined risk assessment in the birds & mammals area than already indicated by the assessment of individual active substances for the considered 15 products in the first tier risk assessment. In the case of aquatic organisms, stricter application restrictions (i.e. larger no-spray-zones to adjacent surface waters or higher requirements regarding drift-reducing nozzles) were indicated in several cases with the number of cases depending on the implementation approach that was followed. For terrestrial organisms, the consideration of mixture toxicity is already implemented in many cases by using toxicity data for the product in the current risk assessment scheme ('whole mixture approach'). Yet, problems have been identified in the absence of such data for combination products, namely the usage of higher-tier studies available for only one of the mixture components as a basis for an authorization decision. Other approaches may still be developed e.g. striving to identify the component(s) relevant for risk assessment. However, these may also be hampered by a frequent lack of data for the technical grade active substance (a.s.) in the terrestrial hazard assessment.

The potential influence of formulation additives on the toxicity of PPP was evaluated using a set of 15 mono-formulations (provided by UBA) in addition to a literature review. By comparing the toxicity of the technical a.s with that of the formulated a.s., the evaluation aimed to identify the frequency of increased toxicity and formulation types that may be related to such a phenomenon. Overall, the findings for the selected mono-formulations and a

previous analysis of more than 100 combination products (Coors & Frische 2011) demonstrated that a toxicity of formulation additives was of relevance mostly for the organisms that are not very sensitive to the a.s., while they hardly increase the toxicity for the non-target organism that are the most sensitive ones for the a.s. Yet, there were exceptions to this rule of thumb. It was further indicated by this evaluation that among the most frequent formulation types none stands out as being *per se* problematic, but that rather a case-by-case evaluation is advisable. Based on the evaluation and the literature search, the toxicity of formulation additives such as some safeners, solvents, surfactants and other components were identified as cause for enhanced toxicity of formulated a.s., which may principally be accounted for if ecotoxicologically relevant additives are considered in the mixture toxicity prediction. However, if there is influence of formulation additives (such as emulsifying agents) on bioavailability and fate of active substances this would not be covered by the component-based approaches considered here, since these concepts cannot predict such toxicodynamic or toxicokinetic interactions.

A similar evaluation was conducted for three different tank mixtures that had been identified as being frequent among actually applied tank mixtures in agriculture. Again, mixture toxicity consideration by CA was generally possible for the standard endpoints, but limited for long-term or higher-tier endpoints. The fact that the assessment in many risk assessment areas relies on the direct comparison of one (or few) tested application rate(s) to the control treatments complicates or even prevents the usage of these data for the assessment of tank mixtures. Such data provide little information for other application rates of the products and products in tank mixtures are often reduced in relation to the maximum authorized application rates. The assumption of existing risk mitigation measures for the products being applied for the tank mixture together with the assumptions on average reduction of individual product application rates was essential for the finding that safe use of the three studied tank mixture was indicated by the mixture toxicity assessment for all cases that could be assessed. In other words, without these two assumptions a risk would have been indicated for the assessed tank mixtures in more cases. Higher-tier studies were not available for all tank mixture components for endpoints that had been identified as critical in the authorization process of the products. Therefore, risks could not be fully excluded for some of these endpoints, namely honey bees, non-target arthropods and earthworms independently from risk mitigation assumptions.

1.4 Indicators for synergism and low dose mixture effects

As synergistic interactions provoked by components of a mixture may occur and cannot be predicted by either reference model (CA, IA) the question is whether there are currently indicators for such interactions available or in sight. The advent of genomic techniques has raised large expectations that their application can provide a novel perspective on such mechanisms of low dose interactions. After the first decade of experimental studies utilising the novel toxicogenomic tools, the existing mixture toxicity studies that address diagnostic, mechanistic or extrapolation questions were therefore summarised and reviewed. From 2002 to 2011, 41 studies were published with a focus on mixture toxicity assessment by means of toxicogenomic techniques, mainly through multiplexed quantification of gene transcripts, though metabolomic and proteomic analysis of joint exposures have also been undertaken. Among the studied compounds several active substances of plant protection products were investigated. It is now standard to explicitly state criteria for selecting concentrations and provides insight into employed data transformation, and statistical treatment with respect to minimising sources of undue variability. Bioinformatic analysis of toxicogenomic data, by contrast, is still a field with diverse and rapidly evolving tools. The combined effect

assessments are discussed in the light of established toxicological dose-response and mixture toxicity models. For one, receptor-based assays seem to be most advanced towards establishing quantitative relationships between exposure and biological responses. Often transcriptomic responses are discussed based on the presence or absence of signals. As there are yet no consented ways of analysing these type of effects, the currently offered interpretations are ambiguous. Furthermore, the majority of mixture studies are designed for comparing the recorded outcomes against individual treatments. I.e. the focus was to retrieve signals of individual components under mixture exposure. This stands in stark contrast to our existing understanding of biological activity at the levels of chemical target interactions and apical combined effects. Thus at the current state of work, evidence provided is rather anecdotal than systematic and is thus not yet ready for use in regulatory extrapolation practise.

Conclusions from the current knowledge

To summarise the findings from reviewing the current theoretical and empirical evidence as well different regulatory settings, it may be concluded that,

- Despite a situation of a limited data base for mixture toxicity studies and acknowledging several specific aspects relevant to an adequate exposure assessment of biologically active compounds;
- Although there may be technical problems to be encountered when trying to perform mixture assessments with the data available in current documentations for product authorization; and
- Notwithstanding that a lack of reliable empirical indicators for mixture synergism based on the individual components effects is to be acknowledged;

this study could not find reasons to refute a component-based mixture toxicity assessment based on non-interaction reference models (CA, IA) as a reasonable worst case estimate. However, provisions to account for lack of data or conceivable interactive effects may well be taken when dealing with resulting uncertainties.

1.5 Implementation schemes for mixture assessment

Prior to developing specific regulatory approaches for incorporating mixture toxicity assessment in the environmental risk assessment of biocides and PPPs we screened proposed and existing regulatory approaches from the human and environmental risk assessment arena to foster a consistent and harmonised approach. In general, it can be said that:

- Current mixture risk assessments typically use one specific methodology (e.g. whole mixture testing or component-based approach) rather than tiered or optional schemes. Existing mixture risk assessments have not been found to be using additional or specific safety factors to account for combined effects;
- The calculus rule of CA is preferably used compared to few suggested IA applications or schemes that perform addition of effects. Often regulatory schemes utilise a specific format of a point wise mixture extrapolation;
- For the use of data in the considered mixture schemes, effects are often pragmatically aggregated with respect to the considered endpoint and effect intensity, or across species of a taxonomic group. Moreover, even aggregation of values which include uncertainty factors, such as PNECs, is suggested and practised;

- Some schemes include refinement options for interactive effects of mixtures;
- Fragmented and inconsistent approaches for mixture assessment seems to emerge across different areas of risk regulation.

In view of all the pros and cons, the different implementation options (namely safety factors, component-based extrapolations and mixture testing) should not be perceived as alternatives but rather as complementary approaches which may be combined in a stepwise fashion with the aim to make optimal use of the available data and resources for a specific assessment situation. To facilitate a rational approach, we explored the critical factors that determine (i) the practical applicability of different implementation options and (ii) the quantitative differences between different component-based approaches. These are:

- the number of mixture components in an exposure scenario,
- the concentration (or dose) ratio of mixture components,
- the slope of individual concentration (or dose) response curves,
- the modes and mechanisms of action of individual toxicants,
- the type, quantity, and quality of available toxicity data for mixture components,
- the type and quality of hazard or risk indicators to be determined, such as EC50, NOEC or TER values for mixtures for instance,
- requirements concerning the safety and reliability of mixture toxicity estimates,
- information about variability and uncertainty of single substance toxicity data,
- quantitative information about relations between different toxicity endpoints, and
- quantitative relationships between NOEC values and low effect concentrations.

Based on considerations of all these factors, we developed stepwise approaches for both biocide and plant protection products and provided a software tool to allow a consistent utilisation. The proposed schemes are tailored toward the specific assessment situations but follow the above outlined common principles.

Biocidal Products

As detailed above the directive on biocides (98/8/EC) defines standards for the environmental risk assessment of biocides, but there existed no comprehensive guidance for product assessment. The new regulation (EU) No. 528/2012 was agreed in 2012 and will apply from September 2013. Development and drafting of the accompanying guidance documents is still ongoing. Hence, currently there are no detailed guidelines for biocide assessment at hand.

In view of this situation, a flexible tiered approach was developed that accounts for various data situations and shall serve as a template for the future implementation of biocide risk assessment guidelines. The minimum requested set of data that is needed for starting the assessment according to the suggested approach consists of (i) solid and complete information on the product composition, (ii) information on the relative risk ranking of the individual compounds, and (iii) the PEC/PNEC ratio for the most risky compound. That is, for compounds with a lower PEC/PNEC ratio only semi-quantitative data from e.g. QSAR estimates or the classification and labelling of the compound in question are needed.

The suggested scheme hence keeps the data demands as low as possible (i.e. optimizes resource efficacy), while at the same time ensures an protection of the environment adequately accounting for our current state of knowledge. It progresses in a tiered fashion. An initial assessment is already possible with a very limited set of data, using an adequately precautionary approach. In case no indications for a reason for concern are detected, no

further testing or data evaluation is required. If there are potential reasons for concern, additional data can be supplied in order to allow a more detailed assessment.

The suggested approach uses component-based assessments as much as possible, starting with an initial assessment based on the simple sum of PEC/PNECs which are followed by more detailed CA- and IA-based assessments in the higher tiers. This strategy facilitates the re-use of existing data for individual ingredients. All component-based assessments are penalized by an additional assessment factor which was termed "IF" (Interaction Factor), which accounts for possible unexpected interactions (higher mixture toxicity than predicted by Concentration Addition due to toxicokinetic and/or -dynamic interactions). It should be noted that this factor does not account for any compounds in the product that are not included in the mixture assessment.

If ecotoxicological data for the whole product or the resulting environmentally relevant mixture (leachates) are available as well, these data take preference to component-based toxicity predictions.

Plant Protection Products

Legally binding standard criteria for acceptable risks of plant protection products (PPP) to non-target species are laid down in the *Uniform Principles* fixed in Commission Regulation 546/2011, mostly in terms of minimum toxicity exposure ratios (TER) for acute, short-term and long-term endpoints in different groups of species. Corresponding toxicity and exposure data for individual substances are a routine requirement and are determined by standard assays and procedures, at least for active substances and under the new PPP Regulation in the future also for safeners and synergists, but not necessarily for co-formulants.

Complementary to these standard single substance data, corresponding whole mixture testing data are often available for the acute and short-term toxicity caused by direct contact with the PPP under consideration (or with a similar product), but typically not for the standard long-term endpoints listed in the Uniform Principles. In addition to standard endpoints, whole mixture testing data may in specific cases also be available from long term higher tier studies, in particular with terrestrial plants or earthworms. However, in these cases corresponding single substances data may often be missing. All available whole mixture testing data usually refer to the original PPP in concentrated or diluted form, not to mixtures of product ingredients in different proportions, as they may be expected to occur in environmental exposure scenarios as a result of differential transformation and transport processes.

Given this comparatively data rich situation, component-based approaches (CBA) may be used for the following purposes:

i. For assessing the risks of mixtures of active substances that originate either from one and the same combination product or from the intended combined use of different products, so-called tank mixtures, with respect to endpoints for which single substance data but no whole mixture test data are available.
ii. For assessing the risks of mixtures of active substances that originate from a combination product for which whole mixture testing data are available, but which are expected to co-occur in an environmental exposure scenario in a different concentration ratio than in the tested product.
iii. For counter-checking experimental short-term toxicity data obtained by testing of products that contain more than one active substance. Significant differences between observed and calculated joint toxicity may point either to additive toxicity

contributions of co-formulants or to synergistic or antagonistic interactions between product ingredients, or both. Such cases may require in-depth examinations.

iv. For the identification of potential drivers of mixture toxicity in terms of toxic units or TER values, in cases where the comparison between whole mixture testing and CBA confirms that the overall toxicity is largely explainable by concentration additive action of active substances.

Beyond such uses of CBAs as a complementary tool in addition to usual whole mixture testing of products, it may also be considered to actually waive experimental product testing, if the CBA gives a strong indication for the absence of any unacceptable risks. However, in the light of the existing evidence on the accuracy of CBA-based assessments, it is recommended as a minimum requirement that such waiving may only be considered, if single substance toxicity data for non-target species are available for all product ingredients, and not just for those that are active against target species. This may include the information that a substance is non-toxic ("inert") with respect to the endpoint under consideration. More detailed requirements for actual waiving of product testing may have to be worked out.

For the purpose of CBA-based environmental risk assessments of both mixtures of ingredients of a single combination product as well as tank mixtures, a uniform tiered approach has been worked out. The approach calculates an expectable TER-value of a mixture (TERmix) for a given endpoint (e.g. fish acute toxicity) and a given exposure scenario. For different endpoints the approach must be applied separately.

The suggested approach is structured into three main tiers. Each of these tiers serves as a filter. On the one hand, such mixtures are sorted out for which there is no indication of an unacceptable risk for the given endpoint and which therefore do not need any further consideration. On the other hand, however, also such mixtures are identified for which the CBA-based evidence for an unacceptable risk is so strong that these concerns can under no circumstances be ruled out by more advanced and more data intensive CBAs for the same endpoint. This is a major difference to other tiered schemes for mixture risk assessment, and this is achieved by considering the quantitative relationships between (i) single substance toxicity and concentration additive joint toxicity and between (ii) concentration additive and independent joint action.

The lowest tier is a pre-checking step, in which only single substance TER values (TERi) are considered. The next tier makes use of TER values calculated for mixtures (TERmix) under the default assumption of a concentration additive joint action. Highest tier assessments are based on considerations of the available knowledge on MoAs and concentration response relationships; where appropriate, TERmix values are estimated under the assumption of IA or a MM.

Under the PPP regulation, single substance TER values are usually available for any of the assessment endpoints defined in the *Uniform Principles*. As a consequence, the proposed assessment scheme should also be applied separately for any of these endpoints, such as effects on algal growth, effects of long-term exposure on fish, and long-term toxicity to Daphnia for instance. A merging of data across such different taxonomic groups may be necessary for other less data rich situations (e.g. in the case of biocidal products; see above), but it is not necessary and not recommended for PPP assessments.

On each of the three levels of the tiered approach, both types of input data, exposure and toxicity estimates may undergo an iterative refinement process. If in the end the CBA still indicates an unacceptable risk of the mixture of concern, experimental testing of re-constituted mixtures may be considered as a tool for final confirmation, unless practical,

ethical, economical or other regulatory considerations argue against such a decisive experiment.

Critical points of the suggested tiered scheme that may need further elaboration are the following:

- potential synergistic interactions that are not covered, but which may be addressed by optional inclusion of an appropriate assessment factor;
- potential contributions of synergists, safeners and co-formulants to the overall toxicity, which could be easily included if single substance toxicity information would be available;
- potential contributions of metabolites of product ingredients that are formed in the environment and about which only insufficient information may be available to the assessing authority; and
- uncertainty about the applicability of CA or IA to higher tier multi-species toxicity data which are occasionally available for pesticides.

1.6 Conclusions and Outlook

From the review of the available theoretical and experimental evidence it can be concluded that concentration addition is a well supported reference model for a component-based assessment of the combination effects of plant protection products as well as biocides.

However, the implementation of concentration addition into regulatory risk assessment procedures is not a self guiding process. Additional decisions are required for dealing with issues such as data gaps or a heterogeneous data base. Dead lock discussions; such as on the justification of concentration addition for mixtures with unknown modes-of-action or the handling of a heterogeneous data base may be overcome by employing tiered approaches in risk assessment as suggested in this report. These may also be useful to achieve a consistent and harmonized approach across EU member states and different regulatory regimes, respectively.

The approaches suggested here can be directly incorporated into current risk assessment schemes for plant protection products and biocides, as they do not require fundamentally new risk assessment strategies. This is in particular achieved by basing the mixture risk assessment on a set of reasonable default assumptions. Additional data or risk management measures are only requested if the available evidence clearly indicates reason for concern. This minimizes the need for additional resources, especially additional animal tests, while at the same time ensuring an adequate protection of the environment.

Future research into the assessment of combined effects from chemical mixtures should focus on the following

1) Indicators for interactive mixture effects, especially by leveraging the power of modern molecular methods;
2) More and better empirical data on the toxicity of biocide mixtures for other than antifouling products and for marine and terrestrial systems;
3) Mixture studies on levels of biological complexity higher than the individual organism or population (i.e. semi- and field studies like aquatic mesocosms), mixture studies addressing chronic toxicity and exposure studies adequate for an advanced environmental mixture risk assessment;

4) Improved and agreed guidelines on the design, interpretation and documentation of ecotoxicological studies on mixtures.

1B Executive Summary – deutschsprachige Fassung

Das durch das Umweltbundesamt geförderte Vorhaben **‚Ökotoxische Kombinationswirkungen von Stoffgemischen – Relevanz und angemessene Berücksichtigung in der Umweltrisikobewertung von Pflanzenschutzmitteln und Bioziden'** (UBA, FKZ 3709 65 404) befasste sich mit der Relevanz und Möglichkeiten der Berücksichtigung von Mischungstoxizitäten in der Umweltrisikobewertung im Rahmen des Autorisierung von Pflanzenschutzmitteln (PPP) und Biozidprodukten.

Seit dem Beschluss des EU-Ministerrats in 2009 on 'Combination effects of chemicals' (Council conclusion 17820/09) ist zu erwarten, dass in Zukunft Umweltregulationen zu Risikobewertung und -management von Chemikalien eine Berücksichtigung von potentiellen Kombinationseffekten durch Chemikalienmischungen vorsehen werden. Während im Prinzip hinreichende Belege dafür vorliegen, dass Mischungstoxizität ein relevantes Thema für die regulatorische Risikoanalyse darstellt, ist weniger klar wie dieses in der Bewertungspraxis erfolgen könnte.

Mit der Intention der Entwicklung eines kohärenten und konsistenten Weges für die Bewertung der Mischungstoxizität über verschiedene regulatorische Bereiche hinweg, waren die spezifischen Projektziele folgende:

- Das verfügbare Wissen über die Anwendbarkeit von Modellen für die Vorhersage von ökotoxikologischen Mischungseffekten sollte zusammengefasst und die Kernaspekte für ihre Nutzung in Risikobewertungsverfahren identifiziert werden;
- Generische Optionen für eine Mischungsbewertung in Umweltrisikobewertungsverfahren sollten entwickelt und Lücken sowie notwendige Rahmenfestlegungen identifiziert werden;
- Die Arten und Erfordernisse der Mischungsbetrachtung innerhalb der existenten Risikobewertungsverfahren für PSM- und Biozidprodukt-Zulassung sollten spezifiziert werden;
- Spezifische Implementierungsverfahren für eine Mischungsbewertung im Rahmen der Biozid- und PSM-Zulassungsprozesse sowie ein Werkzeug für die konsistente Implementierung sollten entwickelt werden.

Es muss hervorgehoben werden, dass die kumulative Expositionsbewertung, d.h. die multiple Exposition eines Organismus gegenüber demselben Stoff aufgrund verschiedener Quellen und/oder Expositionspfade, außerhalb der hier vorgelegten Betrachtungen standen. Für das Projekt wurden die aufgelisteten Zielstellungen in konsekutiver Vorgehensweise erarbeitet. Das verfügbare Wissen wurde als Beschreibung des aktuellen Standes der Wissenschaft mittels Sichtung zur gegenwärtig verfügbaren Evidenz zusammengefasst. Generische Optionen für eine regulatorische Mischungsbewertung wurden anschließend dargestellt unter Berücksichtigung der zuvor identifizierten Kernprobleme. Dieser Schritt erlaubte die Benennung von zusätzlich erforderlichen nicht-wissenschaftlichen Entscheidungen, um einen Rahmen für die Implementierung von Mischungsbewertungen zu entwickeln. Die Praktikabilität von Mischungsbewertungen innerhalb des Biozidprodukte- und PSM-Zulassungsprozesses wurden anschließend spezifiziert durch eine detaillierte Betrachtung der spezifischen europäischen Regelungen und Verfahren, die Analyse von relevanten Mischungstypen und von stoffgruppenspezifischen experimentellen Evidenzen sowie die erneute Prüfung von ausgewählten verfügbaren Bewertungsentscheidungen. Das erzeugte

Wissen wurde anschließend genutzt, um spezifische Verfahrensweisen vorzuschlagen und ein Werkzeug für die systematische und konsistente Einbeziehung von Mischungsbewertungen für verschiedene Biozid- und PSM-bezogene Bewertungssituationen zu entwickeln. Die entwickelten Vorschläge sind hinreichend detailliert entwickelt, um Situationen von variierender Datenverfügbarkeit zu berücksichtigen und erlauben somit evidenzbasierte Mischungsrisikoentscheidungen.

1.1 Stand des Wissens in der ökotoxikologischen Mischungsbewertung

In verschiedensten wissenschaftlichen Monitoringstudien ist nachgewiesen worden, dass Organismen in ihrer Umwelt eher gegenüber Mischungen von Chemikalien denn gegenüber Einzelsubstanzen exponiert sind. Weiterhin zeigen öko-epidemiologische Untersuchungen, dass Mischungsexpositionen Kombinationseffekte hervorrufen können und das die Ignoranz selbiger zu einer Unterschätzung von schädlichen Effekten führen kann. Weiterhin ist es mittlerweile eine weitverbreitete Ansicht, dass eine umfassende Berücksichtigung von Mischungstoxizitäten durch unmittelbare Beobachtung nur für wenige ausgewählte Fälle erfolgen kann, da das Auftreten von Mischungen in der Umwelt zu variabel und divergent ist, um umfassend experimentell untersucht werden zu können. Daher haben Modelle, die eine Kalkulation erwartbarer Kombinationseffekte auf der Basis des Wissens über die biologischen Aktivitäten der Mischungskomponenten erlauben, selbst in Experimentalstudien zur Mischungstoxizität eine etablierte Stellung für die Bewertung erlangt.

Eine mögliche Basis für die Bewertung und damit regulatorisch nutzbare Vorhersage von Mischungstoxizitäten für Organismen in der Umwelt besteht mit den Modellen Konzentrationsadditivität (CA – Concentration addition) und unabhängige Wirkung (IA – Independent action). Diese Modelle erlauben die Berechnung der erwartbaren Kombinationseffekte basierend auf Konzentrations-Effektinformationen sowie der Kenntnis der Konzentrationen für die Komponenten der betrachteten Mischung. Da diese Modelle keine weiteren umweltsystemabhängigen Informationen benötigen sondern ausschließlich auf stofflich-biologische Interaktionseigenschaften basieren, können sie als generische Konzepte angesehen werden. Um die Anwendbarkeit der Konzepte für komponentenbasierte Extrapolationsansätze innerhalb der Umweltrisikobewertungsverfahren zu diskutieren, wurden die Prämissen, der Geltungsbereich und Unsicherheiten mit Hilfe einer systematischen Literatursichtung erarbeitet. Die Literatursichtung erschloss etwa 800 Referenzen zum Thema, wobei der Fokus für die Evidenzanalyse auf Übersichtsarbeiten zu experimentellen Studien (reviews) und zusammenfassenden Berichten gelegt wurde. Nur für spezielle Aspekte wurden ausgewählte experimentelle Originalarbeiten und diverse andere Berichtsarten zusätzlich berücksichtigt. Während das Interesse in der Evidenzlage zu Mischungstoxizitäten für Pflanzenschutzmittel (in der überwiegend englischsprachigen Literatur mit dem Begriff ‚pesticides' erschlossen) und Biozide im Hinblick auf ökotoxikologische Auswirkungen bestand, wurden daneben eine Vielzahl von Endpunkten, Modellierungsansätzen und generischen Studien betrachtet, z.B. um die Validität der Vorhersagen der Extrapolationstechniken einzuschätzen. Die Hauptergebnisse dieser Untersuchungen zur Anwendbarkeit von Referenzmodellen für die Bewertung und Vorhersage von Kombinationseffekten mit Hilfe eines komponentenbasierten Ansatzes können wie folgt zusammengefasst werden:

- Nicht-Interaktion zwischen den Komponenten der betrachteten Mischung ist eine weithin akzeptierte Grundannahme der Referenzmodelle für die Vorhersage von erwartbaren Kombinationseffekten, wohingegen interaktive Effekte als nicht in systematischer Weise vorhersagbar angesehen werden;

- Konzentrationsadditivität (CA) und Unabhängige Wirkung (IA) werden als wesentliche Referenzmodelle für die komponentenbasierte Kombinationseffekt-prognose beachtet. Sie haben weitverbreitete Anwendung erlangt und beide Referenzmodelle werden als tauglich für unterschiedliche Situationen befunden. Weiterhin beinhalten die Modelle jeweils spezifische zusätzliche Bedingungen für ihre Nutzung;
- Generalisierungen zu den quantitativen Unterschieden zwischen vorhergesagten und beobachteten Mischungstoxizitäten sind für verschiedene Bereiche verfügbar (z.B. aquatische und terrestrische Bioteste, Bioteste mit Vertebraten, Invertebraten oder Pflanzen). Die Mischungsmodelle zeigen dabei eine akzeptable Vorhersagegüte sowohl für verschiedene ökotoxikologische Effektendpunkte als auch für unterschiedliche Mischungstypen einschließlich Mischungen mit Transformationsprodukten. Jedoch ist nicht abschließend geklärt, inwiefern die Vorhersagegüte auch für Mischungen mit Metallen ausreichend ist und für bestimmte Stoffgemische, wie z.B. Stoffmischungen in Biozidprodukten, ist die empirische Evidenz zu Kombinationseffekten noch sehr begrenzt;
- Als Voraussetzungen für die Nutzung der Vorhersagekonzepte gilt, dass die Daten für die Einzelstoffe für vergleichbare Expositionsbedingungen, sowie gleiche biologische Effekte und Spezies vorliegen müssen. Die Wirkungsweise der Mischungskomponenten wird als ausschlaggebender Faktor für die Richtigkeit der Vorhersage betrachtet, obgleich für Gemische mit nur wenigen Komponenten beide Konzepte dazu tendieren quantitativ ähnliche Vorhersagen zu liefern;
- In den meisten Fällen ist die nach CA im Vergleich zu der nach IA vorhergesagten Mischungstoxizität höher, weshalb CA auch als realistisch ungünstigster anzunehmender Fall für nicht interaktive Kombinationseffekte betrachtet werden kann. Die Determinanten für die Differenz zwischen den konzeptabhängigen Vorhersagen sind bekannt und können für spezifische Situationen simuliert und quantifiziert werden;
- Ein begrenztes Wissen liegt hinsichtlich der Kombinationseffekte von Mischungen aus aktiven Wirkstoffen in Bioziden und PSMs mit Formulierungsbeistoffen vor. Das gleiche gilt für Evidenzen zu Kombinationseffekten aus Studien höherer Bewertungsstufen (‚higher tier'; d.h. Feldstudien wie z.B. aquatische Mikro- und Mesokosmen oder Regenwurm Freilandstudien). Indikatoren für Synergismen (d.h. Kombinationseffekte die größer als erwartet ausfallen) fehlen bislang;
- Während Mischungseffekte auch für niedrige Effektkonzentrationen der Mischungskomponenten beobachtet werden, ist die Art und Weise ihrer Bewertung umstritten;
- Die Fragestellung ob Kombinationseffekte für eine gegebene Mischungsrisikobewertung relevant oder wahrscheinlich sind, kann jeweils nur explizit durch entsprechende theoretische oder experimentelle Betrachtungen im Bewertungsprozess geklärt werden.

Aus der primären Literaturanalyse innerhalb des Projektes wurde die Notwendigkeit für zusätzliche vertiefende Betrachtungen abgeleitet, um eine detaillierte Bestimmung folgender Aspekte zu erzielen:

- Die Evidenzen zur Tauglichkeit der Referenzkonzepte CA und IA für Biozidmischungen. Hierfür wurde eine Reanalyse von in diesem Bereich publizierten Mischungsstudien vorgenommen. Hieraus wurde gefolgert, dass weitergehende

systematische Untersuchungen erforderlich sind, da derzeit inkonsistente Berichte zu möglichen synergistischen Interaktionen in diesem Feld vorliegen;

- Evidenzen zu Kombinationseffekten von Wirkstoffen und Formulierungshilfstoffen. Hierfür wurden für 15 PSM deren Zulassungsdaten reanalysiert und Mischungskalkulationen durchgeführt und zusätzlich verfügbare Literatur evaluiert;
- Evidenz zur Existenz von Indikatoren für synergistische Effekte und Kombinationswirkungen bei niedrigen Stoffkonzentrationen. Hierzu wurde eine Literaturanalyse durchgeführt, die die erreichten Fortschritte bei der Nutzung von toxikogenomischen Methoden für die Detektion und Bewertung von Kombinationseffekten untersuchte.

1.2 Optionen für die regulatorische ökotoxikologische Mischungsbewertung

Für die Frage, wie die gemeinsame Toxizität von Inhaltsstoffen von Biozid- oder Pflanzenschutzmitteln (PPP) für Nicht-Zielorganismen in der Umwelt innerhalb der Umweltrisikoabschätzung berücksichtigt werden kann, ergeben sich drei grundsätzliche, zu unterscheidende Optionen:

(i) Die Betrachtung der gesamten Mischung (whole mixture approach – WMA), d.h. die direkte experimentelle Testung der betrachteten Mischung und damit Behandlung dieser wie eine Einzelsubstanz;

(ii) Ein komponentenbasierter Ansatz (component-based approach – CBA), d.h. die Kalkulation der erwartbaren Mischungstoxizität aus den Toxizitätsdaten für die Mischungskomponenten durch Anwendung von Modellen, insbesondere solchen die auf der Basis der Referenzmodelle Konzentrationsadditivität (CA), Unabhängiger Wirkung (IA) oder sogenannten gemischten Modellen (mixed models – MM) arbeiten; und

(iii) Ein Ansatz der zusätzliche Bewertungsfaktoren vorsieht, d.h. die Bewertungsunsicherheit durch Mischungseffekte durch spezifische Sicherheitsfaktoren auffangen will, ähnlich denen die für den Ausgleich von Datenlücken oder Empfindlichkeitsunterschieden in der Chemikalien-risikobewertung eingesetzt werden.

Die Betrachtung der gesamten Mischung (WMA) ist das derzeit einzig verlässliche Vorgehen um Kombinationseffekte zu berücksichtigen, die durch synergistische oder antagonistische Interaktionen hervorgerufen werden und daher nicht durch CA oder IA vorhergesagt werden können. Dieser Ansatz ist allerdings stark limitiert durch technische, ökonomische und ethische Einschränkungen. Komponentenbasierte Ansätze werden im Wesentlichen durch eine beschränkte Verfügbarkeit des Wissens über alle relevanten Mischungskomponenten und insbesondere fehlende Daten zu den Einzelstofftoxizitäten limitiert. Darüber hinaus kann die Auswahl zwischen den verschiedenen Modellen durch nicht erfüllbare Datenanforderungen oder unzureichendes Wissen zu den Wirkweisen (MoA) eingeschränkt sein, die für die beobachtbare Toxizität in den Nichtzielorganismen ursächlich sind. Bewertungsfaktoren können genutzt werden, um (i) für unbekannte Mischungskomponenten mit unbekannten Toxizitäten vorzuhalten oder (ii) zur Absicherung gegen mögliche synergistische Interaktionen, allerdings sind sie wissenschaftlich als quantitative Größen schwer abzuleiten.

Die Überlegungen zu den Anforderungen für die angesprochenen grundlegenden Optionen wie z.B. Datenverfügbarkeit zeigen, dass zusätzliche Setzungen erforderlich sind, um kohärente Herangehensweisen innerhalb eines Regelungsbereiches oder gar harmonisierte

Vorgehensweisen über verschiedene Regulationsfelder hinweg zu schaffen. Diese können weder aus Daten noch aus wissenschaftlichen Überlegungen alleine direkt hergeleitet werden. Sie erfordern vielmehr zusätzliche Definitionen zu den Rahmenbedingungen hinsichtlich des Levels an Akzeptabilität von pragmatischen Setzungen, akzeptablen Risikoergebnissen und der tolerablen Unsicherheit. Einige allgemeine Prinzipien zur Ableitung eines transparenten und kohärenten Rahmens zur Mischungsbewertung im regulatorischen Rahmen wurden in diesem Sinne formuliert.

Die vorgeschlagenen Prinzipien enthalten folgende Grundannahmen:

- Die Anforderungen an die Sicherheitsabschätzungen für Mischungen sollen weder höher noch geringer ausfallen als es für die Bewertung von Einzelstoffen üblicherweise etabliert ist;
- Aus sowohl ethischen wie ökonomischen Gesichtspunkten soll die Bewertung der Umweltrisiken durch Mischungseffekte ohne zusätzliche experimentelle Anforderungen zur Testung ganzer Gemische auskommen;
- Für den Fall, dass alle relevanten Mischungskomponenten als bekannt angenommen und in die Kalkulation aufgenommen werden, wird CA als grundsätzliche ‚default'-Annahme für die Ökotoxizität von Mischungen von Inhaltsstoffen in PPP und Biozidprodukten akzeptiert;
- Das wissenschaftliche Verständnis des CA-Konzeptes verlangt als Eingabedaten Effektkonzentrationen (oder -dosen) die sich auf denselben biologischen Endpunkt in derselben Art unter identischen Testbedingungen beziehen. Für die regulatorische Nutzung sind jedoch unter obigen Setzungen pragmatische Vereinfachungen und zusätzliche Annahmen unvermeidbar. Das kann sich auf die Zusammenstellung von Datensätzen beziehen, die für verschiedene Testbedingungen, Endpunkte und Spezies vorliegen aber auch die Nutzung von NOEC-Werten als Surrogate für quantitative Schätzungen von geringen Effektkonzentrationen erfordern. In solchen Fällen sollte eine Betrachtung des potentiellen Fehlers, die durch derartige Abweichungen vom ursprünglichen Konzept eingeführt werden, erfolgen und transparent gemacht werden. Wo möglich können diese dann schrittweise beseitigt werden;
- IA und gemischte Modelle (MM) erfordern im Vergleich zu CA erheblich mehr Daten und beinhalten ein größeres Risiko die tatsächliche Mischungstoxizität zu unterschätzen. Daher sollten die Nutzung von IA und MM auf Fälle beschränkt werden, in denen Wissen zur Wirkweise und den Konzentrations-Effekt-Beziehungen der Mischungskomponenten eine Nutzung dieser Modelle unterstützt;
- In Abhängigkeit von der spezifischen Bewertungssituation, können komponenten-basierte Mischungstoxizitätsschätzungen durch einen spezifischen Bewertungsfaktor ergänzt werden, der für potentiell synergistische Interaktionen vorhält.

In Fällen in denen CBA-basierte Bewertungen auf ein inakzeptables Risiko hinweisen, kann die experimentelle Testung der Mischung als letzte Option zur Klärung betrachtet werden.

1.3 Relevanz einer Mischungstoxizitätsbewertung in der Biozid- und PSM-Produktzulassung

Vor einer näheren Detaillierung der spezifischen Verfahren der Mischungsberücksichtigung im Verfahren der Risikobewertung für Biozide und Pflanzenschutzmittel wurden folgende Fragen untersucht:

(i) Welche Mischungstypen müssten in zukünftigen, um Kombinationseffekt-betrachtungen erweiterten Risikobewertungen Berücksichtigung finden;

(ii) Inwiefern trägt der gegenwärtige EU-Rechtsrahmen der Berücksichtigung von Mischungsbewertungen Rechnung;

(iii) Würden Mischungsbewertungen mit den verfügbaren Referenzmodellen plausibel sein und wären sie auf der Basis von typischerweise in Risikobewertungen verfügbaren Informationen durchführbar?

(iv) Würde die Berücksichtigung von Mischungstoxizitäten absehbar einen großen Einfluss auf die Zulassung von Pflanzenschutzmitteln (PSM) nehmen?

Biozidprodukte

Bei Bioziden sind zumindest drei grundsätzlich verschiedene Mischungstypen zu unterscheiden:

a) Biozidprodukte, die aus einer Mischung verschiedener Wirkstoffe bestehen;

b) Biozidprodukte, die eine Mischung darstellen aus einem (oder mehreren) Wirkstoff(en), gemischt mit weiteren biologisch aktiven Substanzen oder Stoffen, die die biologische Aktivität der Wirkstoffe durch Wechselwirkung mit deren Aufnahme oder Biotransformation verändern;

c) Biozidprodukte, die mit anderen Biozidprodukten oder Stoffen anthropogenen Ursprungs oder Stoffen natürlichen Ursprungs gemeinsam in Oberflächenwasser, Grundwasser, Boden oder Sediment auftreten.

Mischungen des letzten Typus wurden hier nicht berücksichtigt, da sie außerhalb des gegenwärtigen Fokus der Biozidrichtlinie liegen und potentiell eher Gegenstand der Wasserrahmenrichtlinie oder ähnlicher medienorientierter Regelungen wären.

Da Daten und Dokumentationen zur Zulassung von Biozidprodukten (Mischungen aus entweder verschiedenen Wirkstoffen oder Mischungen von Wirkstoffen mit anderen biologisch wirksamen Stoffen) aus erster Hand nicht für eine Sichtung und Analyse der zugrundeliegenden empirischen Evidenz verfügbar waren, wurde in diesem Projekt ein generisches Verfahren für die Biozidbewertung entwickelt. Dieses beinhaltete:

- Einen kurzen Überblick zu existierenden und absehbaren regulatorischen Anforderungen an die Mischungsbewertung von Bioziden, und
- Eine kritische Sichtung der publizierten Mischungsstudien zu Biozide. Der wissenschaftliche Stand des Wissens wurde zusammengestellt und kritisch gewürdigt wobei der Fokus auf Holzschutz- und Antifoulingmitteln lag, d.h. zwei Produktgruppen mit potentiell hohem Umwelteinfluss.

Regulatorische Anforderungen

Mischungen und Substanzen finden sich neu definiert in der EU Verordnung Nr. 528/2012[2] in Übereinstimmung mit Artikel 3 der Regulierung (EC) No 1907/2006 (REACH). Artikel 3(1)a aus EU No 528/2012 erkennt weiterhin die Tatsache an, dass Biozidprodukte Mischungen von Wirkstoffen enthalten können. Und in Annex VI, "Common principles for the evaluation of dossiers for biocidal products", in den Punkt 53 und 54 heißt es schließlich *"In each of the areas where risk assessments have been carried out, the evaluating body shall combine the*

[2] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products.

results for the active substance together with the results for any substance of concern to produce an overall assessment for the biocidal product itself. This shall also take account of any cumulative or synergistic effects. For biocidal product containing more than one active substance, any adverse effects shall also be considered together to produce an overall assessment for the biocidal product itself."

Ein 'bedenklicher Stoff' ('substance of concern') ist in diesem Zusammenhang definiert als "*any substance, other than the active substance, which has an inherent capacity to cause an adverse effect, immediately or in the more distant future, on humans, in particular vulnerable groups, animals or the environment and is present or is produced in a biocidal product in sufficient concentration to present risks of such an effect*" (Art 3(1)f of Regulation (EU) No 528/2012).

Artikel 8.3 (Bewertung von Anträgen, "Evaluation of applications") der EU-Verordnung Nr 528/2012 liest sich wie folgt: *"Where the evaluating competent authority considers that there are concerns for human health, animal health or the environment as a result of the cumulative effects from the use of biocidal products containing the same or different active substances, it shall document its concerns [...] and include this as part of its conclusions."* Die neue Verordnung des Europäischem Parlaments und des Ministerates trat am 27. Juni 2012 inkraft. Sie ersetzt die gegenwärtig gültige Biozid-Direktive (98/8/EC) und wird zum 1. September 2013 der derzeitige Biozidrichtlinie (98/8/EG) ablösen.

Die aktuellen regulatorischen Entwicklungen auf dem Gebiet der Biozide folgen mithin den früheren auf dem Gebiet der PSM Risikobewertung oder weisen sogar darüber hinaus. Mithin werden für die Bewertung von Mischungseffekten bei Bioziden pragmatisch umsetzbare, wissenschaftlich solide und hinreichend protektive Vorgehensweisen benötigt.

Evidenz zu Synergismen mit Biozidmischungen

Eine Literatursuche mit Hilfe von Scopus identifizierte 961 ‚peer-reviewed' Studien zur gemeinsamen Toxizität von Bioziden im Allgemeinen und zur Mischungsökotoxikologie von Antifouling-Substanzen (11 Biozide) und Holzschutzmitteln (25 Biozide) im Besonderen. 89 dieser Publikationen wurden für eine detaillierte Analyse ausgewählt. Die am häufigsten analysierten Mischungen enthielten Kupfer, Tributylzinn und/oder Irgarol. Diese lagen üblicherweise als binäre Gemische mit anderen Bioziden oder mit anderen üblichen Umweltkontaminanten wie PAKs, PCBs oder Schwermetallen vor. Die Ökotoxikologie der individuellen wie auch der gemischten Biozide wurde mit Hilfe der Standardbatterie an vorrangig limnischen Biotesten (Algenwachtumsinhibition, Bakterienrespirations- oder -wachstumstest, Kurzzeit-Fischtoxizität, Kurz- und Langzeittoxizität gegenüber Daphnien etc.) untersucht. Studien zu Mehrkomponentengemischen sind demgegenüber stark unterrepräsentiert. Weiterhin besteht ein deutlicher Mangel hinsichtlich der Effekte von Biozidmischungen auf terrestrische Systeme, wie sie für Mischungen von Bioziden der Produktgruppe 21 (Holzschutzmittel) relevant wären. Ähnliches gilt für Auswirkungen auf die marine Umwelt und die betreffenden Biozidmischungen innerhalb der Produktart 21 (Antifouling).

Daten für 382 verschiedene Mischungen wurden in größerer Detaillierung reanalysiert (davon 336 Zweikomponentengemische, 32 Dreikomponentengemische, 7 Vierkomponenten-gemische, 1 Sechskomponentengemisch und sechs 12-Komponentengemische), die je mindestens ein Biozid beinhalten. Die wesentlichen Befunde dieser Übung können wie folgt zusammengefasst werden:

- Konzentrationsadditivität ist das am häufigsten genutzte Referenzmodell zur Bewertung experimenteller Daten zur gemeinsamen Toxizität von Bioziden, gefolgt von Unabhängiger Wirkung (Response Addition) und Effektsummation;
- Das empirische Wissen zur Ökotoxizität von Biozidmischungen ist immer noch limiert. Nur 50% der Biozidwirkstoffkandidaten, die sich gegenwärtig im Prozess des Reviewprogrammes der EU befinden, waren bislang auch Gegenstand von Mischungsuntersuchungen. Insbesondere wurde nach unseren Funden bislang nur eine Studie durchgeführt, die realistische Biozidgemische (im Sinne der verwendeten Mischungskomponenten, -zusammensetzung, und Konzentrationen) untersucht hat;
- Verschiedene Fälle von starken synergistischen oder antagonistischen Mischungstoxizitäten finden sich in der Literatur beschrieben. Jedoch können diese auf nur einige wenige Publikationen zurückgeführt werden. Nach Ausschluss dieser Mischungen mit starken Abweichungen von nicht-interaktiven Vorhersagen liegt das Verhältnis zwischen vorhergesagten und beobachteten Mischungs-EC50-Werten für die 89 untersuchten binären Gemische bei 1,02; und für die Dreistoffmischungen bei 1,12. Die Daten indizieren daher, dass CA unter den oben getroffenen Einschränkungen die Kombinationseffekten für Gemische aus Antifoulingsubstanzen genauso gut vorherzusagen vermag wie dies für die nachstehend erörterten Pestizidgemische angenommen wird;
- Mit einigen wenigen Ausnahmen wiesen die Komponenten der untersuchten Gemische unähnliche oder unbekannte Wirkweisen oder Mechanismen in den betrachteten Organismen auf und entstammten verschiedenen chemischen Gruppen;
- Die Dokumentation der Mischungsstudien in den analysierten Publikationen variiert dramatisch. In der Mehrheit der Fälle ist eine konsistente, quantitative Bewertung sowie ein Vergleich der Ergebnisse verschiedener Publikationen erschwert durch Limitierungen entweder im Studiendesign, der Dokumentation oder durch die Nutzung unterschiedlicher Verfahren der Bestimmung der Abweichungen zwischen vorhergesagten und beobachteten Mischungseffekten. Dies betrifft zum Beispiel Situationen in denen die Ergebnisse ausschließlich graphisch präsentiert werden (z.B. als Isobolen) oder in denen die Toxizitätsdaten zu den Mischungskomponenten einzeln oder zum Gemisch nicht angegeben werden. Insbesondere Studien die Unabhängige Wirkung nutzen, beurteilen die Vorhersagegüte dieses Modells vorzugsweise mit der beobachteten Mischungstoxizität an einer a priori festgelegten Konzentration. Dieses Vorgehen macht den studienübergreifenden Vergleich unmöglich. Verbesserungen im Studiendesign und der Datendokumentation sind daher dringend geboten, um klarere Schlussfolgerungen für die Risikobewertung vornehmen zu können.

Pflanzenschutzmittel (PSM)

Pflanzenschutzmittel (PSM) liegen in verschiedenen Mischungstypen vor, die allesamt von der Anwendung von PSM in der gleichen Kultur herrühren können, nämlich:

a) Alle Pflanzenschutzmittel als Produkte, da sie in aller Regel aus einem (oder mehreren) Wirkstoff(en) zusammen mit weiteren Formulierungshilfsstoffen bestehen, die selber biologisch aktiv sein können oder die Aufnahme oder Biotransformation der aktiven Substanz modifizieren;

b) PSM, die Mischungen von Wirkstoffen enthalten, sogenannte Kombinationsprodukte;

c) PSM, die vor ihrer Anwendung gemischt werden (Tankmischungen);

d) PSM, die in serieller Anwendung eingesetzt werden (Spritzkalender).

In dieser Studie werden Mischungen von Wirkstoffen die zusammen eingesetzt werden, also Kombinationsprodukte (d.h. Produkte, die mehr als einen Wirkstoff enthalten), und Tankmischungen betrachtet. Weiterhin wurden auch Monoformulierungen (also Produkte die aus einem Wirkstoff und den begleitenden Formulierungshilfstoffen bestehen) betrachtet, um den Einfluss von Formulierungshilfsstoffen auf die Ökotoxizität der Wirkstoffe zu beurteilen. Die Expositionsmodellierung war außerhalb der Betrachtungen dieser Untersuchung, weshalb über grundsätzliche Überlegungen hinausgehendes zu seriellen Applikationen ausschlossen wurde. Gleichfalls wurden Optionen für eine detaillierte Expositionsabschätzung wie sie im Risikobewertungsprozess – für den Fall, dass andernfalls ein Risiko nicht ausgeschlossen werden kann – vorgesehen sind, wurden hier ebenfalls nicht bei den evaluierten PSM Mischungen betrachtet. Dieses hätte zusätzliche Expositionskalkulationen erforderlich gemacht, ohne etwas an der Metrik der Kombinationseffektbetrachtungen zu ändern.

Ein Schlüsselaspekt im Hinblick auf die Betrachtung der Mischungstoxizität in der Risikobewertung ist, dass es nach derzeitigem Vorgehen erforderlich ist zu definieren auf welche Mischungszusammensetzung sich eine bestimmte Risikobewertung bezieht. Gegenwärtig sind zwei Mischungen für ein Produkt (nämlich die Mischung der Komponenten wie sie im angewendeten Produkt vorliegt oder die Mischung wie sie in der Umwelt erwartet wird) geläufig und werden in verschiedener Weise in den unterschiedlichen Risikobewertungsfeldern (Vögel und Säugetiere, aquatische Organismen und terrestrische Organismen) und Verfeinerungsschritten genutzt. Um die Analyse strukturieren zu helfen und das Verständnis für die Implementierung der Mischungstoxizität im gegenwärtigen Risikobewertungsverfahren zu fördern, wird hier eine entsprechende Matrix ausgewiesen.

Regulatorische Anforderungen

Die Analyse des rechtlichen Hintergrundes umfasst die verschiedenen aktuellen Europäischen und nationalen Verordnungen und Richtlinien sowie eine Reihe von Durchführungs- und Positionspapieren. Zusammengefasst wird nach der EU Verordnung EC No 1107/2009 zur Vermarktung von PSM, die die vormalige Richtlinie 91/141/EEC ersetzt, im Hinblick auf die menschliche Gesundheit explizit gefordert "[to take] *into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available*". Während sich im Hinblick auf die Umweltrisikobewertung weder in der Verordnung noch in der vorhergehenden Richtlinie keine derartig explizite Anforderung findet, wird in beiden Dokumenten verlangt, dass PSM "*in the light of current scientific and technical knowledge*" bewertet werden und dass "*Member States shall ensure that use of plant protection products does not have any long-term repercussions for the abundance and diversity of non-target species*". Nach einem Positionspapier des EFSA Panel on Plant Protection Products and their Residues (PPR 2010) wird darüber hinaus sogar die Anforderung für die Risikobewertung auf eine weitergehende Berücksichtigung von "[...] *multiple stress by the use of multiple plant protection products, being applied at the same time (e.g. tank mixtures) or in sequence* [...]" gestellt. Weiterhin fordert Artikel 29 der Verordnung EC No 1107/2009 im Hinblick auf die Zulassung von PSM das "*Following these principles* [Uniform principles for evaluation and authorisation of a PPP], *interaction between the active substance, safeners, synergists and co-formulants shall be taken into account in the evaluation of plant protection products*." Es lässt sich also gut begründen, dass analog zum Zulassungsprozess bei Bioziden sowohl die biologische als auch die regulatorische Relevanz von Kombinationswirkungen bei PSM erkannt ist und den gegenwärtigen Stand des Wissens

abbilden. Mithin sollten sie im Bewertungsprozess Berücksichtigung finden, jedoch fehlen für die technische Durchführung noch entsprechende Verfahrensentwicklungen und Dokumentationen, die dies adäquat umsetzt.

Eine erarbeitete Übersicht zu gegenwärtig angewandten oder vorgeschlagenen Vorgehensweisen zur Berücksichtigung von Mischungstoxizitäten innerhalb von Umweltrisikobewertungen zeigt, dass diverse Behörden in Mitgliedstaaten sehr aktiv auf diesem Gebiet sind. Jedoch erweisen sich die vorliegenden Dokumente oftmals als uneindeutig, unvollständig und über die Mitgliedsstaaten hinweg gesehen als inkonsistent.

Mischungsbewertung auf der Basis von Daten die typischerweise in der gegenwärtigen Produktzulassung verfügbar sind

Zunächst wurde eine Analyse zur gegenwärtigen Implementierung in Deutschland durchgeführt. Hierfür wurden die Risikobewertungsberichte für 15 Kombinationsprodukte, die durch das Umweltbundesamt (UBA) ausgewählt wurden, betrachtet. Diese Analyse zielte darauf einen Überblick zu schaffen, welche Daten in den verschiedenen Risikoprüfbereichen typischerweise verfügbar wären, um eine Mischungsbewertung durchzuführen. Weiterhin zielte die Analyse darauf die Aspekte zu identifizieren, die innerhalb der gegenwärtigen Risikobewertungsschemata zu potentiellen Komplikationen für die Mischungsbetrachtungen führen können, um Hilfestellung für spezifische Implementierungen entwickeln zu können. Durch die Anwendung bestehender oder neu entwickelter Vorgehensweisen zur Berücksichtigung der Mischungstoxizität bei ausgewählten Kombinationsprodukten wurden im letzten Schritt die Konsequenzen für eine zukünftige Zulassung von PSM beleuchtet.

Im Ergebnis konnte gezeigt werden, dass die verfügbare ökotoxikologische Datenlage für die einzelnen Wirkstoffe in PSM überwiegend hinreichend homogen und komplett für die rechtlich geforderten Standardtestendpunkte wie zum Beispiel akute Toxizitätsuntersuchungen für Vögel und Säugetiere, akute Toxizität für Daphnien und Fische und Algenwachstumsinhibition vorliegen, um eine Berücksichtigung der Mischungstoxizität mit dem Model der Konzentrationsadditivität (CA) vorzunehmen. Für Daten zu chronischen Effekten sind üblicherweise lediglich ‚no observed effect concentrations/level' (NOEC/NOEL) verfügbar, die den strikten Anforderungen für die CA-Modellnutzung damit nicht genügen. Weiterhin werden in der Risikobewertung bei PSM-Wirkstoffen vielfach Studien zu chronischen oder ‚higher tier' Studien (höherstufige Prüfungen, d.h. Feldstudien wie z.B. aquatische Mikro- und Mesokosmen und Regenwurm-Freilandstudien) genutzt, die dann oftmals nur für einzelne Wirkstoffe eines Kombinationsproduktes durchgeführt wurden. Dieses schränkt die Anwendung der Mischungsbetrachtungen ein, solange nicht Daten aus unterschiedlichen Ebenen hinsichtlich biologischer Organisation und Komplexität zusammengeführt werden, was implizit auch durch die gegenwärtige Nichtberücksichtigung bereits Praxis ist. Als Konsequenz müssten in derartigen Fällen so deutlich unterschiedliche Endpunkte wie beispielsweise die NOEAEC (no observed ecological adverse effect concentration) aus einer Mesokosmosstudie und der EC50 eines Algenwachstumsinhibitionstestes zusammengeführt werden, um eine Mischungstoxizitätsabschätzung vorzunehmen, eine Herangehensweise die im Vergleich zu den wissenschaftlichen Voraussetzungen der Referenzmodelle fragwürdig ist. Die Verfeinerungsoptionen in der Risikobewertung hinsichtlich der Expositionsabschätzungen führen zu einer weiteren Erschwernis in der Mischungstoxizitätsbetrachtung. Eine Implementierung der Mischungsbetrachtungen basierend auf den bislang üblicherweise verwendeten Herangehensweisen (und für die begrenzte Anzahl an bisher betrachteten Beispielen) führte in den betrachteten Fällen für den Regelungsbereich Vögel und Säugetiere

nicht zu einer häufigeren Notwendigkeit für eine Verfeinerung der Risikobewertung als dies durch die individuellen Wirkstoffe bereits indiziert war. Diese Aussage gilt für die 15 betrachteten Produkte auf der Ebene der ‚first tier' Bewertung. Im Falle der aquatischen Organismen waren striktere Anwendungsbeschränkungen in mehreren Fällen angezeigt (d.h. erhöhte Abstandsauflagen bei Anwendung in der Nähe von Gewässern oder höhere Anforderungen an die Nutzung von driftreduzierenden Düsen), wobei die Anzahl von der Art der verwendeten Implementierung abhing. Für terrestrische Organismen ist die Betrachtung der Mischungstoxizität bereits in vielen Fällen dadurch implementiert, dass im gegenwärtigen Risikobewertungsschema die Toxizität des Produktes Eingang findet (‚whole mixture approach'). Allerdings, können bei Fehlen von Produktdaten Probleme erwartet werden, da die Zulassungsentscheidung in Fällen der Nutzung von ‚higher tier' Studien wiederum ausschließlich auf Einzelstoffuntersuchungen beruht. Andere Herangehensweisen können noch entwickelt werden, die sich beispielsweise auf die Identifizierung der für die Risikobewertung relevanten Mischungskomponenten stützen. Als grundlegende Schwierigkeit ist jedoch zu berücksichtigen, dass oftmals Untersuchungen für Wirkstoffe (a.s.) in technischer Reinheit auf dem Gebiet der terrestrischen Gefährungsbewertung fehlen.

Der mögliche Einfluss von Hilfs- und Beistoffen der Produktformulierung auf die Toxizität von PSM wurde für einen Satz von 15 Monoformulierungen (wiederum durch das UBA ausgewählt und als Dokumentation zur Verfügung gestellt) sowie eine Literaturübersicht untersucht. Durch Vergleich der Toxizitätsangaben für Wirkstoffe in technischer Reinheit mit denen für das formulierte Produkt zielte die Bewertung darauf die Hypothese zu prüfen, dass die Häufigkeit einer erhöhten Toxizität in Abhängigkeit vom Formulierungstyp steht. Die Befunde für die ausgewählten Monoformulierungen zusammen mit einer bereits vorliegenden Analysen von mehr als 100 Kombinationspräparaten (Coors und Frische 2011) zeigte, dass die Toxizität von Formulierungsadditiven vor allem bei den Organismen zum tragen kommt die nicht besonders sensitiv auf den a.s. reagieren. Demgegenüber ist kaum eine gesteigerte Toxizität bei den Nichtzielorganismen zu finden, die besonders sensitiv auf den Wirkstoff ansprechen. Allerdings gab es Ausnahmen zu dieser einfachen Daumenregel. Weiterhin zeigte die Datenauswertung, dass von den häufigsten Formulierungstypen *per se* keine als problematisch auffiel. Daher scheint eine fallspezifische Betrachtung sinnvoll. Basierend auf diesen Betrachtungen sowie der Literaturanalyse, konnte die Toxizität von Vertretern aus speziellen Gruppen von Formulierungshilfsstoffen wie ‚Safeners', Lösungsmitteln, Tensiden und weiteren als Ursache für eine verstärkte Toxizität des formulierten Produktes identifiziert werden. Im Prinzip ließen sich diese durch Einschluss ökotoxikologisch relevanter Zusatzstoffe in die Mischungstoxizitätsbewertung für das formulierte Produkt abbilden. Ist jedoch ein Einfluss von Formulierungshilfsstoffen (wie beispielsweise bei Emulgatoren) auf die Bioverfügbarkeit oder den Verbleib des Wirkstoffes gegeben, wäre dies nicht mit den hier betrachteten komponentenbasierten Ansätzen abgebildet, da die Referenzmodelle nicht geeignet sind toxikodynamische oder toxikokinetische Interaktionen der genannten Art abzubilden.

Eine ähnliche Evaluation wurde für drei verschiedene Tankmischungen durchgeführt, die unter den heute in der landwirtschaftlichen Praxis üblichen als häufig angewendete identifiziert wurden. Wiederum erwiesen sich Mischungsbetrachtungen nach CA im Allgemeinen für Standardendpunkte als durchführbar, wohingegen sie für Langzeit- oder ‚higher tier' Untersuchungen nur eingeschränkt möglich waren. Der Umstand, dass in vielen Risikoprüfbereichen die Bewertung auf dem direkten Vergleich von einer (oder wenigen) untersuchten Applikationsrate(n) gegenüber Kontrollansätzen beruht, erschwert oder verhindert die Nutzung dieser Daten für die Bewertung von Tankmischungen. Die vorhandenen Daten enthalten wenige Informationen für andere Applikationsraten des Produktes und Produkte in Tankmischungen werden oftmals in ihren Aufwandsmengen

gegenüber der erlaubten maximalen Aufwandmenge reduziert eingesetzt. Um zu dem Schluss zu gelangen, das die Anwendung der drei betrachteten Tankmischungen unter Mischungstoxizitätsgesichtpunkten als sicher betrachtet werden konnte, musste angenommen werden, das sowohl die Auflagen zur Risikominderung für die einzelnen Produkte eingehalten werden, als auch dass die Aufwandmengen gegenüber den zulässigen Maximalaufwandmengen reduziert werden. In anderen Worten ohne diese zwei Zusatzannahmen kann durch die Analyse ein zusätzliches Risiko für die betrachteten Tankmischungen identifiziert werden. ;Higher tier' Untersuchungen waren nicht für alle Komponenten der Tankmischungen und die jeweiligen im Zulassungsprozess identifizierten kritischen Endpunkten verfügbar. Daher konnte ein Risiko für einige dieser Endpunkte wie Honigbienen, Nichtziel-Arthropoden und Regenwürmer nicht ausgeschlossen werden, und zwar unabhängig von den Annahmen zu Risikominderungsmaßnahmen.

1.4 Indikatoren für Synergismus und Niedrigdosismischungseffekte

Da synergistische Interaktionen durch die Komponenten einer Mischung auftreten können und diese mit keinem der beiden Referenzmodelle (CA, IA) vorhersagbar sind, ergibt sich die Frage ob es gegenwärtig verfügbare Indikatoren für derartige Interaktionen gibt oder diese in Aussicht stehen. Mit der Einführung von genomischen Techniken in die Toxikologie wurden große Erwartungen geweckt, dass diese neue Aufschlüsse im Hinblick auf die Mechanismen der Interaktionen bei vorliegenden Niedrigdosen ermöglichen. Nach nunmehr einer Dekade in der Experimentalstudien mit den neuen toxikogenomischen Verfahren durchgeführt wurden, konnten daher die vorliegenden Mischungsuntersuchungen zu diagnostischen, mechanistischen und extrapolativen Fragestellungen zusammengefasst und gesichtet werden. Von 2002 bis 2011 wurden 41 Studien veröffentlicht, die einen spezifischen Fokus auf Mischungsbewertungsfragen mit Hilfe von toxikogenomischen Techniken legten, insbesondere durch die Anwendung von multiplex Gentranskriptquantifizierungen, sowie metabolische und proteomische Analyse von Mischungsexpositionen. Unter den untersuchten Chemikalien finden sich auch verschiedene Wirkstoffe, die in Pflanzenschutzmitteln Anwendung finden. Methodische Standards in derartigen Untersuchungen sind die explizite Ausweisung von Kriterien für die zur Untersuchung ausgewählten Expositionskonzentrationen und Transparenz hinsichtlich der zur Minimierung der Fehlervarianz eingesetzten Datentransformations- und statistischen Auswerteverfahren. Im Gegensatz dazu finden sich bei der bioinformatischen Analyse von toxikogenomischen Daten viele unterschiedliche Methoden und insgesamt liegt hier ein Feld mit erheblichen Entwicklungen vor. Die gefundenen Kombinationswirkungsbewertungen aus den vorhandenen Studien werden im Hinblick auf die in der Toxikologie etablierten Dosis-Wirkungsbeziehungen und Mischungstoxizitätsmodelle diskutiert. Rezeptorbasierte bioanalytische Verfahren scheinen dabei am weitesten im Hinblick auf die Erfassung von quantitativen Beziehungen zwischen Exposition und biologischen Antworten entwickelt. Transkriptomische Antworten finden sich hingegen vielfach schlicht als Anwesenheit oder Abwesenheit von Signalen ausgewertet. Da bislang keine konsentierten Verfahren zur Analyse derartiger Effekte existieren, sind die vorliegenden Interpretationen bedauerlicherweise oftmals uneindeutig. Weiterhin wurde in der Mehrzahl der vorliegenden Studien ein Design verwendet, welches die Mischungsreaktionen ausschließlich gegen die Beobachtungen für die Einzelbehandlungen vergleichen lässt. D.h. es wurde auf das Wiederfinden von Einzelstoffsignalen unter Mischungsbelastung fokussiert. Diese Sichtweise steht im grundsätzlichen Gegensatz zum existierenden Verständnis zur Betrachtung von Kombinationswirkungen auf der Ebene von Stoff-Zielmolekül (target) Wechselwirkungen und von Kombinationseffekten auf höheren biologisch integralen Ebenen wie Wachstum und

Entwicklung. Zusammenfassend musste festgestellt werden, dass die bisherigen Befunde damit eher anekdotische Belege für die Existenz von Kombinationseffekten liefern und noch keine systematischen Aussagen oder gar Nutzungen für regulativ wünschenswerte, extrapolative Verfahren erlauben.

Schlussfolgerungen aufgrund des gegenwärtigen Wissenstandes

Als Schlussfolgerungen aus den Übersichten zu gegenwärtig vorliegenden theoretischen und empirischen Befunden und zu verschiedenen regulatorischen Rahmenbedingungen lässt sich festhalten, dass:

- Trotz einer limitierten Datenbasis an Mischungstoxizitätstudien und in Anerkennung von spezifischen Aspekten hinsichtlich der adäquaten Expositionsbewertung von biologisch wirksamen Stoffen (Wirkstoffen);
- Weiterhin in Anerkennung von möglichen technischen Problemen beim Versuch der Durchführung von Mischungsbewertungen auf der Basis von in regulatorischen Dokumentationen zur Produktzulassung gegenwärtigen verfügbaren Daten; und
- Akzeptierend, dass es einen Mangel an empirischen Indikatoren aus der Einzelstoffbewertung für synergistische Mischungseffekte gibt;

konnte diese Untersuchung keine Gründe finden, einen komponierten-basierten Ansatz der Mischungstoxizitätbewertung basierend auf den Nichtinteraktionsmodellen (CA, IA) als vernünftige ‚worst case'-Abschätzung abzulehnen. Allerdings, müssen Vorkehrungen getroffen werden, um mit Situationen von Datenlücken oder vorstellbaren interaktiven Effekten umzugehen und diese bei den Betrachtungen zu Unsicherheiten zu berücksichtigen.

1.5 Implementierungsschema zur Mischungsbewertung

Vor der Entwicklung spezifischer regulatorischer Vorgehensweisen zur Einbeziehung von Mischungsbewertungen in die Umweltrisikobewertung von Bioziden und PSM wurden existente und vorgeschlagene regulatorische Verfahrensweisen aus der verschiedensten Bewertungen für humane und Umweltrisiken gesichtet, um einen möglichst konsistenten und harmonisierbaren Vorschlag zu entwickeln. Generalisierend kann festgehalten werden, dass:

- Heutige Mischungsrisikobewertungsverfahren sich typischerweise auf eine bestimmte Methodologie festlegen (z.B. Mischungstestung oder komponenten-basierte Prognose) und seltener gestufte (‚tiered approaches') oder optionale Schemata vorsehen. Die exklusive Nutzung von zusätzlichen oder spezifischen Bewertungsfaktoren für Kombinationseffekte wird in keinem der gesichteten Mischungsrisikobewertungsverfahren vorgesehen;
- Vorzugsweise werden erwartete Mischungseffekte mit CA errechnet und vergleichsweise wenige Verfahren schlagen die Anwendung von IA oder Effektaddition als Referenzmodell vor. Vielfach wird lediglich eine punktweise Mischungsextrapolationsbetrachtung vorgesehen;
- Bei der Nutzung von Einzelstoffdaten werden in den betrachteten Mischungsschemata oftmals pragmatische Aggregationen hinsichtlich der betrachteten Endpunkte und Effektintensitäten vorgenommen. Ebenso wird vielfach über Spezies einer taxonomischen Gruppe hinweg aggregiert. Darüber hinaus werden auch Werte in der Mischungsbetrachtung vorgeschlagen und genutzt die Unsicherheitsfaktoren beinhalten, wie z.B. PNECs;

- Einige Verfahren beinhalten Verfeinerungsoptionen zur Berücksichtigung von bekannten interaktiven Mischungseffekten;
- Über die verschiedenen Felder der Risikoregulation sind fragmentierte und inkonsistente Herangehensweisen für die Mischungsbetrachtung festzustellen.

Angesichts der Vor- und Nachteile sollten die verschiedenen Implementierungsoptionen einer Mischungsbewertung (nämlich Bewertungsfaktoren, komponentenbasierte Extrapolationen und Mischungstestung) nicht als alternative sondern als komplementäre Ansätze verstanden werden, die sich in schrittweisem Vorgehen für eine optimale Nutzung der jeweils verfügbaren Daten- und Ressourcensituation in einer spezifischen Bewertungssituation nutzen lassen. Um ein gut begründbares Verfahren zu entwickeln, wurden die kritischen Faktoren untersucht, die (i) die praktische Anwendbarkeit der verschiedenen Implementierungsoptionen und (ii) die quantitativen Unterschiede zwischen verschiedenen komponentenbasierten Verfahren bestimmen. Diese sind:

- Die Anzahl der Mischungskomponenten in einem Mischungsszenario;
- Die Konzentrations- (oder Dosis) Verhältnisse der Mischungskomponenten;
- Die Steigung der individuellen Konzentrations- (oder Dosis-)Response Beziehungen;
- Die Wirkweisen und -mechanismen der Einzelstoffe;
- Die Art, Quantität und Qualität der verfügbaren Toxizitätsdaten für die Mischungskomponenten;
- Die Art und Qualität der Gefährdungs- oder Risikoindikatoren die zu bestimmen sind, wie z.B. EC50, NOEC oder TER-Werte für die Mischungen;
- Anforderungen hinsichtlich der Sicherheit und Verlässlichkeit der Mischungstoxizitätsabschätzungen;
- Informationen zur Variabilität und Unsicherheit der Einzelstofftoxizitätsdaten;
- Quantitative Informationen zu den Beziehungen zwischen verschiedenen Toxizitätsendpunkten; und
- Quantitative Beziehungen zwischen NOEC-Werten und niedrigen Effektkonzentrationen.

Basierend auf Betrachtungen zu all diesen Faktoren, wurde ein schrittweises, einheitliches Vorgehen sowohl für Biozide als auch für Pflanzenschutzmittel entwickelt. Gleichfalls wurde eine Software zur Verfügung gestellt, die eine konsistente Nutzung gestattet. Das vorgeschlagene Vorgehen erlaubt die zielgerichtete Bewertung für spezifische Bewertungssituationen und folgt dabei den oben ausgeführten generellen Prinzipien.

Biozidprodukte

Die derzeitige Biozidverordnung (98/8/EG) definiert rechtsverbindliche Standards für die Umweltrisikobewertung von Bioziden, allerdings existiert keine umfassende Handlungsanleitung für die Produktbewertung. Die zukünftige Verordnung (EG) Nr. 528/2012 trat 2012 inkraft und ist ab September 2013 anzuwenden. Die Entwicklung der diese begleitenden Leitfäden dauert an. Daher sind gegenwärtig hierfür keine detaillierten technischen Leitfäden zur Biozidbewertung verfügbar.

Angesichts dieser Ausgangslage wurde ein flexibles stufenweises Verfahrensschema entwickelt, dass für unterschiedliche Datenlagen tauglich ist und als Vorlage für die zukünftige Implementierung von Biozid-Risikobewertungsrichtlinien nutzbar ist. Der erforderliche Minimaldatensatz, um eine Bewertung mit dem vorgeschlagenen Verfahren durchzuführen, erfordert (i) belastbare und vollständige Informationen zur Zusammensetzung des betrachteten Produkts, (ii) Informationen zum relativen Risiko der einzelnen Komponenten und (iii) das PEC/PNEC Verhältnis für die Komponente mit dem höchsten

Risiko. Das heißt für alle Komponenten mit einem geringeren PEC/PNEC Verhältnis werden im ersten Schritt lediglich semi-quantitative Daten z.B. aus QSAR-Abschätzungen oder aus der Einstufungs- und Kennzeichnungsinformation für die in Frage stehenden Komponenten benötigt.

Das vorgeschlagene Verfahrensschema hält also die Anforderungen an zu liefernde Daten so gering wie möglich (es optimiert damit die Ressourceneffizienz) und sichert gleichzeitig einen adäquaten Schutz der Umwelt. Die Umweltrisikobewertung für Mischungen erfolgt stufenweise. Die initiale Bewertung ist bereits mit einem sehr limitierten Satz an Daten möglich, indem ein hinreichend konservativer Vorsorgeansatz gewählt wird. Sofern damit keine Indikatoren über das Vorliegen von Besorgnis erkennbar sind, ist auch keine weitere Testung oder Bewertung erforderlich. Sofern jedoch Besorgnis indiziert wird können zusätzliche Daten geliefert werden, um ein weitergehende Bewertung zu ermöglichen.

Der Verfahrensvorschlag nutzt weitestgehend komponentenbasierte Abschätzungen, beginnend mit einer initialen Bewertung, die auf der einfachen Summe von PEC/PNECs besteht und fortschreitend mit weitergehenden CA und IA-basierten Bewertungen in den folgenden Bewertungsstufen. Alle komponentenbasierten Bewertungen werden mit einen zusätzlichen Bewertungsfaktor belegt, der hier „IF“ (Interaction Factor) genannt wird. Dieser soll mögliche unerwartete Interaktionen (höhere Mischungstoxizität als nach Konzentrationsadditivität erwartet infolge von toxikokinetischen oder -dynamischen Interaktionen) berücksichtigen helfen. Es muss allerdings betont werden, dass hiermit keine Stoffe Berücksichtigung finden, die nicht in der Mischungsbewertung bereits enthalten sind beispielsweise aufgrund einer unvollständigen Beschreibung der Produktzusammensetzung.

Sofern ökotoxikologische Daten für das Produkt oder eine resultierende umweltrelevante Mischung (‚Leachat') ebenfalls verfügbar sind, sind diese Daten einer komponentenbasierten Prognose der Mischungstoxizität vorzuziehen.

Pflanzenschutzmittel (PSM)

Rechtsverbindliche Standardkriterien zum akzeptierbaren Risiko von Pflanzenschutzmitteln für Nichtzielorganismen sind in den *‚Uniform Principles'* der Verordnung 546/2011 der EU-Kommission niedergelegt. Diese erfolgen weitgehend als minimale Toxizitäts-Expositions-Verhältnisse (TER) für akute, Kurzzeit- und Langzeit-Endpunkte für verschiedene systematische Gruppen. Entsprechend gehören Toxizitäts- und Expositionsdaten für Einzelsubstanzen zu den Routineanforderungen der Zulassung und werden nach Standardbiotesten und -verfahren zumindest für die Wirkstoffe erzeugt. Unter der neuen PSM-Verordnung wird dies in Zukunft auch für ‚safener' und Synergisten gelten, nicht notwendigerweise jedoch für andere ‚co-formulants'.

Komplementär zu diesem Standard an Einzelstoffdaten, sind oftmals akute und Kurzzeittoxizitätswerte für eine Exposition durch direkten Kontakt mit einem PSM für das Produkt oder ein zumindest ähnliches Produkt vorhanden. Allerdings gilt dies typischerweise nicht für die Langzeitendpunkte entsprechend der in den ‚Uniform Principles' niedergelegten Standards. Zusätzlich zu den Standardendpunkten können in speziellen Fällen auch Daten aus der experimentellen Testung der Gesamtmischung in ‚higher tier' Langzeitstudien vorliegen. Dies gilt insbesondere für terrestrische Pflanzen oder Regenwürmer. Jedoch können in diesen Fällen auch Daten für die Einzelstoffe fehlen. Die verfügbaren Gesamtmischungsergebnisse sind im Regelfalle für das Originalprodukt in konzentrierter oder verdünnter Form erzeugt worden, nicht aber für Stoffe in unterschiedlicher Gemischzusammensetzung wie sie in der

Umwelt nach Deformulierung und infolge unterschiedlicher Transformations- und Transportprozesse in Expositionsszenarien erwartet werden.

Angesichts dieser vergleichsweise datenreichen Situation lassen sich komponentenbasierte Ansätze der Mischungsbewertung zu folgenden, unterscheidbaren Zwecken einsetzen:

i. Um das Risiko einer Mischung an aktiven Substanzen zu bewerten, das entweder der Mehrfachnutzung eines Produktes oder der Mischnutzung verschiedener Produkte, sogenannte Tankmischungen entstammt, jeweils in Bezug auf Situationen und Endpunkte in denen Test-Informationen über die Einzelsubstanzen nicht aber die Mischung vorliegen.

ii. Um das Risiko zu bewerten, das von einer Mischung an Substanzen aus einem Kombinationsprodukt ausgeht, für welches Gesamtmischungsinformationen verfügbar sind, für welches aber eine geänderte Zusammensetzung in den Umweltexpositionsszenarien erwartet wird.

iii. Zur Prüfung experimenteller Kurzzeittoxizitätswerte, die durch Testung von Produkten mit mehr als einer aktiven Substanz erzeugt wurden. Signifikante Unterschiede zwischen beobachteter und erwarteter Gemischtoxizität können entweder als Hinweise auf Toxizitätsbeiträge durch ‚Co-formulants' oder synergistische oder antagonistische Interaktionen zwischen anderen Inhaltstoffen verstanden werden. Derartige Fälle können erweiterte Untersuchungen erforderlich machen.

iv. Für die Identifikation von möglichen Treibern der Mischungstoxizität in Form von ‚toxic units' oder TER-Werten und zwar in Fällen bei denen ein Vergleich zwischen der Gesamtmischungstoxizität und den CBA bestätigt, das die Gesamttoxizität zum Gutteil durch ein konzentrationsadditives Zusammenwirken der Wirkstoffe erklärbar ist.

Neben diesen Nutzungen von komponentenbasierten Vorgehensweisen als Werkzeug, welches die üblichen Untersuchungen an Produkten ergänzt, könnte ebenfalls erwogen werden Anforderungen an die experimentelle Testung von Produkten optional in den Fällen zur Streichung vorzusehen, in den eine komponentenbasierte Mischungstoxizitätsabschätzung starke Hinweise auf die Abwesenheit von als unakzeptabel verstandenen Risiken ausweist. Angesichts der vorliegenden Evidenzen zur Genauigkeit von komponentenbasierten Abschätzungen ist jedoch zu empfehlen, dass als Minimalanforderung bei Erwägung eines Prüfungsverzichts Toxizitätsangaben für Nichtzielorganismen für alle Produktbestandteile vorliegen müssen und nicht nur für die Komponenten, die biologisch im Zielorganismus als aktiv betrachtet werden. Dies mag Informationen einschließen, dass ein Stoff sich nicht toxisch („inert") in Hinsicht auf den betrachteten Endpunkt verhält. Zusätzliche detaillierende Anforderungen zu den Voraussetzungen für einen Prüfungsverzicht könnten ausgearbeitet werden.

Für die Zielstellung einer komponentenbasierten Risikobewertung sowohl von Mischungen von Stoffen in einem einzigen Kombinationsprodukt als auch in Tankmischungen wurde eine einheitliche, stufenweise Vorgehensweise erarbeitet. Nach diesem Vorschlag wir ein erwarteter TER-Wert für ein Gemisch (TERmix) für einen gegebenen Endpunkt (z.B. akute Fischtoxizität) und ein gegebenes Expositionsszenario berechnet. Unterschiedliche Endpunkte müssen jeweils separat betrachtet werden.

Die vorgeschlagene Vorgehensweise sieht drei Hauptschritte vor, wobei jede Stufe als Filter dient. Auf der einen Seite werden die Mixturen von der weiteren Betrachtung ausgenommen, für die sich keine Indikation für ein unakzeptables Risiko für den gegebenen Endpunkt ergibt.

Auf der anderen Seite jedoch werden die Mischungen identifiziert, für die der komponentenbasierte Ansatz ein unakzeptables Risiko indiziert, das selbst durch ein mehr an Daten für denselben Endpunkt nicht ausgeschlossen werden kann. Hierin liegt ein Hauptunterschied zu anderen vorgeschlagenen Verfahrenvorschlägen zur Risikobewertung von Mischungen. Er wird erreicht durch die Betrachtung der quantitativen Beziehungen zwischen (i) der Einzelstofftoxizität und konzentrationsadditiver Mischungstoxizität und zwischen (ii) konzentrationsadditiver und unabhängiger Mischungswirkung.

Die erste Stufe ist eine Vorabprüfung in welcher nur die Einzelstoff-TER-Werte (TERi) betrachtet werden. Auf der nächsten Stufe werden die TER-Werte genutzt, um einen TERmix-Wert für die Mischung unter der Annahme von konzentrationsadditiver Kombinationswirkung zu berechnen. Auf der höchsten Stufe werden Betrachtungen über das verfügbare Wissen zur Wirkweise und die Konzentrations-Wirkungs-Beziehungen der Komponenten angestellt; wo angemessen können dann TERmix-Werte unter der Annahme von Unabhängiger Wirkung oder mit gemischten Modellen (MM) kalkuliert werden.

Unter der PSM-Verordnung sind die TER-Werte für die Einzelstoffe für jeden in den *Uniform Principles* definierten Bewertungsendpunkt verfügbar. Entsprechend sollte das vorgeschlagene Bewertungsschema auch jeweils separat für jeden dieser Endpunkte angewendet werden, wie z.B. Effekte auf das Algenwachstum, Effekte bei der Langzeittoxizität gegenüber Fischen, oder Effekte der Langzeitexposition gegenüber Daphnien. Eine Zusammenführung von Daten, die für verschiedene taxonomische Gruppen erhoben wurden, mag notwendig werden für Situationen in denen weniger Daten verfügbar sind (wie oben gezeigt mag dies insbesondere für Biozide zutreffen), es scheint hingegen nicht notwendig oder empfehlenswert für die PSM-Bewertung.

Auf jeder der drei Ebenen des stufenweisen Vorgehens können beiden Arten von Eingangsinformationen, sowohl zur Exposition als auch zu den Effektschätzungen einer iterativen Verbesserung unterworfen werden. Sofern an dessen Ende die komponentenbasierte Bewertung immer noch ein unakzeptables Risiko für die betrachtete Mischung ausweist, kann die experimentelle Prüfung der entsprechend zusammengesetzten Mischung als Instrument für die letztliche Bestätigung vorgesehen werden, sofern nicht praktische, ethische oder andere regulatorische Erwägungen einer finalen Entscheidung per Experiment entgegenstehen.

Kritische Punkte der vorgeschlagenen, stufenweisen Vorgehensweise die weitere Ausarbeitungen erfordern könnten sind:

- Potentiell synergistische Interaktionen sind nicht abgedeckt, diese könnten mit dem optionalen Vorsehen eines angemessenen Bewertungsfaktors adressiert werden;
- Potentielle Beiträge von Synergisten, Safenern und Formulierungshilfsstoffen zur Gesamttoxizität sind ebenfalls nicht berücksichtigt, sofern sie nicht durch entsprechend verfügbare Toxizitätsinformationen für die entsprechenden Komponenten mit einbezogen werden;
- Potentielle Toxizitätsbeiträge durch Metaboliten von Produktbestandteilen, die in der Umwelt entstehen und zu denen möglicherweise keine ausreichenden Informationen bei den bewertenden Behörden vorliegen werden nicht erfasst; und
- Unsicherheit besteht bei der Anwendbarkeit von CA und IA auf Toxizitätsdaten aus ‚higher tier'-Untersuchungen, wie sie gelegentlich für Wirkstoffe vorliegen.

1.6 Schlussfolgerungen und Ausblick

Aus der Sichtung der verfügbaren theoretischen und experimentellen Evidenz kann geschlossen werden, dass Konzentrationsadditivität ein gut unterstütztes Referenzmodell für

eine komponentenbasierte Bewertung der Kombinationseffekte von Pflanzenschutzmittelprodukten und Bioziden darstellt.

Die Implementierung von Konzentrationsadditivität in regulatorische Verfahren zur Risikobewertung ist jedoch kein sich selbsterschließender Prozess, da zusätzliche Entscheidungen gefällt werden müssen, etwa wie mit Datenlücken oder heterogenen Datenlagen umgegangen werden soll. Sackgassen die Bewertungen verhindern, wie die Diskussion über den Umgang mit Stoffmischungen bei denen die Wirkweisen der Komponenten unbekannt sind oder bei denen nur heterogene Daten verfügbar sind, können durch eine stufenweise Vorgehensweise bei der Bewertung vermieden werden. Hierfür finden sich konkrete Vorschläge in diesem Bericht. Diese mögen darüber hinaus nützlich sein, um eine konsistente und harmonisierte Vorgehensweisen über die verschiedenen EU Mitgliedsstaaten und verschiedene Regulierungen zu erzielen und eine Fragmentierung von regulatorischen Ansätzen zu vermeiden.

Die hier vorgeschlagene Vorgehensweise kann direkt in die gegenwärtigen Verfahren der Risikobewertung von Pflanzenschutzmitteln und Bioziden übernommen werden, da sie keiner fundamental neuen Bewertungsstrategie bedürfen. Dies wird insbesondere dadurch erreicht, dass die Risikobewertungen für die Mischungen auf einen Satz begründbarer Standardannahmen (‚default assumption') gestützt werden. Zusätzliche Daten oder Risikomanagementanforderungen werden nur erforderlich sofern die verfügbare Evidenz klare Hinweise auf ein zusätzliches Risiko indizieren. Dies minimiert die Notwendigkeit des Einsatzes von zusätzlichen Ressourcen insbesondere zusätzlichen Tierversuchen und sichert gleichzeitig einen adäquaten Schutz der Umwelt.

Offene Fragen für die Forschung hinsichtlich der Bewertung von Kombinationswirkungen durch Chemikaliengemische lassen sich auf folgende Aspekte fokussieren:

1) Gewinnung von Indikatoren für interaktive Mischungseffekte, wobei insbesondere die Möglichkeiten neuerer molekularbiologischer Methoden ausgelotet werden könnten;
2) Erzeugung von mehr und qualitativ verbesserten Daten zur Toxizität von Biozidgemischen die aus anderen Produktarten als dem Antifoulingbereich stammen und die auch marine und terrestrischen Ökosysteme abbilden;
3) ‚Higher tier'-Mischungsstudien für biologische Komplexitätsstufen oberhalb der Individuen- oder Populationsebene (d.h. Feldstudien bspw. in aquatischen Mesokosmen), Mischungsstudien die sich mit chronischer Toxizität befassen sowie Expositionsuntersuchungen, die für eine fortgeschrittene Umwelt-Mischungsrisikobewertung geeignet sind;
4) Verbesserte und konsentierte Richtlinien zu Design, Interpretation und Dokumentation von ökotoxikologischen Studien zu Gemischen.

2 Introduction

Organisms in the environment encounter exposure against various anthropogenic influences. Among these are chemicals emitted directly or indirectly into the environment during production and use of products or as waste. Impairment of ecosystem functions and services for which protection goals (e.g. populations of non-target species, integrity of ecosystem) are formulated has meanwhile been demonstrated to emerge from mixture exposure rather than from individual compounds. This stands in contrast to the traditional way of performing environmental risk assessment for chemicals which pursues a compound by compound strategy.

Since the conclusion of the EU Council of Ministers in 2009 on 'Combination effects of chemicals' (Council conclusion 17820/09) it is apparent that environmental regulations dealing with the assessment and management of risks for adverse biological effects from chemical exposure will progress toward including the consideration of potential combined effects resulting from exposure to chemical mixtures. While in principle there is sufficient evidence that mixture toxicity is an issue for regulatory risk assessment, the means on when and how to deal with this novel issue in the prospective risk assessment of chemicals are less clear.

The project 'Ecotoxic combination effects of substance mixtures' founded by the Federal Environment Agency (UBA, FKZ 3709 65 404) therefore dealt with the specific relevance and means to account for mixture toxicity within a prospective environmental risk assessment for pesticide and biocidal products. With the intention to generate a coherent and consistent approach suitable for the assessment of mixture toxicity across different regulatory areas and specifically for the pesticide and biocide authorization processes, the specific project goals were:

- To summarise the available knowledge on the applicability of mixture models for the prediction of ecotoxicological mixture effects and identify key aspects for their use in risk assessment approaches;
- To develop generic options for mixture assessments within environmental risk assessment schemes and identify gaps and necessary framework settings;
- To specify the types and needs of mixture considerations within the existing risk assessment frameworks for pesticide and biocide product registration;
- To develop specific implementation schemes for mixture assessment within the biocide and pesticide product authorization process and provide a tool for its consistent implementation.

The project approached these objectives in a consecutive manner. The available knowledge was summarised through a comprehensive review of currently existing scientific evidence. Generic options for regulatory mixture assessment were reflected by consideration of the hereby identified key issues. The latter step allowed transparency on necessary decisions that can not be based on scientific arguments solely but requires additional framework settings for a regulatory strategy. The practicability of mixture assessment within the biocide and pesticide product authorization process were specified through detailed consideration of the specific European regulatory frameworks, an in depth analysis of types of mixtures potentially under scrutiny, analysis of specific experimental evidence, and where possible the selected re-analysis of available regulatory documentation. The generated knowledge was subsequently

used to develop schemes for a systematic and consistent inclusion of mixture assessments for the different biocide and pesticide product related aspects. The schemes needed to become sufficiently detailed to deal with situations of varying data availability and at the same time allow evidence-based mixture risk decisions. The established scheme was also documented in forn of a software tool.

Among the reasons why mixture assessment has been so difficult in the past stands that mixture occurrence may be considered as highly variable and difficult to anticipate on the exposure and combined effects side. With respect to the pesticides and biocides assessment or the exposure situation may be regarded as comparatively simple because on the one hand, both product groups are often used in open environmental applications, i.e. the exposure by the product in its mixture composition already represents an environmentally occurring mixture. On the other hand, where modifications of the original product composition are expected to be important in determining environmental exposure scenarios, these are already worked out for the relevant individual compounds. Consideration of mixture exposure may thus be based on the information already available from these settings. On the combined effect side, key for the hazard assessment and thus potential basis for a regulatory usable approach to mixture toxicity in environmental organisms is to find generic approaches that may be considered valid for general applications. Again whole mixture testing and safety factors are two principles that are already established means that may also be employed in this context. Additionally, there are models discussed in environmental science and beyond that allow the calculation of expected combined effects purely based on effect information for the components of a mixture of concern. Most prominent among these are the models of concentration addition (CA) and independent action (IA). Again, if considered suitable, the required information to perform such model-based combined effect assessment may in principle be already available from the existing dossiers for chemical compounds assessment.

A major issue when striving to provide coherent and consistent approaches to mixture toxicity assessment is the use of a suitable terminology. Here we try to avoid confusion by explicitly introducing central terms at place of first use in the text and clear indication of different connotations when occurring elsewhere in the document. Further, a glossary of central terms can be found in the appendix. In this document we understand mixture as a term designated for a situation where an organism is experiencing simultaneous or sequential exposure to different chemicals that may provoke combined effects. This is in contrast to an understanding where one and the same compound are considered that through aggregated/cumulated[3] consideration of various (i) sources or (ii) exposure routes may lead to an increase in the exposure concentration relevant for assessment. On the effect side, we use to term interaction to signify situations where the combined effect provoked by a mixture exposure is different from what is expected as derived from the mixture components effects due to alterations of concentration effect relationships of one compound by another. This stands in contrast to various other usages where the term interaction is used to define (i) any effect of a mixture, (ii) any effects that provokes a specific biological response, (iii) any effect that is different from the effect of one of the components at the same concentration as in the mixture, or (iv) any effects as different from a predefined model (for reference and more explanation please refer to Altenburger et al. 2012).

[3] In the European mixture risk assessment discussion the terms aggregated and cumulative exposure are used mostly synonymously in the above defined sense (e.g. EC DG-SANCO, SCHER, SCENIHR, SCCS. 2012), while in the North American and international discussions other connotations prevail.

3 State of the art in mixture toxicity assessment

It has been demonstrated through various studies that organisms in their environment are typically confronted with mixtures of chemicals rather than that chemical exposure is against individual compounds (Reinert et al. 2002, Bonvin et al. 2011, Schäfer et al. 2011). Also, eco-epidemiological evidence and experimental studies show that exposure against mixtures as present in the environment may provoke combined effects and that ignoring these will underestimate resulting adverse biological outcomes (Altenburger et al. 2004, Posthuma and de Zwart 2006, Vaj et al. 2011). Moreover, it is now a widely held belief that accounting comprehensively for mixture toxicity via direct observation is at least various laborious (ATDSR 2001, 2004). It may indeed prove an impossible task for all mixtures that may occur in the environment as these are too variable and divergent (Altenburger and Greco 2009, Kortenkamp et al. 2009). Thus, for risk considerations two principles routes in assessment may be followed: either a mixture typical for that of concern is considered as an entity which often is called a 'whole mixture approach' or mixtures are inspected focussing on their relevant constituents which may be called 'component-based approach' (Teuschler 2007).

The whole mixture approach is often utilised when site-specific exposure of organism in the environment is to be assessed, such as in whole effluent testing or sediment contamination evaluation. To establish causality between specific contaminations and unwanted biological effects various means to deconstruct those often ill-defined mixtures are suggested (Feron and Groten 2002, Bakker et al. 2007). This line of thinking is also present in prospective risk assessment, e.g. when product testing is required in pesticide assessment instead of active substance testing or when bridging between evaluations for products of different composition is at stake.

The component-based approach toward mixtures stems for experimental studies on mixture toxicity. Here, models that allow formulation of hypothesis of expected combined effects on the basis of knowledge about the components biological activities are an established means for the mixture assessment (Bödeker et al. 1990, Altenburger et al. 2003, Andersen and Dennison 2004). Consequently, the application of such model-based mixture considerations have been suggested for use in assessment of possible combined effects in human and environmental risk assessment (US-EPA 1986, Calamari and Vighi 1992, Chévre et al. 2005): Moreover, it is become an adopted extrapolation practise in specific cases of human risk assessment such as consideration of mixtures of structurally related compounds such as dioxins or PCBs (van den Berg 2006).

The component-based models for mixture effects allow the calculation of expected combined effects typically purely based on effect information for the components of a mixture of concern. As they do not require any further system dependent information, e.g. about the environmental compartment, but rely on individual compound-bioassay interaction properties. Thus, they can be considered as generic extrapolation tools suitable for application in prospective chemical risk assessment, where environmental system properties are scarcely available.

In order to discuss the applicability of the component-based models for extrapolation purposes within prospective environmental risk assessment schemes for plant protection and biocidal products, the literature analysis was focussed to answer the following questions:

- What are the conceptual premises and limitations of models for component-based extrapolation of combined effects?

- What is the evidence for the predictivity of component-based extrapolations regarding mixtures of active substances used in pesticides and biocides and where are major knowledge gaps?

The literature analysis was based on about 800 references retrieved by a systematic literature search. The analysis is biased towards consideration of reviews of experimental studies and summary reports while only few original experimental studies but various other types of 'grey' reports, such as EU opinion papers, were considered at this stage. While the interest was in mixture toxicity evidence for pesticides and biocides with respect to ecotoxicological effects, a variety of endpoints, modelling approaches and generic studies, e.g. on validating the predictivity of extrapolation techniques were also considered. The major findings from these efforts concerning the applicability of the reference models for the assessment and prediction of combined effects using a component-based approach is therefore summarised in the following.

3.1 Non-interaction models as a default assumption for expected combined effects

Thinking and experimentation on the combined effects from the exposure to mixtures of compounds dates back several decades (Bödeker et al. 1992). Major progress in environmental toxicology resulted from the introduction of receptor-based thinking of pharmacology. Particularly, reference models to formulate expectable combined effects are compared against experimental observations (Kortenkamp et al. 2009). Key was the hypothesis derived from the so-called sham experiment and the categories of target sites and modes of action. The 'sham' experiment is a thought experiment wherein the simplest mixture is a mixture of an individual compound with itself (Berenbaum 1981). Clearly, the expectation for the responses from such a mixture experiment is that increasing doses due to mixture exposure should lead to increasing effects. Moreover, the concentration-effect relationship, as derived from dilution-type experiments for that compound, should be retrieved irrespective of how many fractions are applied in the dosing regime. The usefulness of this idea is convincing when thinking about compounds interacting with the same molecular target site. Under the name of dose or concentration addition it became a widely accepted reference model in pharmacological research and environmental toxicology and applies to all mixtures of compounds that act according to a common mode of action.

For mixtures of compounds that provoke their biological action through different target sites, responses are expected to be independent according to the statistical idea of independence. The derived reference model is called independent action, or response addition. The latter term avoids misunderstanding as the combined effect of a mixture of independently acting compounds is still expected to be quantitatively larger than that of any of the components alone. The guiding assumptions and the models for the relationship between the components individual effects and their expected combined effects are provided in Table 1. The two alternative reference models, however, provide quantitatively accurate predictions of the joint effects only if the mixture components do not modify each others concentration effect outcome. In cases where interaction between the mixture components occur observable responses may deviate to be larger or smaller than expected for either concentration additive or independent action effects (Table 1). For interactive combined effects, however, currently there are no generic models available to describe, let alone predict, the effect outcomes.

Table 3.1 Extrapolation concepts for mixture toxicity prediction (reference models) (modified after Bödeker et al. 1992, Faust et al. 2003)

Concentration Addition (LOEWE Additivity)

Suggested for: same site of action;
similar mode of action

Formula:

Binary $c_1/EC_{x,1} + c_2/EC_{x,2} = 1$

Multiple $$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{F_i^{-1}(x_i)} \right)^{-1}$$

Independent Action (BLISS Independence, Response Addition, Effect Multiplication)

Suggested for: different sites of action
dissimilar modes of action

Formula:

binary $E(c_{1,2}) = E(c_1) + E(c_2) - E(c_1)\,E(c_2)$

multiple $$X = 1 - \prod_{i=1}^{n} \left(1 - F_i(p_i \bullet (ECx_{mix}))\right)$$

Abbreviations used

c_i concentration of substance i in the mixture (for CA at the ECx of the mixture)
ECx effect concentration at the response level x
F function describing the relation between concentration and response for the individual component
p_{Si} fraction of substance i in the mixture
X expected combined response
mix mixture

3.2 Mixture toxicity predictions for pesticides

The investigation of combined effects emerging from pesticide mixtures have been the subject of an array of experimental studies. Deneer (2000) summarised results from experimental investigations on the joint action of pesticide mixtures in aquatic organisms published in the time period from 1972-1998. In particular, emphasis was placed on comparing experimentally observed combined effects with component-based predictions derived from the reference model of Concentration Addition. The results were collected for toxicity assays using apical endpoints in fish, crustaceans, insects, molluscs, and algae. In vitro, and enzyme studies were not considered. Also, studies applying i.p. or oral exposures to organisms where this is not the natural route of exposure were not included in the analysis. The author additionally applied some measures to filter out studies were an independent reanalysis of the data was not possible or where mixtures with non-pesticidal compounds such as metals were included, but

otherwise the assessment of the original paper was utilised in the analysis. In total at that time 26 studies covering 202 pesticide mixtures were identified. Supposedly, most studies dealt with the active ingredients of pesticide compounds though no explicit statement can be retrieved from the review. Typically, insecticide mixtures were studied using fish or crustacean species, 27 and 12 mixtures compared to only 3 insecticide mixtures that were being investigated using an algae species. By contrast, 38 out of a total of 51 herbicide mixtures were investigated using algal assays as opposed to organisms form another group. Fungicide mixtures, at that point, were rarely investigated for combined effects.

For 186 of the 202 mixtures retrieved by Deneer (2000) the reported combined effect concentrations were within a factor of two of the effects that could be calculated by concentration addition. 11 mixtures showed an effect higher than that while 5 had less effect than a factor of two from the CA prediction. Two mixtures of insecticides (quinalphos, phenthoate and deltamethrin, carbaryl) showed a more than 10fold higher toxicity than expected by CA in a fish, respectively, mollusc assay, while one mixture (amitrol, glufosinate-ammonium) showed a more than 10fold lesser toxicity in an algal assay. No further pattern e.g. with respect to the mode of action could be retrieved in this analysis.

Belden et al. (2007) in a more recent effort undertook to analyse the quantitative deviations of pesticide mixture findings in relation to the predictions derived from both concentration addition and independent action by using a uniform approach. The authors made additionally up for studies were one component was tested at concentrations without an individually detectable effect for the response under observation as a third group and called this synergistic interaction (SI) and here they analysed the shift of the dose-response curve for the sole individually active component. The retrieved and analysed literature comprised that of the Deneer review plus studies that appeared in the period between 1998-into 2005. The authors provide a compilation of the studies retrieved that is available for reanalysis. Only studies that employed active ingredients were included in the analysis, while product based investigations were not accounted for. Additional criteria to avoid undue bias from individual studies were employed. All in all 45 studies with 303 experiments dealing with pesticide mixture assessments were retrieved for consideration. Based on the knowledge about the intentional biological action in relation to the species used for effect analysis, 207 of the investigated mixtures were classified as conform to the CA assumption, while 37 were associated with IA and 59 were categorised in the SI group. Moreover, 11 of the studies did investigate multiple mixtures.

127 different active ingredients were investigated in the retrieved mixture studies. There is a bias for certain compounds, namely atrazine and chlorpyrifos in the available pesticide mixture evidence with their occurrence in 17 % and 15 % of the mixture experiments. Taxonomic groups were as before (Deneer 2000) mainly fish, amphibians, bivalves, insects, crustaceans, green algae and duckweeds. The species dominating in the reported experiments were *Scenedesmus vacuolatus*, *Lemna minor* and *Chironimus tentans* with 27 %, 19 % and 18 % of the experiments, respectively. In the SI experiments nearly all work is related to the enhancing effect of triazines on insecticide activity. The effects reported derive from short-term co-exposure which often is equal to acute effects in *in vivo* assays using standard biological endpoint such as growth or lethality. The summary assessment of Belden and coworkers (2007) is based on the calculation of a model deviation ratio (MDR) which calculates a ratio between a reference model dependent expected combination effect and the reported observed mixture response. The reanalysis for the IA model proved difficult in some cases when adequate information was lacking to perform this calculation.

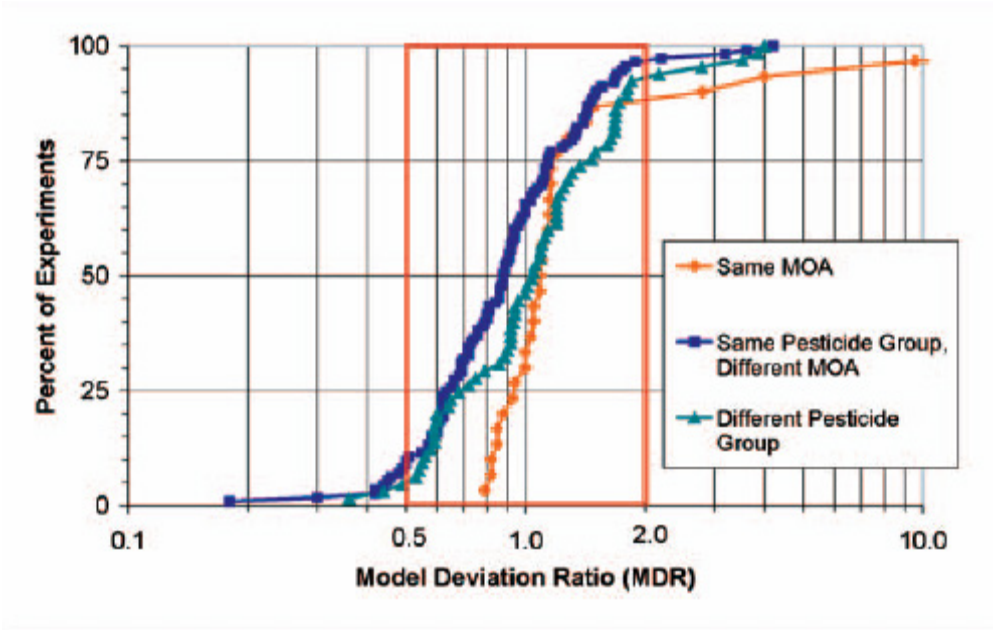


Figure 3.1 **The cumulative distribution of model deviation ratios (MDR) for concentration addition experiments using pesticides**
Grouped by mixtures with the same mode of action ([MOA or MoA]; n = 30, indicated by diamond symbol), mixtures of the same pesticide use group and different MOA (n = 113, indicated by square symbol), and mixtures of different pesticide use groups and different MOA (n = 64, indicated by square symbol). The double-lined box delineates values that are within a factor of 2 from the predictive model. An MDR of 1.0 indicates perfect fit to the model. Greater than 1 indicates greater toxicity than expected and less than 1 indicates less toxicity than expected (adapted from Belden et al. 2007)

For CA 88 % of all mixtures had MDR values within the range of 0.5 and 2.0 irrespective of their intentional biological targets (see figure 1 from Belden et al. 2007). However, in the group of mixtures were the components had different biological targets, CA tended to overpredict the observed combination effects in about 50 % of the cases. Conversely, IA tended to underestimate the combined toxicity observed for most cases of mixtures composed of components with similar targets. For the multiple mixture studies considered it appears that they tend to show less deviations from reference model predictions as compared to the findings for binary mixtures. In the SI category more than 20 % of the studies showed a deviation from the expectation of an unaltered concentration response function by a factor of greater than 2. While this finding on the one hand shows that interactive combination effects may indeed occur, the authors (Belden et al. 2007) rightly point out, that the mixtures investigated are heavily biased towards those where metabolic activation was known to occur.

3.3 Biocides in mixtures

Investigations on combined effect from mixtures involving compounds that are used as biocides are present in the literature though to a considerably lower extent than for pesticides. Also, no reviewing or otherwise summarising publication was retrieved. As can be seen from a series of publications the particular emphasis is for active ingredients used in antifouling biocides, possibly due to the recent international ban of ship paints using organotin

compounds, while for other biocide products there seems to be very little evidence on mixture questions.

The most recent work that we retrieved in our initial literature search (until 2009) is from Bellas (2008) summarising an investigation on mixtures of the antifouling biocides zinc pyrithione, chlorothalonil and seanine in an embryo-larval bioassay with sea-urchin. While some deviations from the mixture predictions derived from CA and IA were detected, CA overestimated the toxicity of the mixtures studied by a factor of 1.6 only. This finding would be in line with the general tendency reported for pesticides and summarised in the previous chapter.

However, a larger number of studies carried out for mixture effect investigations on antifouling active compounds do report their observations as interactive and synergistic response compared to reference models. Reports cover zinc pyrithione and copper studied in a diatome and a polychaete larvea assay (Bao et al. 2008), irgarol, diuron and copper in sea urchin (Manzo et al. 2007), zinc pyrithione and copper pyrithione in brine shrimp (Koutsaftis and Aoyama 2007), irgarol and cadmium (Koutsaftis and Aoyama 2006), and zinc pyrithione and copper in red sea bream and toy shrimp (Mochida et al. 2006).

By contrast, Arrhenius and coworkers (2006) reported for mixtures from antifouling products composed of the herbicidal compounds irgarol, seanine and TBT less than expected combined effects in an algal growth assay when compared against expectations from both reference models CA and IA.

The repeatedly reported synergistic effects in several bioassays for some biocide mixtures were thought to require more in depth follow-up analysis in this project to identify possible causes. Mochida et al. (2006) and Bao et al. (2008) offer an explanation for mixtures involving zinc pyrithione whereby the exchange of the zinc ion against copper has been inferred as causative for the increase in observed mixture effect. At the same time it has to be acknowledged that many active substances used in biocidal products resemble or are identical to organic compounds used in pesticides. Our first hypothesis was that the reported interaction may possibly be due to metal specific potencies. Therefore, as a first step the current state of understanding of combined effects involving metals was considered in some more detail (see Appendix 2 for the full report).

Looking into the available empirical evidence for combined effect observations from mixture exposure with metals a comprehensive review has been provided by Norwood and colleagues (2003). These authors collated, inspected, recalculated and summarised reported experimental evidence on metal mixture effects on aquatic biota. They analysed some 100 original communications beginning from the mid-seventies, and a total of 22 different metals were included in the analysis of 249 mixtures and their combined effect on 77 different aquatic species. The mixtures so far being investigated experimentally for combination effects are biased towards binary mixtures, with some ternary and only few other multiple mixtures. Also, from the 22 different metals included in mixture investigations Zn, Cu(II), Cd, Hg, and Ni account for over 80 % of the metals employed in all studies reported.

The species employed in metal mixture toxicity investigations stem from systematically diverse groups though not surprisingly there is a bias for organisms with well established standard test protocols, e.g. for algae, fish, and invertebrates. Different test protocols imply different testing conditions with respect to media composition, exposure duration, and effect observation. Media composition varied from natural fresh- and saltwater to artificial media. Biological responses observed covered various effects from short-term functional responses such as sodium flux rate or photosynthetic rate to structural responses on histopathological

observation levels or community structure measures. Also, different life and development stages have been investigated in these mixture effect studies (Norwood et al. 2003).

Given this heterogeneity in the evidence base, the general trends that have been elucidated are quite striking (Table 2). A little more than a quarter of the mixtures are being assessed in the original communication as being in agreement with the idea of an additive combination effect, while for another quarter more than additive effects are described. The remaining almost half of observations claimed to have detected less than additive combination effects. It has to be stated that there is no clear provision as to which concept of additivity – concentration addition or response or even effect addition – has been utilised in the original literature. Moreover, the criteria upon which deviation from the non-interactive 'additive' response are assessed are not stated. The results are however more or less reproduced with a more stringent reanalysis of the data performed by Norwood and colleagues (2003) themselves. They undertook, whenever accessible, statistical testing of the authors observations against either concentration addition or what they call effect addition, which we understand to be identical to what we call independent action here.

Table 3.2 **Summary of published observations for metal mixture effects** (modified after Norwood et al. 2003)

	NO. OF METALS IN MIXTURE	LESS THAN ADDITIVE	STRICTLY ADDITIVE	MORE THAN ADDITIVE	TOTAL TESTS	COULD NOT TEST
	2	69	42	45	156	14
	3	7	6	5	18	4
	4	1	0	0	1	2
	5	3	0	3	6	2
	6	1	3	2	6	1
	7	0	0	0	0	1
	8	1	1	0	2	0
	10	0	0	1	1	1
	11	1	0	0	1	0
AUTHOR	TOTAL	89	58	63	210	12
INTERPRETATION	PERCENT	42.4	27.6	30.0	100.0	5.7

The larger number of deviations of observed combination effects from the predictions derived by either concentration addition or independent action is hard to dispute, despite the fact that it is based on a qualitative rather than a quantitative statement. Previous reports have eluded to the issue that qualitative combined effect assessments based on biological effect variation inherent to the specific experimental study they derive may be more sensitive and specific but at the same time with less scope for inference to an extrapolative use than quantitative assessments based on the deviation between expected and observed mixture effects (Grimme

et al. 1994, Belden et al. 2007). In any case, these indications were taken to inspect the ecotoxicological literature for possible processes or mechanisms that might be causative for the apparent interactive potencies of metals in mixtures. From this, at least three different lines for consideration emerge.

Firstly, we have to acknowledge that organism co-evolved with metal occurrence in the environment and in fact in the process some elements became essential for biochemical functioning. Thus most organisms with their cellular systems seem to have evolved typical reaction patterns upon metal exposure that may perhaps best be envisaged from the perspective of essential metals. Cellular homeostasis is maintained for essential metals by means of regulating uptake and utilisation. During low availability, metabolism will regulate towards most efficient uptake of the required metals, while during higher than optimal metal presence, sequestration and elimination mechanisms will become more prominent. There is evidence for several compounds that the concentration of free ions in the cytosolic environment is maintained at very small amounts, e.g. by shielding the charge through weakly chelating compounds. Metal-protein interactions with specific proteins perform essential functions in uptake, storage and elimination of metals. For iron for instance, we know specific transporters such as the divalent metal transporter 1 (DMT1), ferretin as a storage protein, and other iron carry proteins such as transferrin, ferroportin or haphaesti, the latter catalysing the oxidation of Fe^{2+} to Fe^{3+} (Valko et al. 2005). Thus, exposure to metals may be regarded as a stress situation, whereby the biosystem will react with regulated responses up to the point where the system is overloaded either due to too high concentration of bioavailable free ions or too long exposure duration against a metal. A whole line of discussion subsequently diverts into the different intracellular mechanisms of actions of metals which might give rise in themselves to various hypothetical types of interaction (Wang et al. 2008). A central role here is the understanding of oxidative stress and how and where the different metals do eventually provoke oxidative stress within a cell. This situation is clearly distinguishable from that for most organic contaminants where this elaborate level of potential adaptive responses has at least not yet been described. No straightforward hypothesis what this could mean in term of mixture responses has however been developed.

Secondly – in contrast to many xenobiotic organic substances – metals, essential or non-essential, as prevalently charged species are not able to passively pass through cell membranes, but seem to enter cells and tissues actively via the various ion transporters and ion channel proteins invented during evolution. There is a high number and substantial variability in transporter proteins to be acknowledged at least when comparing aggregated biological systematic units. Ion transporter proteins for instance make up for more than 40 % of all transporter types in primates, while accounting for merely 12 % in plants and less than 2 % in protozoa (Ren and Paulson 2005). Again while it is therefore not too surprising that the uptake kinetics of metals observed for organisms or cells are specific for individual metals and may vary greatly between species there is no specific implication for mixture toxicity outcomes suggested in the literature.

Thirdly, it has been known for long that the milieu conditions play a major role in determining the apparent toxic effect of metals on organisms. Water chemistry with factors such as pH, water hardness or the occurrence of other ions in the exposure medium will influence the redox state and speciation of the metals. In consequence, the prevalent metal species will determine the potential for molecular interactions such as sorption or reactivity and thus subsequently also determine the toxic properties. Next to speciation also complexation or chelation of cations by organic substances such as humic acids or polymers such as polyphosphates may affect apparent biological outcomes from metal exposure. For the combined effect of metal mixtures all these processes may be regarded as potential

confounders for the precision of predicting the combined effect of a metal mixture from the components activity, as each metal will be affected differently by changes in any of these factors (Campbell and Tessier 1996). One can try and provide adequate consideration of the major influences through modelling. The free ion activity model (FIAM) and the biotic ligand model (BLM) are among the more successful attempts to capture the influence of milieu factors on the toxicity of metals. The basic assumption of the FIAM being that it is the free ion that eventually determines the biological effect of metals and if therefore the ambient concentration of a metal can be corrected for the other metal, the resultant toxicity should be an expectable value purely dependent on the concentration of the free ion. The BLM is a formalised way to incorporate the impact of water chemistry on metal speciation, to estimate the availability of free metal ion in the water phase on the one hand when accounting for organic and inorganic metal complexation, and the binding to a biotic ligand in competition to other ions on the other hand. The biotic ligand is typically thought of as a membrane-related macromolecular structure such as a transporter protein. If that structure has a relevant biological function, e.g. transport of essential metals, then it can also be regarded as a primary site of toxic action and it may be used to model short-term toxic effects (Paquin et al. 2002). An example would be hypocalcemia believed to be caused through blockage of Ca uptake e.g. by Co, Zn or Cd. Historically, this approach has been developed for mono- and divalent metals and in particular Cu, Ag, Ni with fish as receptor species in mind, i.e. the cation transporters at the fish gill surface are envisaged as the primary biotic ligands (Paquin et al. 2002). This thinking is currently extended and developed for various metals and other non-fish species also. To our knowledge both models have not been extended to the study of the combined effect from metal mixtures though. For metal mixtures the ambient concentration in any case seems to be an unreliable indicator of expectable combined effect, which in turn might rear firm conclusions on a general trend towards non additive behaviour or metal mixture premature.

One logic alternative would thus appear to head for estimates of internal concentrations or biological doses as a basis for a toxicity assessment which might be less prone to confounding factors (Norwood et al. 2007). However, this would mainly be of value for chronic types of effect that are clearly linked to intracellular mechanism of toxic action.

Thus, from the above mentioned studies and considerations of modes of interaction no conclusions on plausible mechanistic explanations for a systematic deviation of mixture effects for biocides involving metal components could be drawn. Within this project we therefore opted to step back and undertake a more detailed analysis of the existing mixture studies using biocides which was subsequently carried out and is documented in chapter 6.

3.4 Specific issues – Co-formulants[4]

A specific aspect for the environmental risk assessment of plant protection and biocidal products is the use of co-formulants that may need mixture considerations when the assessment is else based on the active substances only. Co-formulants are chemically diverse and are typically classified by their function for the product, such as antioxidant, emetic, dispersing agent, emulsifier, dye, antifreeze, adhesive, preservative, solvent, wetting agent, synergist, propellant, stabilizer, safener and others.

Observational findings on specific mixtures of active substances and co-formulants are in the literature and in particular toxicokinetic interactions of active substances with synergists and

[4] For a specific experimental study on the effect of co-formulants in biocidal products see part 2 of this report.

safeners have been discussed (Gressel 1993). No review summarising the findings and knowledge on co-formulant behaviour in mixtures with active substances with respect to environmental assessment could be identified. The only pattern analysis retrieved refers to findings of a previous project from the UBA where five selected anionic, non-ionic and cationic surfactants in mixtures with pesticides had been screened for their combined effects on algae (Figure 3.2).

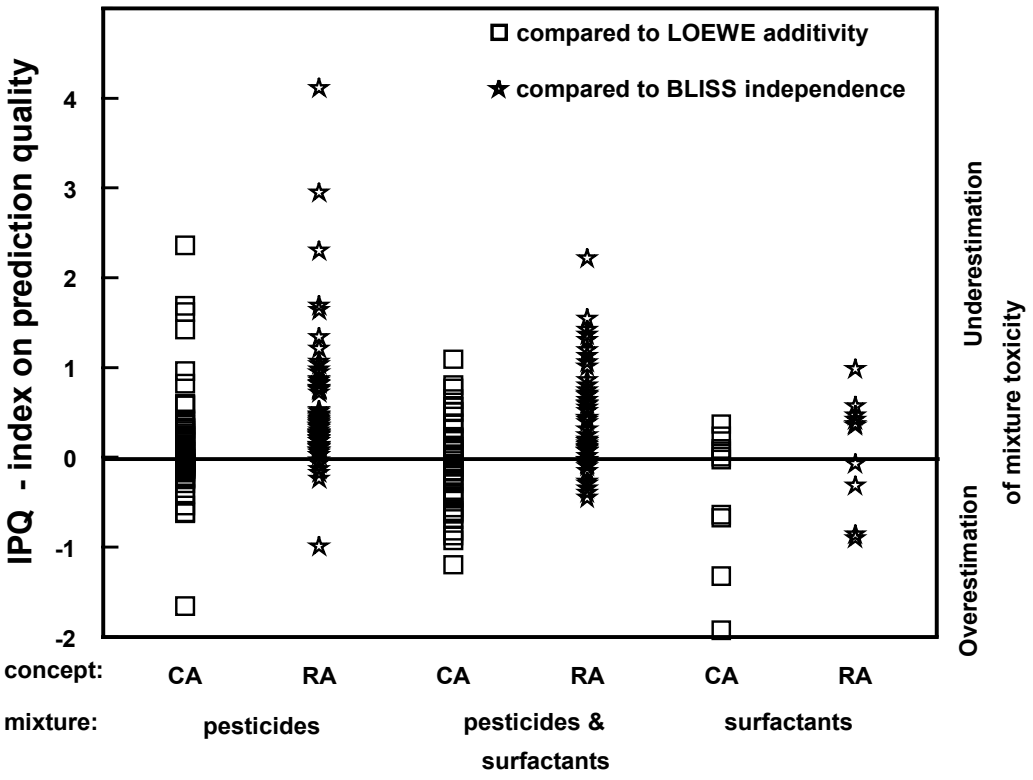


Figure 3.2 Comparison of predicted and observed mixture toxicities using the linearising scale index on prediction quality
Predicted toxicities for the different binary mixtures are calculated on the basis of concentration addition (CA) and independent action (IA) which is also called response addition (RA), respectively, using the concentration response relationships for the single substances. The observed effect is the inhibition of algal reproduction, reported here as the statistically estimated EC50 value. (Modified after Altenburger et al. 1996)

The authors (Altenburger et al. 1996) summarise their findings as follows: "Substances were selected from pesticides and surfactants on the basis of their relevance for aquatic systems […] and with respect to different modes of action in order to generate mixtures of expected similar and dissimilar acting substances […]. Concentration response relationships of 14 pesticides and 5 surfactants and 137 binary mixtures of these were established after experimental testing on the basis of Weibull distribution functions […] deciding whether an observed mixture toxicity is in accordance with the predictions derived from either concept, statistical means of variance calculation have been used for these data and discussed in a

comparative study on the consistency of assessment approaches employing the biometrical models isobolographics, toxic unit summation, universal response surface analysis, independent action and effect multiplication […]. Accounting for 20 % variability of responses at EC50-level about 80 % of all pesticide/pesticide mixtures and 60 % of all surfactant/pesticide mixtures observed were compatible with the predictions derived from the concept of concentration addition. This compared to 43 % and 46 % of the respective mixtures assessed as being in agreement with response addition.

In conclusion, while for mixtures of surfactants and active substances the same component-based perspective for mixture assessment may seem suitable, there is a great lack of systematic studies to be acknowledged. For this reason within this project regulatory data made available for 15 plant protection products were reanalysed to identify whether co-formulants are an issue in product mixture assessment (see Chapter 7).

3.5 Specific issues – Higher tier testing

The evidence on mixture toxicity and its predictability using reference models is overwhelmingly based on studies with individuals and populations of aquatic organisms. Thus, though a large body of evidence useful for mixture assessment has been accumulated (Kortenkamp et al. 2009) several areas are clearly less well studied. Among the major gaps appear the field of so-called higher tier studies, which is taken to mean effect investigations at level of biological complexity beyond the species level. In chemical risk assessment these are typically approached by e.g. aquatic micro- or mesocosm studies or (semi-)field studies like earthworm field studies. Pioneering studies have been identified that undertook to study community responses upon pesticide or biocide mixture exposure (Backhaus et al. 2004, Arrhenius et al. 2004, 2006, Knauer et al. 2010). In these it could be shown that the reference models of concentration addition and independent action may be suitable for combined effect prediction of clearly defined community responses. However, these were specifically designed investigations on combined effects assessment and thus for many available higher tier studies it may be expected that the reported information about observed effect concentrations is highly aggregated toward identifying the sensitive responses only. For mixture effect predictions which are based on calculating the combined effect for defined biological observations, this may contribute to a heterogeneous data base for mixture assessment. Moreover, higher tier studies are also done in order to refine for fate aspects of the exposure against compounds. This consequently may affect the exposure concentration input in the mixture assessment but will not alter the question for the predictability of the reference models. It has therefore been excluded from the considerations in this project.

3.6 Concept related considerations for the application of component-based mixture effect extrapolation

Implicit to the application of the above discussed reference models of Concentration Addition and Independent Action is the use of data for the same exposure setting and effect observation parameter (in a regulatory context this would often be called endpoint, while in a statistical regression model context we may speak of response variable) for the mixture components. This may therefore be considered as a precondition for valid application of either concept in experimental studies, though there are no systematic investigations available as to what that means in operationally relevant detail. For the use of mixture toxicity models in risk assessment this might consequently impose certain difficulties as available data for individual compounds are currently not produced with this in mind, that is they can be expected to show

some heterogeneity in this respect. To illustrate the resulting difficulties, e.g. reported growth inhibition data in algal effect determination may be considered the same effect observation for different compounds though the actual observations may rely on measurements that are as different as microscopic cell counting or fluorometric cell suspension quantification. Similarily, growth rate may be considered a different and incompatible observation compared to biomass yield though the actual measurement might be identical and only subsequent data treatment varies. So, here one might foresee needs for future technical improvements as well as for pragmatic decisions when it comes to the question of the usability of a heterogeneous data base in mixture risk assessment.

Furthermore, the mode-of-action of mixture components is widely believed to be connected with specific models (see table 3.1) and has stipulated much efforts notably in human mixture risk assessment to define what information might be necessary to become accountable for rational choice of the 'correct' mixture reference model (US-EPA 2002). However, in ecotoxicology the empirical evidence did not show the mode of action of components to be of major relevance for the precision of combined effect predictions for either of the reference models (Deneer 2000, Belden et al. 2007), although a slightly more conservative mixture prediction when using concentration addition may be found (Faust et al. 1993, 1994).

As there may be no straightforward available information as to when to employ either of the two reference models for a given mixture it is useful to look at the quantitative differences between the model-dependent predicted combined effects. The determinants of the differences between the combined effect predictions are known and comprise among others the number of mixture components and the slope of the individual concentration-response relationships (Drescher and Boedeker 1995). The difference between the mixture effect predictions from either reference model can be simulated or even quantified for specific situations and amounts to a maximal distance in predicted mixture toxicity effect concentration that is equal to the number of the mixture components (Faust 1999). Empirically, it has been summarised that in many cases CA provides a higher mixture toxicity prediction compared to IA, which is why CA would seem a reasonable worst case models for non interactive combined effect prediction (Kortenkamp et al. 2009).

In an assessment context it is of interest to establish criteria that allow defining which components need to be included in a mixture assessment and which may be left out. The question of whether a combined effect is likely or of relevance for a given mixture can, however, only be answered by explicit consideration for the mixture of concern. There are suggestions to eliminate components from mixture assessment considerations based on certain effect or toxic unit contributions (EIFAC 1987, Price and Han 2011). These approaches, first of all require availability of suitable information for all possible mixture components which underlines the above statement of the unavoidability of an explicit mixture consideration. Moreover, these approaches ignore the fact that point wise estimates violate the dilution principle of the 'sham combination' i.e. any addition will lead to an increase in effect and the question of whether that is measurable or deemed a significant contribution depends among others on the slope of the concentration-response relationship. This information, however, is not included in point wise considerations. Furthermore, as before the results of an estimation of an individual components contribution to a mixture depends on the chosen reference model and will lead to potentially ambiguous results (Altenburger et al. 2004) which should warn against indiscriminate use.

Another aspect of defining the mixture components that may be relevant in a combination effect context is that of mixture toxicity at low level of effect for the individual components. There has been generic experimentation to answer the question of whether chemical mixtures with their components present at only low effect concentration level may still provoke

combined effects and whether these are still predictable by either of the reference models. The experiments have recently been summarised by Kortenkamp et al. 2007. In their majority these were performed with an ecotoxicological perspective and several groups of chemicals were investigated for apical effects. The low level of exposure was typically operationalised with respect to NOEL (No observed effect levels). The major conclusion to be drawn for the context of this study is that NOELs of individual compounds cannot be regarded as concentrations that may safeguard against the occurrence of mixture toxicity. This has even been acknowledged in a recent statement by European Union Scientific Committees on Health (EU SC-Health 2011) though the statement raises the issues that in human risk assessment the safety factors applied to NOEL values might compensate for mixture effects and that different target tissues need to be included in mixture assessment.

3.7 Indicators for synergism and low dose mixture effects

Synergistic interactions provoked by components of a mixture cannot be predicted by either reference model (CA or IA) as these are based on the assumption of non-interactive combined effects (see above). Synergistic interactions may occur and have indeed been reported for mixtures of pesticides and their combined effects on wildlife (Thompson et al. 1996). The question therefore is, if they are not systematically predictable using component-based models do we currently have empirical indicators for such interactions available or in sight.

The advent of genomic techniques has raised large expectations that their application can provide a novel perspective on such mechanisms of low dose interactions. After the first decade of experimental studies utilising the novel toxicogenomic tools, the existing mixtures toxicity studies that address diagnostic, mechanistic or extrapolation questions were therefore summarised and reviewed. From 2002 to 2011, 41 studies were published with a focus on mixture toxicity assessment by means of toxicogenomic techniques, mainly through multiplexed quantification of gene transcripts, though metabolomic and proteomic analysis of joint exposures have also been undertaken. The biological systems studied so far include typically short-term exposure of rodent or fish species with *Danio rerio* being the single most prominent species employed in the investigations. Other sentinel systems and in particular plant studies are much less used. Among the studied compounds several active substances of plant protection products were investigated. For one, receptor-based assays seem to be most advanced towards establishing quantitative relationships between exposure and biological responses. Often transcriptomic responses are discussed in those studies based on the presence or absence of signals. As there are yet no consented ways of analysing these effects, the interpretations are ambiguous. Furthermore, the majority of mixture studies are designed for comparing the recorded outcomes against individual treatments. I.e. the focus was to retrieve signals of individual components under mixture exposure. This stands in stark contrast to our existing understanding of biological activity at the levels of chemical target interactions and apical combined effects. Thus at the current state of work, evidence provided is rather anecdotal than systematic, but the field is rapidly evolving, specifically with the help of bioinformatic tools.

3.8 Conclusions

In view of

- A clearly less extensive data base for mixture toxicity studies with biocides as compared to plant protection products; and
- The acknowledgement of several specific aspects relevant to exposure assessment of biocidal compounds;
- The current lack of reliable empirical indicators for mixture synergism based on the individual components effects;

it is concluded, that currently there is no reason to refute a component-based mixture toxicity assessment based on non-interaction reference models (CA, IA) as a reasonable worst case estimate. However, provision to account for conceivable interactive effect may well be taken when dealing with remaining uncertainties.

4 Generic options

4.1 Aims and approach

Aims

This section provides an outline of generic options for the assessment of hazards and risks of chemical mixtures in a regulatory context. Advantages and disadvantages of different implementation options are compared in terms of data and knowledge requirements and the expectable reliability of resulting assessments. The aim is to provide a common platform for the subsequent development of specific implementation proposals for both biocidal products and plant protection products (PPPs) that are each tailored to the different specific data and assessment situations (*see* sections 6.2 and 7.2).

Options considered

Three basic options are considered in this chapter:

(i) the experimental testing of the toxicity of a mixture of concern, the so-called *whole mixture approach* (section 4.2),

(ii) the calculation of the expectable toxicity of a mixture of concern on the basis of toxicity data for individual mixture components by applying appropriate concepts for predictive mixture toxicity assessments, the so-called *component-based approach* (section 4.3), and

(iii) the safeguarding against potential combination effects within the procedures for single substance hazard assessments by means of a special factor, the so-called *assessment factor approach* (section 4.6).

For the second option, the component-based modelling approach, three different assumptions were alternatively considered as a potential starting point:

(ii-a) the assumption of a concentration-additive joint action of all mixture components (CA),

(ii-b) the assumption of a fully independent joint action of all mixture components (IA), and

(ii-c) the assumption of an intermediate type of joint action.

The latter situation, an intermediate type of joint action (ii-c), may result from heterogeneous combinations of substances that contribute to a common endpoint neither by strictly identical nor by fully dissimilar and independent modes and mechanisms of action. Their expectable joint toxicity may either be described in terms of a *prediction window*, i.e. the range between the two extreme assumptions of fully concentration-additive or fully independent action. Or it may be specified by means of a *mixed model* (e.g. Olmstead and LeBlanc 2005), which presupposes that the constituents of a multi-component mixture can be unambiguously grouped by their modes and mechanisms of action: concentration-additive action of constituents is assumed within such groups, while independent action is assumed for the overall joint toxicity of the groups as a whole.

For the practical application of the concept of concentration addition (option ii-a) in a regulatory context, a number of different approaches have been suggested in the literature. For

pragmatic reasons, these CA-based regulatory approaches usually include simplifying or additional assumptions, and hence they deviate more or less from the principal assumptions that are inherent to the original scientific concept of CA. As a result, such CA-based approaches may differ with respect to both the suitability for specific assessment purposes and the quantitative mixture toxicity estimates that are derived from their application. These aspects were included in the comparative considerations of different implementation options, in so far as they have an actual or potential relevance for the ecotoxicological assessment of PPPs or biocidal products (section 4.5).

Main questions addressed

The comparative analyses of the outlined options build on the review of the state of the art in the preceding chapter 3. Some of the considerations concerning the implementation of existing scientific knowledge into regulatory approaches are resumed here and examined in more detail. Thereby, the focus is on three main questions:

(i) Which factors determine knowledge and data requirements, as well as expectable reliability of different implementation options for specific assessment situations? Which factors are crucial, which are less important?

(ii) Which factors determine the quantitative differences between predictions of mixture toxicities under the alternative assumptions of concentration addition (CA) and independent action (IA)? How large are these differences? (section 4.4)

(iii) Which factors determine the quantitative differences between predictions derived from the original scientific concept of CA and the numerous pragmatic extrapolation methods that have been derived from this concept for regulatory purposes (CA-based approaches)? How large are these differences? (section 4.5)

Aspects of comparing different implementation options for specific assessment situations

When comparing the advantages and disadvantages of different implementation options in the context of PPP and biocidal products authorisation, we focussed on two major aspects:

(i) the practical applicability of suggested procedures, and

(ii) the achievement of an adequate level of protection from unwanted combined effects of chemicals (as specified below).

Economic criteria, in contrast, such as potential direct and indirect costs for applicants and regulatory authorities, were not in the forefront of considerations at this stage.

The protection level provided by a specific methodology for mixture risk assessments was considered to be “adequate”, if it appears to be equivalent to the corresponding safety requirements and risk assessment principles that have been established for single substances under the PPP or biocidal product regulations.

For comparative considerations of different implementation options in a specific regulatory setting, an array of questions must be addressed. Basically, they can be structured into four main topics, which, at least in part, are mutually dependent:

(i) *Definition of the mixture of concern*

Are the nature, the number and the concentration ratio of mixture components well defined? Or is it the task to assess a complex mixture where the composition is not or only insufficiently known? Which data and knowledge about the mixture and its

components must be available, so that a certain implementation option becomes applicable at all?

(ii) *Output requirements: specification of indicators for mixture toxicity and cumulative risks*

Are there specific well-defined indicators or descriptors of hazards or risks of chemical mixtures that shall be determined? And if so, which indicators are feasible to be determined in a specific regulatory setting? Important examples for such indicators and assessment criteria that are frequently used in the regulatory practice are effect concentrations (ECx, in particular EC50), NOECs, and risk quotients such as PEC/PNEC or TER. What are the specific endpoints for which such indicators shall be determined? Which of the available methodologies are at all suited to estimate such indicators for mixtures?

(iii) *Requirements concerning safety and reliability of assessments*

What are the accuracy and the precision that is required in estimating toxicity or risk indicators for mixtures? Which margins of error could be tolerable or not tolerable in specific regulatory context? Are the same margins of error acceptable for both over- and underestimations of hazards and risks? Or is it more important to ensure safety from unwanted combined effects than to avoid any erroneous over-estimation of mixture risks?

(iv) *Input requirements: necessary data and knowledge*

Which data and knowledge must be available for an assessment, if specific hazard or risk indicators (point ii) shall be estimated for a given type of mixture (point i) with an acceptable degree of safety or uncertainty (point iii) and with a given methodology? Or vice versa, what kinds of assessment criteria (point ii) can be determined for what kinds of mixtures (point i) by means of a specific methodology, if a concrete set of input data is given, and what is the resulting safety or uncertainty of the assessment (point iii)?

4.2 Experimental determination of mixture toxicity (*Whole Mixture Approach*)

Suitability for the determination of hazard and risk indicators

By experimental testing of dilution series of a given mixture of substances, both effect concentrations and NOEC values can be determined in the same way as this is usually done for single chemicals. Risk quotients for mixtures can be derived from such experiments, if in the exposure scenario of concern the concentration ratio of mixture components is exactly the same as it was in the experiments. Therefore, this approach is suitable for the assessment of acute toxicity in the case of direct contact with a given mixture of chemicals, such as a biocidal product or a tank mixture of PPPs for instance, or a complex environmental sample, such as a sewage treatment plant effluent for example.

Data and knowledge requirements

The mixture is examined just like a single chemical. Therefore, no knowledge about the composition of the mixture is required. Neither the nature, nor the number nor the concentration ratio of components must be known. Furthermore, neither toxicity data for

individual constituents nor any knowledge about their modes and mechanisms of action must be available.

Safety and reliability of assessments

Safety and reliability of resulting assessments do not differ from single substance assessments. Due to its applicability without any presuppositions regarding both the composition of the mixture and the modes of joint action of its components, whole mixture testing is

- the only safe way to determine the toxicity of complex mixtures with unknown or incompletely known composition, and
- the only safe way to determine synergistic or antagonistic joint effects that may result from toxicokinetic or toxicodynamic interactions of known mixture components.

Practical limitations

The experimental testing can only be performed for selected concentrations and concentration ratios of mixture components, irrespective of whether these are known or not known. As a consequence of distribution and transformation processes in the environment, however, the mixture to which non-target organisms may become actually exposed is only conditionally comparable with the original composition of the respective PPP or biocidal product.

In addition, if the scope of the assessment is not limited to a single product but includes mixtures that may result from the joint or sequential application of different products, then astronomically large numbers of different possible combinations of ingredients of these products can easily result. From 100 different chemicals, for instance, already more than 10 to the power 23 different combinations of substances can be generated. Furthermore, the concentration ratio and the total concentration of components of these mixtures can vary infinitely. Thus, from the start, the practical feasibility of the experimental whole mixture approach is severely limited by the laws of combinatorics. In addition, economic aspects of time and costs, as well as ethical aspects of animal welfare come into play here, both arguing against additional experimental testing of mixtures within the regulatory framework of risk assessments for PPPs and biocidal products. Thus, the experimental approach must necessarily remain confined to selected samples and cases.

Consequences

From the consideration of this background, it becomes clear that the option of experimental whole mixture testing is no real general alternative to the option of the modelling approach, but it should rather be considered as a complementary tool, that can be applied in specific circumstances for specific purposes. In particular, experimental whole mixture testing should be employed in situations, where well-founded suspicions for synergistic interactions require clarification, or where results of predictive modelling are considered to require experimental validation because they indicate unacceptable risks.

4.3 Modelling of the expectable toxicity of a chemical mixture (*Component-Based Approach*)

At least in principle, the component-based modelling approach offers a reasonable chance to achieve realistic hazard and risk assessments of chemical mixtures without additional animal experimentation and with relatively low expenditures in terms of both time and costs. In accordance with the terms of reference, it was therefore a core objective of this project to examine the usability and the effectiveness of this option.

Suitability for the determination of hazard and risk indicators

Basically both concepts, CA and IA, allow to calculate expectable effect concentrations for mixtures that contain individual substances in a given concentration ratio (see Tab. 3.1). In principle, calculations of expectable effect concentrations of a mixture can be performed for any effect level X and are not confined to the EC50 of a mixture (X = 50 %), provided that the necessary input data are available (see below). With no change in input requirements, these calculations can easily be done for any concentration ratio of mixture components. This is the big advantage over experimental testing.

As expectable effect concentrations of mixtures can be calculated for any mixture ratio, it is hence also possible to calculate risk quotients for any exposure scenario that defines concentrations and concentration ratios of mixture components. If a concentration additive joint action of mixture components is assumed, the formula for concentration addition can be directly transformed into a risk quotient for mixtures. The algebraic equivalent of concentration addition usually used for this purpose is the so-called *toxic unit summation* (TUS) (Sprague 1970), also denoted as *sum of toxic units* (STU) (Backhaus & Faust 2012). It is defined by the equation

$$TUS = \sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i} \qquad [4.1]$$

wherein c_i denotes the concentrations of individual substances in a mixture scenario or in a real existing mixture; ECx_i indicates equi-effective concentrations of single substances *i* (i = 1...n), such as the EC50 values of mixture components for example. The quotients c_i / ECx_i have been named *toxic units* (TU). Toxic units represent a scale transformation of the concentration variable in a concentration response function. Absolute concentrations of substances are set in relation to their toxic potencies. To this end they are expressed as fractions or multiples of equi-effective single substance concentrations. In practical applications, X = 50 % ($EC50_i$) is most often used as the reference value. However, in principle STU values can also be calculated for any other effect level X, provided the corresponding single substance data are available. If the sum of toxic units is one (STU= 1), the total effect of the mixture is expected to equal the value X. If the sum of toxic units is larger or smaller than 1, the total effect is expected to be larger or smaller than X, respectively.

For risk assessments of mixtures under the European PPP regulation, the summation of so-called TER values (toxicity exposure ratios) on the basis of EC50 values has been suggested as an indicator. Mathematically, this is simply the reciprocal of the corresponding STUTUS. Toxicologically, however, this is strictly speaking only true, if the EC50 values that enter the calculation refer to identical toxicity endpoints, determined under identical test and exposure conditions. Otherwise, it is an approach that is based on the assumption of concentration additivity, but which should not simply be equated with CA (see section 4.5).

The concepts for component-based mixture toxicity modelling operate with effects and effect concentrations as input and output parameters. Therefore, a prediction of NOEC values for mixtures, in the meaning as they are usually derived from experimental test data, is not possible, at least not with the original mathematical formulation of the concepts. Nevertheless, estimates of expectable NOEC values for mixtures can be calculated, if simplifying additional assumptions are made. Corresponding extrapolation procedures that have been suggested for regulatory purposes are all based on the assumption of a concentration additive joint action (see section 4.5), not on independent action.

Data and knowledge requirements

- *Exactly defined mixture*

  In contrast to the experimental whole mixture testing, the modelling approach requires an exact definition of the composition of the mixture of concern: nature and number of mixture components as well as their concentration ratio must be defined.

- *Single substance toxicity data*

  For all chemicals that are present in a mixture, data on their individual toxicity must be available. This is the second important difference between the modelling and experimental approach.

- *Common (eco)toxicological endpoint*

  Both concepts for prediction, concentration addition and independent action, require the definition of a common (eco)toxicological endpoint to which all mixture components contribute, be it by assuming similar modes and mechanisms of action (CA), or by dissimilar ones (IA). What is calculated is the intensity or frequency of an effect, which in principle could also be caused by each of the single substances, if present in sufficiently high concentrations. As a consequence, the individual toxicity data that are entered into the equations for calculating their joint toxicity must refer to the same endpoint, ideally determined in the same assay under identical conditions of exposure.

  For pragmatic reasons, mixture extrapolation approaches that have been proposed for regulatory purposes deviate more or less from this demand for strictly identical test endpoints. Thereby, an additional source of errors is introduced into the modelling approach. Hence, the question arises, whether and how the magnitude and the direction of potentially resulting prediction errors could be estimated (see section 4.5)?

- *Concept-dependent data requirements*

  The exact data requirements for the prediction of effect concentrations of mixtures are considerably different for CA and IA. In general, calculations under the assumption of an independent joint action of mixture components necessitate much higher data requirements than applications of the formula for concentration addition.

  If concentration addition is assumed, requirements for the necessary single substance toxicity data are only determined by the effect level (e.g. EC50) or the effect range (e.g. EC20 to EC80) for which effect concentrations of mixtures shall be predicted. Under the assumption of IA, in contrast, data requirements additionally depend directly on the concentration ratio of mixture components, and hence indirectly also on the number of mixture components. In all, the data requirements for calculations of IA

increase with decreasing concentration shares of mixture components (p_i) and with an increasing number of mixture components (n).

Data requirements under the assumption of concentration addition

If a concentration additive joint action is assumed, the prediction of effect concentrations of mixtures necessitates that equivalent effect concentrations of single substances are put into the formula (Tab. 3.1). If, for instance, EC50 values are available for all mixture components, the expectable EC50 of the mixture can be readily calculated. Correspondingly, for the calculation of EC10 values of a mixture, EC10 values of single substances are required, and so on. As mentioned before, these calculations of expectable effect concentrations can be done for any concentration ratio of mixture components, without any change in these requirements for necessary input data.

Data requirements under the assumption of independent action

Generally higher data requirements of the concept of independent action result from the fact that the concept does not operate with effect concentrations (ECx_i) but with the intensity or frequency of individual effects ($E(c_i)$). Therefore, no explicit expression can be formulated for the effect concentration of a mixture (ECx_{mix}) that is expectable under the assumption of IA, but the corresponding equation (Tab. 3.1) must be solved numerically in an iterative procedure. For this process it is absolutely necessary, that the effects of single substances can in each case be determined for exactly that individual concentration ($p_i \times ECx_{mix}$) which is present in a mixture that is expected to cause the total effect X under the assumption of IA.

In general, these complicated conditions mean that concentration response functions (F_i) must be available for all the individual toxicants that are present in a mixture. These functions must provide valid estimates of single substance effects in relevant concentration ranges. These relevant concentration ranges, as well as the corresponding individual effects, become lower and lower with decreasing ratios between individual concentrations and the total concentration of mixture constituents. However, the smaller the individual effects are that are entered into the calculation, the higher are the statistical data requirements that have to be met for a proper estimation of such low individual effects.

The average ratio between individual concentrations and the total concentration of mixture constituents decreases with an increasing number of mixture components. As a consequence, the data requirements for a valid use of the IA model increase. This can for the example be illustrated for a situation where the individual effects of the constituents contributing to the total effect of a mixture are assumed to be identical. IA would predict a total effect of 50 %, if two substances are combined in concentrations that would each cause around 30 % individually. If the number of mixture components is increased to 10, however, the same total effect of 50 % is already expected to occur, if the individual effects entered into the IA formula each have a value of only 6.7 %. For these ranges of individual effects, valid estimates of corresponding effect concentrations are required. If this requirement cannot be met, the IA concept cannot provide valid predictions of mixture toxicity. With multi-component mixtures with high numbers of constituents, these data requirements may often be unfulfillable in practice.

Data requirements under the assumption of a mixed model

Mixed models which calculate estimates of multi-component mixtures toxicities by a combination of CA and IA have the same data requirements as the basic concepts, in addition to that, however, detailed knowledge on the modes and mechanism of actions of all mixture components is needed, allowing to separate them into independent sub-groups of similarly acting substances.

Safety and reliability of assessments

Comparative analyses of the predictive value of both concepts, concentration addition and independent action, have been performed in systematic ecotoxicological test series in the lab with sample mixtures that included up to 50 components. The evidence resulting from these experimental studies can be summarised as follows[5]:

(i) For a given combination of toxicants, the assumption of CA usually results in the prediction of a higher mixture toxicity than the alternative hypothesis of IA. This means, if CA is assumed, the risk to underestimate the actual mixture toxicity is usually lower than with the alternative assumption of IA.

(ii) Concentration addition indeed provides quite reliable estimates of the toxicity of multi-component mixtures that are composed from substances that share a common mode of action. This holds true for both, groups of substances that specifically interfere with a common molecular target site, as well as for groups of unspecifically acting environmental chemicals, in particular non-polar organic chemicals that exhibit so-called “base line toxicity” via a so-called “narcotic” mode of action.

(iii) Independent action has a high prognostic value in the case of multi-component mixtures of substances that all have strictly different specific molecular mechanisms of action.

(iv) For heterogeneous mixtures of substances with partly similar and partly dissimilar specific or unspecific or unknown modes of action, it seems reasonable to assume that they usually show an intermediate toxicity within the “prediction window” that is delimited by the predictions that can be derived from the alternative concepts of CA and IA.

(v) According to all empirical evidence, joint actions that are much stronger or much weaker than expectable by either of the two concepts, i.e. clear synergistic or antagonistic effects, are obviously exceptional situations and not at all the rule. For multi-component mixtures of substances at low effect concentrations (at or near NOAEL) synergistic toxicities that exceed the level predicted by CA by more than a factor of 4 have not been observed (Boobis et al. 2011).

[5] See chapter 3 and the reviews given by Kortenkamp et al. (2009) and Backhaus et al. (2010)

Selecting a concept for prediction

The outlined status of empirical findings alternatively offers three basic options for the development of regulatory modelling approaches to ecotoxicological mixture toxicity assessment:

- case by case selection of the most appropriate concept or use of a "mixed model",
- general use of IA as a default assumption, or alternatively
- general application of CA as a guiding rule for predictive hazard and risk assessments of chemical mixtures.

Case by case selection of the most appropriate concept

A case by case approach accounts for the differences between the available prognostic concepts with their mutually exclusive mechanistic assumptions. The selection of that concept which is expected to provide the most accurate prediction for a given mixture of toxicants must be based on an assessment of the similarity or dissimilarity of the mechanisms of action. For heterogeneous mixtures of partly similarly and partly dissimilarly acting substances, this approach leads to the use of so-called *mixed models*, which assume concentration additive action within sub-groups of mixture components, but dissimilar action amongst these groups.

From a purely scientific perspective, the case by case approach may appear to be optimal. From a regulatory perspective, however, the problem of this approach lies in the need for sound criteria and detailed knowledge for grouping of all relevant substances by their modes and mechanisms of action. Hence, this approach requires sound criteria for a corresponding classification of chemicals. However, knowledge on the mechanisms of action of environmental pollutants is rather scarce, in many cases even completely absent. Active ingredients of PPPs and biocidal products may just represent an exemption from this rule; however, usually the knowledge about mechanisms of action refers to the intended effects in target organisms. For non-target organism with a different physiology this knowledge is often not applicable, or with strong limitations only.

Independent action as a default assumption

The use of a single concept as a default assumption may circumvent all the difficulties associated with a proper mechanistic classification of toxicants. In particular in the field of human toxicology, many authors tend to advocate for IA as an appropriate default assumption (see Streffer et al. 2000, chapter 2; Kortenkamp et al. 2009). The argument is that competitive interaction with a common molecular receptor site may be an exception but not the rule for toxicants occurring jointly in the environment. Typically their modes and mechanisms of action may differ.

On the other hand, IA has been demonstrated to provide accurate predictions of multi-component mixture toxicity only in such experimental cases where all mixture components were well known to interact specifically with strictly different molecular target sites. Under realistic environmental exposure scenarios, however, such an ideal-typical situation might be a rare situation too.

Typically, organisms may be confronted with heterogeneous mixtures of specifically acting toxicants, non-specifically acting chemicals, as well as multi-site inhibitors. Existing experimental evidence gives good reasons to assume that such mixtures may often be more potent than predicted by IA. Thus, the use of IA as a default assumption might bear a strong

risk of underestimating the actual mixture toxicity. This clearly conflicts with the paradigm of making *realistic worst case assumptions* in regulatory risk assessments.

Another counter argument comes from the practical limitations already mentioned before: the proper application of IA for multi-component mixtures requires reliable statistical estimates of low toxic effects of individual mixture constituents in low concentration ranges. Data typically generated for regulatory purposes in standard eco-toxicity assays do hardly meet this requirement.

Concentration addition as a default assumption

Finally, CA may be considered as a general default assumption, irrespective of modes of toxicant action and the insufficient knowledge about it. This option may be justified as a pragmatic approach under guidance of the precautionary principle, because

(i) CA is a more conservative approach than IA, clearly bearing a lower risk of underestimating the actual mixture toxicity, and because

(ii) data requirements for a proper application are much easier to fulfil, while on the other hand,

(iii) synergistic effects exceeding the CA expectation occur only exceptionally.

With these arguments, concentration addition has been recommended as a reasonable worst case assumption for the purpose of regulatory hazard and risk assessments (Bödeker et al. 1993, Kortenkamp et al. 2009). Similarly, the recently published WHO/IPCS framework (Meek et al. 2011) suggests dose addition as a default tier zero assumption for all components co-occurring in an exposure scenario and potentially contributing to a common adverse health outcome in humans.

The main counter argument against this approach is that unsound mechanistic assumptions may potentially result in vastly over-protective mixture toxicity assessments, conflicting with the principle of proportionality in the regulatory management of chemicals risks. However, the existing empirical evidence does not support this concern. In published experimental studies on multi-component mixture toxicity the quantitative differences between both predictions, IA and CA, have been reported to be remarkably small, at least from a regulatory perspective. For different types of mixtures with up to 20 components, predictions of EC50 values derived from the two concepts differed by no more than a factor of five (Kortenkamp et al. 2009).

To put this empirical margin of prediction differences into context, it may be noted that it appears to be relatively small in comparison to other uncertainties in toxicity estimates that are usually handled in the regulatory assessment and management of chemical hazards and risks. Experience from ring trials has shown considerable variability in single substance toxicity data from standardized assays with the same species. As a consequence, if not differing by more than a factor of five, ecotoxicity data for active ingredients of PPPs in technical purity have long been considered to be regulatory equivalent (EC DG-SANCO 2005). However, in a recent update of that guideline, the factor accounting for the variability of ecotoxicological test results has been reduced from 5 to 3 (EC DG-SANCO 2011a).

In addition to that, enormous uncertainties are routinely bridged in the next step, when it comes to the necessary extrapolation from such laboratory data to real environmental conditions and to other species or populations. Typically, this is done by applying standard assessment factors of 10, 100, or 1000. Compared to that, potential errors in extrapolations from single substance to mixture toxicities that do not exceed a factor of 5 may be regarded as

being negligible for regulatory decision making. Accordingly, if indeed not being substantially larger, over-estimations of actual mixture toxicity that may result from the suggested use of CA as a default approach could be judged to be not disproportionate and hence acceptable.

However, it may be questioned, whether the available observations of relatively small prediction differences do really represent typical situations in ecotoxicology. Their validity may be suspected to be restricted to the special mixtures, conditions and toxicity endpoints which have actually been tested. This argument cannot be sufficiently invalidated by further experimentation only. Additionally, other complementary approaches are needed in order to strengthen or falsify the view that the default assumption of CA is a reasonable and scientifically sound approach, and not a disproportionate and vastly over-protective one. Otherwise, a consensus about acceptability or non-acceptability of this implementation option might not be achievable.

This leads to the principal question, which are the factors that determine the quantitative differences between IA and CA? And, how large are the quantitative differences that may occur in dependence from these factors under realistic assessment situations?

4.4 Prediction differences between IA and CA

Problem formulation

The two basic concepts for predictive mixture toxicity assessments, CA and IA, are associated with mutually excluding assumptions about modes and mechanisms of toxicant action: similar action in case of concentration addition and dissimilar action in case of independent action. This categorical distinction may evoke false expectations about the quantitative differences between both predictions. Intuitively, it may be assumed that mutually excluding mechanistic assumptions should result in clearly different predictions, also mutually excluding each other as being correct or incorrect for a given mixture.

This, however, is a wrong connotation. In fact, *a priori* there is no fixed relation between the predictions of mixture toxicity that may be derived from the application of the two alternative formulas. The effect concentration of a mixture calculated under the assumption of concentration addition (ECx_{CA}) can be smaller, equal to, or larger than the corresponding alternative prediction based on the assumption of independent action (ECx_{IA}) (Drescher and Bödeker 1995). Thus, the question arises:

- What is the quantitative error that may result, if one of the concepts is erroneously applied in a situation, where in fact the other one would provide the correct mixture toxicity estimate?

A quantitative measure for prediction differences between IA and CA

In the literature, it is often explicitly or implicitly assumed that CA predicts lower effect concentrations than IA, i.e. a higher mixture toxicity. The fact, that at least theoretically also the reverse situation may occur, is rarely acknowledged. However, as a rule, all available empirical evidence indeed supports the assumption that CA typically predicts a higher mixture toxicity than the alternative hypothesis of IA (see section 4.3). Practical relevance has also been demonstrated for situations in which both concepts provide almost identical predictions and which both are in agreement with the actually observed mixture toxicity (e.g. Backhaus et al. 2004). To our knowledge, however, there is no convincing experimental example, where IA does not only predict a significantly higher toxicity than CA, but where

this prediction is indeed also the more accurate one. Thus, the practical relevance of this theoretically possible situation has not been demonstrated.

Given this empirical background, the differences between both predictions are in the following described by means of the ratio

$$ECx_{IA} / ECx_{CA}$$

This quotient gives the factor by which the effect concentration predicted by IA (ECx_{IA}) differs from the corresponding prediction based on CA (ECx_{CA}). In the case of identical predictions, the ratio is 1. In the typical case, where IA predicts a relatively lower toxicity than CA, the ratio is larger than 1. In the opposite situation it takes values between 0 and 1. In other words:

- The ratio ECx_{IA} / ECx_{CA} gives the factor by which concentration addition over-estimates the actual mixture toxicity in a situation where independent action would in fact provide the correct estimate.

Factors determining the ratio between IA and CA

The general mathematical formulations of IA and CA can be transformed into expressions for the prediction of effect concentrations of mixtures (Tab. 3.1). With the resulting two formulae, the parameters that determine the ratio ECx_{IA} / ECx_{CA} are completely defined. These are simply the variables for which input data have to be entered into the formulae for obtaining the alternative mixture toxicity predictions ECx_{IA} and ECx_{CA}. There are four crucial factors:

- the number of mixture components n,
- the slope of the individual concentration response curves, defined by the concentration response functions F_i ,
- the concentration ratio of mixture components $p_1 : p_2 : ... : p_i$, and
- the effect level X under consideration.

Maximal prediction differences

Although the ratio ECx_{IA} / ECx_{CA} is fully determined by these four parameters, it is unfortunately not possible to express it as an explicit function of these parameters. This nasty situation results from the fact that the effect concentration of a mixture that is predicted by IA (ECx_{IA}) is only given by an implicit expression that cannot be turned into an explicit function. Hence, it is also impossible to set up an explicit equation for the ratio ECx_{IA} / ECx_{CA}.

Nevertheless, it is possible to examine the ratio ECx_{IA} / ECx_{CA} for the existence of limit values. Thereby the question can be answered, whether the ratio ECx_{IA} / ECx_{CA} may take any value, or whether extreme values exist that cannot be exceeded under a given constellation of determining factors?

As a result, such mathematical analyses have demonstrated the following:

- The ratio between predictions of effect concentrations of mixtures derived from the alternative concepts of IA and CA (ECx_{IA} / ECx_{CA}) cannot take any value, but it is generally confined to a possibility space that is delimited by 0 and n:

$$0 \le ECx_{IA} / ECx_{CA} \le n , \qquad [4.2]$$

with *n* denoting the number of mixture components. The corresponding mathematical proof was first given in Faust 1999.

The practical meaning of this finding is that the ratio may become infinitely small, at least theoretically, but that it can never exceed an upper limit that is simply given by the number of mixture components. For binary mixtures a maximal possible value of 2 can never be exceeded. For mixtures with up to 10 components, it cannot become larger than an order of magnitude. In case of multi-component mixtures with huge numbers of constituents, however, the ratio may also become very large, at least theoretically and if no further restrictions apply.

- The maximal value of *n* can only occur, if the components of a mixture are all present in so-called "equi-toxic" concentrations, more precisely in equal fractions of equi-effective concentrations. Or in other words, if the toxic units of all mixture components are identical. In any other case, the ratio is always smaller than *n*, whereby the maximal possible value is defined by ratio between the sum of toxic units and the highest toxic unit for a single substance in the mixture:

$$0 \le \frac{ECx_{IA}}{ECx_{CA}} \le \frac{\sum_{i=1}^{n} TU_i}{\max\limits_{i\in(1,\ldots,n)} \{TU_i\}} \le n \qquad [4.3]$$

The corresponding mathematical proof was given in Junghans et al. 2006.

This has important practical implications for situations where only one or a few compounds dominate a mixture in terms of toxic units. For example, if one single mixture component already contributes 50 % to the total sum of toxic units, then the ratio ECx_{IA} / ECx_{CA} can never exceed a value of 2, no matter what the total number of mixture components and their toxic units may be.

- If the concentration ratio of mixture components is not fixed, and hence no other general limit values apply than *n*, then the ratio ECx_{IA} / ECx_{CA} tends towards zero, if the individual concentration response curves of all mixture components become infinitely flat, and it tends towards the maximal possible value of *n* (= number of components), if all these curves become infinitely steep. The ratio takes the value of 1, i.e. both predictions are exactly identical, if the concentration response curves of the mixture components can all be described by the following special form of the Weibull function (Drescher and Bödeker 1995):

$$E(c_i) = 1 - \exp(-\exp(\alpha + \log_e(c_i))), \qquad [4.4]$$

wherein α is a location parameter with no influence on the ratio ECx_{IA} / ECx_{CA}.

The last mentioned point, i.e. the finding that the steepness of concentration response curves is a crucial limiting factor for the maximal possible prediction differences between IA and CA, leads to the question:

- What are realistic scenarios for the steepness of all the individual concentration response curves in a mixture, and what may be the resulting limitations of the ratio ECx_{IA} / ECx_{CA}?

As a contribution to the work on this question, the EU project BEAM explored the potential range of practically relevant prediction differences by means of computer simulations for three different standard ecotoxicity assays with algae, daphnids and fish (Faust and Scholze 2003). On the basis of representative sets of concentration response data for a large variety of different chemicals, maximal possible prediction differences were calculated for mixtures

with up to 100 components that could be generated from these substances. As a result, these simulations confirmed that relatively small prediction differences between IA and CA are no singular chance findings of some experimental studies, but represent a typical situation. For the inhibition of algal population growth as well as for the immobilisation of daphnids, prediction differences that exceed an order of magnitude were shown to be highly unlikely to occur, typically they are much smaller. In case of acute fish lethality as an assessment endpoint, however, this holds only true for mixtures with a maximum of twelve components. With larger numbers of components, large prediction differences between IA and CA may occur for certain concentration ratios of mixture components. The causes of this phenomenon are extremely steep concentration response curves that have been reported for many organic chemicals in acute fish toxicity assays.

In general, however, the combined evidence about quantitative prediction differences, resulting from experimental studies, mathematical analyses and computer simulations, supports the use of concentration addition as a pragmatic, precautious and not overly conservative default approach to the predictive assessment of ecotoxicological hazards and risks of chemical mixtures. If a lower protection level is chosen, this should be justified by specific toxicological knowledge about the mixture of concern.

4.5 Prediction differences between the original concept of concentration addition (CA) and pragmatic CA-based approaches

As outlined in section 4.3, data and knowledge requirements of the modelling approach are usually lower for predictions based on CA than for predictions of independent joint actions. Nevertheless, for the regulatory practice they may still be unfulfillable. As a consequence, for regulatory use, a number of pragmatic approaches have been derived from the original CA concept. Partly they have already become established procedures under specific pieces of legislation in the EU or in the US. A selection of some prominent examples is given in Tab. 4.1.

A common feature of these approaches is that they basically make use of the CA formula as a calculation rule, but either they use input data that deviate more or less from the strict requirements of the original concept, or they make additional simplifying assumptions about the individual concentration response curves of mixture components, or both. In the following considerations, they are therefore collectively denoted as *CA-based* approaches.

Two questions are addressed in this section: (i) what types of simplifications and/or additional assumptions are explicitly or implicitly introduced by CA-based approaches? And (ii), what could be the resulting quantitative prediction differences between the pragmatic CA-based approaches and the original CA concept?

Table 4.1 Examples of pragmatic generalizations of CA for regulatory purposes

Approach	Assessment term		Notes
CA *Concentration (Dose) Addition*	$E_{mix} \leq x$ if	$\sum_{i=1}^{n} \frac{c_i}{ECx_i} \leq 1$	$\frac{c_i}{ECx_i} = TU_i = ToxicUnit$
PODI *Point of Departure Index*	No significant effect if	$\sum_{i=1}^{n} \frac{EL_i}{POD_i} \leq 1$	EL = Exposure Level POD = LOEL, NOAEL, NOEC
HI *Hazard Index*	No reason for concern if	$\sum_{i=1}^{n} \frac{EL_i}{AL_i} \leq 1$	EL = Exposure Level AL = Acceptable Level = ADI, DNEL, …
PEC/PNEC Summation	No unacceptable risk if	$\sum_{i=1}^{n} \frac{PEC_i}{PNEC_i} \leq 1$	PEC = Predicted Environmental Concentration PNEC = Predicted NEC

Basic types of CA-based approaches

When comparing CA-based approaches with the basic assumptions and requirements of the original scientific concept, four main types of pragmatic deviations or simplifications can be seen:

(i) *Not strictly identical toxicological endpoint*

Regulatory requirements for the experimental characterisation of certain hazardous properties of chemicals, such as fish toxicity for instance, usually leave some room regarding the selection of test species and exposure conditions and the exact testing criteria and methodologies, and they are subject to changes in adaptation to technological progress. As a result, typically only heterogeneous data sets may be available for a component based mixture risk assessment.

As a consequence, pragmatic regulatory CA-based approaches cannot do otherwise than using single substance toxicity data that do not refer to strictly identical toxicological endpoints under identical exposure situations. In principle, this kind of deviation from the original concept is therefore included in almost all suggested CA-based approaches, explicitly or implicitly. The degree of the deviation, however, varies considerably. In the mildest form it may only mean to use data for different test conditions or endpoints in the same species. A step further go approaches that aggregate toxicity data for different species within taxonomic groups (such as algae for instance) or which include the use of data from *higher-tier* multi-species studies. And the most pragmatic way is to go across all borders between endpoints and species by simple summing up all kinds of PEC/PNEC ratios, an approach that has been repeatedly suggested for the derivation of water quality objectives for mixtures of chemicals (Calamari and Vighi 1992, Vighi et al. 2003).

(ii) *NOEC instead of ECx*

No observed effect concentrations (NOEC) or *no observed (adverse) effect levels* (NO(A)EL) are well established descriptors of substance toxicities in chemicals regulation. Despite a long lasting debate about the shortcomings of the NOEC concept and the replacement of NOEC values by so-called *benchmark concentrations* or doses, NOECs and NO(A)ELs continue to play a central role. Hence, this is the type of single substance toxicity data that is currently widely available as a potential input for component-based mixture toxicity assessments. And on the output side, it appears also attractive to get indicators of mixture toxicity that have the same format as single substance data and can be handled in the same way.

As a consequence, many suggested CA-based approaches use NOEC or NO(A)EL values instead of effect concentrations (ECx) as input variables, and correspondingly they generate estimates of NOEC or NO(A)EL values as output. Thus, this is the second important type of pragmatic alterations of the original CA concept.

Examples from the field of human mixture risk assessment are the so-called *point of departure index* (PODI) (Wilkinson et al. 2000), which calculates either with NO(A)ELs or with benchmark concentrations or doses (BMD), and the so-called *margin of exposure approach* (MOE). A corresponding approach from the field of ecotoxicological risk assessment is the summation of so-called NOEC-based TER values (toxicity exposure ratios), which has been suggested for use under the PPP regulation (see section 7.1.3). MOE and the summation of NOEC-based TER values refer to different regulatory arenas. In principle, however, they both simply represent the reciprocal of a corresponding PODI.

NOECs are usually defined as the highest tested concentrations at and below which the toxicity parameter under observation does not depart in a statistically significant way from control values. Hence, NOEC values denote concentrations for which low effects can neither be quantified nor safely excluded to occur. In ecotoxicological standard tests, effects smaller than 10 % can usually not reach statistical significance. NOEC values determined in such assays typically correspond to effect levels between 10 and 30 % (Moore & Caux 1997), depending on the experimental and biological variance, the number and spacing of test concentrations, and the number of replicates and controls. The worse the data situation, the higher is the resulting NOEC.

Given this background, the replacement of ECx values with NOECs in the CA formula and the resulting estimation of NOECs for mixtures can only be justified with a simplifying pragmatic assumption, that is to say that NOEC values are approximately equivalent to a uniform low effect level X, such as an EC10 for instance.

This leads to the question, what the magnitude of quantitative errors might be, that can be expected to result from this simplifying approach? For the time being, no well-founded answer can be given. The problem has not yet been systematically addressed in the literature and needs further investigations.

(iii) *Extrapolation factors included in single substance toxicity data*

For the purpose of deriving regulatory *acceptable levels* (AL), experimental effect concentrations or NOEC or NO(A)EL values are usually multiplied with so-called

assessment factors, *uncertainty factors*, or *extrapolation factors*. In general, they aim to take account of potential sensitivity differences between individuals and between species and to cover differing exposure conditions. In the specific context of ecotoxicological assessments under REACH, they shall address the uncertainties arising from (i) intra- and inter-laboratory variation of toxicity data, (ii) intra- and inter-species variations (biological variance), (iii) short-term to long-term toxicity extrapolation , and (iv) laboratory data to field impact extrapolation (ECHA 2008, p.17). 100 is generally said to be the standard extrapolation factor, but depending on the data situation and the specific regulatory context, the actually applied factors may indeed vary between 1 and 10 000.

Some CA-based approaches enter such *acceptable levels* of individual toxicants into the CA formula with the aim to derive a corresponding estimate for an acceptable level of a mixture. A prominent example for such an approach is the so-called *hazard index* (HI) (Teuschler and Hertzberg 1995). A practical advantage of this approach is that AL values may be readily available to regulators, while it may be demanding to retrieve the original toxicological data behind them. However, with extrapolation factors being included in the input data, a third source of potential prediction differences between the original CA concept and the pragmatic CA-based approach is introduced. As an alternative to this approach it is therefore discussed to calculate first a mixture toxicity indicator that does not include any assessment factors, such as the PODI for instance, and then to apply a single assessment factor to the calculated mixture toxicity value, just as it is done in single substance assessments (Wilkinson et al. 2000). If the extrapolation factors that are included in AL values are the same for all mixture components, both approaches yield the same result, otherwise they differ. The question how large these prediction differences could be has not yet been systematically investigated in the literature.

(iv) *Assumption of parallel concentration response curves: the equivalency factor concept*

The so-called equivalency factor concept is a fourth type of regulatory approaches that can be regarded as being equivalent to the CA concept, if additional simplifying assumptions are made. The approach means that the concentrations of all relevant mixture components are expressed in terms of an equivalent concentration of an index component. The result is identical with the assumption of CA, if the concentration response curves of all mixture components can be assumed to be parallel.

Examples for the application the equivalency factor concept are the so-called *relative potency factor* (RPF), and the so-called *toxic equivalence factor* (TEF) which is a special case of the RPF.

Also in this case the question arises, how large the quantitative prediction differences by CA and the *equivalence factor* approach could be, if the explicit or implicit assumption of parallel concentration response curves is not fulfilled in reality? And again it has to be stated that systematic investigations on this point are missing.

Summary of factors determining the prediction differences between CA and CA-based approaches

In principle, all CA-based approaches have one thing in common: as a replacement for actually equivalent effect concentrations (or doses) of single substances (ECx_i^{actual}) surrogate values are entered into the concentration addition formula, which are assumed to give an acceptable approximation of equi-effective concentrations ($ECx_i^{surrogate}$).

In summary of the above outlined typology of pragmatic simplifications and assumptions that can be found in suggested CA-based approaches, either alone or in combination, the following types of such assumed approximations of equi-effective concentrations (or doses) ($ECx_i^{surrogate}$) can be distinguished:

- ECx values for not strictly identical endpoints or differing testing conditions,
- NOEC values which may be assumed to represent low equi-effective concentrations (e.g. EC10),
- extrapolated ECx, NOEC, NO(A)EL or PNEC values which may include differing extrapolation factors,
- ECx values that have been derived from a reference compound under the assumption of parallel concentration response curves, and
- values generated by any possible combination of these approximation procedures.

If such surrogate data for equi-effective concentrations ($ECx_i^{surrogate}$) are entered into the CA formula, the result consequently is an assumed approximation of an equi-effective concentration of the mixture that would be expectable under the assumption of a concentration additive joint action ($ECx_{CA}^{approximative}$). With this notation, the potential prediction difference between the original CA concept and CA-based approaches could be defined by the quotient

$$ECx_{CA}^{\text{approximative}} / ECx_{CA}^{\text{actual}}$$

Systematic investigations into all the determining factors and the resulting magnitude of this ratio are missing. However, specific examinations of the differences between PEC/PNEC summations for all aquatic organism and toxic unit summations for taxonomic groups of aquatic organism have been conducted (see below). And in addition to that specific case, it is also possible to give a general definition of the maximal possible prediction differences between CA and CA-based approaches as outlined in the following.

Maximal prediction differences between CA and CA-based approaches

Under the given presuppositions and definitions, the question of maximal possible prediction differences can be answered as follows:

- The difference between mixture toxicity predictions derived from the original CA concept and pragmatic CA-based approaches is in no case larger than the highest difference between the actual effect concentration of any of the individual substances in the mixture and the corresponding surrogate data used in the calculation.

The mathematical proof for this proposition is instantaneously given by an often unregarded but highly important feature of the concept of concentration addition:

- Mathematically, the prediction of an effect concentration of a mixture (ECx_{CA}) as it is given by the equation

$$ECx_{CA} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{ECx_i}} \quad [4.5]$$

is nothing else than the weighted harmonic mean of the equivalent effect concentrations of individual substances (ECx_i), whereby the fractional shares p_i, by which the individual substances contribute to the total concentration of toxicants in the mixture, constitute the weighting factors (Scholze et al. 2003). The harmonic mean is rarely used in pharmacology and toxicology, but it is one of the three classic mean values that were already defined in ancient times.

By means of averaging, random errors or systematic errors and uncertainties of individual data are not added up or even multiplied. On the contrary, the aim of calculating mean values is to reduce such errors and uncertainties. This is a fundamental mathematical theorem. In general, the reliability of the result increases with the number of data that are included in the calculation of a mean. In the very worst case, all errors of individual data have the same quantity and direction. Then, the error of the mean would be the same as the maximum error that is inherent to the individual data. In all other situations it is smaller.

From this it follows that

- Quantitative estimates of the maximal possible prediction difference between CA and CA-based approaches can be given if indications are available about the maximal possible differences between actually equivalent effect concentrations of single substances and the corresponding surrogate data that are used in the CA-based approach.

This maximal span of prediction differences may be further confined by other determining factors.

Summation of PEC/PNEC ratios as a justifiable CA-approximation

The summation of PEC/PNEC ratios as an indicator of cumulative risks of toxicants in the aquatic environment is a special example of CA-based approaches. It was originally suggested by Calamari and Vighi (1992) for the derivation of water quality objectives.

Predicted no effect concentrations (PNEC) as defined under REACH and other pieces of EU legislation may be based on toxicity data from different types of aquatic species, typically algae, daphnids, or fish, and different types of endpoints such as acute EC50 or chronic NOEC for instance. As a consequence, PEC/PNEC summation violates the principle that data entered into the CA formula should refer to the same biological endpoint in the same species. Therefore, it could be argued that PEC/PNEC summation should not be used in mixture toxicity assessments.

As an alternative, a summation of toxic units (STU) may be considered, where mixture risk indicators are first calculated for a common endpoint within each taxonomic group of aquatic species only. As a second step, then the most sensitive one is selected and an extrapolated mixture risk quotient for an environmental compartment is calculated by applying an assessment factor, just as it is usually done for single substances.

The STU approach is undoubtedly scientifically more sound but the practical applicability suffers from insufficient data availability, and it may additionally suffer from problems with

agreeing on an appropriate assessment factor in case of inhomogeneous data situations, i.e. if the necessary matrix of toxicity data includes partly acute and partly chronic data for instance.

As a way out of this dilemma, a tiered approach has been suggested where PEC/PNEC summation is used as an initial worst case estimate. Only if this indicates possible reasons for concern, the STU approach, which may require the generation of further data, is recommended as a second step (Backhaus and Faust 2012).

Obviously, such a strategy is only sensible, if the PEC/PNEC summation provides mixture risk indicators that are on the one hand more conservative than the STU approach (or equal), but on the other hand also not vastly over-protective and hence not inacceptable as a reasonable filter. Indeed, it can be shown that both requirements can be fulfilled under realistic assessment situations. For a given data matrix, mixture risk quotients calculated by PEC/PNEC summation (i) can in fact never be smaller than those calculated by the STU approach and (ii) cannot exceed the corresponding STU quotients by a factor that is larger than the number of different species groups and/or endpoints that are included in the calculation. Considering a typical assessment situation where the so-called base set of data (EC50 values for algae, daphnids, and fish) is available under the REACH regulation, this means that in fact the risk quotients derived from both methods cannot differ by a factor of more than just 3, at the maximum. The mathematical proof for these relationships was given in the published supplementary information to Backhaus and Faust (2012).

In view of such quantitative considerations, the European Commission's scientific committees have refined their view on the issue. When drafting their recent opinion on mixture toxicity, they initially just stated that „*a combination of PNECs may be misleading*" (EC DG-SANCO, SCHER, SCENIHR, SCCS 2011c). After public consultation, however, this was rephrased into: *"... a combination of PEC/PNEC ratios is less scientifically correct than the sum of TUs. However, it has been proved slightly more conservative and, in some cases, more easily applicable. Therefore, for pragmatic reasons, it may be used as a first-tier conservative approach*" (EC DG-SANCO, SCHER, SCENIHR, SCCS 2011b).

4.6 The assessment factor approach for safeguarding against combination effects of chemicals

Problem formulation

As explained in the preceding sections, the predictive assessment of the toxicity of chemical mixtures by means of modelling approaches is strictly dependent on two crucial prerequisites. These are

(i) the definition of the mixture of concern in terms of the number and nature of components and their concentrations and concentration ratios, and

(ii) the availability of quantitative information about the individual toxic potencies of mixture components in relation to a common toxicological endpoint.

Depending on the specific assessment and exposure situation, both requirements may be not or at least not sufficiently fulfillable.

The experimental testing of the mixture toxicity (section 4.2) may also be no useful alternative, for instance if a new substance is intended to be released into the environment in the future, but it is unclear how resulting scenarios of co-exposure of organisms to this new substance in combination with other already present pollutants might look like.

In single substance hazard and risk assessments, it is common practice to bridge uncertainties and knowledge gaps by means of so-called extrapolation, assessment, safety, or uncertainty factors. Therefore, it appears to be a self-suggesting idea to apply this approach also to the mixture toxicity problem. Could the risk of unwanted mixture effects not simply be managed by the introduction of a special "mixture uncertainty factor" (MUF) into the standard procedures for single substance assessments?

Such a MUF may be proposed as a very pragmatic way to account for the fact that a single substance that is released into the environment might become part of a multi-component mixture, whose cumulative impact on humans and the organisms in the environment can be higher than that of each individual constituents[6].

Up to no now, however, regulatory applications of this approach do almost not exist. A rare example is the procedure for the derivation of environmental quality criteria in the Netherlands. This procedure includes the application of a factor of 100, which is explicitly defined as safety margin for protection from combination toxicity (van Vlaardingen and Verbruggen 2007, p.109). The factor is used to derive a so-called *negligible concentration* (NC) from a so-called *maximum permissible concentration* (MPC). For ecotoxicological endpoints, the MPC is conceptually equivalent to a PNEC. The 100fold lower NC is the target value, i.e. the guideline for the long-term environmental quality to be achieved.

Before discussing the issue further, it needs to be clearly stated that assessment factors that are currently used under the EU framework of chemicals regulation are in general not meant to actually account for mixture effects. Indeed, in the old Technical Guidance Documents in the section about assessment factors for the derivation of PNEC values for the protection of aquatic wildlife, it was written that "additive, synergistic and antagonistic effects from the presence of other substances may also play a role" (EC 2003, Part II, p. 99). However, in the current guideline for chemical safety assessments under REACH, this statement has been omitted in the context of the justification of assessment factors (ECHA 2008, Chapter R10, p.17).

Thus the question is:

- What would be an appropriate magnitude for a special mixture uncertainty factor, if it should ensure an adequate protection from hazardous combined effects according to the current state of science?

Data and knowledge requirements

At a first glance, it might appear that the *assessment factor approach* could be a way to circumvent all the difficulties that regulators may face when trying to perform mixture hazard and risk assessments by means of *whole mixture approaches* or *component-based approaches*. However, if a scientific justification for an adequate magnitude of such a mixture uncertainty factor shall be given, it turns out that this is again not possible without making reference to both, a cumulative exposure scenario and a suitable model for joint action.

[6] An recent example for such proposals is given in a declaration of the Nordic Council of Ministers (2012) who stated: *"In order to deal with substances that are regulated under different regulatory regimes (e.g. cosmetics, industrial chemicals, pesticides etc.), it is as a starting point proposed to allow only a part (e.g. 10 %) of the "safe dose" within each area of regulation. This would be an easy and cost-effective way to decrease the risk of effects due to cumulative exposure of simultaneous exposure to chemicals with similar effects from different routes, e.g. food, water, and the environment"*. The word "assessment factor" is avoided in this statement, in effect however, the proposed approach is the same as applying an additional factor of 10.

Two questions have to be addressed, whereby the answers are in part mutually dependent:

(i) How could a default mixture exposure scenario be defined, which then could already be reflected in regulatory assessments of single substances by means of a corresponding default *mixture uncertainty factor*?

(ii) Which kind of joint action should be assumed to occur in such a standard scenario: concentration addition, independent action, or a *mixed model*?

Justifying a MUF under the assumption of concentration addition

For reasons that have been explained in detail in sections 4.4 and 4.5, the assumption of a concentration-additive action of mixture components appears to be justifiable as a precautious but not inadequately overprotective regulatory approach. Under this premise, the problem may be specified as follows:

- How large should a mixture uncertainty factor be for safeguarding against unwanted combination effects under the assumption of a concentration additive joint action of mixture components?

The mathematical formulation of the CA concept (Tab 3.1) implies that the expectable overall effect of a mixture will never exceed a certain critical effect level X, if the concentrations of all components are smaller than 1/n times the effect concentration (ECx_i) of each individual toxicant that would cause the same effect x if applied singly. For example, the total expected effect of a mixture of ten compounds (n = 10) will always be smaller than 10 %, if the concentrations of all components are smaller than 1/10 of the corresponding individual EC10 values.

Conversely, the expectable total effect of the mixture is in any case higher than the critical level X, if at least one mixture component is present in a concentration which alone already causes an effect that exceeds the critical level X ($\geq ECx_i$).

If, however, the concentrations of all mixture components (c_i) are in an intermediate range between $1/n$ –fold and 1/1-fold of the equivalent individual effect concentrations ($1/n \times ECx_i \leq c_i \leq ECx_i$), then it depends on three factors whether the assumption of concentration addition results in the expectation of a total effect that exceeds the critical level X. These are:

- the assumed number of mixture components n,
- the assumed mixture ratio $p_1 : p_2 : ... : p_i$, and
- the steepness of the individual concentration response curves as defined by concentration response functions F_i.

As detailed in the preceding section 4.5, pragmatic generalisations of the concept of concentration addition have been developed for regulatory purposes, where so-called *points of departure*, i.e. LOEL, NOAEL, or NOEC values, are used in the calculation instead of effect concentrations. For the derivation of a *MUF*, this approach can be adopted, if the pragmatic simplifying assumption is made that NOECs or other *points of departure* denote a uniform effect level (e.g. 10 %) for a uniform endpoint, such as mortality of specific species under specific test conditions for instance. Given these premises, the aforementioned relationships between single substance concentrations and expectable concentration additive effects of the mixture apply in an analogous way. The resulting conditions under which significant total effects of the mixture are expectable or not expectable, respectively, are summarised in Tab. 4.2.

Table 4.2 Conditions for the occurrence of significant effects under the assumption of a concentration additive joint action of mixture components
Simplifying assumption: NOECs represent a uniform effect level (e.g. EC10) for an identical toxicological endpoint

Concentration of mixture components (c_i)	**Significant effect expectable?**
One or more $c_i > NOEC_i$	YES
$1/n \times NOEC_i \leq c_i \leq NOEC_i$	depends on ▪ n (number of components) ▪ concentration ratio ▪ slope of concentration response curves
all $c_i \leq 1/n \times NOEC_i$	NO

As explained in section 4.5, examples of mixture risk indicators that are based on the adaption of the CA concept for NOEC values or similar *points of departure*, are the *point of departure index* (PODI) of the US-EPA or the summation of NOEC-based TER values under the EU PPP regulation, whereby the TER summation is conceptually equivalent to the reciprocal of the PODI. By definition, these indicators do not give a reason for concern for any statistically significant or regulatory unacceptable mixture effects, if they do not exceed a value of 1 (PODI), or do not fall below a value of X (TER summation), with X being a standard assessment factor as defined by the *Uniform Principles* fixed in Commission Regulation (EU) No 546/2011 (see Tab. 7.21 in section 7.2). According to the conditions defined in Tab. 4.2, this trigger value will never be reached if the concentrations or doses of all mixture components are not larger than the individual POD divided by the number of mixture components ($\leq 1/n \times POD_i$). If the individual concentrations are between 1/n and 1/1 of the individual PODs, it will depend on three factors whether an unwanted level of mixture toxicity is indicated or not:

(i) the number of mixture components,

(ii) their actual concentrations or doses in the chosen exposure scenario, and

(iii) their toxic potencies in terms of the magnitude of individual PODs.

Conclusions

As a consequence of these considerations, a default MUF of *n* may be considered as an appropriate precautious measure for safeguarding against unwanted mixture effects, whereby *n* is the number of chemicals that are assumed

(i) to be simultaneously present in a default exposure scenario and

(ii) to contribute to a common (eco)toxicological endpoint.

This approach could by justified as an adequate precautionary measure for the protection from potential mixture effects. However, the problem then is to establish a consensually acceptable default value for the number of mixture components *n* that may be reasonably assumed to contribute to an overall mixture effect in a default standard scenario.

When discussing this problem, it has to be taken into consideration that the assumption of a concentration additive joint action of mixture components does not necessarily mean that all mixture components contribute equally to the overall effect. On the contrary, there are empirical examples where a few components were shown to dominate the overall mixture toxicity in a realistic exposure scenario while the contributions of all other substances were marginal only (see for e.g. Junghans et al., 2006, Kortenkamp and Faust 2010). If such findings could be demonstrated to have a general validity in a certain regulatory setting, this could provide a basis for the definition of an adequate MUF.

4.7 Conclusions for the development of specific approaches for specific regulatory settings

This general outline of the available implementation options aimed to provide a basis for the development of specific proposals for the implementation of mixture toxicity and mixture risk assessments in specific regulatory settings, such as the ecotoxicological assessment of PPPs or biocidal products. To this end, it was important to identify the critical factors that determine the

(i) applicability or non-applicability of different approaches in a specific assessment situation, and the

(ii) quantitative differences between the results of different possible assessment approaches.

As a starting point for the rational development of specific implementation approaches, a specific assessment situation should be characterized in terms of these critical factors. For which factors is any information available? Where does this information allow to specify factors or to confine factors to a limited range?

In summary, the comparative outline of implementation options yields the following checklist of such critical factors:

- number of mixture components in the assessment scenario,
- concentration ratio of mixture components in the assessment scenario,
- slope of concentration response curves of individual mixture components in the assessment scenario,
- modes and mechanisms of action of individual mixture components in the assessment scenario,
- type, amount and quality of available toxicity data for individual mixture components in the assessment scenario,
- type and quality of hazard or risk indicators that shall be determined for the mixture of concern (e.g. EC50, NOEC or sum of TERs for a mixture),
- requirements concerning safety and reliability of mixture hazard and risk assessments (safeguarding against under-estimations, avoidance of over-estimations),
- availability of information about the variability or statistical uncertainty in single substance toxicity data,

- availability of information about quantitative relations between different relevant toxicity endpoints (*endpoint to endpoint* and *species to species extrapolations*),
- availability of information about the quantitative relation between NOEC values (or other *points of departures*) and low effect concentrations.

The comparative evaluation of the advantages and disadvantages of the different basic implementation options leads to the conclusion that these should not merely be considered as alternatives, but rather as complementary approaches. For any specific type of regulatory assessment situation it should be examined how different options could most effectively be combined as elements of a decision tree in a tiered approach.

5 Common Principles

The reflections about the requirements for any of the generic options outlined in the preceding chapter 4 demonstrate that for generating coherent approaches within a regulatory field or even more so for harmonised approaches across different regulatory arenas, there is a need for additional settings. These cannot be derived from either data or scientific knowledge alone. Rather additional framework definitions are required with respect to the level of acceptable pragmatism, the requirements for using available or calling for additional data, the acceptable risk assessment outcomes and the acceptable uncertainty. Considering not only the scientific state of the art (chapter 3) and the available implementation options (chapter 4), but also taking into account the general principles of European chemicals legislation, we propose a first set of common principles for devising a transparent and coherent framework of mixture assessment regulations.

The suggested principles include:

- Environmental safety requirements for mixtures should neither be higher nor lower than the corresponding requirements for single substances;
- Procedures for the environmental safety assessments of mixtures should follow the principle of a tiered approach. For any specific type of regulatory assessment situation it should be examined how different methods and criteria could most efficiently be combined in a tiered decision tree. To this end, the methodologies should insure that proceeding from lower to higher, more data demanding tiers is only required, if there is a substantial chance that this may alter the regulatory conclusion in terms of acceptability or unacceptability of mixture risks.
- As far as possible, assessments of environmental risks for mixture effects should be implemented without additional experimental testing of whole mixtures, both for ethical and economic reasons;
- CA is considered to provide a reasonable default assumption for the joint eco-toxicity of environmental chemicals, including PPP and biocidal product ingredients, provided that there are reasons to assume that all relevant mixture components are included in the calculation;
- As input data, the original scientific concept of CA requires effect concentrations (or doses) that refer to the same biological effect in the same species under identical test conditions. For regulatory use, however, pragmatic simplifications and assumptions are unavoidable. This may refer to the merging of data for different test conditions, endpoints and species and to the use of NOEC values as a surrogate for quantitative estimates of low effect concentrations. In any case, the potential additional errors that may be introduced by such deviations from the original concept should be made transparent. Where possible they should be removed in a stepwise manner;
- IA and mixed models (MM) are much more data demanding and bear a higher risk of underestimating the actual mixture toxicity than CA. Therefore, the use of IA and MM should be restricted to situations where knowledge about MoAs and dose response relationships of mixture components supports the proper usage of these models;
- Depending on the specific assessment situation, component-based mixture toxicity estimates may be complemented by specific assessment factors to account for potential synergistic interactions that may result in more-than-additive joint effects.

- Where CBA-based assessments point to an unacceptable risk, experimental testing of the mixture may be considered as an ultimate option for clarification, unless practical, ethical, economical or other regulatory considerations argue against such a decisive experiment.

Based on considerations of critical factors identified in the outline of generic implementation options (section 4.7), we developed proposals for different tiered approaches for both PPP and biocidal products (sections 6.2 and 7.2). They are each tailored to the specific assessment situations but they conformably follow the guiding principles set out above.

6 Biocides

Biocidal products are preparations containing one or more active substances, which are *"intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means"* (Biocidal Products Directive 98/8/EC, BPD). Biocides are hence closely related to agricultural pesticides and are – due to their high biological activity and potential exposure – of inherent environmental concern. In fact, biocidal products are only allowed to be put on the market of the European Union, if it can be convincingly demonstrated that no unacceptable risks for the environment result from their intended use.

The authorization of biocides and biocidal products is regulated in the EU according to the rules and procedures laid down in the BPD. A key precondition for the authorization of a biocidal product is the inclusion of its active substances in the "list of permitted active substances", provided as Annex I or IA of the BPD ("positive list"). Only products that contain active substances that are listed in Annex I/IA are allowed on the EU-market, and their risk to man and the environment is then assessed at the national level, complemented by the mutual recognition of authorizations between the EU member states.

In 2012 the European Parliament and the EU Commission and Parliament adopted the new regulation (EU) No 528/2012 on the authorization of biocidal products (BPR). It will replace and repeal the BPD and will implement a new EU-wide, harmonized system for the authorization for biocidal products. The EU-wide authorization system will be applied for low-risk biocidal products and products that have similar use conditions throughout the EU. All other biocidal products are expected to still be subject to authorization by the individual member states. The BPR provisions will also apply to existing active substances being evaluated under the BPD review program.

Biocidal products are usually multi-component mixtures of one or more active substances plus a range of co-formulants that serve different purposes (stabilizers, coloring agents, emulsifiers, solvents, diluents, etc.). Additionally, metabolites and degradation products might be formed during and after use of a biocidal product. The overall ecotoxicity of a biocidal product might hence be significantly different from that of each individual ingredient(s) and therefore needs to be assessed during the product authorization phase. In fact, article 19(2) of the BPR states that *"The evaluation […] shall take into account the following factors: […] (d) cumulative effects; (e) synergistic effects.*" This is further elaborated in Annex VI (common principles for the evaluation of biocidal products) which states that the risks associated with the relevant individual components of the biocidal product shall be assessed, taking into account any synergistic effects.

The aim of the following text is therefore twofold. First, an overview on the predictability of the mixture ecotoxicity of biocides is presented, with the aim to analyse the following question: Is the predictive power of Concentration Addition (CA) and (IA) for mixtures of biocides sufficiently high, so that either of these classical concepts can be used as tools for biocide mixture toxicity assessments in a regulatory context?

Only very limited details on how mixture effects should actually be considered during the registration of a biocidal product are provided in the current Technical Note for Guidance on Product Evaluation (ECB, 2008). In fact, there is currently no agreed guidance available among the European Member States on how to assess the mixture effects from the ingredients

of biocidal products, hindering the mutual recognition of authorizations between member states.

The second aim of the present text is hence to fill in this gap and suggest a tiered approach for the adequate consideration of mixture effects during the authorization procedure of biocidal products.

6.1 Literature review on the predictability of mixture effects of biocides

Only peer-reviewed literature that is monitored by Scopus (SciVerse, Elsevier, 2010) was included in the study. The main database query was conducted in Jan 2010, with a follow-up query in Oct 2011, using the following search string:

(

TITLE (biocid* OR disinfect* OR preservativ* OR slimicid* OR rodenticid* OR avicid* OR molluscicid* OR piscicid* OR acaricid* OR repellant* OR attractant* OR pheromon* OR antifoul* OR enbalm* OR taxiderm* OR insecticid*)

OR

KEY (biocid* OR disinfect* OR preservativ* OR slimicid* OR rodenticid* OR avicid* OR molluscicid* OR piscicid* OR acaricid* OR repellant* OR attractant* OR pheromon* OR antifoul* OR enbalm* OR taxiderm* OR insecticid*)

)

AND (TITLE (mixture OR synergist* OR antagonist*)

OR KEY (mixture OR synergist* OR antagonist*))

AND (TITLE (tox* OR ecotox* OR efficacy) OR KEY (tox* OR ecotox* OR efficacy))

695 publications were retrieved in total by this search string (Oct 2011).

In parallel a specific search for the biocides of product groups 8 (wood preservatives, 25 compounds) and 21 (antifoulants, 11 compounds) that are currently in the European review programme was established. The corresponding search string was

CASREGNUMBER (*CAS-Number of the compound in question*)

AND (TITLE (mixture OR synergist* OR antagonist*)

OR KEY (mixture OR synergist* OR antagonist*))

AND (TITLE (tox* OR ecotox* OR efficacy)

OR KEY (tox* OR ecotox* OR efficacy))

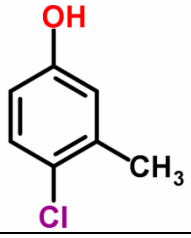
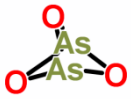
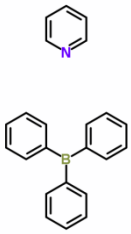
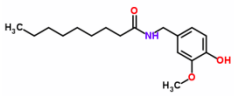
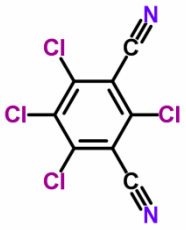
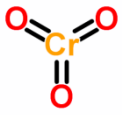
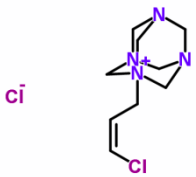
339 references were retrieved.

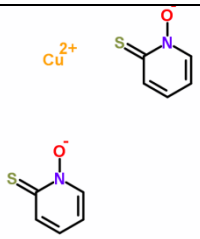
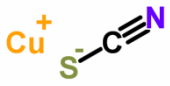
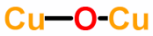
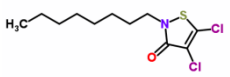
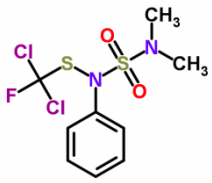
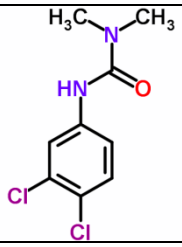
Both sets of references where finally merged and a list of 813 unique references was produced, which was the basis of all subsequent review work.

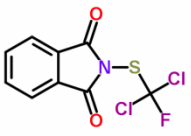
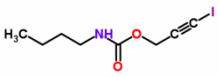
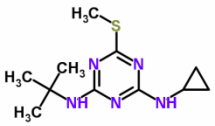
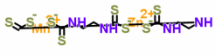
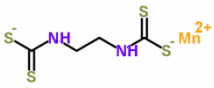
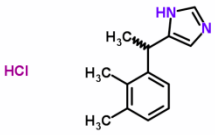
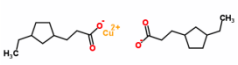
As it turned out that antifoulants are by far the most intensively studied group of biocides, a separate list of all compounds that are either currently on the market, are within the EU-wide review programme or are discussed as novel antifoulants in the scientific literature was established (Table 6.1).

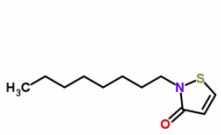
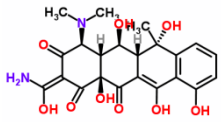
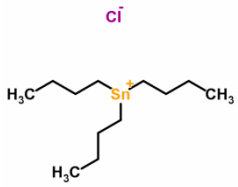
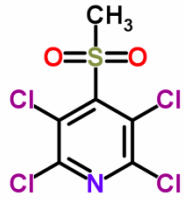
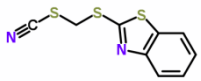
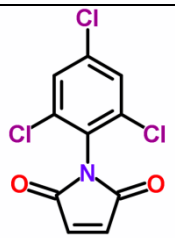
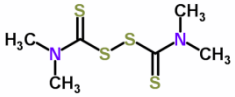
The recent literature on the ecotoxicology of those compounds for the years 2010 and 2011 was then manually scanned for mixture toxicity studies involving those compounds.

Table 6.1 Antifouling biocides considered in the review

Common name	CAS-no	IUPAC	Structure	Reference (to AF use)	Analysed as mixture component?
4-chloro-meta-cresol	1321-10-4	4-chloro-3-methylphenol		Konstantinou, 2006	no
Arsenic trioxide	1327-53-3			Konstantinou, 2006; Hellio & Yebra, 2009	no
Borocide, PTPB	971-66-4	pyridine; triphenylborane		Thomas, 2001; Konstantinou, 2006; Hellio & Yebra, 2009	yes
Capsacine	618-92-8	(E)-N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide			no
Chlorothalonil	1897-45-6	2,4,5,6-tetrachlorobenzene-1,3-dicarbonitrile		Voulvoulis et al., 1999; Konstantinou, 2006; Hellio & Yebra, 2009	yes
Chromium trioxide	1333-82-0	trioxochromium		Konstantinou, 2006	no
cis1-(3-chloroallyl)-3,5,7-triaza-1-azonia adamantane chloride	51229-78-8	not found		Konstantinou, 2006	no

Common name	**CAS-no**	**IUPAC**	**Structure**	**Reference (to AF use)**	**Analysed as mixture component?**
Copper pyrithione	14915-37-8	copper 1-oxidopyridine-2-thione		Thomas, 2001; Konstantinou, 2006; Hellio & Yebra, 2009	yes
Cu2+ (metallic Cu)	7440-50-8	copper(2+)		Voulvoulis et al., 1999	*not included in the analysis*
Cu, copper thiocyanate	1111-67-7	copper(1+) thiocyanate		Voulvoulis et al., 1999; Konstantinou, 2006; Hellio & Yebra, 2009	*not included in the analysis*
Cu, cuprous oxide	1317-39-1			Voulvoulis et al., 1999; Konstantinou, 2006	*not included in the analysis*
DCOIT	64359-81-5	4,5-dichloro-2-octyl-1,2-thiazol-3-one		Voulvoulis et al., 1999; Konstantinou, 2006;	yes
Dichlofluanid	1085-98-9	N-[dichloro(fluoro)methyl]sulfanyl-N-(dimethylsulfamoyl) aniline		Voulvoulis et al., 1999; Konstantinou, 2006; Hellio & Yebra, 2009	yes
Diuron	330-54-1	3-(3,4-dichlorophenyl)-1,1-dimethylurea		Voulvoulis et al., 1999; Konstantinou, 2006;	yes

Common name	**CAS-no**	**IUPAC**	**Structure**	**Reference (to AF use)**	**Analysed as mixture component?**
Fluorofolpet	719-96-0	2-[dichloro(fluoro)methyl]sulfanylisoindole-1,3-dione		Thomas, 2001; Hellio & Yebra, 2009	no
LPBC, Polyphase		3-iodoprop-2-ynyl N-butylcarbamate		Zhou et al., 2006	yes
Irgarol 1051	28159-98-0	2-N-tert-butyl-4-N-cyclopropyl-6-methylsulfanyl-1,3,5-triazine-2,4-diamine		Voulvoulis et al., 1999; Konstantinou, 2006; Hellio & Yebra, 2009	yes
Mancozeb	8018-01-07	zinc; manganese(2+);N-[2-(sulfidocarbothioylamino)ethyl]carbamodithioate		Thomas, 2001	no
Maneb	12427-38-2	manganese(2+); N-[2-(sulfidocarbothioylamino)ethyl]carbamodithioate		Voulvoulis et al., 1999; Hellio & Yebra, 2009; Arai et al. 2009	yes
Medetomidine	86347-15-1	5-[1-(2,3-dimethylphenyl)ethyl]-1H-imidazole hydrochloride		Hellio & Yebra, 2009	no
NACS	1338-02-9	copper 3-(3-ethylcyclopentyl)propanoate		Konstantinou, 2006	No

Common name	**CAS-no**	**IUPAC**	**Structure**	**Reference (to AF use)**	**Analysed as mixture component?**
Octhilinone	26530-20-1	2-octyl-1,2-thiazol-3-one			No
Oxytetrcycline	79-57-2			Konstantinou, 2006; Hellio & Yebra, 2009	No
TBT	1461-22-9	tributyl(chloro)stannane		Voulvoulis et al., 1999	yes
TCMS pyridine ¤	13108-52-6	2,3,5,6-tetrachloro-4-methylsulfonyl-pyridine		Voulvoulis et al., 1999; Thomas, 2001; Konstantinou, 2006; Hellio & Yebra, 2009	No
TCMTB	64441-45-8	1,3-benzothiazol-2-ylsulfanylmethyl thiocyanate		Voulvoulis et al., 1999; Hellio & Yebra, 2009	yes
TCPM, IT-354	13167-25-4	1-(2,4,6-trichlorophenyl)pyrrole-2,5-dione		Konstantinou, 2006	No
Thiram	137-26-8	dimethylcarbamothioylsulfanyl N,N-dimethylcarbamodithioate		Voulvoulis et al., 1999; Konstantinou, 2006; Hellio & Yebra, 2009	yes

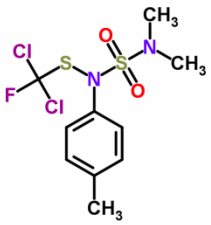
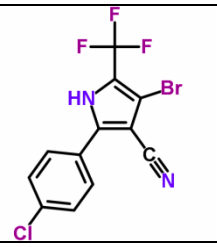
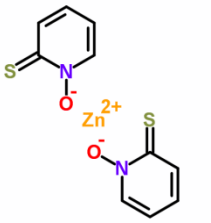
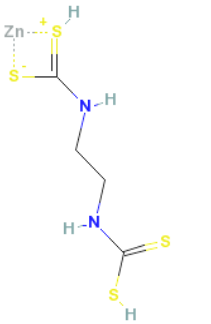
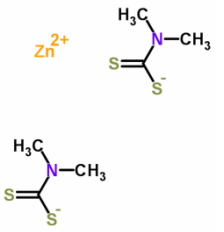
Common name	**CAS-no**	**IUPAC**	**Structure**	**Reference (to AF use)**	**Analysed as mixture component?**
Tolylfluanid	731-27-1	N-[dichloro(fluoro)methyl]sulfanyl-N-(dimethylsulfamoyl)-4-methylaniline		Thomas, 2001; Hellio and Yebra 2009	No
Tralopyril	122454-29-9	4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile		Hellio & Yebra, 2009	No
Zinc pyrithione	13463-41-7	zinc 1-oxidopyridin-1-ium-2-thiolate		Voulvoulis et al., 1999; Konstantinou, 2006; Hellio & Yebra, 2009	yes
Zineb	12122-67-7	[2-(dithiocarboxyamino)ethylamino]-sulfoniumylidenemethanethiolate; zinc		Voulvoulis et al., 1999; Konstantinou, 2006; Hellio & Yebra, 2009	yes
Ziram	137-30-4	zinc N,N-dimethylcarbamodithioate		Voulvoulis et al., 1999; Konstantinou, 2006;	yes

Table 6.2 List of mixture studies that were included in the meta-analysis

1 Arrhenius Å, Grönvall F, Scholze M, Backhaus T, Blanck H. 2004. Predictability of mixture toxicity of 12 similar-acting congeneric inhibitors of photosystem II in marine periphyton and epipsammon communities. Aquatic Toxicology. 68:351-367.

2 Arrhenius Å, Backhaus T, Grönvall F, Junghans M, Scholze M, Blanck H. 2006. Effects of three antifouling agents on algal communities and algal reproduction: mixture toxicity studies with TBT, Irgarol, and Sea-Nine. Arch. Environ. Contam Toxicol. 50:335-345.

3 Backhaus T., Faust M, Scholze M, Gramatica P, Vighi M, Grimme, LH. 2002. The joint action of phenylurea herbicides is equally predictable by Concentration Addition and Independent Action. Environmental Toxicology and Chemistry. 23:258-264.

4 Backhaus T, Arrhenius Å, Blanck H. 2004. Toxicity of a mixture of dissimilarly acting substances to natural algal communities: predictive power and limitations of independent action and concentration addition. Environ. Sci. Technol. 38:6363-6370.

5 Bao VWW, Leung KMY, Kwok KWH, Zhang AQ, Lui GCS. 2008. Synergistic toxic effects of zinc pyrithione and copper to three marine species: Implications on setting appropriate water quality criteria. Marine Pollution Bulletin. 57:616-623.

6 Bellas J. 2008. Prediction and assessment of mixture toxicity of compounds in antifouling paints using the sea-urchin embryo-larval bioassay. Aquatic Toxicology. 88:308-315.

7 Bonnemain H, Dive D. 1990. Studies on synergistic toxic effects of copper and dithiocarbamate pesticides with the ciliate protozoan Colpidium campylum (Stokes). Ecotoxicol. Environ. Saf. 19:320-326.

8 Chesworth JC, Donkin ME, Brown MT. 2004. The interactive effects of the antifouling herbicides Irgarol 1051 and Diuron on the seagrass Zostera marina (L.). Aquat. Toxicol. 66:293-305.

9 Cima F, Bragadin M, Ballarin L. 2008. Toxic effects of new antifouling compounds on tunicate haemocytes I. Sea-Nine 211 (TM) and chlorothalonil. Aquat. Toxicol. 86:299-312.

10 DeLorenzo ME, Serrano L. 2003. Individual and mixture toxicity of Three pesticides; atrazine, chlorpyrifos, and chlorothalonil to the marine phytoplankton species Dunaliella tertiolecta. Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes. 38:529-538.

11 DeLorenzo, ME, Serrano L. 2006. Mixture toxicity of the antifouling compound irgarol to the marine phytoplankton species Dunaliella tertiolecta. Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes. 41:1349-1360.

12 Eullaffroy P, Frankart C, Biagianti S. 2007. Toxic effect assessment of pollutant mixtures in Lemna minor by using polyphasic fluorescence kinetics. Tox. Env. Chem. 89:683-696.

13 Fernandez-Alba, AR, Hernando, MD, Piedra L, Chisti Y. 2002. Toxicity of single and mixed contaminants in seawater measured with acute toxicity bioassays. Analytica Chimica Acta. 456:303-312.

14 Gatidou G. Thomaidis NS. 2007. Evaluation of single and joint toxic effects of two antifouling biocides, their main metabolites and copper using phytoplankton bioassays. Aquatic Toxicology. 85:184-191.

15 Granmo Å, Ekelund R, Sneli JA, Berggren M, Svavarsson J. 2002. Effects of antifouling paint components (TBTO, copper and triazine) on the early development of embryos in cod (Gadus morhua L.). Mar. Pollut. Bull. 44:1142-1148.

16 Hernando MD, Ejerhoon M, Fernandez-Alba AR, Chisti Y. 2003. Combined toxicity effects of MTBE and pesticides measured with Vibrio fischeri and Daphnia magna bioassays. Water Research. 37:4091-4098.

17 Knauert S, Escher B, Singer H, Hollender J, Knauer K. 2008. Mixture toxicity of three photosystem II inhibitors (atrazine, isoproturon, and diuron) toward photosynthesis of freshwater phytoplankton studied in outdoor mesocosms. Environmental Science & Technology. 42:6424-6430.

18 Knauert S, Dawo U, Hollender J, Hommen U, Knauer K. 2009. Effects of Photosystem II Inhibitors and Their Mixture on Freshwater Phytoplankton Succession in Outdoor Mesocosms. Env. Tox. Chem. 28:836-845.

19 Koutsaftis A, Aoyama I. 2006. The interactive effects of binary mixtures of three antifouling biocides and three heavy metals against the marine algae Chaetoceros gracilis. Environmental Toxicology. 21:432-439.

20 Koutsaftis A, Aoyama I. 2007. Toxicity of four antifouling biocides and their mixtures on the brine shrimp Artemia salina. Sci. Tot. Env. 387:166-174.

21 Manzo S, Buono S, Cremisini C. 2008. Predictability of copper, irgarol, and diuron combined effects on sea urchin Paracentrotus lividus. Arch. Env. Cont. Tox.. 54:57-68.

22 Mochida K, Ito K, Harino H, Kakuno A, Fujii K. 2006. Acute toxicity of pyrithione antifouling biocides and joint toxicity with copper to red sea bream (Pagrus major) and toy shrimp (Heptacarpus futilirostris). Environ. Toxicol. Chem. 25:3058-3064.

23 Molander S, Dahl B, Blanck H, Jonsson J, Sjöström M. 1992. Combined effects of Tri-n-butyl Tin (TBT) and diuron on marine periphyton communities detected as pollution-induced community tolerance. Arch. Env. Cont. Tox.. 22:419-427.

24 Padros J, Pelletier E, Reader S, Denizeau F. 2000. Mutual in vivo interactions between benzo[a]pyrene and tributyltin in brook trout (Salvelinus fontinalis). Env. Tox. Chem. 19:1019-1027.

25 Padros J, Pelletier T, Ribeiro CO. 2002. In vivo metabolic interactions between benzo[a]pyrene and tributyltin in arctic charr (Salvelinus alpinus): A long-term study. Drug Metabolism Reviews. 34:183.

26 Rodin, VB, Zhigletsova SK, Kobelev VS, Akimova NA, Kholodenko VP. 2005. Efficacy of individual biocides and synergistic combinations. Int. Biodet. and Biodeg.. 55:253-259.

27 Santos MM, Reis-Henriques MA, Vieira MN, Sole M. 2006. Triphenyltin and tributyltin, single and in combination, promote imposex in the gastropod Bolinus brandaris. Ecotox. Env. Saf. 64:155-162.

28 Schmidt K, Staaks GBO, Pflugmacher S, Steinberg CE. 2005. Impact of PCB mixture (Aroclor 1254) and TBT and a mixture of both on swimming behavior, body growth and enzymatic biotransformation activities (GST) of young carp (Cyprinus carpio). Aquatic Toxicology. 71:49-59.

29 Teather K, Jardine C, Gormley K. 2005. Behavioral and sex ratio modification of Japanese Medaka (Oryzias latipes) in response to environmentally relevant mixtures of three pesticides. Env. Tox. 20:110-117.

30 Teisseire H, Couderchet M, Vernet G. 1999. Phytotoxicity of diuron alone and in combination with copper or folpet on duckweed (Lemna minor). Env. Poll. 106:39-45.

31 Vasseur P, Dive D, Sokar Z, Bonnemain H. 1988. Interactions between copper and some carbamates used in phytosanitary treatments. Chemosphere. 17:767-782.

32 Wang, CG, Zhao Y, Zheng RH, Ding X, Wei W, Zuo ZH, Chen YX. 2006. Effects of tributyltin, benzo[a]pyrene, and their mixture on antioxidant defense systems in Sebastiscus marmoratus. Ecotox. Env. Saf. 65:381-387.

33 Wu YQ, Wang CG, Wang Y, Zhao Y, Chen YX, Zuo ZH. 2007. Antioxidant responses to benzo[a]pyrene, tributyltin and their mixture in the spleen of Sebasticus marmoratus. Journal of Environmental Sciences-China. 19:1129-1135.

34 Zheng RH, Wang CG, Zhao Y, Zu ZH, Chen YX. 2005. Effect of tributyltin, benzo(a)pyrene and their mixture exposure on the sex hormone levels in gonads of cuvier (Sebastiscus marmoratus). Env. Tox. Pharm.. 20:361-367.

35 Zhou X, Okamura H, Nagata S. 2006. Remarkable synergistic effects in antifouling chemicals against Vibrio fischeri in a bioluminescent assay. Journal of Health Science. 52:243-251.

An overview of those 30 organic compounds (plus 3 copper compounds that were not further considered) that are currently on the market as antifouling biocides or that are discussed in the scientific literature as possible candidate substances is given in Table 6.1. 16 of those compounds have been included in at least on mixture study. It should be emphasized here that of those compounds only diuron, irgarol and other members from the classes of phenylureas and *s*-triazines[7] share a similar mechanism of action, which has been thoroughly investigated only in algae and higher plants. Hence, the vast majority of the mixtures discussed below have dissimilar modes and mechanisms of action.

5 publications that were not picked up by the above listed search were manually added to yield a final pool of 35 studies that concern mixtures completely or in partly comprising antifouling biocides (Table 6.2). These publications provided details on 382 different mixtures (in this context a mixture is defined by a specific number and type of compounds, a particular mixture ratio and biological endpoint). By far the majority of studies analysed binary mixtures (88 %, see table 6.3). The 6 studies with mixtures of 12 components all concern the algal toxicity of a phenylurea mixture (including diuron) with an identical qualitative composition, but tested in different mixture ratios and with different algal bioassays (Arrhenius et al. 2004; Backhaus et al. 2002).

[7] Which were mixed with Diuron and/or Irgarol in some studies

Table 6.3 Number of mixture components and analysed mixture studies

Number of components	Total	CA	CA+MDR	IA	IA+MDR
any	382 (35)	198 (18)	117 (12)	128 (11)	24 (8)
2	336 (28)	138 (12)	84 (8)	133 (7)	9 (2)
3	32 (9)	27 (7)	19 (5)	8 (3)	8 (3)
4	7 (2)	7 (2)	7 (2)	0 (0)	0 (0)
6	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
12	6 (2)	6 (2)	6 (2)	6 (2)	6 (2)

"Total": total number of mixtures analysed, "CA": number of mixtures whose toxicity was compared to CA, "CA/MDR": number of mixtures for which an MDR in relation to CA could be calculated, "IA": number of mixtures whose toxicity was compared to IA, "IA/MDR": number of mixtures for which an MDR in relation to IA could be calculated. Values in parentheses provide the number of publications from which the studies were compiled.

All studies investigated "artificial" mixtures, that is, the specific composition, concentrations and mixture ratios did not reflect environmentally realistic exposure scenarios (nor the specific composition of commercial biocide products) in terms of number of components, mixture ratios or concentrations. Instead the mixtures were composed to either provide a systematic overview for (i) an *a prior* defined mixture that was selected because of mechanistic reasons or chemical similarity (e.g. "mixtures of PSII-inhibiting compounds"), (ii) an *a priori* defined group of compounds ("how good is the predictive power of CA for binary mixtures of these booster antifoulants?") or (iii) a specific biological endpoint / bioassay ("does CA work for predicting mixture effects on the embryotoxicity in sea urchins?").

The test species are listed in Table 6.4. Although antifouling biocides are almost exclusively used in a marine setting, several studies have been conducted with limnic species, which is at least partly due to the dual use of some compounds. Diuron for example is not only used as an antifouling biocide, but also as agricultural herbicide. Common ecotoxicological endpoints such as bioluminescence, growth, reproduction, mortality were used in the majority of studies.

Table 6.4 List of test organisms employed for the reviewed tests on the ecotoxicity of antifoulant mixtures

Organism	Habitat	Details / Common name
Artemia salina	Marine	Crustacean (brine shrimp)
Bolinus brandaris	Marine	Gastropod
Botryllus schlosseri	Marine	Ascidian
Chaetoceros gracilis	Marine	Microalgae
Colpidium campylum	Limnic	Ciliate

Organism	**Habitat**	**Details / Common name**
Cyprinus carpio	Limnic	Fish (carp)
Daphnia magna	Limnic	Crustaceen
Dunaliella tertiolecta	Marine	Green algae
Elasmopus rapax	Marine	Amphipod
Gadus morhua L.	Marine	Fish (cod)
Heptacarpus futilirostris	Marine	Crustacean (toy shrimp)
Hydroides elegans	Marine	Polychaete (tube worm)
Lemna minor	Limnic	Vascular plant
Navicula forcipata	Marine	Diatom
Oryzias latipes	Limnic	Fish (japanese medaka)
Pagrus major	Marine	Fish (red sea bream)
Paracentrotus lividus	Marine	Sea urchin
Periphyton communities	Marine	Natural biofilm communities
Photobacterium phosphoreum	Limnic	Marine bacterium
Phytoplankton communities	Limnic	Natural phytoplankton communities
Salvelinus alpinus	Limnic	Fish (archtic charr)
Salvenius fontinalis	Limnic	Fish (brook trout)
Scenedesmus vacuolatus	Limnic	Green algae
Sebastiscus marmoratus	Marine	Fish (false kelpfish)
Selenastrum capricornutum	Limnic	Green algae
Serratia marcescens	Limnic	Bacteria
Thalassiosira pseudonana	Marine	Diatom
Vibrio fischeri	Limnic	Marine bacterium
Zostera marina	Marine	Vascular plant

6.1.1 The predictive power of Concentration Addition

After an initial scan of the publications it was decided to use the ratio predicted to observed EC50 (= Model Deviation Ratio, (Belden et al. 2007)) as a measure of the predictive power of CA, as this allowed a quantitative analysis of most studies. However, for only 84 out of the 134 mixtures whose toxicity was compared to toxicity expectation according to CA in one way or another, such quantification could be carried out. In the remaining studies the results were either presented only graphically (usually as isobolograms, e.g. (Cima et al. 2008)), or in the form of Könneman's Mixture Toxicity Index (MTI), which is defined as

$$MTI = \frac{\sum_{i=1}^{n} \frac{c_i}{EC50_1}}{\max_{i\in(1,\ldots,n)}\left(\frac{c_i}{EC50_1}\right)}$$

(Könemann 1981). That is, without additional information on the single substance EC50 values or the mixture composition, the MTI cannot be back-calculated to a ratio of predicted/observed $EC50_{Mix}$.

6.1.1.1 Binary mixtures

An analysis of the predictive power of CA for 84 binary mixtures is given in Figure 6.1. For this purpose, the ratio CA-predicted to observed $EC50_{Mix}$ (the MDR) was back-calculated from the data given in the publications and the studies were then ranked in ascending order. The average (arithmetic mean) of the ratio predicted/observed EC50 of all studies is 2.0 (median = 0.95). This means, that in average CA predicts a 2 times lower toxicity than observed, while the median indicates a very good predictive power of the concept. This discrepancy results from the extreme ends of the distribution, those studies that discovered severe deviations between observed and predicted EC50-values.

In fact, these are only two studies (highlighted in Figure 6.1), conducted by Fernandez-Alba and colleagues and by Koutsaftis & Aoyama (Fernandez-Alba et al. 2002; Koutsaftis and Aoyama 2006). If those two studies are omitted, the average (arithmetic mean) of predicted to observed EC50 is 1.02, while the median is still somewhat lower at 0.8.

This obviously begs the question what makes these two studies special. Fernandez-Alba and her colleagues investigated a whole range of two-compound mixtures, comprising irgarol plus either seanine, chlorothalonil, diuron, dichlofluanid or TCMTB[8]. They employed very typical ecotoxicological bioassays with *Daphnia magna* (mortaility), *Vibrio fischeri* (bioluminescence inhibition) and *Selenastrum capricornutum* (growth inhibition). The studies were implemented in a classical fixed-ratio design.

[8] 2-thiocyanomethylthiobenzothiazole

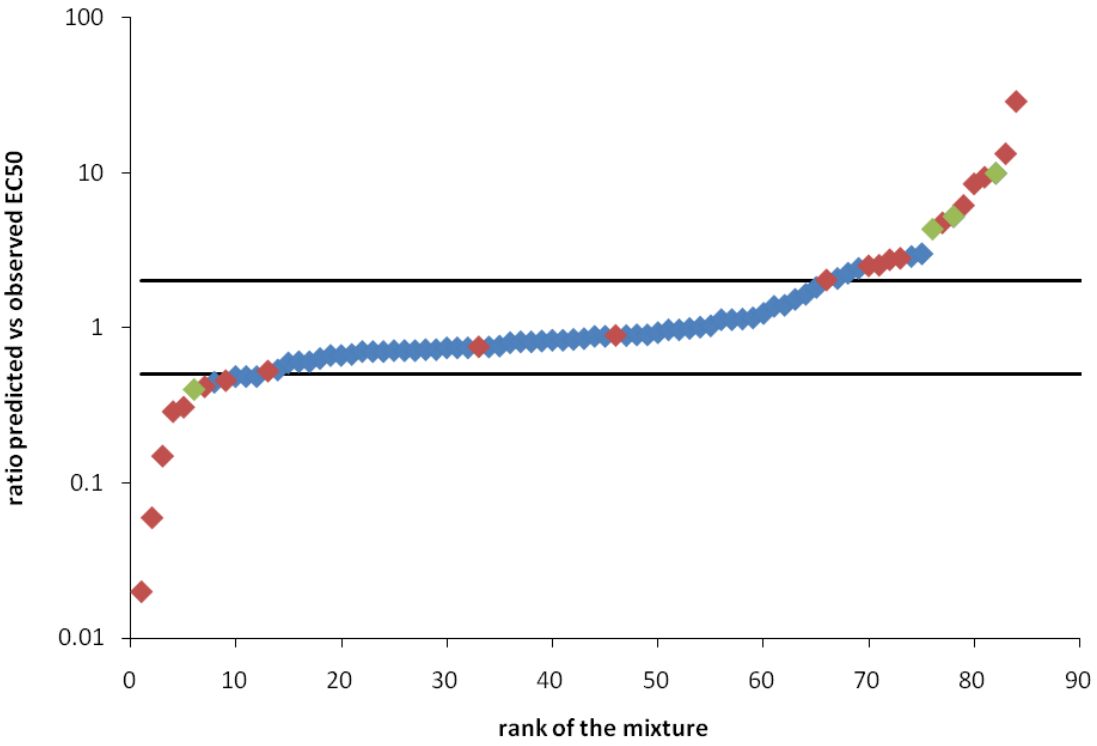


Figure 6.1 Predictive power of CA for binary mixtures of antifoulants
Y-Axis: ratio of CA-predicted vs observed EC50 (MDR). X-Axis: rank of the mixture (1-84). Thick horizontal lines indicate a ratio of predicted to observed EC50 of 2, respectively 0.5. Red symbols: data from (Fernandez-Alba et al. 2002); green symbols: data from (Koutsaftis and Aoyama 2006); blue symbols: data from all other studies.

What makes the study somewhat special is that Fernandez-Alba and her co-workers mixed the compounds in an equi-molar mixture ratio, arguing that this results in *"an equal theoretical probability of competition of the various biocides for the binding sites of target molecules"*. As the test concentrations all refer to aqueous concentrations outside the test organism, this approach, however, ignores any differences in bioaccumulation between the different compounds.

Furthermore, this design results in extremely unbalanced mixtures in terms of the toxicity contribution of the mixture components (their individual TUs). An example from the experiments with daphnids illustrates the point. Here, an equimolar mixture of Irgarol (reported EC50 = 10 mg/L, 40 µmol/L) and Chlorthalonil (reported EC50 = 0.07 mg/L, 0.026 µmol/L) results in a TU of 6.4E-4 for Irgarol and a TU of 0.9994 for Chlorothalonil at the CA-predicted EC50 of 0.052 µmol/L (total mixture concentration). That is, the mixture is from a toxicological perspective almost completely driven by Chlorothalonil, assuming equal uptake and distribution of the compounds in the organism. However, using an equimolar ratio might perhaps help pinpointing chemical interactions in the aqueous phase (growth media) of the experiment.

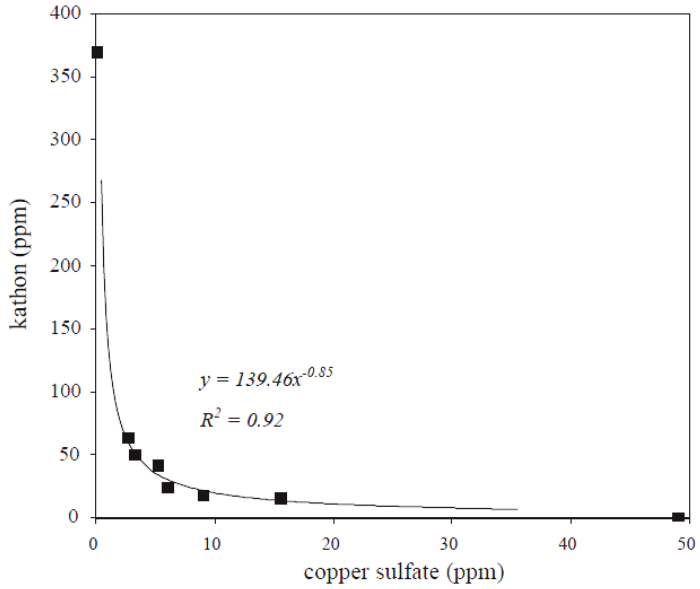


Figure 6.2 More than additive mixture effects from a combination of copper and kathon
Given are the results of a range of concentration-response analyses with mixtures at different ratios, which are plotted as the EC90-isobole (solid line). It should be noted that the concentration-scale on both axes is linear, not logarithmic. From Rodin et al. (2005).

Additionally, the documented EC50 values of the study show unusually high dynamics, in particular with respect to the results in the algal assay. Here the toxicity was determined after an incubation time of 30 hrs and 72 hrs. After 30 hrs the EC50 for Chlorothalonil was reported to be 42.4 mg/L, while after 72 hrs it dropped by a factor of more than 6 000 to an EC50 of 0.0068 mg/L (table 2 of the publication). Similarly, after 30 hrs the EC50 for Irgarol was recorded at 15.5 mg/L, while after 72 hrs it dropped by a factor of more than 1 500 to 0.01 mg/L (same table). Although slightly more lipophilic, Irgarol is a classic PSII inhibiting s-triazine, a group of compounds which usually acts very rapidly and whose toxicity does not show any strong changes over time. Additionally, the maximum water solubility for the compound is reported at 7 mg/L (CIBA speciality chemicals, safety data sheet for irgarol), implying that a good part of the experiments was conducted at nominal concentrations above water solubility. Finally, it should be noted that the authors determined the LOEC for Diuron – another classical PSII-inhibiting herbicide with a known high toxicity to algae – at 23 mg/L (100 µmol/L) and could not determine an EC50 at all, indicating a rather unusually low toxicity in the conducted experiments.

Koutsaftis and coworker investigated the predictive power of CA in a bioassay with the marine alga *Chaetoceros gracilis* (Koutsaftis and Aoyama 2006). The mixtures comprised combinations of Diuron, Irgarol, Zn-Pyrithione, Cadmium, Copper and Zinc. Interestingly, the highest deviations (a tenfold lower EC50 that expected by CA) was observed for a mixture of Diuron and Irgarol, two compounds who are well known to have an identical mechanism of action in algae. The authors speculate that the synergistic toxicity occurs because *"the toxicological behavior (i.e., the complex sequence from exposure to toxicokinetic and toxicodynamic) of two classes of chemicals can be quite different"* and quote a study from Gramatica and colleagues as a reference for the argument (Gramatica et al. 2001). It should also be noted that IA, which would be the concept of choice for a mixture of non-interacting,

dissimilarly acting substances, also failed to predict the joint action of Diuron and Irgarol. The mixture was assessed to also provoke higher effects than expected by IA.

Irgarol as well as Diuron also had a mixture toxicity higher than CA when mixed with Cadmium (ratio predicted to observed EC50 was 3.3, respectively 5).

Rodin and his colleagues (2005) investigated the joint action of copper and kathon (a broad group of biocides, in which Seanine is the most prominent antifoulant) on colony growth of Serratia marcescens, using the classical isobole-analysis (Figure 6.2.). As can be seen, the combined action of both compounds is clear more-than-additive, i.e. synergistic over a range of tested concentrations. The mechanistic reason for this pattern is unclear.

6.1.1.2 Three compound mixtures

36 3-compound mixtures were included in the total pool of analysed antifoulant mixtures (table 1), and the toxicity of 5 of those mixtures was not compared to CA. One study in which the experimental toxicity of a mixture of Cu, Diuron and Irgarol was compared to the CA prediction could not be quantitatively analysed (Manzo et al. 2008), as the mixture did not show any discernible concentration-response relationship (Figure 6.3). Hence, the ratio of predicted to observed EC50 was smaller than 0.1. Two studies by Knauert and colleagues, who analysed a mixture of Atrazine, Isoproturon and Diuron did not allow to calculate a ratio between the predicted and observed EC50-values of the mixtures, but the studies demonstrated clearly that the mixture behaved as expected, according to CA (Knauert et al. 2008; Knauert et al. 2009) – i.e. the ratio prediction/observation is at or close to one.

The ratios of all ternary mixtures that could be quantitatively analysed are plotted in Figure 6.5. The average of these studies is 1.12 (median = 0.63). The maximum observed synergistic deviation from the CA-expected toxicity (factor 3.7) was observed for a mixture of Irgarol, TCMTB and Dichlofluanid, again taken from the publication by Fernandez-Alba and colleagues (Fernandez-Alba et al. 2002). The highest antagonism (factor 0.01) has been recorded in the previously mentioned study by Manzo and colleagoues (Manzo et al. 2008).

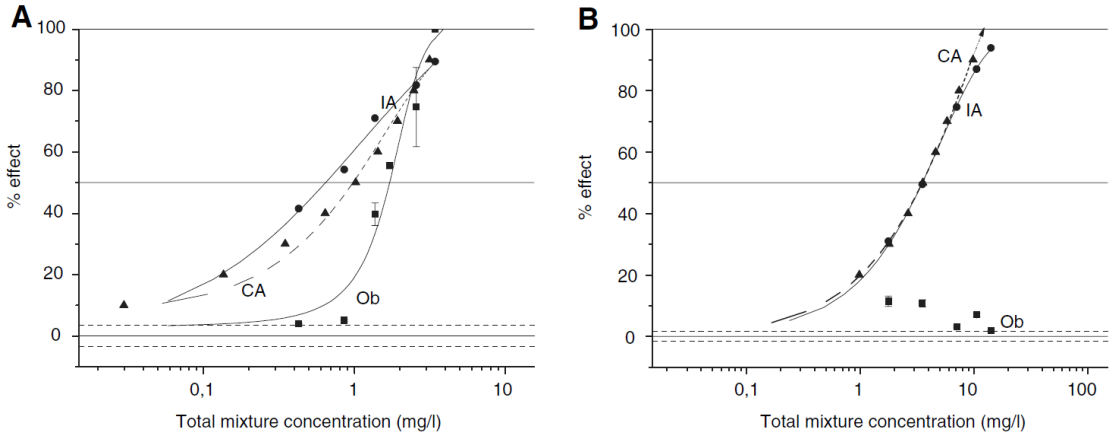


Figure 6.3 Predicted and observed toxicity of a three compound mixture of Copper, Irgarol and Diuron
Mixture ratio EC50 of the mixture components; Endpoints: (A) Embryotoxicity, (B) Spermiotoxicity to the sea urchin *Paracentrotus lividus* after 48-50 hrs exposure. Solid line/dots: Prediction according to IA; Dashed line/triangles: prediction according to CA; Solid line/squares: Experimental observation and fit to the data. From Manzo et al. (2008).

6.1.1.3 Mixtures with more than 3 compounds

The MDR of the ternary mixtures that were investigated in the compiled studies is given in Figure 6.4. A four compound mixture of Irgarol, TCMTB, Dichlofluanid and Seanine has been tested by Fernandez-Alba and Coworkers towards algae, daphnids and bacteria (Fernandez-Alba et al. 2002), with ratios of predicted to observed EC50 values ranging from 6 to 0.4. A 6 compound mixture of TBT, Diuron, KCN, DBMIB[9], Hydroxylamine and CCCP[10] (all compounds that inhibit the photosynthesis, but by different molecular mechanisms of action) was investigated by Backhaus et al. CA predicted the EC50 with a factor of 0.75, i.e. a slightly lower than predicted mixture toxicity was observed (Backhaus et al. 2004). It should be emphasized here, that the 4- as well as the 6-compound mixture were composed of dissimilarly acting substances and was rather well predicted by IA.

The same group of researchers also investigated the algal toxicity of 12-compound mixtures of PSII inhibiting phenylureas, including Diuron (Arrhenius et al. 2004; Backhaus et al. 2002). Independent on whether a single species of algae or natural microalgal communities were investigated, CA predicted the experimental EC50 within a MDR of 0.6-1.5.

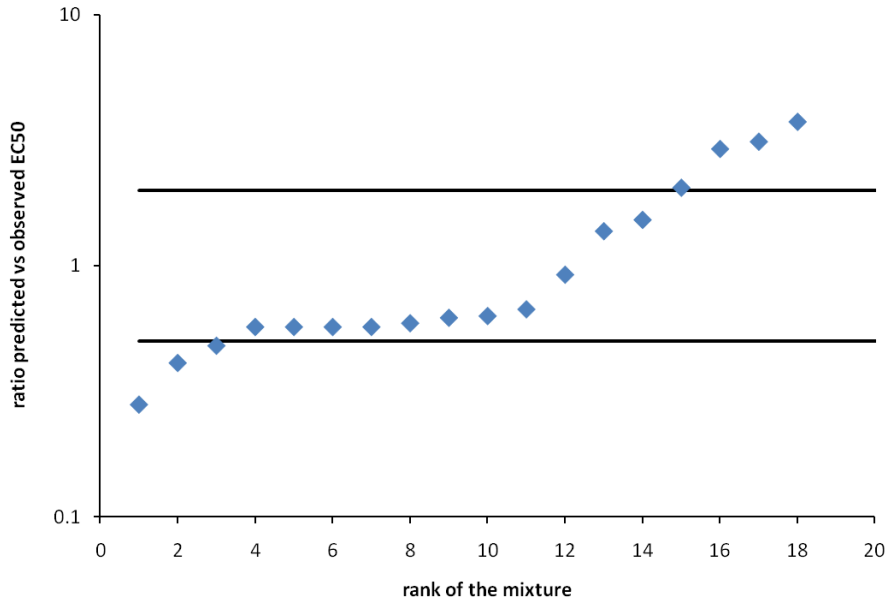


Figure 6.4 Predictive power of CA for ternary mixtures of antifoulants
Y-Axis: ratio of CA-predicted vs observed EC50 (MDR). X-Axis: rank of the mixture (1-18). Thick horizontal lines indicate a ratio of predicted to observed EC50 of 2, respectively 0.5. Blue symbols: data from the selected studies, ranked in ascending order.

[9] Dibromothymoquinone
[10] Carbonyl cyanide 3-chlorophenyl-hydrazone

6.1.2 Independent Action

Independent Action, also termed "Abott's Formula" in several publications, was applied to 160 mixtures. However, only for a very few mixtures the ratio of predicted to observed EC50 (MDR) could be calculated. This concerns the two aforementioned studies on 12-compound mixtures of phenylureas which were investigated in algae and algal communities by Arrhenius, Backhaus and their coworkers (Arrhenius et al. 2004; Backhaus et al. 2002). As the test compounds come from one chemical class and share a similar mechanism of action, it was not expected that IA actually provides an adequate prediction of their joint action. However, in the study from 2002 it turned out that CA and IA actually predict identical toxicities and IA therefore described the experimental data equally well. The fact that both concepts predicted similar toxicities is rooted in the particular steepness of the concentration-response curves of the mixture components, see discussion in (Backhaus et al. 2002), (Drescher and Boedeker 1995).

The same group published a study on the mixture toxicity of various 2-compound mixtures in bioassays with single species and communities of microalgae. The mixtures were composed of TBT, Diuron and/or Seanine, as well as the resulting three compound mixture (Arrhenius et al. 2006). The MDR with respect to IA of the 3-compound mixture was recorded to be 0.67 and 0.78, depending on the mixture ratio. The MDR for the various binary mixtures varied between 0.6 and 0.9.

The biggest deviation, again, was observed in the study by Manzo (see Figure 6.3.), as the mixture did not show any toxic effect at the tested concentrations, which implies a strong antagonism (MDR<0.1) with respect to IA as well as CA.

Several studies conducted an analysis of the predictive power of IA by calculating the ratio of predicted to observed effects of the mixture (RI, ratio of inhibition), in contrast to the ratio of predicted to observed effect concentrations (MDR). The RI, however, is seriously limited in two aspects. One problem stems from the limited scale (0-100 %) on which the RI is applied. That is, if either the predicted or the observed effect is at 100 %, or if the predicted effect is at 0 %[11] , the RI does not serve as a quantitative measure, as it becomes severely biased (see example in figure 6.5.). Additionally, the RI does not allow a comparison across different publications, if it is not based on the same reference point (such as e.g. a comparison with the mixture effect that is predicted to occur at a concentration that experimentally causes 50 % effect). Even if this would be the case, varying steepnesses of the individual concentration-responses curves might introduce a severe bias (figure 6.5.). Hence, a quantitative comparison in parallel to the MDR-based comparisons in figures 1 and 3 was not conducted for the MRI. Typical RI values are for example between 0.6 and 2.8 (Chesworth et al. 2004) or 0.4-9 (Eullaffroy et al. 2007).

6.1.3 Summary

The empirical evidence on combination effects of biocides is, compared to the state of the art in the area of plant protection products, still extremely limited. Huge data gaps exist, both in terms of the biocides actually included in combinations studies as well as the specific environments studied (especially soil, sediments, marine environments). At the time of the literature review (2011) we could not identify any study that systematically investigated the joint action of biocides with other relevant compounds present in biocidal products (e.g. preservatives, surfactants). Recently, however, a first study was published that analysed

[11] The same bias would occur if no effect is observed. However, then the RI cannot be calculated.

combination effects of wood preservatives, including the combined effects of active ingredients and formulation additives (Coors et al, 2012).

Analogous to studies on combination effects of plant protection products, Concentration Addition is the most widely used reference concept for predicting and assessing the mixture toxicity of biocides, followed by Independent Action (Response Addition, Abotts Formula) and Effect Summation. Claims of "strong synergistic" or "antagonistic" mixture toxicities can be traced back to only a very few publications. Excluding these results yields an average ratio of predicted to observed mixture EC50 of 1.02 for 89 analysed binary mixtures and of 1.12 for the analysed ternary mixtures. This is a first indication that CA might perform equally well for biocides as for pesticides.

The documentation of the mixture studies in the analysed publications varies dramatically. In the majority of cases a consistent, quantitative assessment and comparison of the results from different publications is hampered by limitations in either the study design or its documentation, or by use of different approaches to quantify deviations between predicted and observed mixture toxicities. Improved study designs and data documentation are therefore urgently needed in order to allow firmer conclusions for the environmental risk assessment of biocide mixtures.

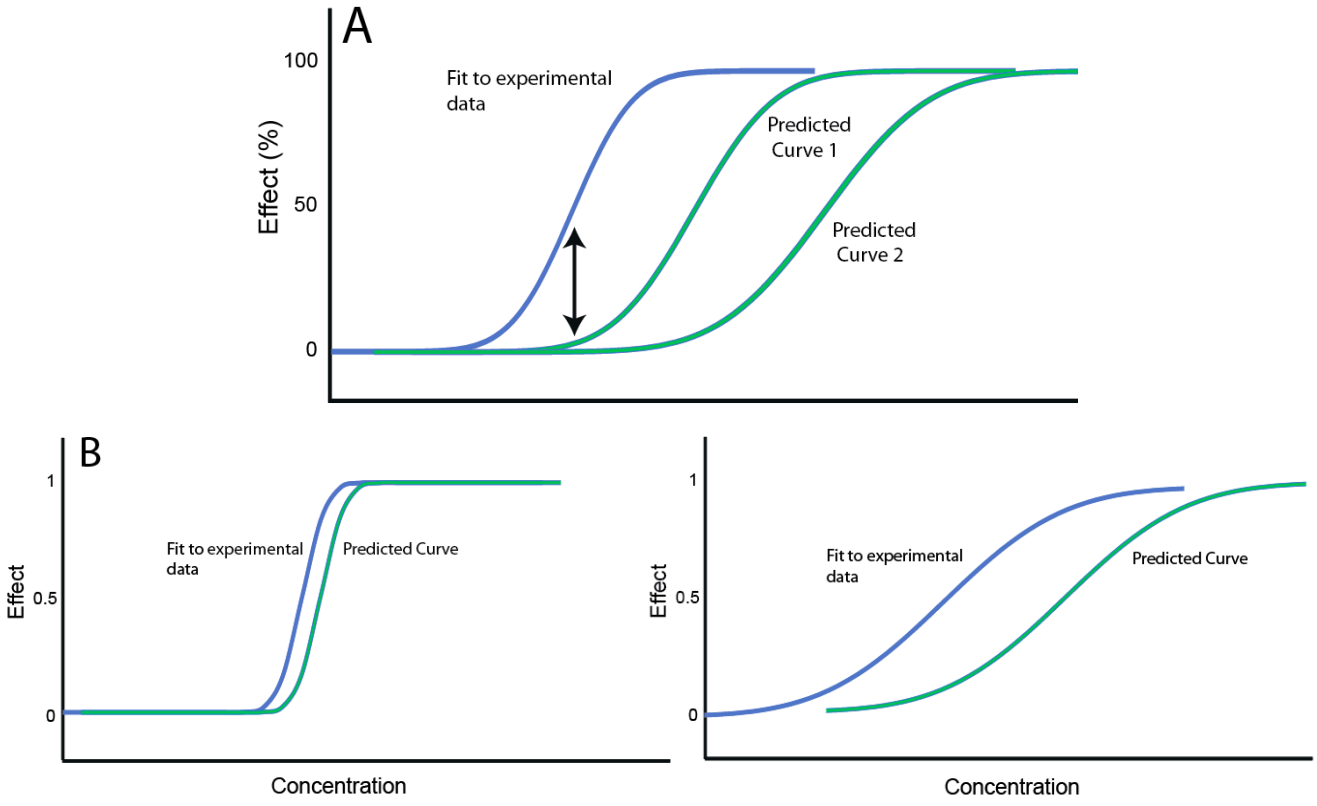


Figure 6.5 Limitations of the RI in an effect-based comparison of experimental and predicted mixture toxicities

A) Both predictions (green curves) underestimate the observed toxicity (blue curve), the second predicted curve clearly has a worse predictive power. However, the RI, if based on a comparison between observed 50 % and the

effect predicted at the same concentration (indicated by the vertical arrow), comes to a similar numerical value for both situations.
B) In both of the depicted situations the RI, if based on the concentration that was experimentally determined to cause 50 % effect, has the same numerical value (5.0) – despite the fact that the mixture toxicity prediction is obviously far better in the left figure.

6.2. Regulatory environmental mixture toxicity assessment in the context of the biocidal product authorization in the EU

In summary, although there is currently substantially less evidence available on biocide mixtures (compared to e.g. mixtures of industrial chemicals or plant protection products), there is currently no strong evidence that would suggest that CA and/or IA are not applicable to biocide mixtures.

In view of the different options for the environmental risk assessment of a chemical mixture (see chapter 4), we suggest the following approach for biocidal product assessment (Figs 6.6, 6.7). It was developed to accommodate various data situations, acknowledging that the initially available data might be quite different for the different products covered by the BPD / BPR.

We base the suggested strategy on component-based approaches as far as possible, as the use of non-testing approaches is already stressed in the BPR in particular with respect to ecotoxicity data for animals. It also facilitates the re-use of existing data for individual ingredients, a factor likely to be increasingly important in the future as the BPR will promote data sharing between applicants. However, the direct testing of a product should be regarded as the "gold standard" for the assessment of acute toxicities or if tests with environmentally realistic mixtures (based on an exposure modeling or monitoring) indicate synergistic interactions. Any component based approach requires that all “relevant” compounds are included in the assessment, i.e. biologically active chemicals that are present at sufficiently high concentrations. Within the framework of biocide legislation such compounds are termed “substances of concern”, i.e. constituents of the biocidal product other than the active ingredient that have “*an inherent capacity to cause an adverse effect on humans, animals or the environment and is present or is produced in a biocidal product in sufficient concentration to create such an effect.*” (98/8/EC; Art 2e). If no ecotoxicological information is at hand for such substances, the only risk assessment option is the direct biotesting of the biocidal product or the resulting environmental mixture, respectively.

A CA-based assessment is a predictive approach and its accuracy is therefore potentially impacted by several confounding factors. These are (i) the stochastic uncertainty of the input data, (ii) the possible amalgamation of single substance data from different species, biotests and endpoints (iii) the non-consideration of relevant ingredients, (iv) the non-consideration of the competing concept of Independent Action, and (v) possible chemical, toxicokinetic and/or dynamic interactions.

6.2.1 Stochastic uncertainties

Uncertainties in the input data can stem from e.g. inaccurate measurements or an inherently high variability in the data from a particular bioassay. However, as long as there is no systematic bias in the input data, the stochastic uncertainty of the CA-predicted EC50 is always equal to or smaller than the uncertainty of the most uncertain single substance EC50

value. Hence, if the single substance data are of sufficient quality for the respective single substance risk assessment, no special consideration is needed for a mixture toxicity assessment using CA.

6.2.2 Using data from different bioassays and endpoints

CA links the toxicity of individual substance to their joint action. The concept implicitly assumes that all single substance toxicity data are recorded for the same species, in the same bioassay and using the same endpoint. In practice, however, the available single substance data might have been recorded under slightly different conditions, using different endpoints or even stem from different species. The following rules should provide guidance for the application of CA in this context: (i) Toxicity data from different phyla, ecotoxicological endpoints and acute/chronic data should not be combined unless their ecotoxicological implications are similar and adequate assessment factors are considered, see discussion on PEC/PNEC summation below; (ii) different endpoints should only be combined, if their ecotoxicological meaning is similar, e.g. EC50 values based on growth rate and final biomass; (iii) data from acute and chronic studies should not be combined; (iv) if data from more than one species/endpoint are at hand, the most sensitive endpoint of the available endpoints/species should be selected for each compound.

6.2.3 Non-consideration of relevant ingredients

Obviously, if toxic compounds are not considered in a component-based assessment, the calculated risk will be an underestimation of the actual risk of the biocidal product. It is, however, impossible to provide a general estimate of the magnitude of such an underestimation, as this depends on the concentration and ecotoxicological potency of the compounds that are erroneously not included in the assessment. Therefore, special care has to be taken to ensure that all toxic ingredients are included in a component-based assessment of a biocidal product.

6.2.4 IA in the context of biocide authorization

The mixtures that make up a biocidal product will usually not be composed of either only strictly similarly or of only strictly dissimilarly acting compounds. Hence, the application of either CA or IA is inherently biased. From the available evidence (see review in Kortenkamp 2009), it is to be expected that the application of CA to a mixture of not entirely similarly acting compounds will lead to a slightly cautious mixture assessment (slight overestimation of risk). Accordingly, using IA for a mixture of at least partly similarly acting substances would often lead to a risk underestimation. Consequently, should IA be used during the authorization of product, an applicant would need to prove that IA adequately describes the toxicity of the assessed biocidal product. This, however, is only possible by comparing the IA-prediction to experimental data for the product for each considered endpoint. It might therefore be easier and less resource demanding to limit the experimental work to testing the whole product and to omit a component-based analysis using IA. If IA is considered due to dissimilar modes or mechanisms of action of the ingredients, it should be checked prior to any experimental work whether the actual mixture ratio allows for the possibility that IA might indeed lead to a different regulatory outcome of the assessment, see discussion by Backhaus and Faust (2012).

6.2.5 Synergistic mixture toxicity

CA, as well as IA, is based on the assumption that the compounds in a mixture do not interact, neither chemically nor in their toxicokinetic and -dynamic phases. Such interactions might cause synergisms, i.e. a mixture toxicity that is higher than expected by both concepts. Although comparatively rare in general (Kortenkamp, 2009), several examples of synergistic interactions can be found in the literature. They are mainly restricted to mixtures with a few (usually two) compounds, which is exactly the situation that is relevant within the context of biocidal product authorization. For example, the combination of zinc-pyrithione and copper, two antifouling biocides, shows a clearly higher toxicity than predicted by CA in a range of bioassays, due to the trans-chelation of zinc-pyrithione to the significantly more toxic copper-pyrithione (Bao, 2008). Mixtures of organophosphates and carbamates (insecticides) were consistently more toxic to fish than predicted by CA, despite their similar mechanisms of action (Laetz, 2009). This is most likely caused by the inhibition of organophosphate biotransformation to their inactive dicarboxylic acid derivates by carbamates. An additional example of a synergistic mixture toxicity is provided in Figure 6.2. It shows a strong synergistic interaction beween copper and kathon, two active ingredients for biocidal products. In this example CA underestimates the toxicity by at a factor of approximately 4. However, as outlined in chapter 6.1., such a pattern seems a rare exception.

We therefore suggest to initially penalize CA-based assessments with an additional assessment factor, termed "IF" (Interaction Factor), in particular if no ecotoxicity data for the product in question are at hand. This factor shall account for the possibility of synergistic interactions (higher mixture toxicity than predicted due to chemical, toxicokinetic and/or -dynamic interactions). It should be emphasized that the IF is not meant to account for any of the other potential error sources that were outlined above.

A review on the predictive power of CA for pesticide mixtures concluded that in less than 5 % of the published studies the experimental toxicity exceeded the predictions by a factor of 2 or more (Belden, 2007). A recent re-analysis of data available to the German Federal Environment Agency evaluated the predictive power of CA for commercial pesticide products and came to the conclusion that in 50 % of the cases CA predicted the experimental toxicity correctly within a factor of 2 (Coors & Frische, 2011). These studies do not allow estimating an IF, as it is unclear to which extent each of the factors listed above (stochastic uncertainty, interactions, incomplete consideration of all components present) was responsible for the overall deviations between CA-predictions and observations. But as the general chance of underestimating the risk by more than a factor of 2 seems to be low for the majority of cases, an IF of 2 currently seems sufficiently protective. However, it should be pointed out, that empirical evidence on the joint effects of co-formulants and active ingredients of biocidal products is scarce, and more empirical evidence is urgently needed. Consequently, if available evidence is at hand, the IF might have to be set to a value greater than 2, or the IF could be decreased down to 1 for a specific product if sufficiently justified.

Interactions are highly specific for the test organism, exposure conditions and the compounds involved. As CA is based on the idea of a no-interactions (see state of the art, chapter 3), the concept is principally unable to predict the toxicity of mixtures whose compounds interact. An appropriate IF might, however, be used to ensure that the regulatory CA-based assessment of mixture toxicities is sufficiently protective even against synergistic mixture effects. Consequently, the setting of an IF is not a scientific, but a regulatory decision – driven by the protection level aimed for and the underlying cost-benefit assumptions.

Inert compounds (e.g. water, non-soluble pigments) are chemicals that do not show any toxic effects, even at excessive concentrations. They do not have an impact on the mixture toxicity

assessment, as both concepts assume that they do not contribute to the overall toxicity of the product. However, information on whether such compounds might increase the uptake, inhibit the biotransformation or otherwise interact with biologically active compound of the product in a manner that increases the overall toxicity should be considered on a case-by-case basis.

Inert compounds need to be clearly differentiated from compounds that are not an active ingredient *per se*, as they are not directly effective to the target organism, but still are biologically active. Piperonyl butoxide for example would fall into this group, as the compound itself is not biocidal, but increases the toxicity of other biocides by inhibiting their cytochrome P450-driven metabolization. Such "synergists" might lead to serious risk underestimations, and hence have to be considered specifically in a case-by-case manner.

Requested input data

The outlined strategy keeps the initial data demands a low as possible (i.e. optimizes resource efficacy and limits unnecessary testing), while at the same time ensuring an adequate protection of the environment, according to the philosophy and approaches of the BPD/BPR. The minimum requested set of data for a component-based assessment (Figure 6.6. and 6.7.) consists of (i) solid and complete information on the product composition, and (ii) the PEC/PNEC ratio for the most risky compound, typically the active ingredient. This implies that the PEC/PNEC ratio of all other compounds is known to be lower, which should be demonstrated for each relevant endpoint prior to the assessment. As only semi-quantitative data are needed for this purpose, QSAR-estimates, hazard classification data from classification and labeling according to the CLP Regulation (EC) No 1272/2008, censored toxicity data (e.g. from limit tests) and simple exposure estimates should be sufficient.

The final risk of the product is hence estimated as

$$RQ_{Product} = n \times IF \times \left(\frac{PEC}{PNEC} \right)_{\max} \qquad [6.2.1]$$

n is the number of relevant compounds in the mixture, while the interaction factor *IF* accounts for possible toxicokinetic or -dynamic interactions. Eq. 6.2.1 is providing a first tier worst-case risk estimation, assuming that all compound have a risk quotient equal to $(PEC/PNEC)_{max}$.

This first tier allows an initial precautionary assessment already with a very limited set of data. If there are no reasons for concern (i.e. $RQ_{Product} < 1$), no further testing or data evaluation is required.

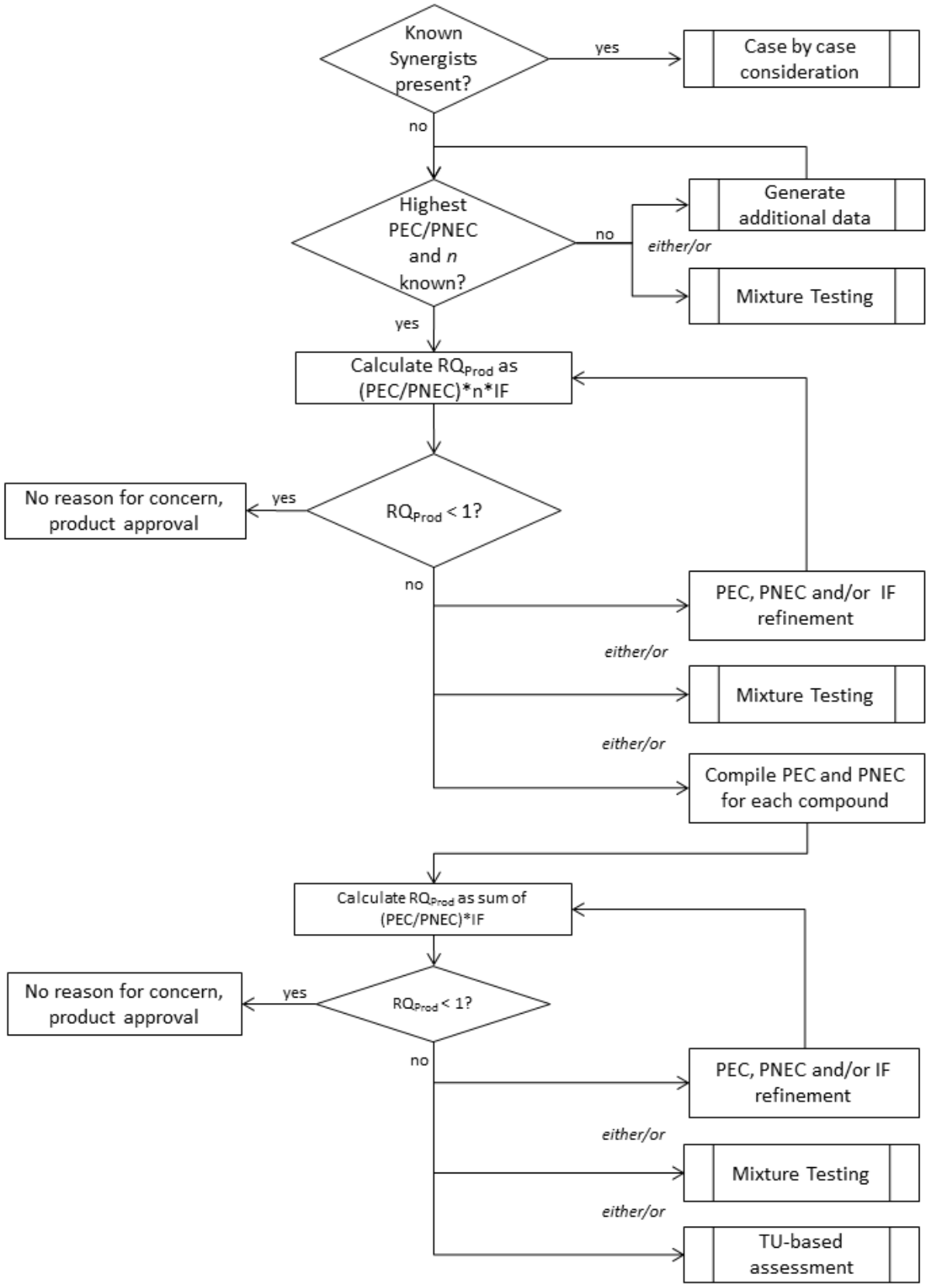


Figure 6.6 Approach for environmental risk assessment of biocide products
PEC = Predicted Environmental Concentration, PNEC = Predicted No Effect Concentration, RQ_{Prod} = Risk Quotient for the Product, TU = Toxic Unit, IF = Interaction Factor, n = number of compounds in the mixture

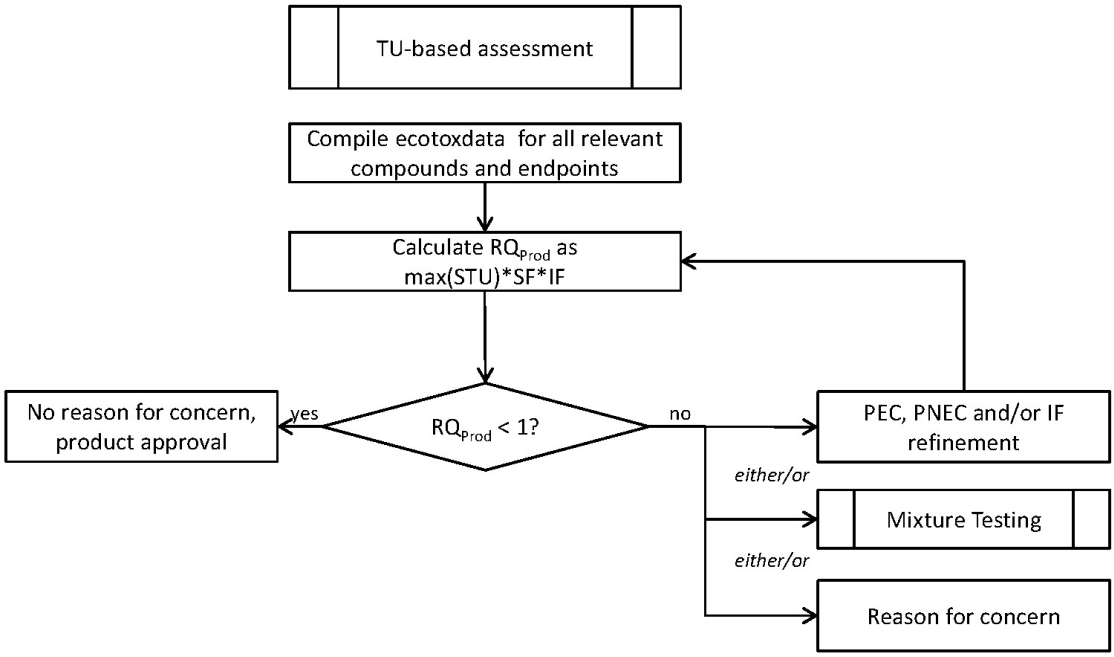


Figure 6.7 Toxic Unit (TU) based approach for environmental risk assessment of biocide products
PEC = Predicted Environmental Concentration, RQ_{Prod} = Risk Quotient for the Product, TU = Toxic Unit, IF = Interaction Factor, n = number of compounds in the mixture, AF = Assessment Factor

Four options exist, if there are potential reasons for concern ($RQ_{Product} > 1$, Fig. 6.6): (i) the PEC and/or PNEC estimate of the most risky compound might be refined by providing additional ecotoxicological data and/or exposure estimates. Such an effort might worthwhile in particular if the PNEC assessment is based only on the so-called base-set of data (short-term toxicity data for algae, daphnids and fish, according to ECHA (2008); (ii) evidence is collected that allows for a better estimate of IF; (iii) the whole mixture (biocidal product) might be subjected to direct biotesting, considering the limitations as outlined above; or (iv) a more detailed component-based assessment is carried out that uses quantitative risk estimates not only for the most risky compound, but for every compound.

If PEC/PNEC ratios are at hand for all relevant ingredients, the risk quotient of the product can be simply estimated by their sum:

$$RQ_{Product} = \sum_{i=1}^{n} \left(\frac{PEC}{PNEC} \right)_i \times IF \qquad [6.2.2]$$

Summing up PEC/PNECs is mentioned in the Technical Notes for Guidance as one option for biocide product assessment (ECB, 2008). However, it should be pointed out that eq. 6.2.2 is fundamentally different from CA (eq. 4.1), as the PNECs from the various compounds might be based on data from completely different endpoints and species. Hence eq. 6.2.2 violates one of the fundamental assumptions of CA. However, it can be proven that eq. 6.2.2 provides a conservative approximation of CA (Backhaus, 2012). Furthermore, it is a major advantage of the PEC/PNEC sum (eq. 6.2.2) that it can be applied even if different amounts of data are

available for the different compounds in the product, for example when an extended data set including chronic ecotoxicity data is at hand for the active ingredient, but only base-set data are available for the other substances of concern. For a more detailed discussion on the use of PEC/PNEC sums see (Backhaus, 2012).

Should eq. 6.2.2 still indicate reason for concern ($RQ_{Product} > 1$), the following options exist: (i) direct product testing (but see discussion above); (ii) a refinement of the PEC- and/or PNEC-values by providing additional information on the exposure and / or hazard characterization of the compounds, especially those that dominate the sum of PEC/PNECs, or (iii) the application of CA in the form of a toxic unit summation as follows

:

$$RQ_{STU} = \max(STU_{endpoint\,1}, STU_{endpoint\,2}, ..., STU_{endpoint\,m}) \times AF \times IF = \max\left(\sum_{i=1}^{n} \frac{PEC_i}{EC50_{i,j}}, \sum_{i=1}^{n} \frac{PEC_i}{EC50_{i,j}}, ..., \sum_{i=1}^{n} \frac{PEC_i}{EC50_{i,m}}\right) \times AF \times IF$$

[6.2.3]

AF denotes the resulting assessment factor, in concordance with the corresponding REACH guidelines (ECHA, 2008). Eq. 6.2.3 calculates the sum of toxic units (STU) for each and every of *m* ecotoxicological endpoints (which are species-specific). The maximum STU then indicates which endpoint for which species is most sensitive to the biocidal product in question and which is hence used for the final assessment (ECHA, 2008). It can be proven that the risk quotient that results from summing up PEC/PNECs is always equal or higher than the maximum STU according to eq. 6.2.3 (Backhaus, 2012). Their precise relationship depends on the ecotoxicological profiles of the compounds in the mixture. In case of dissimilar profiles, the ratio between the application of eq. 6.2.2 and 6.2.3 approaches the theoretical maximum of *m* (number of considered endpoints). If the compounds have almost the same ecotoxicological profiles (which can be expected e.g. for a mixture of simple organic solvents), then the risk quotients from both equations become identical.

The maximum ratio between $RQ_{PEC/PNEC}$ and RQ_{STU} of *m* provides a convenient decision criterion on whether the detailed data collection or production in order to conduct a refined assessment based on RQ_{STU} (eq. 6.2.3) might influence the regulatory outcome: if $RQ_{PEC/PNEC}$ is higher than m, RQ_{STU} will always be above 1, i.e. indicate reason for concern.

Employing eq. 6.2.3 requires that data for all relevant compounds are available for all endpoints, as it would otherwise be impossible to determine the maximum of all organism- and endpoint-specific STUs and an appropriate overall assessment factor (AF). This makes an application of equation 6.2.3 – although it most closely follows the conceptual idea of CA – rather demanding.

A risk quotient exceeding one might be caused by the overestimation that results from the application of CA to a mixture of not entirely similarly acting compounds. Details on how to estimate this possible overestimation are provided by Junghans (2006) and Backhaus (2012). The direct testing of the biocidal product might provide additional insight, given that a substantial risk overestimation by CA is possible, which depends on the number of involved compounds, their toxicity and molar ratio in the mixture. Otherwise there would be a clear indication for a reason for environmental concern, which would call for appropriate risk management strategies.

6.2.6 Conclusions and Outlook

The component based assessment of biocide products is a robust approach to account for mixture toxicity when incorporated into an appropriate tiered scheme. Particularly, it allows to focus attention and efforts on those cases for which mixture effects are of potential

concern, and it initially uses only the available toxicity information of the individual components for this purpose. The presented tiered approach might hence serve as a template for the development of specific guideline documents in support of the new biocide regulation (EU) 528/2012. In view of the novelty of the regulation details of the presented approach might require fine-tuning, as soon as more practical experience has been collected. In particular the use and initial size of the IF might warrent later review and perhaps adjustment.

7 Plant Protection Products

7.1 Relevance of mixture assessment under the PPP regulation

7.1.1 Introduction

Given the discussion in the previous chapters, the objective of considering mixture toxicity at all in the risk assessment of PPP is not further discussed here but assumed as the starting point for further analysis. The aim of the present chapter is the analysis of the relevance of considering mixture toxicity for the risk assessment of plant protection products (PPP), in terms of the current assessment praxis and in terms of the consequences of a changed praxis. The consequences of such a change in the assessment praxis are evaluated using the indication for a refined risk assessment or for stricter risk mitigation measures as a proxy. These surrogate parameters were used, because a full environmental risk assessment of the mixtures selected as examples is beyond the scope of the present study as this would require exposure modelling, consideration of refinement options or high-tier testing.

The analysis takes into account current European and national directives, regulations and supporting guidance documents in order to describe the legal background. The analysis of the current implementation specifically on the national level (Germany) was based on a survey of risk assessment reports that were provided by the Federal Environment Agency, Germany (UBA). The UBA is the evaluation authority responsible for the environmental risk assessment which is then delivered to the Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL) as the competent authority for the national PPP authorization and respective risk management. In addition to the analysis of the current praxis, the consequences of applying mixture toxicity concepts are evaluated and discussed for a number of exemplary PPP in order to inform the development of adequate implementation options. This evaluation was done for the different risk assessment areas (birds & mammals, aquatic and terrestrial organisms) and, as far as possible, for various types of pesticide mixtures: combination products, tank mixtures and serial applications of PPP. Another aspect treated in this chapter is the influence of formulation additives and the relevance of considering them in the risk assessment.

7.1.2 Legislative background

The legislation on the level of the European Union (EU) determines the overall regulatory background for the registration and authorization of plant protection products in a member state. The directive 91/141/EEC first published by the European Commission 1991 (EC 1991) and subsequently amended provides the regulatory framework for the authorization of PPP. PPP are authorized at the national level, i.e. by the competent authorities of the EU member states, and may only contain active substances (a.s.) that have been included on Annex I ("positive list") of the directive 91/141/EEC. The requirements for the data to be submitted for the inclusion of an a.s. on Annex I are specified in Annex II of the directive and those necessary for the national authorization of a product are specified in Annex III. In practice, available information on either a.s. or formulated products (usually mono-formulation, i.e. products with one a.s.) are often "bridged" in order to use the data for Annex II and Annex III requirements. The directive 91/141/EEC generally requires that PPP may only be authorized if (among others) "[...] *it is established, in the light of current scientific and technical knowledge*, [...]" that the use of the PPP has *"[...] no unacceptable influence on the*

environment [...]" (EC 1991). This is further specified in the Annex VI (General Principles on Decision Making) in that "*Member States shall ensure that use of plant protection products does not have any long-term repercussions for the abundance and diversity of non-target species.*" Notwithstanding such implicit requests for considering combination effects of PPP applied simultaneously or sequentially, the directive 91/141/EEC does not specifically mention how combination effects of mixtures of substances in the PPP shall be assessed or even explicitly requires the consideration of such effects in the risk assessment.

Directive 91/141/EEC has been replaced in December 2009 by a new regulation, the regulation EC No 1107/2009 of the European Parliament and of the Council (EC 2009b). This regulation requests that PPP and their residues "[...] *shall have no immediate or delayed harmful effect on human health, [...], taking into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available;* [...]" and thereby explicitly requires the consideration of combination effects for human health. A similar phrase regarding effects on the environment lacks this specification of cumulative and synergistic effects. However, Article 29 of the regulation requests with regard to the authorization of PPP that "*Following these principles* [Uniform principles for evaluation and authorisation of a PPP], *interaction between the active substance, safeners, synergists and co-formulants shall be taken into account in the evaluation of plant protection products*." The regulation further defines the various components contained in a PPP, namely active substances, synergists, safeners, co-formulants, adjuvants and basic substances, and establishes requirements for the approval of all these components (which are in the following here all addressed by the term "formulation additives"). Respective procedures, directives and regulations still have to be developed and implemented.

The regulation EC No 1107/2009 further points out the need of providing new or revising existing technical guidance documents for the evaluation of PPP, which specify the procedures on how to provide the information and conduct the evaluation of PPP as set in the regulation. While such EU guidance documents generally have a non-binding character, i.e. they are not considered as official legislative documents they can be enforced in a legally binding status at a national level. The revised guidance document regarding the environmental risk assessment for birds & mammals, which has been published in December 2009 (EFSA 2009), considers explicitly mixture toxicity in the evaluation of combination products (i.e. PPP with more than one a.s.). The approach specified in this guidance document is described and discussed by using examples of combination products later on in the present study. Guidance documents for evaluating ecotoxicity of PPP in the aquatic and terrestrial compartment are currently under revision. The currently existing guidance documents in the aquatic and terrestrial compartment do not specifically address the issue of mixture toxicity beyond the assessment of formulated products, i.e. representing a whole-mixture approach.

The relevant national law in Germany regarding the authorization and use of PPP is the just recently revised "*Pflanzenschutzgesetz*" (PflSchG 2012) with its related provisions being still under revision (PflSchMGV 1987, PflSchAnwV 1992). The consideration of combination effects between several a.s. or formulation additives is not specifically required in these legislations on the national level to date.

The relevance of mixture toxicity starts to be generally agreed upon at the overarching European legislative level, as expressed for example in the conclusions of the Council of the European Union regarding combination effects of chemicals (EC 2009a). These conclusions state "[...] *the fact that combination effects from exposure to multiple chemicals from single sources or products are recognised in some parts of Community legislation,* [...], *and that agreed methods for assessment need to be further developed*". It is further acknowledged "[...]

that human beings, animals and plants are exposed to many different chemicals from different sources and pathways, and that recent studies indicate that combination effects of these chemicals [...] *can have serious negative implications for human health and the environment*" (EC 2009a). The EFSA Panel on Plant Protection Products and their Residues published an opinion paper in 2010 (PPR 2010) that discusses specific protection goals for the environment to be addressed by the revised guidance documents. Among others, this opinion paper states that "*multiple stress by the use of multiple plant protection products, being applied at the same time (e.g. tank mixtures) or in sequence, should be assessed to identify 'similar residues' in the area of envisaged use*." (PPR 2010, p. 50).

It can be argued, therefore, that the biological and regulatory relevance of combination effects reflects the current scientific knowledge and that such effects should be considered during the authorization process, while technical knowledge on how to adequately take these effects into account needs further development and guidance.

7.1.3 Current praxis of considering mixture toxicity in member states of the European Union

The aim of this chapter is to provide a quick overview on how EU Member States handle the issues of mixture toxicity in the risk assessment for PPP in order to inform the development of implementation options. Several EU member states were working on implementing mixture toxicity considerations and released updated guidance during the project period.

The non-exhaustive overview presented below clearly demonstrates that currently applied approaches vary considerably among EU member states, if mixture toxicity is at all considered in the national authorisation. Additionally, the applied approaches lack in parts clear and unequivocal guidance.

Consistent and harmonised approaches would be highly welcome for both authorities and applicants (for PPP authorization), and are particularly important with regard to the forthcoming mutual recognition of PPP within the EU ("zonal authorisations") as implemented by Regulation 1107/2009.

The Netherlands

For The Netherlands, the most recent evaluation manual is available online at the webpage of the competent authority (College voor de toelating van van gewasbeschermingmiddelen en biociden, www.Ctgb.nl). This manual describes in Appendix C the consideration of combination toxicity (Van Vliet 2010), which has considerably changed compared to an earlier version from April 2006. Furthermore, some background information and general considerations are additionally provided in the chapter on ecotoxicology of this evaluation manual (pages 10/11). In the most recent version of the evaluation manual, it is stated that concentration addition shall be applied to evaluate the toxicity and the related risk of combination products as well as that of tank mixtures listed on the instructions for use of a product. This consideration shall in principle apply for all organisms, i.e. aquatic and terrestrial.

The calculation is based on the Toxicity Exposure Ratios (TER) derived for the individual a.s. and distinguishes between situations where the trigger thresholds are identical for the a.s. and those where they are different. Combined TER values shall only be calculated within groups of acute and chronic toxicity estimates, respectively. It is not clearly stated, however, if

different taxonomic groups shall be combined or not; a question that arises frequently in the case of the aquatic compartment.

In case of identical trigger thresholds for the individual a.s., the Ctgb manual (Van Vliet 2010) suggests the following:

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

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

This formula can be reduced since the trigger values (= threshold values) are identical:

$$TER_{combi} = \frac{trigger}{\sum_i \frac{trigger}{TER_i}} = \frac{1}{\sum_i \frac{1}{TER_i}} \qquad [7.1.1]$$

In case that the individual trigger thresholds are not identical, the Ctgb manual provides the following formula (Van Vliet 2010):

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

with the formula for the TER_{combi} being hence:

$$TER_{combi} = \frac{trigger_{combi}}{\sum_i \frac{trigger_i}{TER_i}} \qquad [7.1.2]$$

However, the trigger to be used for the combination product, the $trigger_{combi}$, is not clearly defined. According to the formula above, the individual trigger values of the a.s. are subsequently divided by each other. The result changes depending on the order of the individual trigger values. Assuming a case with three a.s. in the product for which different trigger values had been set, being 2 for substance 1, 5 for substance 2 and 10 for substance 3. The resulting $trigger_{combi}$ would be 0.04. As it is not defined which a.s. has to be substance 1 etc, the calculation could as well be made using 10 for substance 1, 5 for substance 2 and 2 for substance 3, which results in a considerably different $trigger_{combi}$ of 1.

Note that, if triggercombi were defined as the sum of all individual triggers, TERcombi resembled the harmonic mean of the TERi weighted by the $trigger_i$.

$$TER_{combi} = \frac{\sum_i trigger_i}{\sum_i \frac{trigger_i}{TER_i}} \qquad [7.1.3]$$

However, in both situations of identical or different triggers, the TERcombi must be above the triggercombi to indicate that the risk is acceptable (Van Vliet 2010). This condition reduces the two above formulae to one general formula that includes the special case of identical $trigger_i$:

$$\frac{TER_{combi}}{trigger_{combi}} > 1 \;\Leftrightarrow\; \frac{1}{\sum_i \frac{trigger_i}{TER_i}} > 1 \qquad [7.1.4]$$

The component-based risk assessment for combination products shall always be conducted and then compared to the toxicity of the formulated product, if available (Van Vliet 2010). The evaluation manual further states that the risk assessment shall be based on the smaller TER value of the two, i.e., it calls for the more conservative approach. As a consequence, this approach would not allow refining a risk assessment that had been based on theoretical mixture toxicity calculations by actually testing the combination product.

For a risk assessment based on HQ values instead of TER values (i.e. for bees and non-target arthropods), the Dutch evaluation manual foresees the simple addition of the HQ values derived for the individual a.s. and a comparison to the usual trigger values for these organisms (Van Vliet 2010). Since an HQ is in principle the reciprocal of a TER, this approach is analogue to the approach described above.

Finally, the consideration of combined toxicity can be waived according to the evaluation manual and replaced by a risk assessment for the most toxic substance in the combination product (or tank mixture) if "[...] the difference in toxicity of the active substances for the different species is large (more than a factor of 100) and the calculated PEC-values are in the same order of magnitude" (Van Vliet 2010). Yet, this statement is not unequivocal. It remains for example open if such a large difference in toxicity between the a.s. must be observed for all species and/or endpoints or if an observation for some endpoints or some species would be sufficient.

United Kingdom

The guide for applicants and the respective data requirement handbook as published online (www.pesticides.gov.uk) by the competent authority of the UK (since April 2009: Chemicals Regulation Directorate Pesticides, CRD, as a part of the Health and Safety Executive, HSE) does not specifically refer to or requests the consideration of combination effects in the environmental risk assessment of plant protection products.

The unit formerly responsible for the PPP registration in the UK was the Pesticide Safety Directorate (PSD, also as a part of HSE) that published in March 2009 a guideline on the need of ecotoxicological studies with formulations and their use in the risk assessment (PSD 2009), which is still available from the webpage of the competent authority. This document summarizes the requirements for testing formulated products as given in the respective directive and guidance documents. It also addresses specifically the case of formulation additives, stating that studies with the product will usually be required when the formulation contains "*significant amounts (>10 % w/w) of emulsifiers and solvents*" with regard to aquatic toxicity, toxicity to honey bees, and acute earthworm toxicity (PSD 2009). For non-target arthropods and terrestrial plants, studies should usually be conducted with the formulated product to account for potential effects of formulation additives (PSD 2009).

In chapter 5, guidance on combination products (named here "*Mixed active substance formulations*") is provided (PSD 2009). Consideration of additive effects is explicitly restricted to acute toxicity and to products "[...] *where the toxicological action of component actives are similar*" (PSD 2009). This requirement of similar toxicological action is not further defined, which contains considerable uncertainty both for the authority and for applicants. To prove "similar toxicological action" extensive information on the

pharmacodynamic and pharmacokinetic of the a.s. is deemed necessary (Borgert et al. 2004), which may not be available to a sufficient degree for pesticides. Besides, toxicological actions of an a.s. may differ among different target and non-target organisms.

The consideration of combination effects may be conducted either by formulation testing (i.e. a whole-mixture approach) or by considering additivity based on the concept of concentration addition (i.e., by a component-based approach). The calculated mixture toxicity of the formulated product shall be compared to the initial PEC of the product in soil and water. Which proportion of the substances shall be fed into the calculation of mixture toxicity (i.e. the proportion in the product or the proportion at their PECs) is not specified in the document. In contrast to the Dutch guidance on combination effects, it is further not specified in the British guidance if actual testing of the formulated product can overrule a component-based mixture toxicity risk assessment.

An alternative approach is suggested as particularly suitable for terrestrial vertebrates (i.e., birds & mammals), which requires to calculate an exposure estimate in terms of the most toxic compound based on the toxic unit concept (PSD 2009). The actual calculation is not described in further detail and it is not indicated to which toxicity estimate this exposure estimate shall be compared.

The applicant guidance provided online by CRD has also a section on tank mixtures. CRD distinguishes between "convenience tank-mixes" and "positive tank-mixes". The first term refers to tank mixtures applied in order to save time and effort (i.e. conducting one spray application instead of two), while the latter term refers to tank mixtures that are applied in order to obtain better pest control in terms of efficacy or reduce the application rate of one or more of the tank mixture components. Generally, recommendations of tank mixtures that are to be included in the product labelling need approval from CRD. However, the requirements for this approval comprise currently only data on efficacy and physical, chemical and technical properties of the tank mixture, but apparently no data on environmental risks. As stated explicitly in the applicant guidance, the policy of CRD with regard to tank mixture has been under review at the time of this evaluation.

An update on combined toxicity risk assessments was published online by CRD on June 16, 2011. This update basically refers to the combined toxicity risk assessment proposed by EFSA for the birds & mammals assessment (see later chapter). The CRD approach differs from the EFSA approach in that it proposes a screening step. If one active substance is "*clearly driving the risk assessment*", the Tier I assessment for all a.s. is passed "*with a margin of safety*", or the "*mammalian toxicology assessment identify that a combined assessment was not required*" (CRD 2011, online) a further assessment of combined toxicity is not deemed necessary for a combination product. However, none of the three conditions is further specified, e.g. with regard to the size of the margin of safety or the definition of what means "clearly driving". The update further states that "*combined toxicity should also be considered for other species e.g. aquatic organisms, earthworms, etc in consultation with the formulation guidance document until further notice"* (CRD Regulatory Update 16 June 2011, online).

The Northern zone

The Northern zone with regard to authorization of PPP comprises Denmark, Estonia, Finland, Iceland, Latvia, Lithuania, Norway, and Sweden. These countries issued a "Guidance document on work-sharing in the Northern zone in the registration of plant protection products" (the recently updated version was obtained in December 2011 online at

http://www.kemi.se/en/Content/Pesticides/Plant-Protection-Products/Guidance-and-forms---plant-protection-products/). This guidance is explicitly not legally binding, but intends to improve mutual recognition procedures in the Northern zone. Article 4.6.7 refers to mixture toxicity and states that for combination products either product tests must be available for the risk assessment or component-based approaches must be applied. The stated formula for the component-based risk assessment is basically identical to the one described in the Ctgb document described above, i.e.,

$$\frac{1}{\sum_i \frac{trigger_i}{TER_i}} > 1 \qquad [7.1.5]$$

as condition for approval. This approach should be applied separately for the various taxonomic groups in the aquatic compartment (fish, crustaceans, algae, and aquatic plants). For mesocosm studies, the "*overall NOEC*" should be used for TER calculation.

Germany

There is no official guidance publicly available that describes the consideration of mixture toxicity within the German PPP authorisation process. Since about 2006, however, CA is applied in the risk assessment for birds & mammals and, less frequently, for aquatic organisms on a case-by-case basis according to the following formula:

$$\mathrm{TER(mix)} = \left(\sum_i \frac{1}{\mathrm{TER}(\mathrm{WS}_i)} \right)^{-1} \qquad [7.1.6]$$

with TER(mix) being the TER of the mixture and TER(WS_i) the TER of each individual mixture component. This approach has been presented on various workshops and scientific meetings, also with regard to the evaluation of tank mixtures (e.g., Frische et al. 2007). When applied to the aquatic compartment, the issues pointed out before (i.e., combining different taxonomic groups and levels of organisation such as single species and multispecies tests, combining acute and chronic toxicity estimates, and the choice of the relevant trigger threshold) arise without being currently resolved in a defined guidance.

7.1.4 Plant protection products – Basics of the risk assessment and types of mixtures

The authorisation of PPP depends, among other issues, on an environmental risk assessment. This assessment basically follows a risk-based approach, i.e., it relies on the comparison of an estimated concentration or dose that does (not) affect non-target organisms with the predicted environmental exposure concentration/dose (PEC or DDD) of the substance(s) in question. Typically, the comparison is expressed in a risk quotient that has to meet a defined acceptability criterion (= threshold trigger) in order to indicate acceptable risks. Risk quotients are either above or below the relevant trigger value. Risk quotients are strictly related to the assumptions of the assessed application and thus exposure scenario, while the assumptions may be changed in a refinement of the risk assessment. Because of their scenario- and assumption-based derivation, risk quotients do not represent a measure that quantifies the actual environmental risk, e.g. in terms of the likelihood of the occurrence of unacceptable effects under field conditions. Therefore, a tenfold difference between two risk

quotients does not mean that there is a tenfold higher environmental risk in one of the cases. In some risk assessment areas, e.g. effects on microbial communities, no risk quotients are calculated within the risk assessment but observed effects at agricultural application rates are directly compared with the non-treated controls in laboratory or (semi)field tests and are considered as acceptable if they do not exceed specified limits (e.g. less than 25 % effect compared to the control). It is important to note that for all assessment areas (i.e. birds and mammals, aquatic, soil, etc.) to be considered a tiered risk assessment approach is proposed by the existing legislation with the different tiers being further specified by the technical guidance documents. Hence, a risk assessment that indicates risk at an initial (lower) tier can generally be refined, e.g. by providing more realistic estimates for exposure estimates or by refining the toxicity estimate, e.g. by conducting so-called higher-tier studies such as for example aquatic mesocosm or earthworm field studies.

Different agricultural practices can cause the presence of mixtures of pesticides in the environment:

1. The simultaneous application of different active substances on the same field. This case is represented most obviously in combination products, i.e. PPP that contain two or more active substances. Another source can be tank mixtures, i.e. the mixing of different PPP by the farmer directly ahead of the application ("in the tank"). In both cases, the mixture is released as such into the environment and its composition at that time is principally known.
2. The serial application of different active substances on the same field. The composition of the mixture is at no time known but can only be estimated by fate modelling, because the mixture is only constituted after release into the environment. The serial application of the same active substance on the same field is not really a mixture (combination) effect issue, but rather an exposure issue since it is generally accepted that effects increase with increasing concentrations of the same substance. This specific case is covered in the current praxis of the risk assessment by using a multiple application factor (MAF) in the calculation of the relevant exposure measure (except for bees), which accounts for the potentially increased environmental concentration of the substance due to repeated application. However, this MAF takes only into account repeated applications of an a.s. within the same product, but not repeated applications of the same a.s. by different products.
3. The simultaneous or serial application of the same active substance on different fields. This agricultural practice on a landscape scale can result in higher concentrations of the active substance in adjacent environmental compartments than foreseen within the risk assessment for a defined application scenario related to an individual field as it is currently conducted for each single product.
4. The simultaneous or serial application of different active substances on different fields. This practice can result in mixtures of active substances in various environmental compartments on the landscape scale.

The different types of mixtures outlined above increase from 1 to 4 in terms of their complexity, particularly with regard to the possibility to predict the composition of the mixture (i.e., the identity and concentrations of components) occurring in the environment. Spatial-temporal aspects such as differential fate of the active substances and their transport processes in the environment are determining factors and must be taken into account. Hence, estimating the exposure to pesticide mixtures resulting from such agricultural practices requires sophisticated fate and transport models. While predictive exposure assessment of mixtures is beyond the scope of the present study, it is important to be aware of this complexity. This is because mixture toxicity can only be predicted by theoretical component-

based approaches for clearly defined mixtures. Hence, when it comes to implementing mixture toxicity in the risk assessment it is essential to unambiguously define the mixture to which the assessment relates and use the correct relative proportions of mixture components in the calculation of the mixture toxicity.

In order to facilitate a better understanding of which mixture would be subject for an evaluation if mixture toxicity were to be implemented within the currently applied assessment scheme, a matrix has been developed that relates the composition of the type of mixtures with the different risk assessment areas (Table 7.1). There are in general two types of mixture compositions that are relevant: the mixture components are present in the relative proportions as applied in agriculture (e.g., as applied in a combination product or tank mixture) or the mixture components are present in the relative proportions defined by their individual predicted environmental concentrations (PEC) or doses. The distinction between these two types arises from the current risk assessment scheme for single active substances where the toxicity estimate of a substance is related to its exposure estimate as represented by either directly the agricultural application rate (i.e., grams a.s. or grams product per hectare) or by the predicted concentration in the considered environmental matrix or the dose expected to be taken up by non-target organisms. Hence, while the effect-related site of the mixture toxicity consideration may remain the same, the exposure-related site may change and, thereby, also the identity of the assessed mixture with regard to the proportional composition of its components.

The relationship of the matrix with the four types of mixtures potentially resulting from agricultural practice, as listed in Table 7.1, is illustrated in detail in the following chapters for exemplary cases of PPP mixtures. Each cell of the matrix is discussed in this context with regard to typical data availability, current praxis of the risk assessment and the consequences as well as specific problems when mixture toxicity is taken into account.

The same aspects and general considerations discussed above for mixtures of different actives substances applies for mixtures of active substances and formulation additives with the most important difference that formulation additives are typically not regulated in the same way as active substances according to the current assessment practice. Therefore, consideration of formulation additives will be addressed separately in the last chapter.

Table 7.1 Matrix of mixtures that may be assessed for their environmental risk following the current risk assessment scheme for single active substances
The matrix distinguishes the mixtures based on their composition with regard to the relative proportions of the mixture components and the different risk assessment areas.

Risk assessment area	**Mixture Composition**	
	Components in the relative proportions of applied mixture	**Components in the relative proportions of predicted environmental mixture**
Birds & Mammals	acute toxicity – first tier	acute toxicity – refined
	long-term toxicity – first tier	long-term toxicity – refined
	secondary poisoning	secondary poisoning – refined
Aquatic compartment	spray drift – occasionally *	spray drift, run-off & drainage – first & higher tier
Terrestrial organisms	bees, non-target arthropods, plants, micro-organisms – first & higher tier	–
	earthworms & other soil macro-invertebrates – field and semi-field studies, occasionally long-term studies	earthworms & other soil macro-invertebrates – acute & long-term studies

* if the predicted environmental concentration is calculated for the applied mixture as a whole without using substance-specific fate parameters for the individual components.

7.1.5 Combination products

Currently, only some of the mixtures resulting from simultaneous application of different active substances on the same field (listed as #1 in chapter 3) are subject to authorisation, namely all combination products and those tank mixtures that are specifically announced by the producer on the product labels. The present chapter will focus on combination products, while tank mixtures are discussed in the following chapter.

In order to obtain an overview on data availability and the current praxis of the UBA with regard to consideration of mixture toxicity, 15 risk assessment reports of PPP (selected by UBA) were evaluated. All reports relate to recently submitted requests for product authorization and concern herbicidal or fungicidal combination products used in agriculture, pasture or vineyards. There were no specific selection criteria beyond these considerations. Table 7.2 provides an overview on the evaluated combination products. There were 29 different a.s. present in these 15 combination products, with two of them (metsulfuron and propamocarb) being present in two products The products will in the following be referred to by their number listed in Table 7.2.

Table 7.2 Key information on the 15 evaluated combination products.
Combination products selected for a detailed analysis with regard to implementing mixture toxicity considerations are indicated in bold.

Number	Pesticide Type	Active substances
1	fungicide	epoxiconazole & metconazole
2	fungicide	tebuconazole & prothioconazole
3	fungicide	fluopicolide & propamocarb
4	fungicide	iprodione & thiophanate-methyl
5	fungicide	metsulfuron methyl & tribenuron methyl
6	fungicide	cymoxanil & propamocarb
7	fungicide	folpet & benthiavalicarb isopropyl
8	fungicide	picoxystrobin & cyprodinil
9	herbicide	phenmedipham & ethofumesate & metamitron
10	herbicide	flufenacet & metosulam
11	herbicide	diflufenican & metsulfuron methyl
12	fungicide	fluazinam & metalaxyl-M
13	herbicide	MCPA & dicamba
14	herbicide	terbuthylazine & pethoxamid
15	fungicide	myclobutanil & quinoxyfen

One of the intended uses (e.g. treatment of barley brown rust) was selected for the analysis, usually the supported use with the highest application rate and the most frequent applications. For each product, the same selected intended use was evaluated for the various risk assessment areas.

For each risk assessment area, the current praxis will be shortly described in order to relate the mixture represented by a combination product to the various cells of the matrix shown in Table 7.1. This description is then followed by the analysis of the 15 evaluated products to indicate the typical availability of data, current praxis of mixture toxicity consideration by the UBA, and the consequences of implementing mixture toxicity considerations within the risk assessment.

As outlined in Chapter 3, there are different approaches currently being applied or discussed by Member State authorities in order to consider mixture toxicity in the environmental risk assessment of PPP. They are mostly based on the concept of Concentration Addition (CA), but may differ in some aspects. The consequences of implementing the general approach based on CA will be illustrated by using five of the 15 evaluated combination products as examples. Since data availability and thereby applicability of CA differs among the risk assessment areas, the three areas birds & mammals, aquatic organisms and terrestrial organisms will be addressed separately in the following sub-chapters.

The calculation of the toxicity exposure ratio (TER) for commercial combination products can be conducted based on the CA-concept according to the following formula:

$$TER_{mix} = \frac{toxicity\ estimate\ of\ mixture}{exposure\ estimate\ of\ mixture} \qquad [7.1.7]$$

Under the condition that mixtures of strictly identical composition (identical relative proportions of the mixture components) are considered for the toxicity as well as the exposure estimate, this approach is identical with the summation of reciprocal TER values according to

$$\frac{1}{TER_{mix}} = \sum_i \frac{1}{TER_i} \qquad [7.1.8]$$

with TER_{mix} being the TER of the mixture and TER_i the TER of each individual a.s. in the mixture. These formulae are at the basis of most currently applied or developed approaches and will be specified and applied for the different risk assessment areas in the following.

BIRDS & MAMMALS

In all but one of the 15 evaluated UBA reports, the risk assessment for birds & mammals had been conducted according to the then most current guidance document (SANCO/4145/2000 – final, EC 2000). A revised guidance document has been published on 17 December 2009, which should be applied in submissions since 1 July 2010 (EFSA 2009). In the present study, the analysis of the current praxis regarding the implementation of mixture toxicity relates therefore to the praxis according to a guidance document that has been revised in the meantime. Recommendations for how to consider mixture toxicity in the future, on the other hand, should relate to the most current guidance document published in 2009. The following analysis aims therefore to take both aspects into account.

The risk assessment for birds & mammals follows a TER (toxicity-exposure ratio) approach. According to the former guidance document, the TER is calculated as the ratio between the toxicity estimate (LD_{50} or NOEL) and the exposure estimate (estimated theoretical exposure, ETE) derived separately for each evaluated scenario.

$$TER = \frac{NOEL\ or\ LD_{50}}{ETE}$$

[7.1.9]

ETE is calculated by the following formula:

$$ETE = \frac{FIR}{bw} * C * AV * PT * PD$$

[7.1.10]

with *FIR* being the food intake rate of the species, *bw* the body weight, *C* the concentration of the a.s. in the fresh diet (residue concentration), *AV* the avoidance factor, *PT* fraction of diet obtained in the treated area, and *PD* the fraction of the food type in the diet. To obtain *C* in the case of multiple applications of a product, the residue concentration C_0 is corrected by multiplication with the multiple application factor (MAF).

In the first tier assessment, the parameters *AV*, *PT* and *PD* are set to 1 as worst-case assumptions. The parameters *FIR* and *bw* are obtained from tabulated default data for each specific scenario that has to be considered. Hence, in the first tier assessment the exposure estimate of each a.s. relates to its concentration in the product by a fixed factor *a* that is identical for all a.s. in a product with

$$a = \frac{FIR}{bw} * AV * PT * PD$$

[7.1.11]

The assessment of secondary poisoning of birds and mammals is also based on a TER approach using the respective long-term toxicity endpoint. The dietary exposure is calculated using as substance-specific parameters the bioconcentration factor in fish (BCF_{fish}) and the Kow and Koc (as proxy for the BCF_{worm}) as well as the predicted environmental concentration of the a.s. in surface water (PEC_{sw}) and in soil (PEC_{soil}) for secondary poisoning via fish and earthworms, respectively.

The exposure estimate in the risk assessment for birds & mammals is the amount of the a.s. that is expected to be ingested by the animal. Hence, the ETE_{mix} as the sum of all ETE_i relates to a mixture with the relative proportions of the components as predicted in the environment (compare Table 7.1). In the case of the first tier assessment, however, this mixture composition is identical to the one of the combination product itself with regard to the relative proportions of the mixture components. This is because all ETE_i are related to the concentration of the substance *i* in the product by the very same factor *a*, resulting in no change of the relative proportions of the a.s.

The threshold value for the TER is 10 for acute and short-term toxicity for birds as well as acute toxicity for mammals, while a threshold value of 5 applies to long-term toxicity for birds and mammals as well as secondary poisoning. If the respective TER in the first tier assessment does not exceed these threshold values, a need for a refinement of the risk assessment is indicated.

In higher-tier assessments, the parameters *AV*, *PT* and *PD* can be refined to account in the calculation of *ETE* more realistically for field conditions. Further, the residue concentrations can be refined using e.g. measured data, or tabulated *FIR* and *bw* data can be replaced with data from relevant species. The MAF can additionally be refined using measured residue

("disappearance") data. Under certain defined circumstances, another option for refinement is a reduction of the threshold value from 10 (acute and short-term toxicity) and 5 (long-term toxicity) to lower values.

As a consequence of the refinement of the exposure estimate, the factor *a* that relates the concentration in the product to *ETE* as exposure estimate can be different for each a.s., i.e. *a* is substance-specific. The relative proportions of the a.s. in the resulting mixture ETE_{mix} will therefore differ from the relative proportions of the same a.s. in the combination product unless exposure estimates for all a.s. are refined in the same way. The latter is particularly unlikely in the case of a refinement based on substance-specific fate data. Hence, the mixture to be considered in a refined assessment is the mixture of a.s. in the relative proportions predicted to be environmentally relevant, which is usually not identical to the relative proportions present in the product.

Data availability

For all of the 29 individual a.s. present in the 15 evaluated combination products, the required laboratory data for acute and long-term toxicity towards birds and mammals were available. Only for the short-term risk assessment for birds, which is no longer required under the new regulation, data were not available for all a.s. (Table 7.3). In addition, respective data obtained with the combination product were available in 9 cases for acute toxicity to mammals and once each for acute toxicity to birds and long-term toxicity to birds.

While *C. virginianus* (Bobwhite Quail) and *Rattus sp*. are the most frequently used test species for birds and mammal toxicity tests (based on the selected 15 reports), the overview illustrates that endpoints from various other species can as well drive the risk assessment. In some cases, the toxicity estimate related only to male or female animals because sex-specific toxicity was taken into account. Particularly with regard to the long-term toxicity, non-standardised exposure durations and the use of the most sensitive of several different response variables contributed additionally to data heterogeneity.

Censored data represented a large proportion of acute toxicity estimates. Censored data (i.e. a toxicity estimate given as greater than a certain value) result from the finding that the test substance did not cause more than 50 % mortality at the highest tested concentration or no toxicity was observed in a limit test (test with one single dose, e.g. 2000 mg/kg body weight in the avian acute oral toxicity test (OECD guideline 223) resulting in LD_{50}>2000 mg/kg bw). The proportion of censored data is much lower for the long-term toxicity estimates.

Table 7.3 Summary of toxicity estimates used for the birds & mammals risk assessment

According to the guidance document (EC 2000) together with the number of cases where data obtained with a particular species were used for the assessment and the proportion of censored data for the in total 29 different a.s.

	Toxicity estimate [1]	**Species (number of a.s.)**	**Proportion of censored data**
Acute toxicity to birds	LD_{50} (mg/kg body weight); acute oral exposure	*Colinus virginianus* (24) *Anas platyrhynchos* (2)	58.6 %

		Cortunix japonica (1) *Carduelis chloris* (1) *Phasanius colchicus* (1)	
Short-term toxicity to birds	LD_{50} (mg/kg body weight/day); 5-day dietary exposure	*Colinus virginianus* (20) *Anas platyrhynchos* (7) no data reported (1)	78.6 %
Long-term toxicity to birds	NOEL (mg/kg body weight/day); most sensitive parameter measured in reproduction study; dietary exposure for several weeks	*Colinus virginianus* (19) *Anas platyrhynchos* (11) *Cortunix japonica* (1)	3.4 %
Secondary poisoning of birds	NOEL (mg/kg body weight/day); bioconcentration factors earthworm and fish	*see above*	
Acute toxicity to mammals	LD_{50} (mg/kg body weight); acute oral exposure	Rat (26) Mouse (2) Rabbit (1)	58.6 %
Long-term toxicity to mammals	NOEL (mg/kg body weight/day); most sensitive parameter measured in multigenerational study; dietary exposure for several weeks	Rat (19) Rabbit (9) species not reported (1)	3.4 %
Secondary poisoning of mammals	NOEL (mg/kg body weight/day); bioconcentration factors earthworm and fish	*see above*	

[1]: LD_{50} median lethal dose; NOEL no observed effect level

Current praxis of considering mixture toxicity

In several risk assessment reports, mixture toxicity was considered by the UBA using a CA-based approach according to the following formula:

$$\frac{1}{TER_{mix}} = \sum_i \frac{1}{TER_i}$$

[7.1.12]

with TER_{mix} being the TER of the mixture and TER_i the TER of each individual a.s. in the mixture.

Metabolites or formulation additives were never considered additionally to the parent compounds for mixture toxicity. In two cases the parent compound (prothioconazole and thiophanat-methyl) had been replaced by the main metabolite in the risk assessment.

In the following, the consideration of mixture toxicity in the 15 UBA reports is summarized for the birds & mammals risk assessment. Since one of the key questions to answer in the analysis was the frequency with which the consideration of mixture toxicity would demand a refinement of the risk assessment, Table 7.4 summarizes how often refinements were triggered by the individual a.s. and, as comparison, the number of cases when only the data available for the product itself (representing the whole mixture approach) or only the consideration of mixture toxicity as applied by the UBA had triggered a refinement.

In two cases, a refinement had been triggered only by the consideration of mixture toxicity but not by the assessment of individual a.s. or, if applicable, by the data available for the product itself. One of these two cases was acute toxicity to birds (combination product # 9) and the other one long-term toxicity to mammals (product # 4). The toxicity of the product itself triggered more often a refined risk assessment, both in terms of the absolute number of cases (1 and 4 for acute toxicity to birds and mammals, respectively) and in relation to the number of cases where such an assessment could be conducted (1 out of 1; 4 out of 9 for acute toxicity to birds and mammals, respectively).

Table 7.4 Frequency of a refined risk assessment
Triggered in the first tier assessment by the assessment of at least one of the individual active substances (a.s.) in the combination product, only by the assessment based on product toxicity data and only by the consideration of mixture toxicity in the 15 evaluated combination products with regard to the risk assessment for birds & mammals as conducted by the UBA. Given in brackets is the number of products for which the respective assessment had been conducted by the UBA and, hence, a refinement could have been triggered in the first place.

	Refined risk assessment of the combination product triggered		
	by the assessment of individual a.s.	only by the assessment based on product data	only by considering mixture toxicity
Acute oral toxicity birds	1 (15)	1 (1)	1 (7)
Short-term toxicity birds	0 (15)	0 (0)	0 (3)
Long-term toxicity birds	10 (15)	0 (1)	0 (5)
Acute toxicity mammals	3 (15)	4 (9)	0 (1)
Long-term toxicity mammals	10 (15)	0 (0)	1 (5)

Consequences of implementing mixture toxicity in the risk assessment

The risk assessment according to the revised guidance document (EFSA 2009b) follows the same principle of a tiered approach as outlined in the previous guidance (EC 2000). Yet, the input parameters of the toxicity exposure ratio TER are slightly different now. While the parameters used for the toxicity estimate have not changed (LD_{50} or NOEL), the parameter ETE has been replaced by the *daily dietary dose* (DDD):

$$TER = \frac{NOEL\ or\ LD_{50}}{DDD} \qquad [7.1.13]$$

DDD for the first tier assessment is calculated using the application rate, application frequency and intervals as well as tabulated default values (shortcut values) for indicator and generic focal species that are pre-defined for a large number of applications (intended uses). A multiple application factor (MAF) is derived from the application frequency and the intervals between the applications and is based on 90th percentile residue data (MAF 90) for acute or on mean residue data (MAF mean) for long-term toxicity assessment.

The first tier assessment can be conducted using an Excel tool provided by EFSA (http://www.efsa.europa.eu/de/efsajournal/pub/1438.htm*)* that needs as input parameters the application rate of the a.s. (kg/ha), the number of applications, the interval between applications, and the toxicity estimates for birds and mammals. Finally, the user has to select the crop type, the growth stage at the time of application and the generic focal species from

drop-down lists. A screening step is additionally conducted automatically by the tool using the indicator species related to the selected scenario.

While the consideration of mixture toxicity was no issue in the former guidance document (EC 2000), the revised guidance document (EFSA 2009b) explicitly mentions "*Combined effects of simultaneous exposure to several active substances"* in chapter 2.5. There, it is stated that for the national authorization of products that contain several a.s. the risk assessment must consider the combined effects of the simultaneous exposure for birds and mammals. Further information on how to fulfil this requirement is provided in Appendix B of the revised guidance document. The general scheme of the risk assessment is to calculate a toxicity estimate ($LD_{50\ mix}$) for the mixture as a single virtual compound and relate this value to the exposure estimate for this single virtual compound (DDD_{mix}).

$$TER_{mix} = \frac{LD_{50\ mix}}{DDD_{mix}} \qquad [7.1.14]$$

The calculation of a LD_{50} value ($LD_{50\ mix}$) for the mixture as "virtual compound" according to EFSA guidance can be done by

$$LD_{50\ (mix)} = \frac{1}{\sum_i \frac{P(i)}{LD_{50\ (i)}}} \qquad [7.1.15]$$

with $LD_{50(i)}$ being the LD_{50} of the individual a.s. and P(i) being the proportion of the individual a.s. in the mixture. As stated above, using the proportions of the a.s. in the product instead of the proportions in the DDD_{mix} makes no difference in the case of a first tier assessment. But it can have great impact in case of a refined assessment. The possibly occurring differences between using (wrongly) the a.s. proportions in the product and (correctly) the proportions in the DDD_{mix} in a refined risk assessment for birds & mammals acute toxicity are illustrated in the Annex by using a fictional example. This fictional example clearly demonstrates that none of the two possibilities is *a priori* more conservative.

Hence, great care must be taken to calculate both the toxicity and the exposure estimate by using the same relative proportions of the a.s. in the assessed mixture (i.e., the proportions as given in the DDD_{mix}) in the case of a refined risk assessment. As long as the correct proportions are used on both sides of the quotients, the approach recommended by EFSA (2009b) is identical to the approach already being applied by UBA (previously described).

Key points of the guidance in Appendix B (EFSA 2009b) are listed and interpreted below:

- The concept of concentration addition is used as a default model for acute toxicity (mortality). Alternative concepts may be used on a case-by-case consideration.
- Combined acute effects are calculated based on the LD_{50} values of the individual a.s. and their individual proportions in the mixture. The proportion of each a.s. relates to the summed concentration of all a.s. in the mixture (which is set to 1), hence does not include co-formulants unless they are explicitly considered in the mixture toxicity calculation.
- Co-formulants with known toxicity might have to be included if necessary (i.e. in order to achieve reliable results).
- The assessment of combined toxicity is conducted regardless of whether toxicity data for the product are available or not. The outcome of the assessment is compared to the measured product toxicity. The lower (i.e. more conservative) one of the two values

must be used for the risk assessment unless there is clear evidence that a factor not relevant under environmental conditions caused an unexpected high toxicity of the product in the laboratory test. Consequently, conducting toxicity tests with the product cannot revise decisions based on mixture toxicity considerations, which is not in line with usual practice so far. On the other hand, additional product toxicity tests with vertebrates may be avoided by this approach for new products.

- For each a.s., the LD_{50} identified for the single substance assessment (i.e. the LD_{50} value for the most sensitive species) is used unless "*clear evidence*" demonstrates a specific mechanism of toxicity for the species in question for one a.s. and data on other species are available.
- If different environmental fate parameters are used for each a.s. in a refined risk assessment, the LD_{50} of the mixture is corrected by the multiple application factor (MAF) derived for each individual a.s.
- The contribution of each a.s. to the toxicity of the mixture is assessed by a "tox per fraction" approach. Those a.s. that are found to have "*marginal impact*" on the mixture toxicity (specified as a contribution of less than 10 % to the overall toxicity) may be exempt from the mixture toxicity calculation, resulting eventually in a single substance assessment for the most toxic a.s. However, the guidance for calculating the "tox per fraction" is not unequivocal.
- The use of censored LD_{50} values derived by limit tests is preferred over the use of predicted numerical LD_{50} values (e.g. derived by modelling).
- Targeted studies can be required if synergistic effects are expected.
- Exposure estimates are derived as the sum of the exposure estimates for the individual a.s. To this end, the application rate for the mixture (AR_{mix}) can be calculated based on the application rates of the individual a.s. ($AR_{(i)}$) as:

$$AR_{mix} = \sum_{i} AR_{(i)} \qquad [7.1.16]$$

- If multiple applications occur or if substance-specific MAF values are used in a refined risk assessment, exposure estimates for the mixture as a virtual compound are derived by multiplying the residual concentration after one application with the individual MAF before the values are summed across the individual a.s.
- The consideration of mixture toxicity in the long-term risk assessment for birds and mammals is "*currently not recommended*" (EFSA 2009b), because of unreliability related to using NOEL values instead of EC_x values and the (expected) frequent non-identity of the biological endpoints used for the individual a.s. However, the guidance document recommends performing a mixture-toxicity based risk assessment on a case-by-case basis for products that contain a.s. that are "*acting in the same way on a defined molecular target*" (EFSA 2009b). Particularly mentioned as example are aromatase inhibitors. The guidance document further provides a description of a "*simple approach*" to be used when considering mixture toxicity in the long-term assessment. This approach uses the NOEL value of the most toxic a.s. in the mixture (decided based on the individual NOEL values expressed on a molar basis). The application rate(s) of the less toxic a.s. ($AR_{(i\ adjusted)}$) are expressed in kg/ha related to the most toxic compound after re-calculation based on the individual molecular weights (MW_i and $MW_{most\ toxic}$):

$$AR_{i\ adjusted} = \frac{MW_{most\ toxic} * AR_i}{MW_i}$$

[7.1.17]

A combined toxicity assessment according to the new EFSA guidance (EFSA 2009b, outlined above) was applied to a selected number of the 15 combination products. This evaluation was only conducted for the first tier assessment, but for all generic focal species relevant for the chosen intended use of the PPP. The refinement options in the birds & mammals risk assessment have greatly changed in the new guidance document (EFSA 2009b) and it is beyond the scope of the present study to conduct a complete new refined risk assessment for all a.s., which would be necessary in order to fully assess combined toxicity for the products. The Excel sheets used to conduct the risk assessments and details on the relevant input parameters and the resulting TER are provided in the confidential Annex of the present report, while the main results are summarized below.

Again, the frequency of an indication of a refined risk assessment was taken as a proxy for the relevance and consequences of implementing mixture toxicity into the risk assessment.

The consideration of acute mixture toxicity according to the revised guidance document (EFSA 2009b) was possible with the available data for all of the five selected products.

Table 7.5 Summary of TER_{mix} values in the birds & mammals first tier risk assessment for 5 selected combination products
Shown is the number of products for which a refined risk assessment for at least one generic focal species is indicated by either the TER of at least one individual a.s. in the product, only by the TER of the combination product itself, or only by the TER_{mix} calculated according to the guidance document (EFSA 2009b). Given in brackets is the total number of products for which the evaluation was performed (toxicity data for the product were not always available).

Endpoint	Refined risk assessment for at least one generic focal species triggered by	Number of Products
Acute Toxicity Birds	at least one of the a.s.	3 (5)
	also the combination product	1 (1)
	only the combination product	0 (1)
	also the mixture toxicity consideration	3 (5)
	only the mixture toxicity consideration	0 (5)
Long-term Toxicity Birds	at least one of the a.s.	2 (2)
	also the combination product	- (0)
	only the combination product	- (0)
	also the mixture toxicity consideration	2 (2)
	only the mixture toxicity consideration	0 (2)
Acute Toxicity Mammals	at least one of the a.s.	3 (5)

	also the combination product	2 (3)
	only the combination product	1 (3)
	also the mixture toxicity consideration	3 (5)
	only the mixture toxicity consideration	0 (5)
Long-term Toxicity Mammals	at least one of the a.s.	2 (2)
	also the combination product	- (0)
	only the combination product	- (0)
	also the mixture toxicity consideration	2 (2)
	only the mixture toxicity consideration	0 (2)

Two products among the five that were selected for the closer analysis here fulfilled the condition of "similarly acting mixture components" (EFSA 2009b) to some degree as the a.s. were from the same mode-of-action group based on the classification scheme of HRAC (2008) and FRAC (2008). The first of these two products contains two triazole fungicides (FRAC group G1) and the second one contains two herbicides from the group of synthetic auxins (HRAC group O). For both products mixture toxicity was therefore considered also for the long-term risk assessment according to the most recent guidance document (EFSA 2009b). Triazoles as a group are suspected to act as aromatase inhibitors and thereby exhibit potentially endocrine disrupting effects in vertebrates (Sanderson 2006). Hence, the first product clearly qualifies for considering mixture toxicity in the long-term risk assessment for bird and mammals according to EFSA (2009b). A more specific risk assessment, detailed in the guidance document in Chapter 5 "Special Topics" may be needed for this product because of potential endocrine-disrupting effects. However, mixture toxicity is not an aspect of this specific risk assessment (EFSA 2009b) and therefore the topic of endocrine disruption is not further considered in the present study. As the mode of action of synthetic auxins in birds may be questioned as being specific and relevant, i.e. affecting a "*defined molecular target*", which is "*actually driving the risk assessment*" as required in the guidance (EFSA 2009b), the consideration of mixture toxicity in the long-term risk assessment of the second product may in fact not be required. However, it is conducted here to serve as another example. Because potency differences between the a.s. in the product are not taken into account (the NOEL for the most toxic a.s. is used), the 'simple approach' of EFSA (2009b) must be considered as very conservative and may call for a refinement that includes potency estimates as mentioned in the guidance documents (EFSA 2009b). However, potency estimates for various a.s. regarding the same endpoint may be rarely available; at least they were not available for the here evaluated a.s. For both assessed products, a refinement for the long-term risk assessment for birds as well as mammals was already triggered by the individual a.s. (Table 7.5). Hence, the mixture toxicity assessment could not trigger a refinement that had not already been indicated as required by the conventional assessment.

There was one case where the estimate for acute (mammal) toxicity of the combination product itself (#14) would trigger a refinement without a risk being indicated by any of the a.s. or the mixture toxicity consideration. This might indicate that the toxicity of the combination product was underestimated by CA. Yet, a comparison between the predicted and the observed toxicity of the formulated combination product was comprised, as in many other cases, by a comparison being based on censored data only. The reason for this is that the same limit concentration or dose holds usually for the a.s. as well as for the formulated product. This leads to censored data for toxicity estimates that can hardly be compared to CA-predicted estimates, because the actually tested doses (or concentrations) of the a.s. in the

formulated product are too low. In the case of #14 for example, the acute mammal toxicity of the product was given as LD_{50} > 300 mg product/kg bw, translating into LD_{50} > 0.13 mg sum a.s./kg bw, while the CA-predicted toxicity estimate of the product was with LD_{50} = 1325 mg sum a.s./kg bw about factor 10,000 higher. Since the highest tested dose of the product in terms of a.s. is far below the predicted toxicity estimate, there is no evidence at all that the product is actually more toxic than predicted.

Overall, the consideration of acute mixture toxicity in the first tier assessment according to EFSA (2009b) indicated a need for a refined assessment only for combinations products for which already the individual risk assessment for at least one of the a.s. triggered a refinement (Table 7.5). Hence, there were no apparent consequences of considering mixture toxicity with regard to the frequency of the need for a refined risk assessment in this small data set. Actual consequences for authorization could only be evaluated, however, after conducting the required refined risk assessment. Note that the two products (#4 and #9) where the mixture toxicity consideration by UBA had triggered a refined risk assessment (but not precluded authorization) were not included in the five selected products here.

To summarize, the consideration of mixture toxicity in the birds & mammals risk assessment is generally possible with available data, at least in the first tier assessment. Based on the small data set evaluated here, such an approach appears to indicate only for a limited number of cases a need of a refined risk assessment that would not already be required in a conventional risk assessment focussing on the individual active substances contained in the combination products.

AQUATIC ORGANISMS

The risk assessment for aquatic organisms in the 15 evaluated UBA reports was generally following the most recent guidance document (EC 2001). Table 7.6 provides an overview on the key data requirements for aquatic organisms according to this guidance document and the respective directive. These requirements apply basically to the a.s. to be included on Annex I of the directive (or now: regulation). In practice, however, tests conducted with the formulated a.s. (i.e., tests with the products) can be used in reasoned cases for the assessment of the a.s. according to Annex II. The guidance document mentions particularly the case of mesocosm studies conducted with formulated products that may also be used to evaluate the a.s. with regard to Annex I inclusion (EC 2001, section 2.5.2).

Table 7.6 Data requirements for the risk assessment of plant protection products for aquatic organisms.

	Toxicity Estimate [1]
Standard data requirements	
Daphnia acute toxicity	EC50 in mg a.s. /l or mg product/l
fish acute toxicity	LC50 in mg a.s. /l or mg product/l
algal growth inhibition	EC50 in mg a.s. /l or mg product/l
Further data requirements triggered e.g. by persistence, multiple application, or mode of action	
acute toxicity other aquatic invertebrates	EC50 in mg a.s. /l or mg product/l

chronic *Daphnia* toxicity	NOEC in mg a.s. /l or mg product/l
chronic fish toxicity (various test designs)	NOEC in mg a.s. /l or mg product/l
toxicity to sediment-dwelling organisms (e.g. *Chironomus*)	NOEC in mg a.s. /l or mg product/l
growth inhibition of *Lemna* (other macrophytes)	EC50 in mg a.s. /l or mg product/l
Higher-tier studies triggered by outcome of standard risk assessment	
e.g. mesocosm or microcosm studies	NOEAEC in mg a.s. /l or mg product/l
Probabilistic risk assessment, e.g. species sensitivity distribution	e.g. HC5 in mg a.s. /l or mg product/l

[1] *EC50: median effect concentration; LC50: median lethal concentration; NOEC: no observed effect concentration; NOEAEC: no observed ecologically adverse effect concentration*

The data requirements for formulated PPP are set out in Annex III of the directive (EC 1991) and are described in more detail in the guidance document (EC 2001). Annex III states that for ecotoxicological studies the "[...] *information provided, taken together with that for the active substance(s), must be sufficient to permit an assessment of the impact on non-target-species (flora and fauna) of the plant protection product, when used as proposed*." (EC 1991, Annex III 10 (i)) and further acknowledges that information submitted for the a.s. can be evaluated for that purpose in practice. For aquatic organisms, the standard data set (Table 7.6) is in principle required for PPP that can contaminate water (EC 1991, Annex III 10.2.1). Yet, this requirement can under certain conditions (as detailed in the guidance document) be reduced to a test with only the most sensitive species. Such a reduction of the requirements does not apply if "... *the acute toxicity of the plant protection product cannot be predicted on the basis of the data for the active substance which is especially the case if the formulation contains two or more active substances or formulants such as* [...]" (EC 1991, Annex III 10.2.1). Hence, the ability to reliably predict the acute toxicity of PPP by mixture toxicity concepts based on the information on the individual a.s. (i.e. component based approaches such as CA and IA) would in principle allow reducing the need of testing formulated PPP, including combination products.

The risk assessment for the aquatic compartment as conducted currently by UBA in the national authorization procedure follows a TER approach, but it is not conducted separately for distinct taxonomic groups or acute and long-term toxicity as it is done for birds & mammals. Instead, the aquatic risk assessment is conducted using selected endpoints from the available data set, generally the endpoint(s) with the lowest toxicity estimate (such that if a safe use is indicated by the assessment for this most sensitive endpoint, all other organism are expected not to be at risk, too). The typically available data set (both for a.s. and formulations) includes at least the standard endpoints algal growth inhibition, *Daphnia* acute toxicity and fish acute toxicity. Further data and assessments (e.g. for chronic toxicity, water plants, or sediment-dwelling organisms) may be required depending for example on the persistence of the a.s., the mode of action of the a.s. or the number of applications of the product within a year. With the selected endpoint(s), an initial risk assessment is performed. If a risk is indicated, the initial risk assessment is usually followed by a refined assessment using higher-tier studies as provided by the applicant. The trigger threshold (acceptability criterion) for the TER depends on the used endpoint; it is the lower the more environmentally realistic and reliable the assessment is deemed.

On the exposure side, the three scenarios *spray drift*, *run-off* and *drainage* are each assessed separately based on the respective predicted environmental concentration (PEC). The risk assessment uses in all cases a PEC as exposure estimate. The PEC is calculated with substance-specific data (e.g. persistence, volatilisation, photolytic stability, and deposition) and indication-specific data (e.g. time of application, interception, and applied amount of product) for each a.s. for each of the three scenarios *spray drift*, *run-off* and *drainage*. As a consequence, the environmental mixture composed of the a.s. at their respective PEC will generally differ from the relative composition of the combination product. Hence, the mixture to be assessed for aquatic organisms relates within the current standard risk assessment scheme generally to the relative proportions of the a.s. defined by the PEC and not to the relative proportions as present in the product. This is the case for the standard risk assessment using acute and chronic toxicity estimates as well as for a refined risk assessment using higher-tier studies, e.g. mesocosm studies.

The effect assessment can be based on a toxicity estimate derived for the product itself, which represents the whole-mixture approach in case of combination products. Direct entry of the product into surface waters is considered in the *spray drift* scenario, but usually not in the *run-off* and *drainage* scenario. Therefore, the toxicity estimate for the product is usually not used in the latter two scenarios. If the toxicity estimate of the combination product is used in the *spray drift* assessment, the estimation of the respective exposure estimate is a critical step that can be dealt with in different ways. PEC values can be calculated for the product as a whole by assuming implicitly identical fate parameters (half-life, volatilisation etc.) for all a.s. in the product, or the PEC of the product can be calculated as the sum of the individual PEC_i by assuming substance-specific fate parameters for all a.s. in the product. In the first case, the relative proportions of the a.s. in the assessed environmental mixture are identical to the relative proportions in the product. This situation represents therefore an exception to the general rule that mixtures of a.s. in the relative proportions of their PEC are assessed for aquatic organisms. While it did occur among the evaluated UBA reports (examples are #8 and #12), it was not always unequivocally clear to which relative proportions of the a.s. the PEC estimates for the product related.

Data availability

Data on acute and chronic *Daphnia* toxicity, acute fish toxicity and algal growth inhibition were available for all individual a.s. contained in the 15 combination products. This enables in principle the prediction of concentration-additive mixture toxicity for all these endpoints. Toxicity data for the products were frequently available for acute *Daphnia* and fish toxicity as well as for algal growth inhibition, allowing in principle for a comparison between expected and observed product toxicity for these basic standard endpoints that represent three trophic levels. However, in only five of the 15 combination products, one or more of these three standard endpoints was actually driving the aquatic risk assessment. Other endpoints such as chronic toxicity to fish or *Daphnia* or mesocosm studies were often identified as the relevant endpoints for the aquatic risk assessment (details are provided in the confidential Annex).

The endpoints that were used in the UBA risk assessment reports (i.e. the endpoint for which the relevant TER values were calculated) differed among the products and comprised almost all possible endpoints from acute *Daphnia* toxicity to chronic *Daphnia* or fish toxicity, algal or *Lemna* growth inhibition and microcosm studies (Table 7.7). Only in a few cases TER values were calculated for all available endpoints instead of only for the most sensitive ones.

If a risk assessment was conducted for each of the individual a.s. in a product, different endpoints were usually most sensitive for the different individual a.s. and, hence, used for the risk assessments. An example of high heterogeneity was product #2 for which the EC_{50} of an

algal growth inhibition test was used for one a.s. and a NOEC of a fish sexual development study was used for the other a.s.

In some cases, the data for the individual a.s. were derived from tests with mono-formulations containing the respective a.s. In these cases, additives contained in the mono-formulation (and which likely differ from the additives in combination products with these a.s.) may influence the toxicity and thereby distort the mixture toxicity prediction for the combination products.

In 5 of the 15 combination products, the relevant endpoints (i.e. those with the lowest toxicity estimate) were similar for all a.s. (Table 7.7), i.e. either chronic fish toxicity, chronic *Daphnia* toxicity or *Lemna* growth inhibition were used in the risk assessment of all a.s. in a given combination product. However, the three cases of similar endpoints for chronic fish toxicity involved different fish species, different study designs and different response variables and represent thereby a relatively larger heterogeneity within this endpoint than toxicity to *Daphnia* reproduction or *Lemna* growth.

The relevant toxicity endpoints differed among the a.s. contained in the other 10 combination products. These dissimilar endpoints included heterogeneity with regard to trophic levels, with regard to acute/chronic toxicity and with regard to the complexity level (single species/multispecies tests). In half of these cases, data were not available for both a.s. for each of the relevant endpoints. In the other half of the cases, data availability would in principle allow assessing mixture toxicity for each of the relevant endpoints separately. The threshold values applied in the UBA report differed among the dissimilar endpoints in several cases, typically when acute and chronic endpoints were identified as relevant for the different a.s. Toxicity estimates obtained for the combination product were occasionally also considered for the *spray drift* scenario (data not shown here).

Table 7.7 Relevant toxicity endpoints in the aquatic risk assessment of the 15 evaluated combination products

As identified in the assessment of the UBA, considering only the spray drift scenario (different endpoints were used in the run-off and drainage scenario for two products). Shown is the number of products with the same combination of relevant endpoints, either similar (total of 5 products) or dissimilar (total of 10 products). In addition, it is indicated if toxicity data for the relevant endpoint were also available for the respective other active substance.

Endpoint Combination		**Endpoint available for other a.s.**	**Identical TER thresholds**	**Number of products**
Similar Endpoints				**5**
	Chronic fish toxicity (NOEC)	Yes	Yes	3
	Chronic *Daphnia* toxicity (NOEC)	Yes	Yes	1
	Lemna growth inhibition (EC50)	Yes	Yes	1
Dissimilar Endpoints				**10**
	Algal growth inhibition (EC50)	Yes	Yes	2

	Lemna growth inhibition (EC50)			
	Fish chronic toxicity (NOEC) Fish acute toxicity (EC50)	Yes	No	1
	Chronic toxicity *Daphnia* (NOEC) Chronic toxicity sediment-dwellers (NOEC)	No	Yes	1
	Algal growth inhibition (EC50) Acute toxicity aquatic invertebrate (EC50)	Yes	No	1
	Algal growth inhibition (EC50) Chronic toxicity *Daphnia* (NOEC)	Yes	Yes	1
	Lemna growth inhibition (EC50) Chronic fish toxicity (NOEC)	No	No	1
	Lemna growth inhibition (EC50) Microcosm/Mesocosm (NOEAEC)	No	No	2
	Chronic toxicity *Daphnia* (NOEC) Microcosm (NOEC)	No	No	1

Current praxis of considering mixture toxicity

In most cases, combination effects were not mentioned or even considered in the UBA risk assessment reports. In several cases, the expected toxicity based on concentration addition was compared with the observed product toxicity. Depending on the outcome of the comparison, either the toxicity data for the product were used or the assessment was based on individual a.s. and deemed protective for the mixture for various reasons.

Yet, results from a precursor project (Coors 2009, Coors & Frische 2011) indicated that the deviation of the concentration-additive mixture toxicity prediction from the observed product toxicity (as expressed by the model deviation ration MDR) do not generally correlate among endpoints. Hence, a finding of a deviation from concentration-additive behaviour of the a.s. in a product with regard to acute fish toxicity cannot be extrapolated to the expectation of the same behaviour in, for example, algal growth inhibition or chronic fish toxicity. The only two endpoints that showed a significant correlation of the MDR were found to be *Daphnia* and fish acute toxicity. As a conclusion, the above mentioned approach of comparing by default the expected and the observed product toxicity is no safeguard against the occurrence of more-than-additive mixture toxicity in other endpoints (where this comparison may not be possible due to a lack of data).

In three cases among the 15 assessment reports of the combination products, a TER_{mix} was calculated by the UBA as the sum of the reciprocal TER values of the individual a.s. in the product. The exact derivation of the TER_{mix} was not reported in one case, but the resulting

value was also not further considered in the risk assessment. In the second case (product #10), a TER based on an EC_{50} value for *Lemna* growth inhibition was combined with a TER based on a NOEAEC from a microcosm study. The TER threshold values used for the two a.s. differed (10 and 5, respectively). As threshold value for the TER_{mix} the higher of the two, i.e. 10, had been selected in the assessment report. The comparison of the TER_{mix} values for the *spray drift* assessment with the threshold value led to an actually employed risk mitigation measure of a greater buffer zone (5 m with 75 % drift reduction nozzles) than an assessment based on the individual a.s. would have triggered (1 m buffer zone with 75 % drift reduction nozzles). Such stronger risk mitigation measures due to consideration of mixture toxicity by the TER_{mix} approach were not indicated in the assessments regarding the exposure pathways *run-off* and *drainage*. In the third case (product #2), NOEC values for chronic fish toxicity were used for the aquatic risk assessment of both a.s. In both cases a threshold value of 10 had been set, which was also used for the combined-effects assessment. Yet, the NOEC values related to different response variables for the two a.s.: from a 96 d *Fish Early Life Stage* test with *O. mykiss* (for the relevant metabolite of one a.s.) and from a 122 d *Fish Sexual Development* test with *P. promelas* (for the other a.s.). As in the second case described above, stricter risk mitigation measures were employed due to the calculated TER_{mix} value in the *spray drift* assessment (but not for *run-off* and *drainage*) compared to a conventional single-substance assessment.

Hence, among the 15 evaluated combination products were two cases where an explicit consideration of mixture toxicity resulted with regard to *spray drift* contamination of the aquatic compartment in stricter risk mitigation measures than would have been employed in a conventional single-substance risk assessment.

In comparison to mixture toxicity approaches applied by other member states, it has to be noted that at least in one case the TER_{mix} had been calculated by the UBA not separately for different taxonomic groups but combined different taxonomic groups or levels (i.e. *Lemna* and a mesocosm model ecosystem). Further, the selection of the relevant trigger value was not consistent with the approach proposed by other member states.

Consequences of implementing mixture toxicity in the risk assessment

The consequences of implementing mixture toxicity in the aquatic risk assessment are evaluated in terms of the indication for stricter risk mitigation measures than those required for the combination products according to current authorization. Possible risk mitigation measures in the *spray drift* scenario are requirements for the technical equipment (drift reduction nozzles) and for minimum distance to surface waters to abide during application. Hence, indication of stricter risk mitigation measures could for example imply a distance of 10 m instead of 5 m to surface waters or 50 % drift reduction nozzles instead of no requirement for drift reduction nozzles. In the *run-off* and *drainage* scenario, stricter regulations could imply larger buffer zones (e.g. 20 m instead of 10 m) and seasonal application restrictions on drained fields, respectively. The here conducted evaluation did not take into account possible avoidance of stricter risk mitigation measures by performing a refined risk assessment (e.g. by conducting additional higher-tier studies), because this is beyond the scope of the present study.

For aquatic organisms, the formula of the CA concept is specified as

$$TER_{mix} = \frac{EC_{50\,mix}}{PEC_{mix}} \qquad [7.1.19]$$

where $EC_{50\,mix}$ is calculated according to the CA concept with P_i as the proportion of active substance *i* in the mixture at the relative proportions of the individual PEC values

$$P_i = \frac{PEC_i}{\sum_i PEC_i}$$

[7.1.20]

and PEC_{mix} being the sum of the PECs of the individual a.s.

$$PEC_{mix} = \sum_i PEC_i$$

[7.1.21]

Again, care has to be taken not to use the proportions of the a.s. in the combination product (unless the derivation of PEC is identical for the a.s. and, hence, the proportions are identical in the applied and the environmental mixture).

The approach used here exemplarily for considering mixture toxicity involved the calculation of the TER_{mix} as the sum of the reciprocal TER_i of all a.s. in each of the 5 selected combination products (the same as those evaluated more closely in the birds & mammals assessment). In contrast to the recommendation for birds & mammals (EFSA 2009b), toxicity estimates derived by statistical hypothesis testing (e.g. no-observed-effect-concentration, NOEC) were also used for the aquatic risk assessment in the mixture toxicity implementation. Thereby, identical effect levels for NOECs are assumed independently of the test system and the tested concentration range. This is a violation of the assumptions of the CA concept that is discussed in more detail elsewhere (Kortenkamp et al. 2009; Chapter 4, page 73). The calculation of TER_{mix} follows here two different approaches: 1) combining toxicity estimates of the a.s. only within taxonomic groups (i.e., algae, crustaceans, fish, and water plants), and 2) combining toxicity estimates of the a.s. across different taxonomic groups and levels of organisation. For the second approach, two options were evaluated with regard to the threshold for the TER_{mix}: firstly, using the higher (more conservative) trigger value in place the individual TER_i and, secondly, integrating the trigger values into the formula and comparing the result to the threshold value of 1.

$$\frac{1}{\sum_i \frac{trigger_i}{TER_i}} > 1$$

[7.1.22]

This approach is referred to as “combined trigger” in Table 7.8 (and is essentially identical to the PEC/PNEC-summation discussed in chapter 4.

The results of this evaluation are summarized in Table 7.8 for the five selected products. A lack of exposure estimates (and thereby TER_i values) for at least one of the a.s. in a combination product was encountered in more than half of the 15 risk assessment reports, which precluded the evaluation of more products without re-calculation of exposure estimate.

There were several cases among the 5 evaluated combination products where consideration of mixture toxicity was not possible due to a lack of toxicity or exposure estimates for one of the a.s. or where no clear decision was possible based on the available censored data (indicated as n.d. in Table 7.8).

Calculating TER_{mix} values by combining TER_i values across different taxonomic groups was possible for four of the five products in the *spray drift* scenario and for all five products in the *run-off* and *drainage* scenario. For three of the five products (#1, #3, and #14), stricter risk mitigation measures were indicated for at least one scenario when the more conservative of the two trigger values was applied. When the trigger values were combined according to the formula given above, stricter risk mitigation measures were less frequently indicated. These fewer cases were namely product #1 with regard to *run-off* and product #14 with regard to *spray drift*.

When TER_i values were only combined within taxonomic groups, stricter risk mitigation measures were indicated for the same two cases (product #1 *run-off* and product #14 *spray drift*) and, additionally, for product #1 in the *spray drift* scenario, product #3 for the *run-off* scenario and product #14 for the *run-off* scenario. Hence, the approach of combining TER_i values only within taxonomic groups was in terms of the frequency of risk indication between the other two applied approaches.

Table 7.8 Summary of the results of the exemplary implementation of mixture toxicity by different approaches for five selected combination products

A need for stricter risk mitigation measures (stricter application restrictions) based on the consideration of mixture toxicity is indicated by + and no need by -. Shown is for each of the scenarios *spray drift*, *run-off* and *drainage* the number of products for which stricter restrictions would be required compared to the current authorisation. The TER trigger threshold used for the assessment is given in brackets together with the relevant toxicity estimate of each active substance. In all five products, different toxicity estimates for the two a.s. in the product were identified as relevant for the aquatic risk assessment.

Product (#)	Relevant Toxicity Estimate (TER trigger)	Stricter risk mitigation measures indicated by mixture toxicity consideration		
		Spray drift	Run-off	Drainage
1	EC_{50} water plant (10)	n.d.[1]	n.d.[1]	n.d.[1]
	NOEC chronic fish toxicity (50)	+	+	-
	combining different taxonomic groups (50)	+	+	+
	combining different taxonomic groups (combined trigger)	-	+	-
3	EC_{50} algae (10)	n.d.[2]	+	-
	EC_{50} aquatic invertebrate, acute (100)	n.d.[2]	-	-
	combining different taxonomic groups (100)	n.d.[2]	+	+
	combining different taxonomic groups (combined trigger)	n.d.[2]	-	-
8	NOEC chronic toxicity *D. magna* (10)	-	n.d.[3]	-
	EC_{50} microcosm study (2)	n.d.[4]	n.d.[4]	n.d.[4]
	combining different taxonomic groups (10)	-	n.d.[3]	-
	combining different taxonomic groups (combined trigger)	-	-	-

	EC_{50} water plant (10)	n.d.[3]	-	-
13	EC_{50} algae (10)	n.d.[3]	-	-
	combining different taxonomic groups (10)	n.d.[3]	-	-
	NOEAEC mesocosm study (3)	n.d.[4]	n.d.[4]	n.d.[4]
	HC_5 water plant (5)	+	+	-
14	combining different taxonomic groups (5)	+	+	-
	combining different taxonomic groups (combined trigger)	+	-	-

[1]no data required for fungicides; [2]product data used for assessment; [3]no clear decision due to censored data; [4]not determined due to lack of toxicity estimate for one a.s.;

The five cases for which stricter risk mitigation measures were indicated by a within-taxonomic group combined toxicity assessment are described in the following in more detail:

- Product #1, *spray drift* and *run-off* scenario: the assessment was only possible for chronic fish toxicity but not for water plant toxicity due to a lacking toxicity estimate for one of the two fungicides. The TER trigger for chronic fish toxicity was set to 50 in the risk assessment report in order to account for possible endocrine effects. The (typically applied) TER of 10 for chronic fish toxicity would have been met by the TER_{mix} in all three implementation approaches in both scenarios with the result of no need for stricter risk mitigation measures. The TER_{mix} values obtained for combined chronic fish toxicity were with 48 to 49 just below the applied trigger of 50 in the *spray drift* scenario.
- Product #3: The TER_{mix} for algal growth inhibition was with a value of 7 below the trigger of 10 in the *run-off* scenario. The *spray drift* scenario had been regulated based on the algal growth inhibition of the combination product itself. Hence, the component-based approach indicated risk while the whole-mixture approach does not in this specific example.
- Product #14: The assessment for water plants combined an HC_5 derived from water plant EC_{50} values for one a.s. with an EC_{50} value for water plant growth inhibition for the other a.s. The resulting TER_{mix} was with values of 4.4 to 4.6 below the trigger of 5.

A higher-tier study was used in the risk assessment for one a.s. in each of the products #8 and #14. A combined assessment for this endpoint was not possible as such studies were not available for the other a.s. in the product. In both cases the relevant endpoint for the other a.s. was about similar to the most sensitive endpoint in the higher-tier study (aquatic invertebrates or primary producers, respectively). These endpoints could be assessed for combined toxicity and may be seen as sufficient surrogates for the higher-tier endpoint. Yet, the question remains open if and how exactly the higher-tier studies with one a.s. can be used to refine the combined toxicity risk assessment for the combination product.

Overall, stricter risk mitigation measures and, thereby, potential consequences for the authorisation, were indicated in several of the evaluated 5 combination products. In two more products among the 15 combination products where stricter risk mitigation measures had been established in the authorisation due to mixture toxicity considerations (see previous sub-chapter). Hence, based on the evaluated data set of 15 combination products consideration of mixture toxicity can be expected to result in stricter restrictions in the authorisation than expected according to a conventional risk assessment for individual substances only. As the present study shows, the number of concerned combination products will likely depend

strongly on the chosen approach for implementing mixture toxicity considerations in the aquatic risk assessment.

TERRESTRIAL ORGANISMS

For terrestrial organisms, the risk assessment in the 15 evaluated UBA risk assessment reports was conducted according to the most recent guidance document (EC 2002). Table 7.9 summarizes the key data requirements for terrestrial organisms. As in the case of aquatic organisms, the data requirements for formulated PPP are set out in Annex III of the directive (EC 1991), in principle providing the possibility to use data submitted for the a.s. in the risk assessment of the product and the other way round. Particularly tests with the lead formulation on non-target arthropods, earthworm reproduction and soil micro-organisms are mentioned in this context as providing at the same time information for the evaluation of the a.s. (chapter 2.4, EC 2002). Tests with combination products should only be used for the evaluation of the a.s. if there was no effect observed at the top (or limit) dose, because "*otherwise it would be difficult to attribute the toxicity to one or the other substance*" (EC 2002). Furthermore, conditions are specified in the guidance document that allow waiving tests with the lead formulation and use instead data derived from tests with the a.s. (e.g. no repetition of lower tier tests when higher tier tests need to be conducted with the formulation anyway). On the other hand, tests with terrestrial plants should be conducted with formulated products "[...] *because formulations contain, besides the active substance, all those components and co-adjuvants required for maximising biological activity*" (EC 2002, p 32).

As indicated in Table 7.9, the toxicity estimates obtained for bees, non-target arthropods, soil microorganisms and non-target plants are related with or directly tested at the application rate of the product. Hence, the application rate serves as exposure estimate and not a predicted environmental concentration. The same holds if mixtures are to be considered within this framework and, therefore, the mixtures to be assessed for these endpoints are composed of a.s. in the relative proportions as present in the product (see Table 7.1). The relevant application rates for the risk assessment may be adapted to include multiple application factors, drift reduction factors, vegetation distribution factors or be refined in higher-tier assessments. Yet, in all cases these adaptations are applied in the same way to all a.s.; so there is no change in the relative proportions of a.s. in the assessed mixture in comparison to the applied PPP.

The only terrestrial assessment areas where toxicity estimates are (mostly) related to PEC values as exposure estimates are acute and chronic toxicity to earthworms and other soil macro-organisms such as collembolans and mites. For these endpoints the mixtures to be assessed would therefore be composed of a.s. at the relative proportions defined by their respective PECs. Occasionally, toxicity estimates derived in laboratory studies are directly compared to field application rates and not the PEC, if the study design allows such a comparison. This situation is an exception to the general pattern described in Table 7.1 and was not encountered in the 15 evaluated reports. In higher-tier studies with these organisms, the exposure is usually part of the (semi-)field study design (i.e. the envisaged application rates are directly tested) so that the results are not used for a formal TER (or HQ) calculation, but are immediately interpreted in terms of risk by comparison to control treatments (EC 2002, p. 9). Again, mixtures would be assessed in this context with the a.s. being present at the relative proportions given in the product.

Table 7.9 Data requirements for the risk assessment for terrestrial organisms.

Data requirements	Necessity	Endpoint	Risk indicator [1]	No risk expected if
Acute toxicity to bees	standard requirement	oral and contact LD50 in µg a.i/bee or µg product/bee	HQ=spray application rate/LD50	HQ<50
Higher tier tests with bees	triggered by acute toxicity to bees	mortality, behaviour, honey crop, or state of colony	none defined; comparison to control	no significant effects at application rate
Other non-target arthropods (NTA)	standard requirement	glass-plate test with *T. pyri* and *A rhopalosiphi*; LR50 in g a.s./ha or L product/ha	in-field HQ=spray application rate*MAF/LR50; off-field HQ=spray application rate*correction factor*MAF*VDF/(DF*LR50)	HQ<2
Higher tier tests with other NTA	triggered by standard NTA tests	lethal and sub-lethal effects in extended laboratory, aged-residue, semi-field, or field tests	none defined; comparison to control	effects <50 % at application rate and recovery observed
Acute toxicity to earthworms	standard requirement	LC50 in mg a.s./kg soil d.w. or mg product/kg soil d.w.	TER=LC50/initial PEC_{soil} or (if log Kow>2 and 10 % peat in test soil) TER=$LC50_{corr}$/initial PEC_{soil}	TER>10
Long-term toxicity to earthworms	triggered by acute earthworm toxicity or multiple applications or persistence of substance	NOEC in mg a.s./kg soil d.w. or mg product/kg soil d.w.	TER=NOEC/initial PEC_{soil} or (if log Kow>2 and 10 % peat in test soil) TER=$NOEC_{corr}$/initial PEC_{soil}	TER>5
Higher tier tests with earthworms	triggered by long-term earthworm toxicity	abundance, biomass and species composition in field tests	none defined; comparison to control	no significant effects at application rate or recovery observed within one year
N-transformation and C-mineralisation	standard requirement	effect on N-transformation and C-mineralisation in relation to control	none defined; comparison to control	<25 % effect at maximum PEC

Tab.7.9 continued

Data requirements	Necessity	Endpoint	Risk indicator [1]	No risk expected if
Other soil macro-organisms	triggered by persistence of substance, standard NTA, micro-organisms, or long-term earthworm toxicity	collembolan or mite reproduction test or litter bag study (loss of biomass)	for reproduction tests: TER=NOEC/initial PEC_{soil} or (if log Kow>2 and 10 % peat in test soil) TER=$NOEC_{corr}$/initial PEC_{soil} for field test: none defined; comparison to control	TER>5 or (field test) no effects of >10 % at application rate
Non-target plants – TIER 1	standard requirement	screening data on 6 plant species from different taxa tested at highest application rate	none defined; comparison to control	<50 % effects for all species at maximum application rate
Non-target plants – TIER 2	triggered by >50 % effects on at least one species in tier 1; all herbicides	6-10 species; foliar or soil application, effects on emergence, length, and weight as g product/ha or g a.s./ha in e.g. seedling emergence and/or vegetative vigour test	TER=$ER50_{most\ sensitive\ species}$/maximum application $rate_{corrected\ for\ drift\ and\ interception}$ or probabilistic 5 % value (HC5) of ER50 values (SSD, species sensitivity distribution)	TER>5 or ER50 (5 %) < maximum application rate
Non-target plants – TIER 3	triggered in tier 2	effects in semi-field or field tests	none defined; comparison to control	none defined

[1] MAF: multiple application factor; DF: drift factor; VDF: vegetation distribution factor; HQ: hazard quotient; LR50: median lethal application rate; ER50: median effect application rate

Data availability

In the case of the 15 evaluated combination products, data on contact and oral toxicity to bees as well as data on acute toxicity to earthworms were always available for all a.s. and, with very few exceptions, also for the formulated combination products (details are provided in the confidential annex). Other standard data such as first tier non-target arthropods tests (glass plate tests with *T. pyri* and *A. rhopalosiphi*) as well as C- and N-transformation tests were often, but not always available for all a.s. in a product and additionally also for the combination product. Data on long-term toxicity to earthworms and toxicity to plants were occasionally available for the a.s., and in some cases additionally also for the combination product. As an exception, data on long-term toxicity to springtails as well as litter bag studies were available for both a.s. for one product. In many cases, the data available for individual a.s. were derived from tests with a mono-formulation. Hence, an influence of formulation additives cannot be excluded and may, as in the case of the risk assessment for aquatic organisms, influence mixture toxicity predictions.

Current praxis of considering mixture toxicity

In the 15 UBA reports, the risk assessment for the various terrestrial organisms based generally on the data that were available for the combination product. In the few cases where no data for the product were available, data derived for the individual a.s. were used (soil micro-organisms with product #2, acute earthworm toxicity with product #5, and honey bee toxicity with product #11). For two products, TER values were calculated for the a.s. as well as based on product data, as far as available. There were four cases among the 15 where the risk assessment for earthworms was finally based on higher-tier studies with earthworms that had been conducted with a mono-formulation of one of the two a.s., which had been deemed acceptable for the risk assessment of the combination product.

Combination effects were in no case considered in the UBA risk assessment report. In two cases, concentration-additive toxicity was calculated based on a.s. data and compared to the toxicity of the product. In one of these cases, the calculation used the concentration instead of the proportion in the CA formula. In these and two more cases, the report stated that combination effects could be assumed to be sufficiently covered by using the toxicity data derived for the combination product in the risk assessment.

Consequences of implementing mixture toxicity in the risk assessment

There are currently no component-based approaches consistently applied to consider mixture toxicity for terrestrial organisms in the risk assessment of PPP. Given the data availability described earlier, any such approach is hampered by the fact that toxicity estimates for individual a.s. must often be derived from tests with mono-formulations or that they are not available at all.

Generally, mixture toxicity is to a large degree already implemented for terrestrial organisms by the application of a whole-mixture approach, i.e. using test data derived with the combination product for the risk assessment.

As in the case of the aquatic compartment, the question remains open how higher-tier studies with one a.s. can be used in a refinement, particularly with regard to earthworm toxicity. Table 7.10 provides an overview on endpoints reported for earthworms in the 5 selected UBA risk assessment reports to illustrate this problematic. For three of the five products, higher-tier studies are available with mono-formulations of one of the concerned a.s. (in all three cases

for more toxic a.s.). The application rates in these studies covered the ones of the combination products with regard to the most toxic single substance in the combination product, but may be questioned to additionally account for the toxicity of the second a.s., i.e. for the toxicity of the respective mixture.

Table 7.10 Compilation of endpoints for earthworms that were used in the UBA risk assessment reports for five selected combination products

Given are the application rates for the individual active substances and the respective combination product, the reported toxicity estimates for laboratory tests (effects on survival and reproduction, respectively) and results from (semi)field studies that were provided as higher-tier tests. In brackets, the TER values derived in the reports are provided for acute toxicity (TER trigger of 10) and effects on reproduction (TER trigger of 5).

Product (#)	Substance (application rate)	Endpoints for earthworms and related Toxicity Exposure Ratio (TER)		
		Acute, LC_{50} (10)	Reproduction, NOEC (5)	(Semi)field Study (n.a. [1])
1	epoxiconazole (2 × 112.5 g/ha)	n.d.	62 g/ha (0.5)	NOEC at 2 × 125 g/ha [3]
	metconazole (2 × 82.5 g/ha)	n.d.	337.5 g/ha (4)	n.d.
	product (2 × 3 L/ha)	343.7 mg/kg (16.7)	24.1 kg/ha (7.5)	n.d.
3	fluopicolide	n.d.	n.d.	n.d.
	propamocarb	n.d.	n.d.	n.d.
	product (4 × 1.6 L/ha)	>1000 mg/kg (>107)	30 L/ha (10.4)	n.d.
8	picoxystrobin (2 × 178 g/ha)	3.35 mg/kg (5)	0.32 mg/kg (0.29)	no effects at 250 g/ha [3]
	cyprodinil (2 × 660 g/ha)	96 mg/kg (43)	26.6 mg/kg (6.7)	n.d.
	product (2 × 2.2 kg/ha)	37.7 mg/kg (5)	8 mg/kg (0.61)	n.d.
13	MCPA	828 mg/kg (n.d.)	n.d.	n.d.
	dicamba	>1000 mg/kg (n.d.)	n.d.	n.d.
	product (2 × 6 L/ha)	>1000 mg/kg (>26)	56.4 kg/ha (9.9)	n.d.
14	terbuthylazine (1 × 750 g/ha)	105 mg/kg (70)	2250 g/ha (3)	acceptable effects at 1 × 750 g/ha [3]
	pethoxamid (1 × 1200 g/ha)	218 mg/kg (91)	n.d.	n.d.
	product (1 × 4 L/ha)	302 mg/kg (28)	< 4 L/ha (<1)	n.d.

[1]n.a.: not applicable, [2]n.d.: not determined or not reported, [3]tested with mono-formulation,

7.1.6 Tank mixtures

Tank mixtures are defined as a mix of at least two products prepared shortly before use. Annex VI of the directive 91/141/EEC (point 2.1.5) requests that tank mixtures that are proposed as requirement or recommendation on product labels shall "*achieve the desired effects*" and shall comply with the requirements set out regarding efficacy. There are no explicitly stated requirements with regard to an environmental risk assessment. Furthermore, there are currently no specific environmental regulations for the application of tank mixtures that are not recommended or required on the respective product labels. Hence, these tank mixtures are legal as long as the application complies with the respective restrictions for all individual products in the tank mixture.

A sub-category among tank mixtures is the addition of an adjuvant to a PPP shortly before application. Adjuvants are defined in the PPP regulation (EC 2009, Article 2.3 d) as "substances or preparations which consist of co-formulants or preparations containing one or more co-formulants, in the form in which they are supplied to the user and placed on the market to be mixed by the user with a plant protection product and which enhance its effectiveness or other pesticidal properties". This definition is in line with the usage of the term "adjuvant" in most of the published literature (Hazen 2000, Haller et al. 2003, Kudsk et al. 2008, Ryckaert et al. 2007, 2008, Blanco et al. 2009), while occasionally authors use the term also for formulation additives that are already contained in the marketed PPP (Krogh et al. 2003). There is no specific assessment of adjuvants foreseen by the regulation (EC 2009) beyond the possibility that certain co-formulants (which adjuvants by definition contain) can be listed in Annex III with the consequence that they may not be included in a PPP.

Adjuvants are particularly frequently used together with herbicides to enhance their efficacy for example by increasing the contact area of pesticide droplets on leaves or by enhancing the penetration of the leaf cuticula (Hazen 2000, Ryckaert et al. 2008). Due to enhanced spreading of the pesticide, adjuvants can increase the residues found on plants, which may provide on the one hand side higher efficacy but on the other hand also higher consumer risk (Ryckaert et al. 2007). Enhanced pesticide efficacy due to usage of adjuvants can be seen as beneficial as it allows reducing the application rate of the pesticide while obtaining still the desired effect (Kudsk 2008). This saves costs for the farmer and may reduce undesired effects in the environment as long as the adjvant does not also enhance effects on non-target organisms. However, farmers may not always be aware of this option or apply it routinely. Similarly, tank mixtures of herbicidal PPP aiming to optimise weed control (different weed species show differential sensitivity to herbicides) can be economically and ecologically beneficial, but this approach may as well result in an "overkill" situation if application rates of the individual components are not accordingly reduced (Kudsk 2008). Adjuvants generally contain the same substances, namely surfactants and solvents as major groups that will be discussed here in the context of formulation additives. Therefore, adjuvants are not further considered in the context of tank mixtures.

Scenarios for tank mixtures

Empirical data on tank mixtures used in agriculture were provided through the UBA by the Julius-Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Strategies and Technology Assessment in Plant Protection, in Kleinmachnow, Germany. These data were obtained from a network of reference farms for plant protection ("*Netzwerk Vergleichsbetriebe*"), which consists of a number of farms that document the actual application of plant protection products. One key parameter derived from these empirical data is the "*Behandlungsindex*", which is an indicator for the intensity of PPP applications, called

in the following B-index (Roßberg et al. 2002). The B-index is first calculated for each field ("*Schlag*") separately according to this formula:

$$B_{index} = \sum_n \left(\frac{treated\ area}{total\ field\ area} * \frac{application\ rate\ of\ PPP}{maximum\ application\ rate\ of\ PPP}\right) \quad [7.1.23]$$

with *n* being the number of application of PPP from a specified treatment group (insecticides, fungicides, herbicides, molluscicides, and plant growth regulators). The B-index represents the number of PPP application of a specified pesticide type on one field and decreases if application rates were reduced in relation to the maximum application rate (which means the maximum authorized application rate) or if only a part of the field was treated. For example, 5 applications of different or identical insecticidal products at their respective maximum applications rates on the whole field result in the same B-index as 10 such application with the respective applications all reduced by 50 %. PPP applied in a tank mixture are each counted separately.

The B-indices are then averaged across farms to derive a mean B-index for each combination of crop culture and PPP pesticide type and finally summed across treatment groups to obtain a mean B-index for PPP applications on a particular crop.

The data on the application of tank mixtures made available by the JKI cover two crops (winter wheat and winter oilseed rape) in the years 2007 and 2008. The B-indices for these two crops in the years 2007 and 2008 are shown in Table 7.11 together with the number of fields with these crops that were included in the survey and the total number of PPP applications.

Table 7.11 Data on the application of plant protection products (PPP) in winter wheat and winter oilseed rape in 2007 and 2008 and the resulting B-index as compiled from Freier et al. (2008) and Freier et al. (2009).

	Winter wheat		Winter oilseed rape	
	2007	**2008**	**2007**	**2008**
Number of fields in survey	179	205	137	143
Total number of PPP applications	1691	2123	1049	1228
Mean B-index				
herbicides	1.9	2.0	1.6	1.8
insecticides	1.2	1.0	2.3	2.3
fungicides	1.9	2.2	0.6	0.9
plant growth regulators	0.8	1.1	0.9	1.0
total	5.8	6.3	5.4	6.0

Winter wheat and winter oilseed rape belong, together with winter barley, to the three main crop cultures of the network farms in the survey. While thereby representing relevant crop cultures, winter wheat and winter oilseed rape may not be the most relevant crops in terms of intensity of PPP application. Several crops had for example a higher total B-index, e.g. potatoes (20.2), sugar-beets (9.8), orchards (30.3), vineyards (13.8) and hops (13.0) in 2007 (Freier et al. 2008).

Freier et al. (2009) report on the number of herbicidal tank mixture applications (excluding products with glyphosate and the tribenuron-methyl containing product "Pointer") in the three main cultures winter wheat, winter oilseed rape, and winter barley in 2007 in comparison to the number of single herbicidal applications. These data indicate that herbicidal tank mixtures are relatively frequent in winter wheat (135 of 280 applications, i.e. 48.2 %), but less frequent in winter oilseed rape (39 of 260, i.e. 15 %).

More detailed data on tank mixtures in winter wheat and winter oil seed rape were provided by the JKI. These already compiled data contained the number of tank mixture applications (separately for each tank mixture) in 2007-2008 together with the average relative reduction of the maximum application rate for each product in these tank mixtures.

The most frequent tank mixtures of each pesticide type combination are shown in Table 7.12 for winter wheat and in Table 7.13 for winter oilseed rape. The frequency of pesticide type combinations applied as tank mixtures differ to some degree among the two crops. A combination of only herbicides was the most frequent tank mixture in winter wheat, but also relatively frequent in winter oil seed rape. Herbicidal tank mixtures had also been found frequently in an analysis of tank mixture recommendations in a precursor study (Coors et al. 2008). A combination of fungicides and insecticides scored second in both crops, while this combination has only occasionally been found among tank mixture recommendations in a precursor study (Coors et al. 2008). A combination of only fungicides had been found among tank mixture recommendations almost as frequent as combinations of herbicides (Coors et al. 2008), but appeared to occur at a lower frequency among actual applications of tank mixtures in winter wheat and winter oil seed rape. In contrast to the precursor study, the present data set distinguishes between fungicides and growth regulators based on the season of application. Yet, the most frequent fungicide/growth regulator combination for winter oilseed rape contains for example a product that is registered also as fungicide; a combination that would have been counted in the precursor study as fungicidal combination. In contrast, the growth regulators contained in the respective tank mixture combination for winter wheat are indeed only registered as growth regulators but not as fungicides.

Table 7.12 Total number of tank mixture applications separately for treatment group combinations in winter wheat 2007/2008 based on data provided by the Julius-Kühn Institute, Germany

Details for the most frequent tank mixture in each pesticide type combination include the average application rate in relation to the maximum application rate, the active substances of the products contained in the tank mixtures and their pesticidal mode of action group (according to FRAC, HRAC or IRAC).

Pesticide type combination	Total number of applications	Most frequent tank mixture combination (relative application rate)	Active substances of the products contained in the tank mixtures (mode-of-action group)
Herbicide & Herbicide	65	Bacara (0.69) & Cadou (1.0)	diflufenican (F1), flurtamone (F1) & flufenacet (K3)
Fungicide & Insecticide	59	Fandango (0.47) & Input (0.59) & Biscaya (1.0)	fluoxastrobin (C3), prothioconazole (G1) & spiroxamine (G2), prothioconazole (G1) & thiacloprid (4A)
Fungicide & Growth regulator	55	Capalo (0.75) CCC 720 (0.31) & Moddus (0.44)	fenpropimorph (G2), epoxiconazole (G1), metrafenone (U8) & chlormequat (-) & trinexapac (-)
Fungicide & Fungicide	39	Champion (0.53) & Diamant (0.46)	epoxiconazole (G1), boscalid (C2) & fenpropimorph (G2), epoxiconazole (G1), pyraclostrobin (C3)
Growth regulator & Growth regulator	28	CCC 720 (0.33) & Moddus (0.46)	chlormequat (-) & trinexapac (-)
Herbicide & Growth regulator	11	ATLANTIS WG (1.0) & CCC 720 (0.46)	iodosulfuron (B), mesosulfuron (B) & chlormequat (-)

In conclusion, the data on actual agricultural applications of tank mixtures in two crop cultures across two seasons indicate a somewhat greater diversity of tank mixtures than previously described (Coors et al. 2008). While the present data set is arguably not representative for agricultural application of tank mixtures in general (i.e. for the whole variety of crop cultures), it is considered as comprehensive enough to allow selecting tank mixtures scenarios to be further explored here.

Table 7.13 Total number of tank mixture applications separately for pesticide type combinations in winter oilseed rape 2007/2008 based on data provided by the Julius-Kühn Institute, Germany, and details for the most frequent tank mixture in each treatment group.

Pesticide type combination	Total number of applications	Most frequent tank mixture combination (relative application rate)	Active substances of the products contained in the tank mixtures (mode-of-action group)
Insecticide & Growth regulator	161	Karate mit Zeon Technologie (1.0) & Folicur (0.57)	lambda-cyhalothrin (3) & tebuconazole (G1)
Insecticide & Fungicide	108	Biscaya (0.99) & Cantus (0.97)	thiacloprid (4A) & boscalid (C2)
Herbicide & Herbicide	31	Cirrus (0.29) & Nimbus CS (0.59)	clomazone (F3) & metazachlor (K3), clomazone (F3)
Herbicide & Growth regulator	31	Fusilade MAX (0.63) CARAMBA (0.42)	fluazifop-P (A) & metconazole (G1)
Growth regulator & Growth regulator	13	CARAMBA (0.24) & Folicur (0.35)	metconazole (G1) & tebuconazole (G1)

Implementing mixture toxicity considerations

From the tank mixtures listed in Table 7.12 and 7.13, the following ones have been selected as examples for a closer analysis:

- Tank mixture 1: Bacara (0.69) & Cadou (1.0) as example for two herbicides frequently applied as tank mixture in winter wheat
- Tank mixture 2: Champion (0.53) & Diamant (0.46) as example for two fungicides frequently applied as tank mixture in winter wheat
- Tank mixture 3: Biscaya (0.99) & Cantus (0.97) as example of an insecticide & a fungicide frequently applied as tank mixture in oilseed rape and a combination type not considered in previous projects

These tank mixtures comprise in total the following eight active substances (mode-of-action group):

- thiacloprid (4A insecticide)
- boscalid (C2 fungicide)
- epoxiconazole (G1 fungicide)
- fenpropimorph (G2 fungicide)
- pyraclostrobin (C3 fungicide)
- diflufenican (F1 herbicide)
- flurtamone (F1 herbicide)
- flufenacet (K3 herbicide)

Relevant information for these a.s. and the respective products were obtained from the national risk assessment reports provided by UBA. For the product “Cadou” the report of “Cadou SC” was used instead, as “Cadou SC” replaced “Cadou” in 2010. In rare cases,

information was obtained from risk assessment reports for mono-formulations of an a.s., e.g. the PEC value for fenpropimorph in “Diamant”.

The assessment was conducted for the respective scenario (winter wheat and oilseed rape treatment, respectively) based on the maximum application rate of each product. Additionally, the average reduction of the application rates for the products in the tank mixture was taken into account. A key decision was the assumption on applied risk mitigation measures. Since an assessment of pesticide applications not in compliance with the authorization of PPP is clearly beyond the scope of the present study, the least strict required risk mitigation measures for each product in the tank mixture were taken into account. The stricter risk mitigation measure of the two products was then assumed to have been followed for the tank mixture, because otherwise the application would not have been in compliance with authorization of both PPP. For example, a buffer zone of 5 m and usage of 90 % drift reduction nozzles (as required for “Bacara” alone) was assumed in the case of the “Bacara” and “Cadou SC” tank mixture. This resulted for “Cadou SC” logically in lower PEC values than in the single-product application, which requires only a 1 m buffer zone and no drift reduction nozzles.

Another key decision for the calculation of PEC values for the tank mixture application is the assumption of a linear relationship between PEC and application rate, which means that a reduction of the application by 50 % is assumed to result in a reduction of the PEC value by 50 %.

As pointed out in previous chapters, no toxicity estimates (and TER or HQ values) are generally available for several endpoints that would allow assessing mixture toxicity by calculating a TER_{mix}. These endpoints (non-target arthropods, micro-organisms, and soil macro-invertebrates other than earthworms) were therefore not considered for the tank mixtures. The drainage and run-off scenario for the aquatic compartment as well as the birds and mammals risk assessment were also not assessed here, because a re-calculation of exposure estimates for the reduced application rate of the products in the tank mixture would have been required, which was beyond the scope of the present study.

The following tables with the detailed results of the assessments of the three tank mixtures includes the *spray drift* scenario for the aquatic compartment (only endpoints that were found relevant in the risk assessment of the respective a.s.), as well as the assessment for earthworm and non-target plants. Not included in the following tables are endpoints for which the respective TER values of the products were still above the respective threshold values when multiplied with number of mixture components (as first step criterion, see following chapters). The calculations were conducted in analogy to the evaluation performed for combination products.

Tank mixture 1

The results for tank mixture 1 (two herbicides) are shown in Table 7.14. A combination product (Bacara) and a mono-formulation (Cadou) were combined in this tank mixture.

Table 7.14 Tank mixture of the two herbicidal products "Bacara" (on average 0.69 of maximum application rate) and "Cadou SC" (no reduction) applied on winter wheat.

Only spray drift was evaluated for the aquatic compartment with the relevant toxicity estimates for each a.s. indicated in bold. The assumption of a 5 m buffer zone relates to both distance to surface water and to vegetation. Given are the content of the a.s. in the respective products, the application rates, the relevant exposure and toxicity estimates for the a.s. in the individual products as well as for the mixture (combination product and tank mixture) together with the resulting TER (in brackets).

	Bacara	**Cadou SC**	**Tank Mixture**	
Risk mitigation measure	5 m buffer zone and 90 % drift reduction nozzles	1 m buffer zone and no drift reduction nozzles	5 m buffer zone and 90 % drift reduction nozzles	
Application Rate	1 L/ha	0.5 L/ha	1.0 L/ha Bacara + 0.5 L/ha Cadou	0.69 L/ha Bacara + 0.5 L/ha Cadou
Content a.s.				
flurtamone	250 g/L	-	250 g/ha	172.5 g/ha
diflufenican	100 g/L	-	100 g/ha	69.0 g/ha
flufenacet	-	500 g/L	250 g/ha	250.0 g/ha
sum a.s.	350 g/L	500 g/L	600 g/ha	491.5 g/ha
$PEC_{SURFACE\ WATER}$				
flurtamone	0.0475 µg/L	-	0.0475 µg/L	0.033 µg/L
diflufenican	0.0190 µg/L	-	0.0190 µg/L	0.013 µg/L
flufenacet	-	2.31 µg/L	0.0475 µg/L	0.048 µg/L
sum a.s.	0.0665 µg/L	2.31 µg/L	0.1140 µg/L	0.094 µg/L
Toxicity estimate and (TER) for surface water assessment, spray drift scenario				
Algal growth inhibition E_bC_{50} [TER trigger of 10]				
flurtamone	11 µg/L (231)	-	11 µg/L (231)	11 µg/L (333)
diflufenican	**0.25 µg/L (13.2)**	-	0.25 µg/L (13.2)	0.25 µg/L (19.2)
flufenacet	-	1.42 µg/L (0.6)	1.42 µg/L (30)	1.42 µg/L (30)
mixture	0.83 µg/L (12.5)	-	1.00 µg/L (8.8)	1.06 µg/L (11.3)

Tab. 7.14: *continued*

	Bacara	**Cadou SC**	**Tank Mixture**	
Lemna growth inhibition EC_{50} [TER trigger of 10]				
flurtamone	**9.9 µg/L (208)**	-	9.9 µg/L (208)	9.9 µg/L (300)
diflufenican	39 µg/L (2052)	-	39 µg/L (2052)	39 µg/L (3000)
flufenacet	-	2.43 µg/L (1.1)	2.43 µg/L (51)	2.43 µg/L (51)
mixture	12.6 µg/L (189)	-	4.6 µg/L (40)	4.0 µg/L (43)
Microcosm study EAC [TER trigger of 5]				
flurtamone	-	-	-	-
diflufenican	-	-	-	-
flufenacet	-	**12 µg/L (5.2)**	12 µg/L (250)	12 µg/L (250)
$PEC_{INI\ SOIL,\ 2.5\ CM\ DEPTH}$				
flurtamone	0.268 mg/kg	-	0.268 mg/kg	0.185 mg/kg
diflufenican	0.267 mg/kg	-	0.267 mg/kg	0.184 mg/kg
flufenacet	-	0.66 mg/kg [1]	0.660 mg/kg	0.660 mg/kg
sum a.s.	0.535 mg/kg	0.66 mg/kg	1.195 mg/kg	1.029 mg/kg
Earthworm toxicity reproduction NOEC and (TER) with trigger of 5				
flurtamone	0.32 g/kg (1194)	-	0.32 g/kg (1194)	0.32 g/kg (1730)
diflufenican	0.50 g/kg (1873)	-	0.50 g/kg (1873)	0.50 g/kg (2717)
flufenacet	-	8 mg/kg (12.1)[1]	8 mg/kg (12.1)	8 mg/kg (12.1)
mixture	0.39 g/kg (729)	-	14.3 mg/kg (11.9)	12.3 mg/kg (12)
$PEC_{INI\ PLANTS}$				
flurtamone	0.143 g/ha	-	0.143 g/ha	0.099 g/ha
diflufenican	0.057 g/ha	-	0.057 g/ha	0.039 g/ha
flufenacet	-	0.346 g/ha	0.071 g/ha	0.071 g/ha
sum a.s.	0.200 g/ha	0.346 g/ha	0.271 g/ha	0.209 g/ha
Non-target plant toxicity ER_{50} and (TER) with trigger of 5 or 10				
flurtamone[2]	5.5 g/ha (38)	-	5.5 g/ha (38)	5.5 g/ha (56)
diflufenican[3]	2.88 g/ha (51)	-	2.88 g/ha (51)	2.88 g/ha (74)
flufenacet[4]	-	10.5 g/ha (30)	10.5 g/ha (148)	10.5 g/ha (148)
mixture	4.37 g/ha (22)	-	5.16 g/ha (19)	5.46 g/ha (26)

[1]Estimate in assessment report relates to soil depth of 5 cm; [2]Screening test *Chenopodium album;* [3]Vegetative vigour *Brassica napus;* [4]Seedling emergence *Sorghum bicolor*

For earthworms and plants, the calculated TER values of the tank mixture indicated no risk even without consideration of reduced application rates. In the case of the aquatic compartment, *Lemna* and algal growth inhibition and a microcosm study (triggered by risk to primary producers in the standard assessment) were the relevant endpoints for the three a.s. in the tank mixture. The consideration of mixture toxicity for the tank mixture indicated no risk for *Lemna* even without reduction of the application rate and also no risk for algae if the reduced application rate was taken into account. Due to lack of data, such an assessment could not be performed for the higher-tier endpoint "microcosm", but the TER values for this endpoint indicate that no risk is to be expected if the risk mitigation measures that are forced by the the product Bacara (i.e. the product for which no higher-tier endpoint was available) are followed for the tank mixture. The step back to a mixture assessment on a first tier level (i.e., *Lemna* and algae, see above) supports this conclusion of no unacceptable risk for primary producers as long as the risk mitigation measures are obeyed. The situation and resulting conclusion would be different, if buffer zones and requirements for drift reduction nozzles were similar for both products or stricter for the product that was individually assessed on a higher tier.

Tank mixture 2

Tank mixture 2 ("Champion" & "Diamant") may be seen as almost a worst-case scenario because here two combination products are mixed together, resulting in four different a.s. in the tank mixture with one of these a.s. (epoxiconazole) being introduced by each of the two products. However, the application rate of this frequently applied tank mixture was on average considerably reduced for both products (Table 7.15).

HQ values reported for the two combination products with regard to bee toxicity were below 17 for one product and 30 and 39 for the other product (failing the first step criterion of HQ*2<50). No component-based assessment was possible due to lack of data, but a summation of reciprocal product HQ values derived values below 11, i.e. well below the trigger of 50.

Table 7.15 Tank mixture of the two fungicidal products "Champion" (on average 0.53 of maximum application rate) and "Diamant" (on average 0.46 of maximum application rate) applied on winter wheat

Only spray drift was evaluated for the aquatic compartment with the relevant toxicity estimates for each a.s. indicated in bold. Given are the content of the a.s. in the respective products, the application rates, the relevant exposure and toxicity estimates for the a.s. in the individual products as well as for the mixture (combination product and tank mixture) together with the resulting TER (in brackets).

	Champion	**Diamant**	**Tank Mixture**	
Risk mitigation measure	1 m buffer zone and 50 % drift reduction nozzles	5 m buffer zone and 50 % drift reduction nozzles	5 m buffer zone and 50 % drift reduction nozzles	
Application Rate	1.5 L/ha	1.75 L/ha	1.5 L/ha Champion + 1.75 L/ha Diamant	0.795 L/ha Champion + 0.805 L/ha Diamant

Content a.s.				
boscalid	233 g/L	-	349.50 g/ha	185.2 g/ha
epoxiconazole	67 g/L	43 g/L	175.75 g/ha	87.9 g/ha
fenpropimorph	-	214 g/L	374.50 g/ha	172.3 g/ha
pyraclostrobin	-	114 g/L	199.50 g/ha	91.8 g/ha
sum a.s.	300 g/L	371 g/L	1099.25 g/ha	537.2 g/ha
$PEC_{SURFACE\ WATER}$				
boscalid	1.858 µg/L	-	0.367 µg/L	0.195 µg/L
epoxiconazole	0.486 µg/L	0.112 µg/L	0.208 µg/L	0.103 µg/L
fenpropimorph	-	1.394 µg/L	1.394 µg/L	0.641 µg/L
pyraclostrobin	-	0.190 µg/L	0.190 µg/L	0.087 µg/L
sum a.s.	2.344 µg/L	1.696 µg/L	2.159 µg/L	1.026 µg/L
Toxicity estimate and (TER) for surface water assessment, spray drift scenario				
Algal growth inhibition E_bC_{50} [TER trigger of 10]				
boscalid	1.34 mg/L (721)	-	1.34 mg/L (3651)	1.34 mg/L (6872)
epoxiconazole	1.19 mg/L (2449)	1.19 mg/L (10625)	1.19 mg/L (5721)	1.19 mg/L (11553)
fenpropimorph	-	327 µg/L (234)	327 µg/L (234)	327 µg/L (510)
pyraclostrobin	-	152 µg/L (800)	152 µg/L (800)	152 µg/L (1747)

Tab. 7.15: *continued*

	Champion	**Diamant**	**Tank Mixture**	
mixture	1.31 mg/L (557)	302 µg/L (178)	362 µg/L (168)	371 µg/L (362)
Lemna EC_{50} [TER trigger of 8 or 10]				
boscalid	n.a.	-	-	-
epoxiconazole	**4.3 µg/L (8.8)**	4.3 µg/L (38)	4.3 µg/L (21)	4.3 µg/L (42)
fenpropimorph	-	n.a.	-	-
pyraclostrobin	-	n.a.	-	-
mixture	-	-	-	-
Fish acute toxicity LC_{50} or (for pyraclostrobin) HC_5 of LC_{50} [TER trigger of 100 or 20]				
boscalid	LC_{50} >2.7 mg/L	-	(>1000)	(>1000)

epoxiconazole	LC_{50} >2.15 mg/L	LC_{50} >2.15 mg/L	(>1000)	(>1000)
fenpropimorph	-	2.11 mg/L	(>1000)	(>1000)
pyraclostrobin	-	**5.9 µg/L (31)**	5.9 µg/L (31)	5.9 µg/L (68)
mixture	>2.5 mg/L (>1094)	>51.5 µg/L (>30)	>65 µg/L (>30)	>67 µg/L (>65)
$PEC_{INI\ SOIL}$				
"Champion"	1.5 L/ha	-	1.5 L/ha	0.795 L/ha
"Diamant"		1.75 L/ha	1.75 L/ha	0.805 L/ha
sum			3.25 L/ha	1.6 L/ha
Earthworm toxicity reproduction NOEC and (TER) with trigger of 5				
"Champion"	**1.5 L/ha (1)**	-	1.5 L/ha (1)	1.5 L/ha (1.9)
"Diamant"	-	≥ 10 L/ha (≥5.7)	-	≥ 10 L/ha (≥5.7)
mixture	-	-	2.8 L/ha (≥0.9)	2.6 L/ha (≥1.6)

Chronic toxicity to earthworms was a critical endpoint for one product, which was authorized based on higher-tier studies. The other product, "Diamant", appeared to carry little risk based on a laboratory study NOEC(reproduction)>10 L/ha. However, this product adds epoxiconazole to the tank mixture. A component-based mixture assessment was not possible due to lack of data for some a.s. contained in the products. A mixture toxicity consideration using the NOEC values for the products indicated risk for the tank mixture. Higher-tier studies for mono-formulations with some of the a.s. or with the combination products might be considered to decide if the risk of the tank mixture is acceptable for earthworms, as it has been the case in the risk assessment of the two individual products. Particularly for epoxiconazole, no available field studies have tested the application rate that results from the here assessed tank mixture. However, a field study with an epoxiconazole mono-formulation, which was used in the authorization of one product, indicating no unacceptable risk at an application rate of two times 125 g/ha, which is higher than the application rate of epoxiconazole alone by the tank mixture.

Fish acute toxicity and *Lemna* growth inhibition were the relevant endpoints used in the aquatic risk assessment of the two products. Since data on *Lemna* are not required for fungicides, they were not available for all a.s. in the tank mixture. As a surrogate, algal growth inhibition was assessed here for the tank mixture and indicated no risk independently from the reduced application rate under the assumed risk mitigation measures. The same is true for fish acute toxicity, for which either an HC_5 value or, for all other a.s., LC_{50} values were used.

Tank mixture 3

The results for tank mixture 3 are summarized in Table 7.16. Both products in the tank mixture contained only one a.s. and the application rate of the two products was almost identical with the maximum application rate (0.99 of "Biscaya" and 0.97 of "Cantus"), resulting in only slight reductions of exposure estimates.

Chronic toxicity to fish was the relevant aquatic endpoint for the fungicide and the respective toxicity of the insecticide was about in the same range. Due to the risk mitigation measures resulting from the toxicity to aquatic invertebrates, however, the TER value related to chronic fish toxicity of the insecticide was so low that no risk for the tank mixture was indicated.

Table 7.16 Tank mixture of the insecticidal product "Biscaya" (on average 0.99 of maximum application rate) and the fungicidal product "Cantus" (on average 0.97 of maximum application rate) applied on oilseed rape
Only spray drift was evaluated for the aquatic compartment with the relevant toxicity estimates for each a.s. indicated in bold. Given are the content of the a.s. in the respective products, the application rates, the relevant exposure and toxicity estimates for the a.s. in the individual products as well as for the mixture (combination product and tank mixture) together with the resulting TER (in brackets).

	Biscaya	**Cantus**	**Tank Mixture**	
Risk mitigation measure	5 m buffer zone and no drift reduction nozzles	1 m buffer zone and no drift reduction nozzles	5 m buffer zone and no drift reduction nozzles	
Application Rate	0.3 L/ha	0.5 kg/ha	0.3 L/ha Biscaya + 0.5 kg/ha Cantus	0.297 L/ha Biscaya + 0.485 kg/ha Cantus
Content a.s.				
thiacloprid	240 g/L	-	72 g/ha	71.28 g/ha
boscalid	-	500 g/kg	250 g/ha	242.50 g/ha
sum a.s.			322 g/ha	313.78 g/ha
$PEC_{SURFACE\ WATER}$				
thiacloprid	0.172 µg/L	-	0.172 µg/L	0.170 µg/L
boscalid	-	7.31 µg/L	1.504 µg/L	1.459 µg/L
sum a.s.	-	-	1.676 µg/L	1.629 µg/L
Toxicity estimate and (TER) for surface water assessment, spray drift scenario				
Fish chronic toxicity NOEC [TER trigger of 10]				
thiacloprid	244 µg/L (1418)	-	244 µg/L (1418)	244 µg/L (1435)
boscalid	-	**125 µg/L (17)**	125 µg/L (83)	125 µg/L (86)
mixture	-	-	131.6 µg/L (78)	132 µg/L (80)
Mesocosm study NOEAEC [TER trigger of 5]				
thiacloprid	**1.57 µg/L (9.1)**	-	1.57 µg/L (9.1)	1.57 µg/L (9.2)
boscalid	-	n.a.	-	-

mixture	-	-	-	-
Acute toxicity aquatic invertebrates EC_{50} [TER trigger of 100]				
thiacloprid	6 µg/L (35)	-	6 µg/L (35)	6 µg/L (35)
boscalid	-	5330 µg/L (729)	5330 µg/L (3543)	5330 µg/L (3653)
mixture	-	-	57.9 µg/L (34)	56.9 (35)
$PEC_{INI\ SOIL}$				
thiacloprid	72 g/ha	-	72 g/ha	71.28 g/ha
boscalid	-	250 g/ha	250 g/ha	242.50 g/ha
sum a.s.	-	-	322 g/ha	313.78 g/ha
Earthworm toxicity reproduction NOEC and (TER) with trigger of 5				
thiacloprid	**56.4 g/ha (0.78)**	-	56.4 g/ha (0.78)	56.4 g/ha (0.79)
boscalid	-	**900 g/ha (3.6)**	900 g/ha (3.6)	900 g/ha (3.7)
mixture	-	-	207 g/ha (0.6)	205 g/ha (0.7)

For both products, the TER value with regard to chronic earthworm toxicity was below the trigger value of 5. Consequently, the respective TER for the tank mixture was below the trigger value, also when taking into account the (slightly) reduced application rates in the tank mixture. In the single-product risk assessment reports, higher-tier studies (earthworm field studies) for the individual products indicated acceptable risk, because application rates of in total 900 g boscalid/ha and 375 g thiacloprid/ha, respectively, produced no significant effects on earthworm populations after one year. While these individual application rates are clearly well above the amount of a.s. applied with the tank mixture, the question remains how such higher-tier studies of the individual products can be used consistently in a mixture toxicity context.

In the risk assessment for bees and non-target arthropods, higher-tier studies were triggered for the insecticidal product and no risk identified in the standard assessment in case of the fungicidal product. Similarly, the toxicity of thiacloprid to aquatic invertebrates triggered a mesocosm study as higher-tier assessment, which was not available for the fungicidal product. Again, the risk of boscalid to aquatic invertebrates was low as indicated by a TER of 729 (Table 7.16). Hence, for all these three risk assessment areas the risk posed by the fungicidal product appears negligible compared to the risk posed by the insecticidal product. Thereby, this tank mixture represents an example where guidance is needed under which circumstances a risk assessment for a mixture may be based on one (or some) of the mixture components only. This is particularly relevant for a fungicide-insecticide combination since such mixtures (neonicotinoid insecticide and azole fungicide) are among the rare examples of reported synergistic interaction (Schmuck et al. 2003).

CONCLUSIONS

The exemplary assessment of three tank mixtures frequently applied in agriculture showed that, in principle, consideration of mixture toxicity is possible. For all three tank mixtures, no risk was identified for the tank mixture in the standard endpoints that were assessable. This was due to the assumed risk mitigation measures, the reduced application rates of the individual products in the tank mixture and the differential sensitivity profile for the a.s., e.g. either fish or algae driving the aquatic risk assessment. In other cases of tank mixtures than the three selected ones, however, the situation may be different. This holds particularly for tank mixtures of a.s. for which the same non-target organisms are very susceptible.

There are further limitations. Namely the unavailability of mixture toxicity implementation concepts for the kind of toxicity data that are typical for a number of endpoints such as non-target arthropods, soil micro-organisms, soil macro-invertebrates other than earthworms, and higher-tier studies. These endpoints were frequently driving the risk assessment, and particularly long-term toxicity to earthworms was relevant in the assessment of individual products in two of the three tank mixtures. In other cases, only one of the two products in the tank mixture was forcing a higher-tier assessment (e.g., bees in the case of the insecticide).

Summarizing, considering mixture toxicity in an assessment of a few, non-representative tank mixtures indicated no risk in the assessable standard endpoints under the assumptions that the described risk mitigation measured and application rate reductions are obeyed. For a number of endpoints, among them several that were critical in the individual product assessments, the risk of the tank mixture could not be assessed with currently available data. Risks resulting from the application of these tank mixtures can therefore not fully be excluded, namely for earthworms and bees.

7.1.7 Serial applications

As outlined previously, serial applications comprise the repeated application of PPP on the same crop culture across a period of time. By this agricultural practice, the same as well as different active substances can be applied. Consequently, the composition of the mixture is highly complex and can only be estimated by sophisticated exposure models. Serial applications of the same product within the period of one year are covered in the environmental risk assessment by taking a multiple application factor (MAF) into account for the exposure assessment. No specific regulations or exposure calculations exist currently for serial applications of different PPP, even if they contain the same active substances (only in some few exceptional cases, e.g. for copper-containing PPP).

As explained previously, exposure estimates are crucial in order to calculate the mixture toxicity based on component-based approaches for a defined mixture. Since deriving exposure assessments was beyond the scope of the present study, the consequences of current serial applications practice can only be generically discussed in the following.

No data on actual and representative serial applications in agriculture were available from any systematic study; therefore the discussion uses an example of a serial application of fungicides in vineyards recommended by local organisations (Hill et al. 2008, Fig. 7.1). The recommendations are very detailed with regard to products and application frequency and intervals. No recommendation is provided regarding the application rate, e.g. the possibility of a reduced application rate. It is important to note that serial applications in agriculture extend beyond the application of PPP from the same category. In this particular example, additional

applications of e.g. herbicides and insecticides should in principle be taken into account, but respective information is currently not available.

Relating the recommendations to active substances contained in the products results in the following pattern:

- First row recommendation a): "Polyram" (metiram), "Cabrio Top" (metiram & pyraclostrobin), "Melody Combi" (folpet & iprovalicarb), "Forum Star" (folpet & dimethomorph), "Universalis" (folpet & azoxystrobin), 2-times "Folpan WG" (folpet)

As this list shows, the a.s. metiram is applied twice in different products and the a.s. folpet repeatedly in a series of four different products. This recommendation example clearly illustrates thereby the types of mixtures potentially resulting from serial applications: the same a.s. applied repeatedly by different products and different a.s. applied together and in series on the same crop. Serial applications of folpet (e.g. 5 times per year for the treatment of fungi in vineyards) are covered in the risk assessment of folpet mono-formulations. In the case of metiram, 8 applications per year in vineyards are for example covered in the risk assessment of "Polyram". Hence, it appears that the here encountered recommendation of a serial application is covered by the risk assessment for individual products with regard to the repeated application of the same a.s. by different products. However, it is open if this holds true for all kinds of serial applications.

- Second row recommendation for treatment of *Oidium*: sulfur, "Cabrio Top" (metiram & pyraclostrobin), "Vento Power" (myclobutanil & quinoxyfen), "Collis" (kresoxim-methyl & boscalid),"Cabrio Top" (metiram & pyraclostrobin), "Topas" (penconazole)

This serial application recommendation does not contain repeated application of the same a.s. by different products, but only by the same product ("Cabrio Top"). It clearly illustrates that combination products are used in serial applications and thereby combines two different types of mixtures.

The calculation of exposure estimates for these two examples of serial application of PPP would be highly complex. GIS based-modelling approaches as described for example by Caj et al. (2011) may help with this challenge. Such models need to take into account repeated application of the same and different a.s. over time and rely heavily on the availability of PPP usage data. The availability of reliable exposure estimates is the pre-requisite to implementing mixture toxicity concepts for the environmental risk assessment of serial applications.

BEISPIELE FÜR MÖGLICHE SPRITZFOLGEN 2008

Beispiele für Peronospora-Spritzfolgen

Die dargestellten Möglichkeiten für Spritzfolgen zeigen nur einen Ausschnitt der Möglichkeiten.

a) **Flaschenweinvermarkter mit knapper Zeitplanung** bzw. Betriebe mit geringer Schlagkraft – Betonung auf tiefenwirksamen Mitteln

1. Vbl.	2.Vbl.	Abgeh. Blüte	1. Nbl.	2.Nbl.	3. Nbl	(4. Nbl.)
Polyram	Cabrio T.	Melody C.	Forum St.	Universalis	Folpan WG	Folpan WG
Polyram	Forum St.	Universalis	Melody C.	Equation PRO	Folpan WG	Delan WG
Polyram	Melody C.	Cabrio T.	Mildicut	Forum Star	Delan WG	Folpan WG

Hier werden Spritzabstände von ca. 13-15 Tagen angestrebt, was hinsichtlich Oidium eine besondere Mittelwahl (z.B. Flint, Vivando, Collis) während der Blüte erfordert.

b) **Minimierung der Mittelkosten** bei Erhaltung der Raubmilben.

Spritzabstand 10-12 Tage – Betonung auf kostengünstige Fungizide

1. Vbl.	2.Vbl.	Abgeh.	1. Nbl.	2.Nbl.	3. Nbl	4. Nbl.
Polyram WG	Polyram WG	Folpan WG	Folpan WG	Folpan WG oder Delan WG	Folpan WG oder Delan WG	Folpan WG

Mit zunehmender Verfrühung der Lesezeitpunkte wird die Beachtung der Wartezeit bei der letzten Peronospora-Spritzung immer wichtiger. Unter den Kontaktfungiziden haben mit 35 Tagen Folpan und das Kupfermittel Cuprozin die kürzeste Wartezeit.

Beispiele für Oidium-Spritzfolgen

Hochgradig oidiumempfindliche Rebsorten /Befallslagen
z.B. PORTUGIESER, DORNFELDER, KERNER

1.VBL.	2.VBL.	Abgeh. Blüte	1.NBL.	2.NBL.	Ab 3.NBL.
SCHWEFEL	Flint	Cabrio Top	Vento P.	Vivando	Systhane
SCHWEFEL	Cabrio Top	Vento Power	Collis	Vento Power	Topas
SCHWEFEL	Collis	Vivando	Discus	Topas	Systhane

Figure 7.1 Suggestions for serial applications of fungicides in vineyards (from Hill et al. 2008)

7.1.8 Relevance of formulation additives for the toxicity of plant protection products

There is currently no regulatory specification in terms of thresholds for acceptable deviations between the ecotoxicological profile of a formulated and a technical active substance. There is, however, a specification on the equivalence of technical materials of active substances that are included on Annex I. This specification relates basically to the impact that changes in production processes may have on the presence and relevance of impurities in the technical material. While this guidance document is only meant to regulate the equivalency of various technical materials with regard to acceptable deviations in (eco)toxicity, it can also provide some insight on the deviations that may potentially be considered acceptable between technical and formulated material. The former version of this guidance document (EC 2009c) related to Directive 91/141/EEC and stated a deviation of factor 5 in experimentally observed ecotoxicity as acceptable, while the acceptable deviation for toxicity was set to factor 2. The most recent version of this guidance document (EC 2011) relates to regulation EC No 1107/2009 and states a factor of 3 as an acceptable deviation between experimentally observed ecotoxicity. This acceptable deviation of factor 3 is explicitly stated not to be interpreted as to be irrelevant for the risk assessment, but only to cover experimental variability (EC 2011, p.13). Newly included in the guidance document is an explicit consideration of mixture toxicity based on the concept of concentration addition to calculate (i.e. to consider theoretically) the ecotoxicity of a new technical material with known impurities (EC 2011, p. 11). Within this so-called generic approach a new material is considered as equivalent, i.e. the effect of impurities is deemed not relevant, if its predicted ecotoxicity does not exceed the ecotoxicity of the reference material by a factor of 2. The recent guidance document also specifies the issue of bridging studies, i.e. studies that shall proof equivalence of different technical materials. Such bridging studies "*must be carried out according to the same test methodology, under identical exposure conditions (e.g. static, flow-through) and with the same test species*" (EC 2011, p.12). However, bridging between different groups of organisms may be acceptable, but only if the same mode-of-action can be assumed in these two organism groups. For example, bridging is not allowed between primary producers and invertebrates in the case of herbicides. This is consistent with the finding that formulation additives (*impurities*) are the more relevant for the toxicity towards an organism the less sensitive this organism is for the active substance (Coors & Frische 2011).

The task of this project part was to assess if and how formulation additives influence the toxicity of a plant protection product (PPP) by using up to 15 PPP mono-formulations as example. There was no data base available that allowed selecting mono-formulations based on their known content of formulation additives. Therefore, a less straightforward approach was applied by selecting products considered in some aspects as representative.

Aspects taken into consideration when selecting the mono-formulations were:

- Information obtained by literature search
- Range and frequency of formulation types and their representation among the different pesticide categories
- Frequency of active substances, separately for major pesticide categories, and their representation among the different formulation types
- Range of different formulation types present in the data base for a given active substance
- Evidence of previous projects regarding the influence of formulation additives

The goal was to select 15 mono-formulations for the further analysis that covered i) different formulation types of a given a.s., ii) the most common formulation type(s) across a range of

different a.s., iii) formulation types (and eventually a.s.) that are expected to be problematic based on available previous information, and iv) active substances from a range of mode-of-action groups from key pesticide categories. Such a selection was expected to enable to some degree an extrapolation of the influence of additives in certain formulation types across a.s. and identify formulation types or pesticides (based on mode of action, chemical structure or use category) that are prone to exhibit increased toxicity in formulated products. However, given the small number of mono-formulations to be assessed, the huge diversity of formulation additives and the general non-availability of information on the identity (and (eco)toxicity) of formulation additives in PPP, the assessment conducted in the present study can only be considered as exemplary.

Similar to enhanced product toxicity, formulation additives may also reduce the toxicity to non-target organisms such safeners contained in herbicides are intended to reduction the phytotoxicity to crop plants. No in depth analysis of such lower-than expected product toxicity is carried out here, because these cases will result in over-protective but not underprotective regulatory decisions. Assuming a normal statistical distribution of deviations, however, deviations from the predicted product toxicity will be equally frequent in both directions.

Based on the database of the BVL (*Bundesamt für Verbraucherschutz und Lebensmittelsicherheit*), there were in total 477 PPP with one active ingredient (i.e. mono-formulations) registered in Germany as of August 2008 (excluding registrations under a different trade name). These 477 products were analyzed in order to provide a concise overview on marketed formulations of PPP and to inform the selection of 15 mono-formulations for the further analysis.

FORMULATION TYPES

Table 7.17 lists the 45 different formulation types present among the 477 mono-formulations registered in Germany (August 2008). Some of the formulation types were only represented by products that are used for the protection of stored products (GE, KN, CP) or uses that are of little relevance in the context of the present study (i.e. rodenticides, repellents, pheromones, glues and sealing waxes). The formulation types PA, BB, TB, CB, CP, and XX were exclusively represented by such use categories (e.g. all *block baits,* BB*,* were rodenticides etc.). But even after excluding these nine formulation types, there remains a large range of very different formulation types among marketed PPP.

The three most frequent formulation types (SC, EC, and WG) were among the five most frequent formulation types in all major categories of pesticides (i.e. categories with > 25 products), except growth regulators that showed a quite different pattern (Figure 7.2). This finding is basically in agreement with Knowles (2008), who stated that the most common formulation types for spray applications are SL (for water-soluble a.s.), EC (for oil-soluble a.s.) as well as WP and SC (for insoluble a.s.).

With regard to the selection of mono-formulations for the further analysis in the present study, it appeared reasonable to cover the most frequent formulation types in each pesticide category, i.e., EC, SC, SL, and WG.

Table 7.17 Formulation types and their (international) code that were present among the 477 mono-formulations registered in Germany in August 2008.

Code	Description	Code	Description
AE	aerosol dispenser	KN	cold fogging concentrate
AL	other liquids to be applied undiluted	LS	solution for seed treatment
AP	other powder	ME	micro-emulsion
BB	block bait	MG	microgranule
CB	bait concentrate	OD	oil dispersion
CP	contact powder	PA	paste
CS	capsule suspension	PR	plant rodlet
DC	dispersible concentrate	RB	bait ready for use
DP	dustable powder	SC	suspension concentrate
DS	powder for dry seed treatment	SE	suspo-emulsion
EC	emulsifiable concentrate	SG	water soluble granule
ES	emulsion for seed treatment	SL	water soluble concentrate
EW	emulsion, oil in water	SP	water soluble powder
FG	fine granule	ST	water soluble tablet
FS	flowable concentrate for seed treatment	TB	tablet
GA	gas	TC	technical material
GB	granular bait	UL	ultra-low volume liquid
GE	gas generating product	VP	vapour releasing product
GR	granule	WG	water dispersible granule
GS	grease	WP	wettable powder
HN	hot fogging concentrate	WS	water dispersible powder for slurry treatment
KK	combi pack solid/liquid		
KL	combi pack liquid/liquid	XX	others

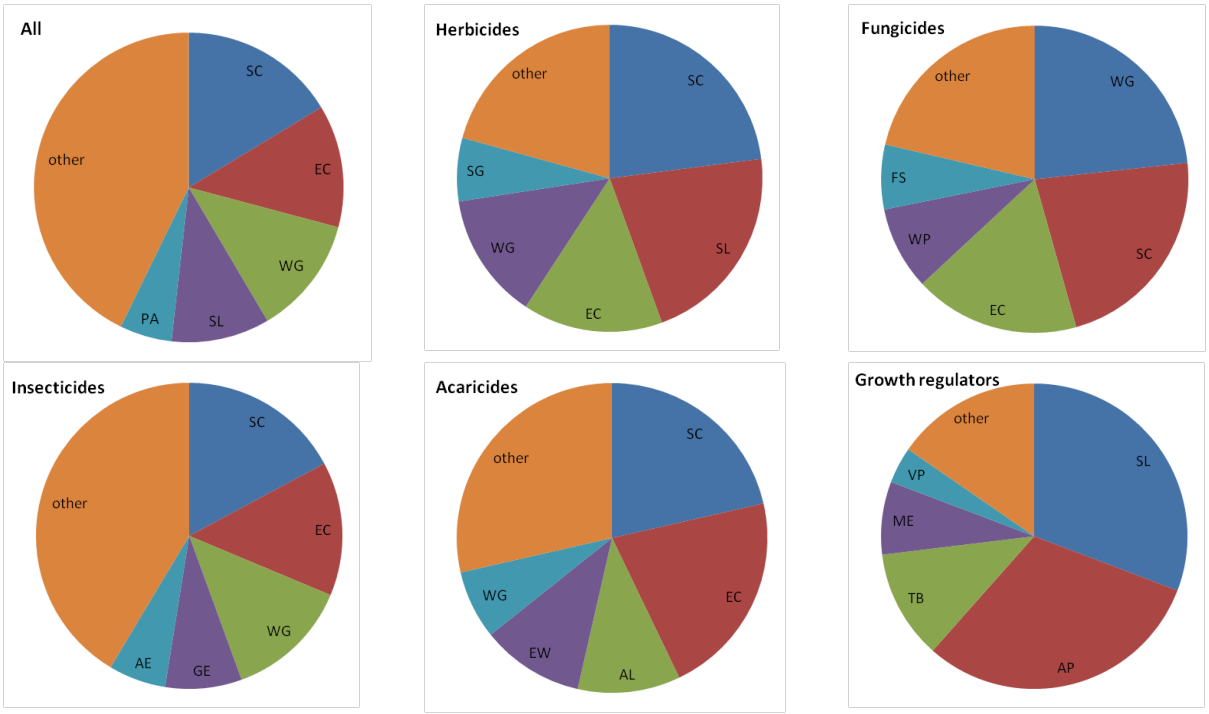


Figure 7.2 Relative frequency of formulation types
In all mono-formulations (477) and in the pesticide categories that are represented with more than 25 products: herbicides (135 mono-formulations), fungicides (103), insecticides (99), acaricides (28), and growth regulators (26). Shown in each chart are always the five most frequent formulation types, while the remaining types are pooled into "other".

Emulsifiable concentrates (EC formulations) have the largest market share in terms of applied volume in a global perspective (Knowles 2008). This formulation type contains typically about 5-10 % of a mixture of emulsifiers, usually a non-ionic and an anionic surfactant (Knowles 2008). In addition to the emulsifying surfactants, EC formulations typically contain organic solvents to increase the solubility of the a.s. in the concentrate (Knowles 2008). EC formulations have been pointed out by the guidance of the competent authority in the United Kingdom as being "[...] often more toxic to non-target species than other formulation types (due to the high level of solvents and surfactants), [...]" (PSD, 2009, p. 17). EC formulations were the second most frequent among the 477 mono-formulations and ranked high in all major pesticide categories, except the growth regulators. In total, there were 61 EC formulations among the assessed 477 products.

Suspension concentrates (SC formulations) are nowadays usually water-based and contain typically wetting and dispersing agents such as for example ethoxylates or polymeric surfactants together with anti-settling agents and preservatives (Knowles 2008).

Solution concentrates (SL formulations) represent concentrates of aqueous solutions of the a.s. that may contain typically some surfactants, wetting agents, anti-freeze agents and water-

miscible solvents (Knowles 2008). Wetting agents are added to improve the efficacy of the product by enhancing the uptake (Knowles 2008). If the active substance can be formulated as SL, this appears to be preferred. The survey of mono-formulation (Table 7.18) shows that for a.s. formulated as SL there rarely exists a product with an alternative formulation type.

Table 7.18 Number of mono-formulations for all a.s. with at least two mono-formulations, shown only for the five major pesticide categories and the seven formulation types selected to be of interest for the further analysis.

	MoA[1]	EC	SC	WG	SL	EW	ME	SE
Herbicides	**HRAC**							
Metamitron	C1		3					
Metribuzin	C1			2				
Phenmedipham	C1		3					
Chloridazon	C1			2				
Chlortoluron	C2		2					
Isoproturon	C2		4					
Bromoxynil	C3	3	1					
Carfentrazone	E			2			1	
Bifenox	E		2					
Glyphosate	G				13			
Glufosinate	H				2			
Pendimethalin	K1		1	1				
Metazachlor	K3		2					
Flufenacet	K3		1	1				
Ethofumesate	N		2					
Mecoprop-P	O				3			
2,4-D	O				3			
Dichlorprop-P	O				2			
MCPA	O				2			
Fungicides	**FRAC**							
Azoxystrobin	C3		3					
Pyraclostrobin	C3	2						
Trifloxystrobin	C3		1	1				
Cyprodinil	D1	1		2				
Quinoxyfen	E1		2					
Iprodione	E3		1	1				
Propamocarb	F4				2			
Prochloraz	G1	1				1		
Propiconazol	G1	2						
Myclobutanil	G1					2		
Sulfur	M2			4				
Mancozeb	M3			2				
Captan	M4			2				
Folpet	M4		1	1				
Chlorothalonil	M5		1	1				
Metrafenone	U8		2					
Insecticides	**IRAC**							
Dimethoat	1B	3						
lambda-Cyhalothrin	3			2				
alpha-Cypermethrin	3		2					
Spinosad	5		2					
Imidacloprid	4A			2				
Thiamethoxam	4A			1	1			
Thiacloprid	4A		1					1
Rape seed oil	n.a.	2				1		
Mineral oil	n.a.	1				2		
Acaricides								
Rape seed oil	n.a.	2				1		
Mineral oil	n.a.	1				2		
Sulfur	n.a.			2				
Growth regulators								
Chlormequat	n.a.				4			

Ethephon	n.a.		3	
Trinexapac	n.a.	1		1

[1] Pesticidal Mode of Action group according to HRAC (2008), FRAC (2008), and IRAC (2008). n.a.: not applicable

Water dispersible granules (WG formulations) replace to some degree nowadays wettable powders and suspension concentrates, because their use is dust-free and requires little amount of liquids (Knowles 2008). The preparation of the formulation is technically very demanding. The formulations contain typically similar additives as SC and WP formulations, namely wetting and dispersing agents together with disintegrating agents (typically a salt) and a water soluble filler (Knowles 2008).

In the course of developing better formulations, the reduction of the content of (volatile) organic solvents has been one goal among others. As alternatives particularly to EC formulations, emulsion formulations such as oil-in-water emulsions (EW), suspo-emulsions (SE) and micro-emulsions (ME) have become more common (Knowles 2008). These contain less or no solvents, but still a considerable amount of surfactants and emulsifiers.

ACTIVE SUBSTANCES

Of all the 221 a.s. in the mono-formulations, 61 were present in 2-7 different formulation types. Yet, this selection covers all pesticide categories and formulation types, i.e. a very diverse set of mono-formulations. In the following, the analysis is restricted to the a.s. from the five major pesticides categories. Among those, 165 were present in the seven preliminarily selected formulation types (EC, SC, SL, WG, EW, ME, and SE). Table 7.18 includes the minority of 57 among these 165 a.s. that were contained in at least two different products, which were then mostly of the same formulation type. Only 15 of these 165 a.s. were formulated in two different formulation types and none was available in three or more different formulation types. Hence, the range of formulation types represented by a single a.s. was very limited, which did not allow to select mono-formulations of a few a.s. to assess the influence of the formulation type independently of the a.s. The reason for this limited variability of formulation types for a given a.s. is that the type of formulation strongly depends on the physicochemical properties of the a.s. (Kudsk 2008).

The herbicide glyphosate is the most frequent a.s. and exclusively formulated as solution concentrate. Because of this little variation and the literature already available for formulated glyphosate (see below), this a.s. was not selected for the further analysis. Bromoxynil is the only herbicide formulated as EC and additionally one SC formulation is available. At the same time, bromoxynil is the only a.s. that is formulated as EC and as SC. Among the fungicides, there is only one a.s. (cyprodinil) for which an EC mono-formulation is available in addition to any other formulation type. Cyprodinil is further the only a.s. formulated as EC and as WG. Few fungicides are formulated as SC and WG formulations and they may serve as candidates to compare these two formulation types. The emulsion formulation types ME and SE are only represented by in total three products, while EW formulations are more frequent, but are not present among herbicides.

In a previous study, a large number of combination products had been assessed for deviations between their predicted mixture toxicity according to the concept of concentration addition and their observed product toxicity (Coors 2009, Coors and Frische 2011). Formulation additives were discussed in that study as one among several factors that caused a higher toxicity of the product than expected given the toxicity of the a.s. contained in the product.

Under the assumption that the type of formulation is often similar in mono-formulations and combination products for a given a.s., these substances (namely sulfonylurea, triclopyr, bromoxynil, and fenpropidin) appeared as potential candidates for the analysis of mono-formulations.

SELECTED MONO-FORMULATIONS AND RESULTS OF THE ANALYSIS

The final selection of mono-formulations is shown in Table 7.19. It covers 10 different a.s from 9 different chemical structure groups. Herbicides are represented by 7 products, fungicides by 5 products, and insecticides by 3 products. EC formulations are represented by 8 products, covering herbicides, fungicides and insecticides as well as 6 different a.s. types of chemical structure. WG formulations are represented by 3 products covering 2 structural groups, but no fungicides. SC and EW formulation types are only represented by 1 product each and two products are OD formulations. There are only three a.s. that are present in different types of formulations.

Table 7.19 Selected 15 mono-formulations for the further analysis.

Number	Active substance	Pesticide category[1]	Mode of action group[2]	Type of chemical structure	Formu-lation type[3]
1	iodosulfuron	H	B	sulfunylurea	WG
2	iodosulfuron	H	B	sulfunylurea	OD
3	prosulfuron	H	B	sulfunylurea	WG
4	triclopyr	H	O	pyridinecarboxylic acid	EC
5, 6, 7	bromoxynil	H	C_3	hydroxybenzonitrile	EC
8	fenpropidin	F	G_2	piperidine	EC
9	fenpropimorph	F	G_2	morpholine	EC
10	prochloraz	F	G_1	imidazole	EW
11	cyprodinil	F	D_1	anilinopyrimidine	WG
12	cyprodinil	F	D_1	anilinopyrimidine	EC
13	dimethoate	I	1B	organophosphate	EC
14	thiacloprid	I	4A	neonicotinoid	OD
15	thiacloprid	I	4A	neonicotinoid	SC

[1] H: herbicide, F: fungicide, I: insecticide

[2] B: inhibitor of branched-chain aminoacid synthesis (ALS inhibitor); K_1: inhibitor of microtubule assembly; O: synthetic auxin; C_3: inhibitor of electron transport at photosystem II; G_1 & G_2: inhibitors of ergosterol biosynthesis through different enzymes; D_1: proposed inhibitor of methionine biosynthesis; 1B: inhibitor of cholinesterase; 4A: agonist of nicotinic acetylcholine receptor

[3] WG: water-dispersible granules; OD: oil dispersion; EC: emulsifiable concentrate; EW: oil in water emulsion; SC: suspension concentrate

There were nine different endpoints for which data were available for the formulated as well as the technical a.s. for at least two of the 15 selected mono-formulations. For each of these endpoints, the deviation ratio (expected toxicity estimate for the product based on the toxicity of the technical a.s. and its content in the product, divided by the observed toxicity estimate for the product) was calculated. Similar to the MDR used for the combination products (Belden et al. 2007, Coors & Frische 2011), a deviation ratio (DR) above 1 indicates that the toxicity of the formulated product is higher than expected. This in turn indicates an influence of the formulation additives either by genuine toxicity or by enhancement of the toxicity of the a.s.

For seven out of the 15 mono-formulations an underestimation of product toxicity by factor 5 and more was found between the toxicity of the technical and the formulated a.s. in at least one of the considered endpoints (Table 7.20). A difference between applying a threshold of 3 or 5 was only detected once, for bee contact toxicity.

Table 7.20 Number of mono-formulations for which the toxicity of the formulated a.s. deviated from the toxicity of the technical a.s. by factor 3 or more, factor 5 or more, and factor 100 or more

Given is in addition the number of analysed products for each considered endpoint (only those endpoints are included for which at least two products could be analyzed). The total number relates to the number of products that showed a deviation by factor 3, 5 or 100 or more, respectively, in at least one of the assessed endpoints.

Endpoint	**Number of analysed products**	**DR < 3**	**DR ≥ 3**	**DR < 5**	**DR ≥ 5**	**DR ≥ 100**
Fish acute toxicity	12	10	2	10	2	2
Fish chronic toxicity	4	3	1	3	1	0
Daphnia acute toxicity	11	8	3	8	3	2
Daphnia chronic toxicity	5	3	2	3	2	1
Algal growth inhibition (E_bC_{50})	11	9	2	9	2	0
Lemna growth inhibition	2	2	0	2	0	0
Bee, oral toxicity	4	1	3	1	3	0
Bee, contact toxicity	4	2	2	3	1	0
Earthworm acute toxicity	6	5	1	5	1	0
Total	**15**	**8**	**7**	**8**	**7**	**2**

The following formulation and pesticide types comprised the seven products with relevant deviation of the technical and formulated a.s. in at least one endpoint:

- WG and OD formulations with iodosulfuron. In both here considered mono-formulations, the safener mefenpyr-diethyl was contained. The reported fish and *Daphnia* toxicity of this formulation additive could in both products almost completely explain the large (>100-fold) observed deviations, which occurred only for the endpoints fish acute toxicity and *Daphnia* acute and chronic toxicity. In the WG formulation, however, an unexplained deviation of about factor 3 to 5 remained in these endpoints, while the about 20-fold deviation in the endpoint algal growth inhibition was hardly influenced by including the (low) algal toxicity of mefenpyr-diethyl. Another product containing the sulfonylurea prosulfuron showed no deviation in algal toxicity (the only assessable endpoint) between technical and formulated a.s. Based on the safety data sheet and the UBA evaluation report, this product does not contain the safener mefenpyr.
- Three of the eight EC formulations showed a more than 3-fold deviation in at least one endpoint. These were the herbicides triclopyr (*Daphnia* acute toxicity) and bromoxynil (bee toxicity) as well as the fungicide fenpropidin (fish, *Daphnia* and algal toxicity). However, other EC formulations such as for example of the insecticide dimethoate showed less than 2-fold deviation in all six assessed endpoints. The other two EC formulations with bromoxynil showed as well no deviation above factor 3. Yet, they could not be assessed with regard to bee toxicity due to lack of other data.
- The insecticide thiacloprid showed more than 3-fold deviations for bee toxicity as OD (DR of 10.7 and 28.4) as well as SC formulation (DR of 5.0 and 1.9). No relevant deviation was detected for fish acute toxicity of the SC formulation, the only other assessable endpoint.

Overall, the findings for the mono-formulations confirm that enhancement of toxicity by formulation additives is of relevance mostly for the organisms that are not very sensitive to the a.s., while they hardly increase the toxicity for the non-target organism that are the most sensitive one for the a.s. An exception to this rule of thumb was the toxicity of fenpropidin and iodosulfuron towards algae as well as the toxicity of thiacloprid to bees (assuming that bees are very sensitive for this insecticide).

It is further indicated that EC and OD formulations may be problematic in that they may enhance the toxicity of technical a.s. mostly to less sensitive but also to very sensitive (algae, bees) non-target organisms. Finally, enhanced toxicity of formulated a.s. can be caused by genuine toxicity of an additive as exemplified by the toxicity of the safener mefenpyr-diethyl to *Daphnia* and fish. Based on material safety data sheets, the SC formulation of thiacloprid was the only one among these seven formulations that did not contain any other hazardous compounds in addition to the a.s. Since no ecotoxicity data were available for the individual hazardous components in the other six products, it could not be verified if all of the higher-than-expected product toxicity could be traced back to genuine toxicity of hazardous components only.

EXAMPLES OF TOXICITY-ENHANCING FORMULATION ADDITIVES

Toxicity of formulation additives is the most basic way by which the toxicity of a pesticidal a.s. can be enhanced in a product compared to the technical material. Other ways are, for example, enhancement of uptake or increased exposure due to decreased degradability of the a.s. In the following, some examples of formulation additives that were identified as causes for enhanced toxicity of formulated a.s. will be discussed briefly.

The safener mefenpyr-diethyl contained in two of the sulfonylurea mono-formulations assessed in the present study was found to account for the higher-than-expected toxicity of the PPP towards fish and *Daphnia*. The safener was in both products identified on the MSDS as hazardous component. A literature search for "mefenpyr" delivered no published information on the ecotoxicity of this formulation additive in herbicides.

Non-ionic surfactants account for a large amount of formulation additives. Some major groups are alkylphenol ethoxylates (APEO), alcohol ethoxylates (AEO) and alkylamine ethoxylates (ANEO). APEO have been banned from PPP in Europe and are nowadays mainly replaced by AEO and ANEO, which can also possess relevant aquatic toxicity (reviewed by Krogh et al. 2003). From the group of ANEO particularly tallow amines have frequently been identified in the literature as being toxic to non-target organisms (Haller et al. 2003, Krogh et al. 2003, Cox and Surgan 2006). Tallow amines are for example contained in the product Roundup, a SL formulation of the herbicide glyphosate, and account for the enhanced toxicity of the formulated a.s. towards cyanobacteria (Lipok et al. 2010), green algae and *Lemna* (Cedergreen and Streibig 2005), and *Daphnia* (Pereira et al. 2009). Tallow amines had been considered in parallel with glyphosate in an environmental risk assessment for glyphosate (Giesy et al. 2000).

In the case of glyphosate it was found that the toxicity of the formulated product could also be greater than that of the pure technical a.s. due to the genuine toxicity of isopropylamine, the counter ion in the glyphosate salt in some formulations (Lipok et al. 2010). Active substances can also become more toxic by a chemical reaction with formulation additives over longer storage periods as illustrated by the synthesis of methamidophos from the (less toxic) organophosphorus insecticide chloramidophos (the intended a.s.) and the additive methanol (Zhou et al. 2009). This example represents another case of enhanced toxicity of the formulation that is related to the active substance.

Next to surfactants, solvents are frequently contained in formulated PPP. They may directly increase PPP toxicity by their genuine toxicity as in the case of diacetone alcohol (DAA) in an ultra low volume (ULV) formulation of an insecticide strongly enhancing the acute oral toxicity to birds (Kitulagodage et al. 2008). Another example was reported by Tisler et al. (2009) who observed for a SL formulation of the insecticide imidacloprid that the technical and formulated a.s. were similar with regard to aquatic toxicity, but that the large amount of solvents (38.4 % v/v DMSO and 37.5 % v/v 1-methyl-2-pyrrolidone) accounted for relevant toxicity and caused higher-than-expected toxicity of the product.

Solvents may also influence the fate of the a.s. and thereby the toxicity if, for example, uptake is increased, degradation is decreased or release is prolonged as in the case of a microencapsulation formulation of a herbicide (Sopena et al. 2009). Another example for fate-related potential enhancement of PPP toxicity are organohydrotalcites as formulation additives that mimic clay minerals and enable slow-release formulations which provide longer efficacy of the herbicide terbuthylazine and reduced loss via leaching (Bruna et al. 2008). The oligosaccharide cyclodextrin is a third example of a formulation additive intentionally increasing solubility and stability of the a.s. in the PPP (Villaverde et al. 2005, Zhou et al. 2008). While it cannot be fully excluded that cyclodextrins influence the toxicity of the product in the environment, a laboratory study found at least no enhanced effect towards *Daphnia* of the cyclodextrin-complexed insecticide chloramidophos (Zhou et al. 2008).

Piperonyl butoxide (PBO) inhibits cytochrome P450 enzymes and thereby affects the metabolisation of various chemicals. PBO is for example used in insecticidal PPP to overcome the metabolic resistance of insects towards pyrethroids, carbamates and

neonicotinoids (Bingham et al. 2008). It is far less efficient in case of target-site resistance. But PBO expresses also synergistic interaction in insecticide-susceptible strains, which indicates that it interferes to a measurable level with background enzyme activity (Bingham et al. 2008). Due to this mode of action, PBO is officially labelled as synergist in formulated pesticides and can also be present in formulated biocides (Moreno et al. 2008). In analogy to its intended function, PBO can be expected to also enhance the toxicity of insecticides to non-target organisms as it was for example shown for the toxicity of the pyrethroids permethrin, sumithrin, and resmethrin to trout (Paul et al. 2005). However, none of the PPP registered in Germany by August 2009 contained PBO and no other synergists are registered.

A little more than half of the mono-formulations assessed in the present study did not show enhanced toxicity of the formulated compared to the technical a.s. in any of the assessed endpoints. Similarly, a number of cases were found in the literature where the toxicity of the formulated and the technical a.s. were about similar. Examples are the insecticide methomyl and the herbicide propanil for which the toxicity to green algae and *Daphnia magna* differed by less than factor 3 between formulated and technical a.s. (Pereira et al. 2009). Similar small deviations were reported for nine out of ten herbicides (the exception was Roundup) with regard to toxicity towards green algae and *Lemna* (Cedergreen & Streibig 2005). A rare example for such comparisons in soil organisms is the study of De Silva et al. (2010) who found less than factor 2 deviation between the toxicity of formulated and technical pesticides (the insecticides chlorpyrifos and carbofuran as well as the fungicide mancozeb) with regard to survival and reproduction of a tropical earthworm species. Several of these above mentioned formulations were of the EC type.

CONCLUSIONS

With regard to the chemical structure of active substances or, more general, the formulation types of PPP, no apparent general rule could be established that would allow identifying in advance PPP with higher ecotoxicity than expected based on the toxicity of the a.s. Hence, a case-by-case evaluation will be necessary during the registration process to identify and eventually consider enhanced toxicity of a PPP. Some formulation types (e.g. EC and OD) as well as PPP containing high amounts of surfactants or solvents or additives known to be problematic particularly appear as candidates for such as closer case-by-case evaluation. The MSDS of PPP are expected to help in identifying such candidate PPP, because problematic substances will usually be identified as hazardous compounds as it was the case for the evaluated products with regard to content of e.g. solvents and mefenpyr. EC and OD formulations appear to have a relatively high incidence of enhanced product toxicity. However, it must be kept in mind that many EC formulations do not fit into this pattern, while at first view unproblematic formulations such as a SC formulation without any hazardous components besides the a.s. can be more toxic than expected (see above, the example of the SC formulation with thiacloprid).

A key question is in which parts of the risk assessment should an enhanced toxicity of the formulation be considered? With regard to standard endpoints, available data (see also the evaluation for the combination products) often allow a comparison between expected and observed product toxicity. This is only for a few standard first tier endpoints (e.g. toxicity to terrestrial plants and non-target arthropods) frequently not possible due to lacking single-substance data. Such a comparison can serve to efficiently identify products that contain problematic formulation additives, which need to be considered in a mixture toxicity approach. If product toxicity is considerably lower than expected, it may be related to antagonistically acting formulation additives or point at flawed toxicity data for the product

(or the a.s.). As apparent from the present evaluation (Table 7.20), toxicity data for chronic endpoints are mostly not available for the a.s. and the product. Besides, the toxicity of the formulated product may not be of relevance for the assessment of chronic toxicity due to differential fate of the a.s. and the formulation additives. Currently, the requirements for chronic toxicity data of the product (e.g. for fish) are not unambiguously defined and their consideration in the risk assessment is not consistent across Member States, but this situation is expected to improve with release of new guidance documents (Creton et al. 2010).

7.2 Implementation of mixture risk assessments under the PPP regulation

This section outlines options for the environmental risk assessment of chemical mixtures in the specific context of the authorisation of plant protection products (PPP) by competent authorities of EU Member States (MS). The outline reflects both the scientific state-of-the-art summarized in chapter 3 and the insights into regulatory practice and problems gained from the relevance analysis in the preceding section 7.1. It is based on the common guiding principles proposed in chapter 5 and, where applicable, it makes use of generic options examined in chapter 4, but it is tailored to the specific assessment situations under the PPP regulation in terms of mixture definitions, available input data, and assessment criteria. With the aim to improve regulatory mixture risk assessments without increasing testing requirements, the outline focuses on the advanced use of component-based approaches (CBA). In doing so, notice is taken of the current fragmentary status of the development of corresponding technical guidelines both on the Community level as well as in some MS, as has been detailed in section 7.1.2. Concerning the available input data, the outline reflects the standard data requirements that have been laid down under Community legislation and additionally the results of further testing as they are typically seen or required by the German Federal Environment Agency (UBA) under national law (see section 7.1). Experience and requirements of competent authorities in other MS may differ in details.

7.2.1 General considerations

Assessment criteria and appropriate approaches

Legally binding standard criteria for acceptable risks of plant protection products (PPP) to non-target species are laid down in the *Uniform Principles* fixed in Commission Regulation 545/2011 (EU 2011), mostly in terms of minimum toxicity exposure ratios (TER)[12] for acute, short-term and long-term endpoints in different groups of species (Tab. 7.21). It is therefore self-suggesting to assess mixtures of PPP ingredients that are combined in a single product, in a tank mix, or in consecutive applications also in terms of corresponding toxicity exposure ratios for mixtures (TER_{mix})[13]. This idea is currently brought forward by some MS and by the

[12] Unfortunately, both, toxicity/exposure ratios (TER) and exposure/toxicity ratios (HQ for bees) are used as risk indicators under the PPR regulation. In addition to this inconsistency within the same law, the use of TER values is unique in comparison to all other pieces of EU chemicals legislation, where risks are always characterized in terms of exposure/toxicity ratios, such as the PEC/PNEC ratio for instance. The PPR Panel has repeatedly called for a harmonization in terms of exposure/toxicity ratios (EFSA 2009a). However, up to now, the inconsistent and confusing use of TER values continues to be prescribed by the law.

[13] For the sake of simplicity, this section focuses on the derivation of TER_{mix}. However, in the case of risk assessments for bees, TER_{mix} has to be replaced by an HQ_{mix}. As already noted, HQ is mathematically simply the inverse of TER (HQ = 1/TER). Hence all calculations can be done analogously and reverse signs apply to comparisons with trigger values. The situation is somewhat different in the special case of risk assessments for other beneficial terrestrial arthropods and for non-target soil micro-organisms, where the acceptability criterion

EFSA birds & mammals guideline (see section 7.1). If worked out well, the approach may ensure a consistent terminology, effective use of already available data, and quantitatively comparable assessment results for both individual substances (TER_i) and mixtures (TER_{mix}). If the same trigger values are applied for both TER_i and TER_{mix}, an equivalent level of protection is established for both single substances and mixtures.

Table 7.21 Legal Standard Requirements for PPP Authorisation *re* Impact on Non-Target Species*
(Uniform Principles; Commission Regulation 545/2011, Annex, Part I, Section C.2.5.2) (EU 2011)

Organism Group	Endpoint	Requirement
Birds and other non-target terrestrial vertebrates	acute and short-term LD 50	TER ≥ 10**
	long-term	TER ≥ 5**
aquatic organisms	fish and Daphnia, acute	TER ≥ 100**
	fish and Daphnia, long-term	TER ≥ 10**
	algal growth inhibition	TER ≥ 10**
honeybees	oral or contact	HQ ≤ 50**
other beneficial arthropods	lethal or sublethal lab tests at max. application rate	≤ 30 % of test organisms affected**
earthworms	acute	TER ≥ 10**
	long-term	TER ≥ 5**
non-target soil micro-organisms	N or C mineralisation after 100 days (lab)	affected by ≤ 25 %**

* requirements for bioconcentration factors omitted in the table

** unless it is clearly established through an appropriate risk assessment that under field conditions no unacceptable impact occurs after use of the plant protection product in accordance with the proposed conditions of use (i.e. by a higher-tier risk assessment)

If used in concordance with individual TER values, TER_{mix} may in general be defined for a given endpoint and effect level *x* as the ratio between a total effect concentration (or dose) ECx_{mix} and a total predicted (or known) exposure concentration (or dose) PEC_{mix}:

$$TERx_{mix} = \frac{ECx_{mix}}{PEC_{mix}}, \qquad [7.2.1]$$

whereby both sides of the risk quotient must refer to the same mixture of *n* compounds in the same concentration (or dose) ratio $p_1 : p_2 \ldots p_n$. The total exposure concentration PEC_{mix}

is not defined in terms of a risk quotient but in terms of a maximum allowable effect (30 % and 25 %, respectively). In principle, CBAs can handle this requirement in an analogous way for mixtures. However, the practical details depend very much on the exact data situation and need further elaboration.

calculates as the simple sum of individual concentrations (or doses) of mixture components PEC_i in an exposure scenario

$$PEC_{mix} = \sum_{i=1}^{n} PEC_i \,, \qquad [7.2.2]$$

and hence the relative proportions p_i of mixture components are given by

$$p_i = \frac{PEC_i}{PEC_{mix}} \,. \qquad [7.2.3]$$

As is done for individual TER values in the case of chronic endpoints, estimates of low effect concentrations (or doses) (e.g. $EC10_{mix}$) may be replaced in the TER_{mix} assessment by no-observed-effect concentrations (or doses) $NOEC_{mix}$ (or $NOEL_{mix}$) as a surrogate (see discussion of this aspect in chapter 4.5, p. 53)

PPPs are intentionally prepared mixtures of known components, and for a given situation of joint exposure to such components (PEC_{mix}) the corresponding mixture toxicity data (ECx_{mix} or $NOEC_{mix}$) that is needed to calculate TER_{mix} can either be experimentally determined by whole mixture testing or by means of a CBA. Hence, the third generic implementation option that has been outlined in section 4.6, i.e. the assessment factor approach, appears to be less relevant in the specific context of PPP authorisation and is therefore not further pursued in this section.

In the preceding section 7.1, component-based sample calculations of TER_{mix} values followed a current practice of the UBA and assumed a concentration-additive joint toxicity of mixture components. However, this should not be misunderstood as a necessary or inseparable conjunction. Where a CBA is taken to estimate the mixture toxicity input data for a TER_{mix} calculation (i.e. ECx_{mix} or $NOEC_{mix}$), this may in principle of course be done with any kind of applicable model. As detailed in chapter 4, the current state of debate suggests CA as a default assumption that should be replaced by refined modelling where necessary and feasible (Kortenkamp, Backhaus, Faust 2009; Meek et al. 2011, EC 2011a). However, as also explained in chapter 4, a simple component-based estimation of a $NOEC_{mix}$ is only possible by pragmatic CA-based approaches that deviate from the original CA-concept by additional simplifying assumptions. Higher tier CBA approaches based on IA or mixed models work for ECx_{mix} estimations only.

Data availability

For the standard assessment criteria (Tab. 7.21), corresponding toxicity and exposure data for individual substances are a routine requirement and are determined by standard assays and procedures (EU 2011), at least for active substances (a.s.) and under the new PPP Regulation (EC 2009) in the future presumably also for safeners and synergists, but not for co-formulants[14].

[14] For the sake of simplicity, we distinguish only between "active substances" (a.s.) and "co-formulants" throughout this section. Active substances require approval on the Community level and inclusion in a corresponding positive list after thorough safety testing and risk assessment. Co-formulants do not. They may be used as product ingredients unless they are included in a negative list of undesirable co-formulants. However, as already detailed in section 7.1, under the new PPP Regulation 1107/2009 (EC 2009), the requirement for authorisation of single ingredients are no longer limited to a.s. only. They have been extended to include also "safeners" and "synergists". During the current transition period, however, testing requirements have not yet

Complementary to these standard single active substance data, corresponding whole mixture testing data are often available to the assessing authority for the acute toxicity caused by direct contact with the PPP or with a similar product in the original or in diluted form, typically for aquatic organisms, plants and non-target arthropods. For other organism groups and for the standard long-term endpoints listed in the Uniform Principles, however, available experimental eco-toxicity data are typically limited to individual a.s. or selected mono-formulations of these a.s., which are deemed to be representative.

In addition to standard endpoints, whole mixture testing data may in specific cases also be available from long term higher tier studies, in particular with terrestrial plants or earthworms. However, in these cases corresponding single substances data may often be missing. All available whole mixture testing data usually refer to the original PPP in concentrated or diluted form, not to mixtures of product ingredients in different proportions as they may be expected to occur in environmental exposure scenarios as a result of differential transformation and transport processes.

Purposes of CBA use

In comparison to biocidal products (see chapter 6) and all other pieces of EU chemicals legislation, the outlined assessment situation is relatively data rich and it already includes some whole mixture testing data. Under these conditions, CBAs may be used as a complementary tool and for a number of purposes:

(i) For assessing the risks of mixtures of a.s. that originate from a single combination product for which whole mixture testing data are available, but which are expected to co-occur in an environmental exposure scenario in a different concentration ratio than in the tested product.

(ii) For assessing the risks of mixtures of a.s. that originate either from one and the same combination product or from the intended combined use of different products (tank mixes) with respect to endpoints for which single substance data but no whole mixture test data are available.

In addition to such uses as a surrogate for whole mixture testing, CBAs may also be used as part of an integrated approach that makes use of both testing and modelling data,

(iii) for counter-checking experimental short-term toxicity data obtained by testing of products that contain more than one a.s.. Significant differences between observed and calculated joint toxicity may point either to additive toxicity contributions of co-formulants or to synergistic or antagonistic interactions between product ingredients, or both. Such cases may require in-depth examinations, and

(iv) for the identification of potential drivers of mixture toxicity in terms of toxic units (reciprocals of TER values), in cases where the comparison between whole mixture testing and CBA confirms that the overall toxicity is largely explainable by concentration additive action of active substances.

Beyond such uses of CBAs as a complementary tool in addition to usual whole mixture testing of PPPs, it may also be considered to actually waive experimental product testing, if the CBA gives a strong indication for the absence of any unacceptable risks. However, in the

been established for safeners and synergists and the data situation regarding non-target toxicity may therefore be the same as for other co-formulants.

light of the existing evidence on the accuracy of CBA-based assessments, it is recommended as a minimum requirement that such waiving may currently only be considered, if single substance toxicity data for non-target species are available for all product ingredients, and not just for those that are active against target species. This does not mean that extensive additional toxicity testing is ultimately required for all co-formulants. A well-reasoned judgement that a substance is non-toxic ("inert") with respect to the endpoint under consideration could be sufficient. More detailed requirements for actual waiving of product testing may have to be worked out.

In the following section 7.2.2, the available implementation options are briefly checked in more detail for each of the specific assessment situations that a competent authority may face in the context PPP authorisation: mono-formulations, combination products, tank mixes, and serial applications. Wherever a CBA may be used in these specific assessment situations as a surrogate for experimental whole mixture testing (points (i) and (ii) above), this should be done in a consistent manner and following uniform principles. To this end, a proposal for a uniform tiered approach is presented in the sub-sequent section 7.2.3.

7.2.2 Assessment situations and corresponding implementation options

Mono-formulations and the eco-toxicological relevance of co-formulants

PPPs containing a single a.s. are usually assessed under the hypothesis that not only the target toxicity but also the non-target toxicity is primarily caused by that active ingredient and not by the co-formulants. Given the usual data situation outlined above, the competent authority basically has three options for checking the validity of this null-hypothesis in the case of a specific product. Where possible, these three options may be applied in concert:

(i) Information retrieval about eco-toxicity of co-formulants

Notwithstanding the stricter rules that in the future may apply under the new PPP legislation to synergists, safeners, and co-formulants (see footnote above), the authority may check all available information sources for indications of critical eco-toxicological properties of relevant co-formulants, in particular material safety data sheets (MSDS) and already existing lists of unwanted substances in PPPs, such as the one published by the German Federal Office of Consumer Protection and Food Safety (BVL)[15]. Where such information gives reasons for concern, different consequences may be drawn on a case by case basis: *(a)* generation of quantitative evidence on the toxicity and the risks of individual co-formulants and use of such data in a CBA, (*b*) further whole mixture testing for the specific endpoints of concern, or (*c*) refusal of authorization on the basis of already existing information (such as inclusion in a negative list, in particular).

(ii) Comparing active substance toxicity and toxicity of mono-formulations

Where the necessary data are available, the authority may compare whole mixture test results obtained with the mono-formulation with corresponding data for the

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http://www.bvl.bund.de/EN/04_PlantProtectionProducts/09_ProductChemistry/PlantProtectionProducts_ListUndesiredFormulants_basepage.html?nn=1414532

individual a.s. in technical purity[16]. As outlined above, this approach is usually limited to some acute standard endpoints and not applicable to the assessment of long-term effects. Where the comparison between a formulated and a non-formulated a.s. reveals significant differences, the authority may choose to base the risk assessment of the PPP on the lower of the two ECx or NOEC values, as a precautionary measure.

(iii) Comparing different mono-formulations of the same active substance

Where only whole product test data, but no information on the individual toxicity of the a.s. are available for the endpoint under consideration, indications on significant contributions of co-formulants to the overall toxicity may only be derivable from comparisons with other mono-formulations of the same a.s., such as the sample formulation that has to be examined as part of the EU authorisation procedure for an a.s., in particular. If significant differences in the toxicity of two different mono-formulations of the same active substance (adjusted to a.s. content) are associated with substantial differences in the co-formulants composition, this points to a significant contribution of co-formulants to the overall toxicity, either in one or in both of the two formulations compared. Otherwise, the hypothesis of no significant contribution of co-formulants to the overall product toxicity cannot be rejected.

Where significant differences in the toxicities of two different mono-formulations of the same a.s. are observed, a detailed case by case evaluation should examine whether this difference may be sufficiently explainable by the differences in co-formulants composition or not. To this end, in general both the quality and the quantity of the co-formulants must be taken into consideration, but specific rules and criteria may need further elaboration. Where such an evaluation allows to identify one or more co-formulants as a plausible cause for the observed differences in product toxicity, single substance testing of the a.s. and/or the relevant co-formulant(s) may be considered as a next step for further clarification. Where significant differences in the toxicity of different mono-formulations of the same a.s continue to be insufficiently explainable by differences in the co-formulants, the authority may choose to base the risk assessment uniformly on the lowest of the available product toxicity data (adjusted to a.s. content), as a precautionary measure.

All quantitative comparisons of toxicity data, both between individual a.s. and mono-formulations (point *ii*) as well as between different mono-formulations (point *iii*), require the definition of a threshold for discriminating between significant and insignificant differences in ECx or NOEC values, not in a strict statistical meaning but in terms of relevance for regulatory risk assessments. As a benchmark, the competent authority may refer to the factor that has been fixed in the Commissions' *Guidance document on the equivalence of technical materials of substances regulated under Regulation (EC) No 1107/2009* (EC 2011b) in order to account for the variability of ecotoxicological test results. Accordingly, data for the same

[16] For the purpose of this comparison, ECx or NOEC-values for the formulation must be adjusted to the content of the a.s. When comparing NOEC values, the potential effect of different experimental designs (in terms of test concentrations and replicate numbers) must be taken into consideration.

species and endpoint that do not differ by my more than a factor of 3 may be considered to be equivalent[17].

Bridging data gaps with similar mono-formulations

Apart from assessing significant differences between mono-formulations (point *iii above*), the competent authority may also be confronted with the reverse task of assessing the toxicological equivalence of mono-formulations. This applies to situations where whole mixture testing data are not available for that mono-formulation that actually needs to be assessed, but for a similar one. Thus the question arises, whether the toxicity data for the similar mono-formulation (adjusted to a.s. content) could be used to bridge the data gap? A priori, such bridging appears to be least critical in situations where the mono-formulations just differ slightly in the a.s. content but not in the nature and contents of co-formulants. However, detailed case-by-case evaluations are needed, and more precise decision rules require further elaboration. In addition to different contents of components, available data for a similar mono-formulation may also refer to a different species and/or endpoint than the one that shall be assessed. Thus, a dual bridging of both different mixture composition and different toxicity endpoints may be required. To this end, guiding rules that have been laid down in the context of assessing the ecotoxicological equivalence of different technical batches of an active substance (EC 2011b) may be applied in an analogous way to mono-formulations.

Combination products

Given the typical data situation outlined above, the competent authority may base ecotoxicological risk assessments (ERA) of PPPs that contain two or more active substances on three different types of information: (i) measured mixture toxicity, (ii) modelled mixture toxicity, or (iii) toxicity of a single "driver" of mixture toxicity, i.e. a single active substance that is assumed to dominate the overall toxicity of the original product and/or of mixtures of product ingredients that may occur in the environment as a result of product use. Where feasible, integrated use may be made of these three types of information:

(i) ERA on the basis of toxicity tests with the whole combination product

Whole mixture test results are usually considered to provide the "golden standard" and are therefore used preferentially, where available.

However, there could be rare situations where the full ecotoxicological potencies of active mixture components are masked in whole product test data due to antagonistic effects of co-formulants or other a.s.. Indications for such a situation may be gathered from comparisons of the experimentally observed product toxicity with the expectable mixture toxicity of the active ingredients, provided that the corresponding single substance data are available for component-based calculations. Where such comparisons provide strong evidence that the whole product is significantly less toxic than expectable on the basis of the active ingredients[18], an evaluation of the potential causes and consequences may be

[17] As already noted in section 4.3, the factor was recently reduced from 5 to 3. The guidance document prescribes that the standard factor of 3 shall be replaced by the actual spacing factor of test doses or concentrations in the respective assay, if this is larger than 3.

[18] For the sake of simplicity, initial calculations of expectable mixture toxicity should be done under the default assumption of CA. If the calculation points to a significantly higher mixture toxicity than actually observed, it

required. As a result of such evaluations, the competent authority may on a case-by-case basis decide to deviate from the principle of using whole mixture data preferentially, and may instead use a CBA as a precautionary measure under special circumstances. Further details of corresponding decision rules may have to be worked out.

(ii) ERA on the basis of mixture toxicity modelling by means of a CBA

Component-based modelling is generally considered to be the second best strategy for mixture risk assessment. And, as outlined above, for many endpoints and exposure situations it is the only possible one. Provided that the necessary input data are typically only available for active substances but not for co-formulants, the CBA can be expected to be sufficiently protective, if two underlying assumptions are correct:

- co-formulants that are not included in the calculation make no significant contribution to the overall toxicity, and
- toxicodynamic or toxicokinetic interactions between product ingredients that cause more-than-additive ("synergistic") effects do not occur.

If there is evidence to the contrary, a case-by-case assessment may be needed.

Thus, proper application of the CBA requires a pre-checking of all available information about ecotoxicological properties of relevant co-formulants as well as synergistic potencies of both co-formulants and a.s.. In addition to information that may be retrievable from the literature, valuable indications for potential underestimations of mixture toxicity by means of a CBA may also be gained from quantitative comparisons between experimental toxicity data for the whole product and corresponding calculations of the expectable toxicity on the basis of data for the single active ingredients. Hence, such calculations should be performed for all endpoints for which both whole product test data and corresponding single active substance data are available to the authority. If, under the assumption of CA, the experimentally determined product toxicity does not significantly exceed the calculated one in any of these test cases, no objections against the use of the CBA for other ratios of the mixture components and/or other endpoints arise from these examinations.

Such comparisons between experimental data and modelling data for the toxicity of combination products require the definition of a criterion for assessing the regulatory significance of quantitative differences. Basically, the problem is the same as for comparisons between different mono-formulations and individual active substances (see above) and between different technical batches of an a.s.. Accordingly, in order to account for the variability of ecotoxicological test results, the competent authority may again use the same guidance value as fixed in Guidance Document Sanco/10597/2003 (EC 2011b). This means that differences between observed and predictable mixture toxicity that do not exceed a factor of 3

should be checked in a second step, whether the difference may be explainable by assuming IA. The data needed for actually calculating IA may usually not be available, but the maximum possible difference between predictions based on CA and IA can be calculated by means of Eq. 4.3 (chapter 4). Hence, the equation provides an estimate of minimal mixture toxicity that could be expectable under any possible no-interaction assumption (CA, IA or a mixed model). If this minimal expectable mixture toxicity is still higher than the experimentally observed one, an antagonistic interaction is the only possible mechanistic interpretation.

may be considered irrelevant for regulatory purposes. For other technical details of comparing measured and modelled toxicity data of combination products, the reader may refer to Coors and Frische (2011).

(iii) ERA on the basis of toxicity tests with a single "driver" of mixture toxicity

From a regulatory perspective, it may be desirable to simplify the CBA as far as possible. It may be argued that there are many assessment situations where there are good reasons to assume that the toxicity of the mixture of concern should be largely explainable in terms of a single active component that dominates the overall potency, which means that the CBA essentially boils down to a single component assessment. As a consequence, a sufficient protection level could already be achievable by simply basing the risk assessment on toxicity data for that single "driver" only.

Experimental evidence in support of this assumption may again be gathered from comparisons between measured and modelled toxicity data of a combination product, for all endpoints where the available data allow to do so. Under the assumption of CA, contributions of individual components to the expectable mixture toxicity are given by the contributions of the individual toxic units (TU_i) to the sum of toxic units ($STU = \Sigma\ TU_i$) (see chapter 4)[19]. Hence, where

- CA has been shown to provide a reliable estimate of the toxicity of a PPP, and
- the largest part of the STU (say ≥90 %)[20] comes from a single active substance,

it can be concluded that this component apparently drives the overall toxicity[21,22]. This holds true for the concentration ratio of compounds and the endpoint that has

[19] For the sake of simplicity, this paragraph describes the identification of mixture toxicity drivers under the assumption of CA in terms of toxic units and not in terms of TER values. The reasons for this approach and the interrelations between both descriptors are as follows. As explained in chapter 4, toxic units (TUx_i) are individual concentrations (c_i) (or doses) of mixture components expressed as fractions of effective concentrations ($TUx_i = c_i / ECx_i$). In case of an environmental exposure scenario, the individual concentrations c_i are denoted as predicted environmental concentrations (PEC_i) and hence TUx_i provides a risk quotient in terms of an exposure/toxicity ratio: $TUx_i = PEC_i / ECx_i$). Under the assumption of CA, these toxic units simply sum up to a corresponding exposure/toxicity ratio for a mixture that contains the components in the ratio of individual PEC values: $TUx_{mix,\ CA} = \Sigma\ TUx_i$. As said before, TER values used under the PPP regulation unfortunately denote reverse risk quotients, i.e. toxicity/exposure ratios, and are hence reciprocals of corresponding toxic units: $TERx_i = 1/TUx_i$. As a consequence, under the assumption of CA, a TER value for a mixture ($TERx_{mix,\ CA}$) does unfortunately not calculate as a simple sum of individual TER values, but as the reciprocal of the sum of the reciprocal individual TER values: $TERx_{mix,\ CA} = 1 / (\Sigma\ 1/TERx_i)$. These confusing double reciprocal operations are avoided by simple TU-based calculations.

[20] Further work is needed in order to justify a specific trigger value.

[21] It is important to notice that the TU-based identification of mixture toxicity drivers strongly depends on the validity of the CA model and the completeness of all relevant input data. If not CA but IA describes the joint action correctly, not the toxic unit but the strength of the individual effect ($E(c_i)$) is the appropriate descriptor for the contribution of a component to the mixture toxicity. For the ranking of mixture components and the identification of a driver, both descriptors may lead to the same but also to different or even opposite conclusions, depending on whether the concentration response curves are parallel or have diverging slopes. If neither CA- nor IA-based calculations provide a correct estimate of the product toxicity, may be due to synergistic or antagonistic interactions and/or due to disregarded contributions of co-formulants, then TU-based identifications of suspected drivers of toxicity can be completely misleading.

[22] The calculation of a "tox per fraction" quotient suggested in Step 1 of Annex B to EFSA's guidance document for birds & mammals (EFSA 2009b) is an approach that is equivalent to a TU-based identification of a mixture toxicity driver. Unfortunately, however, the EFSA document does not adopt the TU-term and its definition from the scientific literature but invents a new one. Confusingly, the new term "tox per fraction" denotes a quotient

actually been tested. To make efficient use of this finding, the authority must have reasons to assume that the same holds also true for other assessment endpoints and for the mixture ratios that result from the calculation of environmental exposure scenarios (PEC_{mix}). To this end, detailed decision rules may need further elaboration.

Tank mixes and serial applications

As already explained in section 7.1.6, tank mixes that are recommended or required on the label of a PPP are subject to authorisation. Others are not. Regarding the non-target toxicity of tank mixes requiring authorisation, the Uniform Principles (EU 2011) do not explicitly specify testing needs and authorisation criteria. However, in view of the German UBA, basically the same principles apply as for mono-formulations and combination products. Hence, all the corresponding considerations in the preceding parts of this section also apply accordingly.

Legal tank mixes not requiring approval may be prepared by PPP users from the range of products that have been authorized for use in the same crops or application areas (such as railways or storage rooms for instance). As has been demonstrated in section 7.1.6, the risks of such tank mixes can in principle be assessed in terms of TER_{mix} by means of a CBA. Yet, this is often not possible without additional data. As the mixture components are applied simultaneously, the total exposure concentration PEC_{mix} may simply be estimated as a sum of the PECs for the individual active substances, and the corresponding mixture toxicity estimate (ECx_{mix} or $NOEC_{mix}$) can be generated from the corresponding data for the individual active substances that are present in a tank mix. Of course this approach functions only for homogenous data matrices, i.e. toxicity data referring to the same endpoint or the same group of endpoints must be available for all relevant mixture components. Therefore it is usually limited to standard endpoints.

For serial applications of different PPPs the assessment situation is somewhat different. Due to the non-simultaneous application, a simple summation of individual PECs may be no appropriate way of calculating PEC_{mix} for a given point in time and space, while the corresponding mixture toxicity estimates could be generated in much the same way as for tank mixes or any other combination of PPP ingredients. Thus, advanced exposure modelling for environmental pesticide mixtures resulting from consecutive applications of different PPPs in the same or in adjacent fields is needed for an appropriate assessment of potentially resulting risks by means of a CBA.

Article 29(6) of the PPP Regulation 1107/2009 (EC 2009) prescribes that "*interaction*[23] *between the active substance, safeners, synergist and co-formulants shall be taken into account in the evaluation of plant protection products*". However, this requirement for taking mixture toxicity into account is confined to the authorisation of single PPPs (including tank mixes required or recommended by PPP producers). As a consequence, it may be argued that there is no explicit legal mandate for assessing the risks of chemical mixtures resulting from tank mixes not requiring approval and from serial applications of different PPPs. On the other

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that is equivalent to the reciprocal of a toxic unit. This also means that the "tox per fraction" quotient is just a special form of a TER value.

[23] Obviously, the legal text does not use the term "interaction" in the specific toxicological meaning of deviations from non-interaction models such as CA and IA, but may be interpreted as a general requirement for taking mixture toxicity into consideration

hand, however, Article 4(3) sets out that the authorisation criterion of "*no unacceptable effects on the environment*" shall be met "*having regard to realistic conditions of use*" of a PPP. Tank mixes and serial applications are obviously an important part of the *realistic conditions of use* of PPPs. Ignoring this reality may therefore result in potential underestimations of actual risks.

Basically, this concern is shared by EFSA's PPR Panel who stated: "*Multiple stress by the use of multiple plant protection products, being applied at the same time (e.g., tank mixes) or in sequence, should be assessed to identify 'similar residues' in the area of envisaged use. Multiple stress from pesticides should also be considered to prevent additive impacts on the abundance and diversity of non-target species*" (EFSA 2010). As detailed above, missing information about realistic exposure scenarios is the major current obstacle to an effective tackling of this problem. Strategies for overcoming this bottleneck therefore need to be worked out.

7.2.3 Tiered component-based approach

For the purpose of CBA-based environmental risk assessments of both mixtures of ingredients of a single combination product as well as tank mixes, a uniform tiered approach has been worked out (Fig. 7.3). The approach can also be applied to mixtures resulting from serial applications, if corresponding exposure estimates become available (see above). The calculations may include data for all mixture components or they may be confined to active substances if these are considered to be the only relevant ones. The approach calculates an expectable TER-value of a mixture ($TERx_{mix}$) for a given endpoint and effect level x and a given exposure scenario PEC_{mix}. For different endpoints the approach must be applied separately.

Main features of the scheme

The suggested approach is structured into three main tiers. The lowest tier (TIER 0) is a pre-checking step, in which only single substance TER-values ($TERx_i$) are considered. The next tier (TIER 1) makes use of TER-values calculated for mixtures under the default assumption of a concentration additive joint action ($TERx_{mix, CA}$). Highest tier assessments (TIER 2a and 2b) are based on considerations of the available knowledge on MoAs and concentration response relationships. Where appropriate, TIER-2-TER_{mix}-values are estimated under the assumption of IA or a mixed model (MM) ($TERx_{mix, IA}$ or $TERx_{mix, MM}$).

CA-based calculations of TER_{mix}-values as they are already performed by the German UBA (section 7.1) are embedded in TIER 1 of the proposed scheme. The introduction of a preceding TIER-0-pre-checking of single substance TER-values is only a simple and minor amendment to this already existing practice. A major difference, however, is made by the addition of a sub-sequent TIER 2. This opens the way for proceeding from the default assumption of CA to IA and mixed models where appropriate and feasible. Thereby, the suggested specific approach for tiered eco-toxicity assessments of PPPs becomes compatible with relevant generic frameworks for regulatory mixture risk assessments, in particular those proposed by WHO/IPCS (Meek at al. 2011) and the EU's scientific committees (EC 2011a). This may help to increase consensual acceptability as well as consistency of approaches across different pieces of EU chemicals legislation and across the disciplinary borders between human and environmental risk assessors. It may be argued that standard data sets prescribed under the PPP regulation will not allow to go from CA-based assessments (TIER 1) to more data and knowledge demanding modelling approaches (TIER 2). However,

there may be exemptions for mixtures of very intensively studied chemicals, or a producer may decide to go for the generation of the missing knowledge. Therefore, this way should not be blocked a priori.

Each of the three suggested tiers is designed to serve as a filter. On the one hand, such mixtures are sorted out for which there is no indication of an unacceptable risk for the given endpoint and which therefore do not need any further consideration. On the other hand, however, also such mixtures are identified for which the CBA-based evidence for an unacceptable risk is so strong that these concerns can under no circumstances be ruled out by more advanced and more data intensive CBAs for the same endpoint. Thereby, pointless efforts are avoided. This resource-efficient support of regulatory decision-making is considered to be a major advancement over other proposed schemes for mixture risk assessment, such as the WHO/IPCS framework (Meek at al. 2011) and the decision-tree suggested by the EU's scientific committees (EC 2011a). This advancement is achieved by considering the quantitative relationships between (i) single substance toxicity and Concentration Addition and (ii) concentration additive action and independent joint action, as has been explained in detail in chapter 4.

The tiered assessment in detail

Not for every mixture a mixture risk assessment actually needs to be conducted. Such cases are therefore sorted out in TIER 0 by a simple examination of TER values for individual mixture components *i* ($TERx_i$).

The first question is whether there are unacceptable risks from one or more individual components already? Where this is indicated by non-exceedance of corresponding trigger values, the mixture also poses an unacceptable risk anyway. It may not be decisive whether co-exposure to the other mixture components further aggravates the overall risk or not. Hence, the assessment procedure can already stop here.

Where all mixture components pass the first examination step (all $TERx_i \geq$ trigger), it may then be asked whether the default assumption of CA could in fact lead to the indication of an unacceptable risk? Or, whether all individual TER-values are so high that they certainly safeguard against any unacceptable risks from a concentration-additive action? Due to the features of the CA model explained in chapter 4, this is the case if all individual TER-values exceed the corresponding trigger values by a factor of *n*, whereby *n* denotes the number of relevant mixture components included in the calculation. Thus, where this criterion is fulfilled (all $TERx_i \geq$ trigger times *n*) the procedure can also be stopped: the mixture fulfills the authorization criteria. Only the remaining cases are carried on to TIER 1.

TIER 1 starts with the actual calculation of $TERx_{mix}$ under the default assumption of CA ($TERx_{mix,\,CA}$). To this end, the transformed CA-formula (Eq. 4.5 in chapter 4) may be plugged into the $TERx_{mix}$ definition (Eq. 7.2.1 above) which gives

$$TERx_{mix}^{CA} = \frac{ECx_{mix}^{CA}}{PEC_{mix}} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{ECx_i}} = \frac{1}{\sum_{i=1}^{n} \frac{1}{TERx_i}}, \quad if\ p_i = \frac{PEC_i}{PEC_{mix}} = \frac{PEC_i}{\sum_{i=1}^{n} PEC_i}. \qquad [7.2.4]$$

Where the resulting value exceeds the trigger value that applies to the given endpoint and effect level *x* (see Tab. 7.28 above), the risk from a concentration-additive action of mixture components is assessed to be acceptably low. Where this is not the case, the next question to

be examined is, whether the alternative assumption of IA or a mixed model could result in an acceptable risk? The answer is obtained by using Eq. 4.3 (chapter 4) which defines the maximal possible difference between corresponding predictions of an effect concentration of a mixture by means of CA and IA, respectively (Junghans et al. 2006). Application of the formula to the problem of TERmix calculations yields the decision criterion

$$TERx_{mix}^{CA} \geq trigger \times \frac{\max\{TUx_i\}}{\sum_{i=1}^{n} TUx_i}, \quad with\ TUx_i = \frac{1}{TERx_i} \quad ? \qquad [7.2.5]$$

Where this criterion is fulfilled, there is a chance that the assumption of IA or a mixed model may finally lead to the conclusion of an acceptable risk. Only in this case it makes sense to carry the mixture on to TIER 2. Otherwise it is sorted out, because it poses an unacceptable risk anyway.

TIER 2 starts with a compilation of (i) available knowledge on the modes of action of mixture components and (ii) data on the concentration (or dose) response relationships of individual mixture components for relevant endpoints in non-target species. Then the first question to be examined (TIER 2a) is whether the knowledge and data indeed support reliable mixture toxicity estimates based on the assumption of IA or a MM. This is the case, if

- the data quality fulfils the model-specific requirements explained in detail in chapter 4, in particular concentration (or dose) response functions F_i are available for all relevant mixture constituents, and
- the MoA is well known for all mixture components, and
- all mixture components can be grouped into types of MoAs that are fully independent from each other.

Where one or more of these three prerequisites for a proper application of IA or a MM are not fulfilled, the TIER 2 assessment must unfortunately be cut short: the unacceptable risk indicated by the assumption of CA in TIER 1 cannot be ruled out.

Where indeed all three prerequisites are fulfillable, the second part of the TIER 2 assessments (TIER 2b) starts with grouping of all relevant mixture components by fully independent MoAs. Then $TERx_{mix}$ is calculated under the assumption of IA or a MM. IA is appropriate if all components of the mixture act by completely dissimilar and fully independent MoAs. Use of a MM is appropriate if there are sub-groups of mixture components which share a common MoA.

Under the assumption of IA, the expected effect concentration of a mixture ($ECx_{mix, IA}$) is only implicitly given by the term

$$X = 1 - \prod_{i=1}^{n} (1 - F_i(p_i \bullet (ECx_{mix}^{IA}))). \qquad [7.2.6]$$

As a consequence, also no explicit expression for the calculation $TERx_{mix, IA}$ can be given. The value for $ECx_{mix, IA}$ must first be determined numerically under the condition pi = PEC/ΣPECi (Eq. 7.2.3). The result must then be divided by PECmix to obtain $TERx_{mix, IA}$. Basically the same applies to the use of a MM.

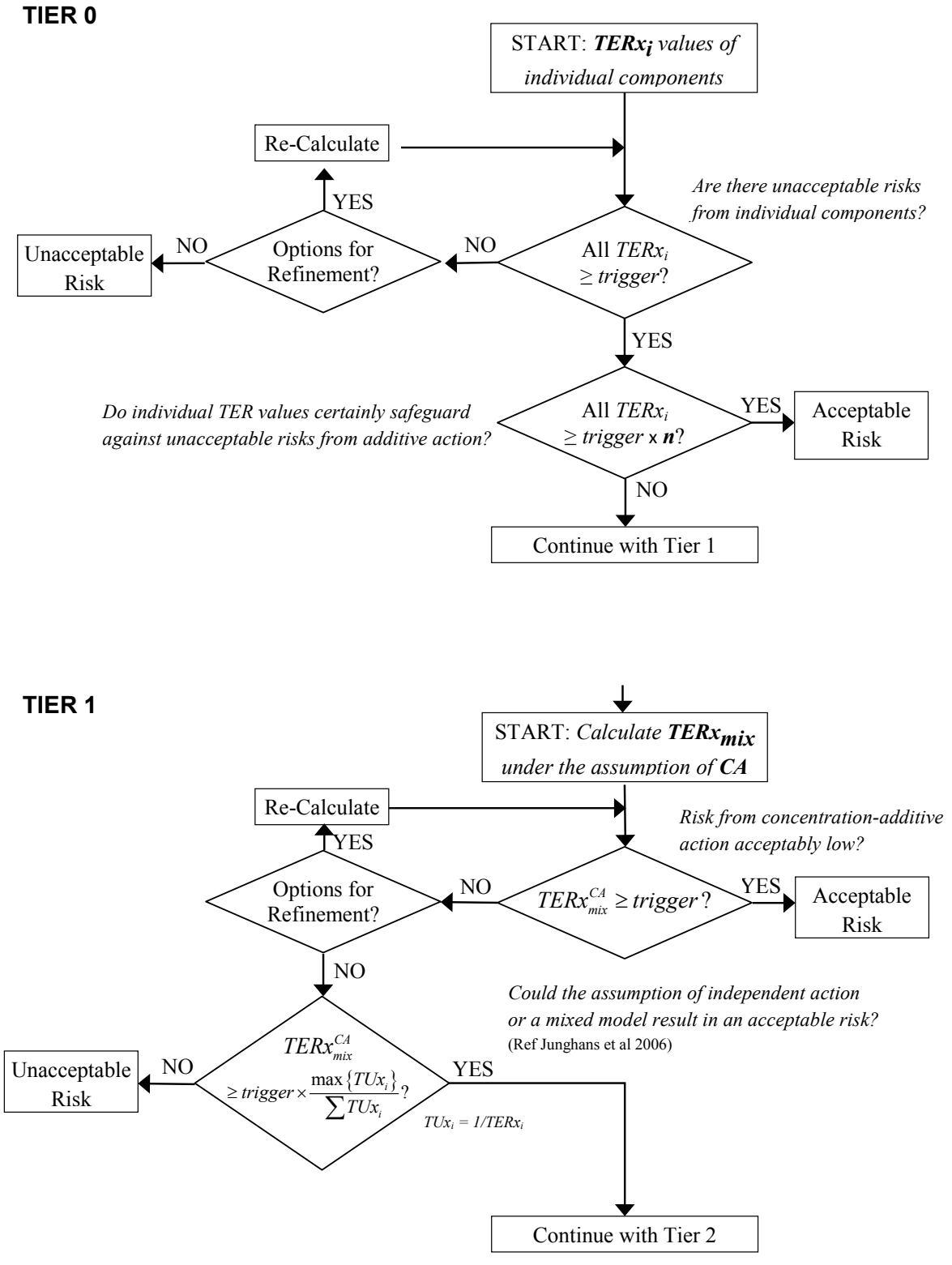
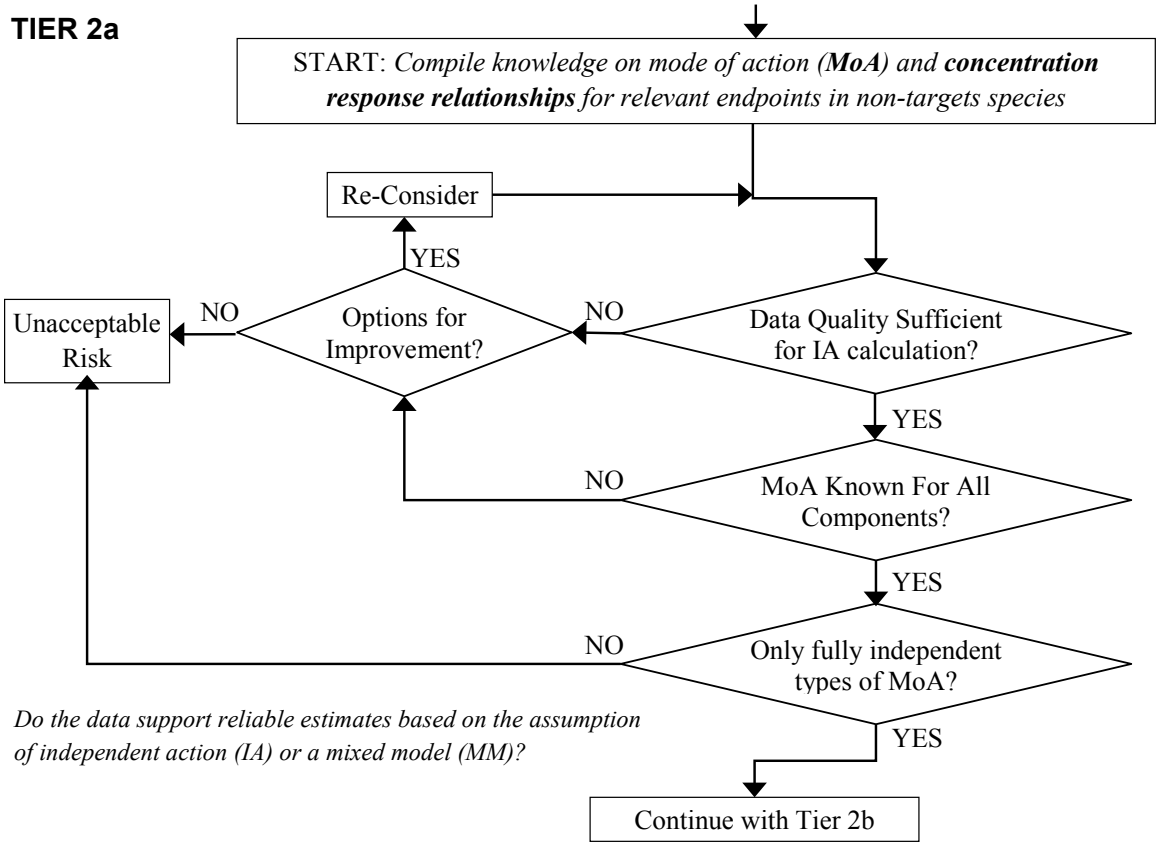


Figure 7.3 Component Based Approach to Ecotoxicological Mixture Risk Assessment under the PPP Regulation

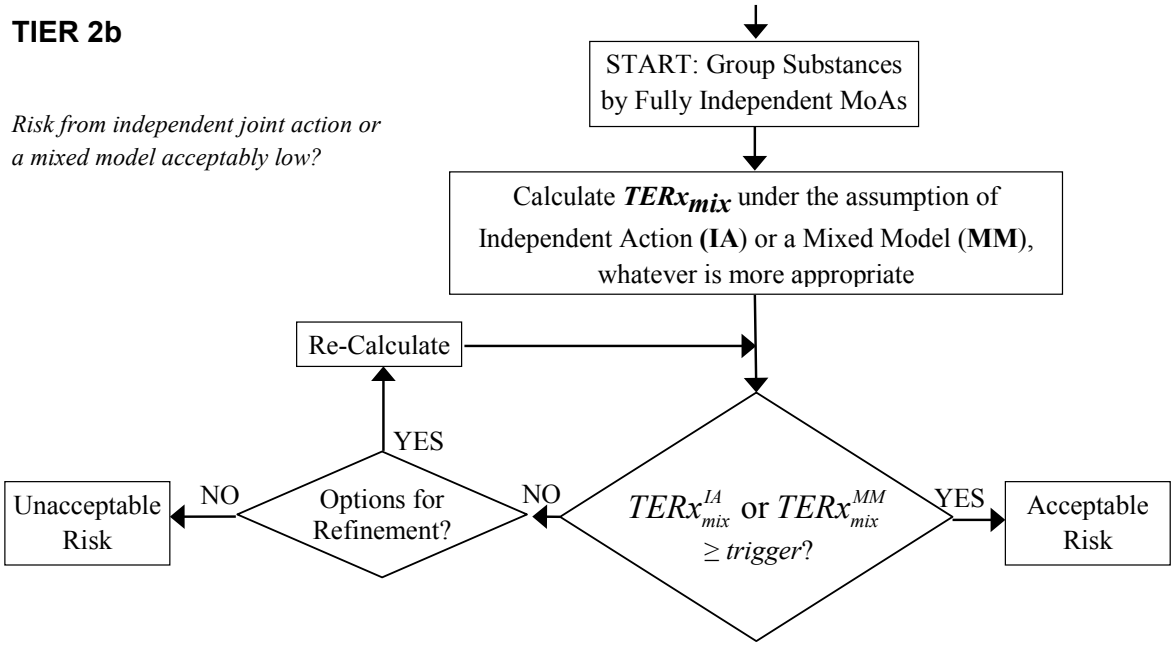
TIER 2a



Do the data support reliable estimates based on the assumption of independent action (IA) or a mixed model (MM)?

TIER 2b

Risk from independent joint action or a mixed model acceptably low?



Use of the scheme

Under the PPP regulation, single substance TER-values are usually available for any of the assessment endpoints defined in the *Uniform Principles* (see above). As a consequence, the proposed assessment scheme should also be applied separately for any of these endpoints, such as effects on algal growth, effects of long-term exposure on fish, and long-term toxicity to Daphnia for instance. A merging of data across such different taxonomic groups may be necessary for other less data rich situations (e.g. in the case of biocidal products; see chapter 6), but it is not necessary and not recommended for PPP assessments. This is not only scientifically more sound, but it also avoids problems that could result from the fact that different trigger values may apply to different endpoints, such algal toxicity and acute toxicity to fish or daphnids for instance (see Tab. 7.21).

In general, the scheme produces TERmix estimates in terms of ratios between predicted effect concentrations (or doses) and predicted exposure concentrations (or doses). Corresponding input data for the toxicity side of the risk quotient are individual effect concentrations in TIER 0 and TIER 1, and individual concentration (or dose) response functions in TIER 2. For TIER 0 and TIER 1 assessments however, NOEC (or NOEL) values may also be used as alternative input data. For TIER 2 this is not possible. As detailed in chapter 4, the use of NOEC values in a CA-based assessment (TIER 0 and TIER 1) can be justified as a pragmatic way of making most effective use of available data for an initial mixture risk assessment. Where this simplified approach points to an unacceptable risk, there is a need for further clarification[24].

Each of the three tiers includes a loop for refinements or improvements. This allows for iterative assessments in case that corrected, improved or extended versions of the necessary starting data or input knowledge become available. Such information may result from both, improved exposure and/or toxicity data for single mixture components. If in the end the CBA still indicates an unacceptable risk of the mixture of concern, experimental testing of re-constituted mixtures may be considered as an ultimate tool for final confirmation, unless practical, ethical, economical or other regulatory considerations argue against such a decisive experiment.

[24] The decision logic and rational introduced here for dealing with individual NOEC values as input information clearly differs from considerations presented in Step 3 of Annex B to EFSA's guidance document for birds & mammals (EFSA 2009b). On the one hand, the EFSA document suggests confining NOEC-based assessments of mixture risks for sublethal and reproductive endpoints to groups of PPP ingredients for which a common MoA has been established. This requires information that is only considered on the highest tier of the scheme proposed here, because this knowledge is often lacking for non-target organisms. Thus, initially the EFSA proposal is less precautious than the approach suggested here. On the other hand, however, the EFSA document suggests as a "*simple approach*" that such assessments should be done by simply summing up the molar concentrations of all components belonging to the same mechanistic group and by simply assuming that they are all as toxic as the most toxic one. This is in most cases clearly more conservative than the default assumption of CA. The suggested *simple approach* agrees with the assumption of CA only in the extreme cases where either the most toxic component dominates the mixture in terms of toxic units or where the potencies of all compounds (in terms of NOEC or ECx values) are almost identical. Hence, in general the *simple approach* can be justified as a CA-based worst case assumption in situations where the concentration (or dose) ratio of mixture components is undefined. However, this does not apply to the scenario considered in the EFSA document. As the suggested *simple approach* starts with summing up concentrations of similarly acting components, this also means that the mixture ratio is well known. Hence, it remains obscure why CA is not applied.

Critical points and room for improvement

The proposed tiered CBA clearly provides a higher protection level than assessing single PPP ingredients as if they were present in isolation. But of course, there is room for improvement. As with any modelling approach, this results from (i) gaps in the necessary input data, (ii) shortcomings of the inherent model assumptions, and (iii) limitations of the applicability domain of the possible output data. More specifically, this means that further efforts should focus on the following critical points:

(i) Gaps in input data: How to deal with co-formulants and metabolites?

The reliability of a CBA critically depends on the inclusion of all relevant mixture compounds in the calculation. Relevant are all components that have the potential for causing the common non-target effect under consideration, if alone present at sufficiently high concentrations (or doses). Unfortunately, the general a priori assumption that only active substances are relevant and all co-formulants are inerts is obviously wrong, as indicated by numerous examples (Coors & Frische 2011, Coors et al. 2012). Where single substance toxicity data are available for co-formulants, this information could be immediately included in the CBA. And where reliable evidence supports the assumption of inertism, such co-formulants could be left out of the calculations for well-founded reasons and not just due to missing information.

With the principle of "*No data, no market*" having been established by REACH Article 5 (EC 2006), the situation of insufficient information about eco-toxicological properties of substances that are used as co-formulants in PPPs may not generally remain unchanged in the long run. Potential incentives for speeding up the process and improving the knowledge base in the midterm may be worth consideration. As an example, waiving of whole product testing could be considered in cases where relevant single substance toxicity information is made available by a PPP producer for all product ingredients and not just for active substances. In the short-term, competent authorities can reduce the risk of underestimations of mixture toxicity by means of the cross-checking between single substance data, whole product test data, and modelling results as detailed in section 7.2.2 above.

After release of PPPs into the environment, the assessment situation becomes more complex due to the biotic and abiotic transformation of product ingredients. In general, the transformation products have been found to be as toxic as or less toxic than the parent compounds, but exemptions to this rule have also been observed (Boxall at al. 2004). As a consequence, the development of appropriate regulatory strategies for dealing with mixtures of parent compounds and degradation products in a systematic way deserves further efforts.

(ii) Shortcomings of model assumptions: How to deal with synergistic interactions?

The proposed scheme builds on the presumption that mixtures are not more toxic than predicted by CA. Potential synergistic interactions that result in more-than-concentration-additive effects are not covered. A careful pre-checking of all available information about any potential interactions between mixture components is therefore essential (see section 7.2.2 above). As detailed in chapter 3, the search for tools that would allow to identify synergistic substance combinations in a systematic way is on-going. Currently and in the near future, such tools are not available. As a consequence, available evidence for synergistic potentials may

largely depend on chance findings or negative episodic experience in the past. This raises concerns that the protection level that is achievable by the proposed tiered CBA may not always be sufficient due to (currently) unpredictable toxico-kinetic or toxico-dynamic interactions between mixture components.

As a way for dealing with this uncertainty, the competent authority may consider to divide model-based mixture toxicity estimates by a special "interaction factor" (IF), either generally or under specific conditions only. As an option, this approach has been explicitly included in the proposed assessment scheme for biocidal products (chapter 6). In the context of TERmix values for PPPs, an equivalent approach would be to raise the trigger values that are considered to ensure a sufficient protection from unacceptable risks (see Tab. 7.21) in the case of model-derived values. One problem with this option is to define the appropriate magnitude and the appropriate conditions for the application of such an IF. Another one is the fact that the starting assumption of a more-than-concentration-additive action can only be ruled out by means of experimental testing, if it should lead to the indication of an unacceptable risk. This means that the option to proceed in the proposed scheme from TIER 1 to TIER 2 would be blocked, unless an experiment has shown that the actual mixture toxicity does in fact not exceed CA. In view of these difficulties, the development of an advanced strategy for dealing with potential synergistic interactions in an appropriate and consensually acceptable way remains a challenging task.

(iii) Limitations of the applicability domain: How to deal with higher tier multi-species assays?

The concepts of CA and IA have been developed and extensively evaluated for single species mixture toxicity assessments. At the current state-of-science, it is unclear whether and under what conditions they are also applicable to endpoints of complex higher tier test systems that are quite frequently used in the context of PPP authorisation, such as microcosms, mesocosms, and earthworm field studies in particular. As detailed in chapter 3, there are some few promising studies, but further research on this topic is clearly required.

Another way of performing risk assessments on a higher tier level is the extrapolation from single species toxicity test results to biotic communities by means of the SSD methodology (species sensitivity distributions). Proposals for applying this methodology to assessments of mixture toxicity have been developed (De Zwart and Posthuma 2005). For this methodology, a lot of data are required which not available by default in the context of PPP authorization (Verbruggen and Van den Brink 2010). Nevertheless, at least in some cases it may be a valuable tool that deserves further attention. As a potential consequence of such data limitations, the competent authority may decide to base a PPP risk assessment not on higher-tier data that may be available for a single mixture component only, but to step-back to lower-tier data which may be available for all relevant mixture components.

7.3 Impact of mixture assessment in PPP authorization

While this Chapter mainly deals with the options and consequences of considering mixture toxicity in the environmental risk assessment on the authorization of PPP, this paragraph briefly touches on the impact on additional efforts and economic aspects. Besides potential beneficial aspects with regard to environmental safety, a change in the risk assessment

approach towards considering mixture toxicity may also invoke costs both for applicants and authorities.

The already high complexity of the environmental risk assessment of PPP will further increase if consideration of mixture toxicity needs to be conducted routinely. As apparent from the examples presented in this study, the calculation of TERmix values for the various risk assessment areas requires careful compilation of toxicity and exposure estimates for the mixture that needs to be considered. Even if the guidance on how to consider mixture toxicity is unambiguous and straightforward, the additional work to be conducted in the context of the complex environmental risk assessment will be considerable. This holds for both the applicant who has to conduct the risk assessment as well as the authority that has to review and approve the risk assessment.

As summarized in the present study, EU Member States are developing or already applying concepts to implement mixture toxicity considerations for combination products. If these approaches are not sufficiently harmonized across Europe, the effort for an environmental risk assessment of a PPP to be authorized in several MS will increase even more. Particularly, the already existing differences in the calculation of exposure estimates across the MS and the regional zones will add to the complexity of the mixture toxicity implementation. In the case of biocides, calculation of exposure estimates is already rather cumbersome and complex given the diversity of product types and related exposure scenarios. However, exposure estimates for all relevant compounds of a biocidal product (active substances and so-called Substances of Concern) have to be derived anyway. Therefore, a basic mixture assessment by CBA should always be possible without further data requirements.

Ambiguous guidance or large differences among MS will hamper logistic and economic planning of the applicant. In extreme cases, the risk of failing authorization may increase. These kinds of cost are hard to quantify in terms of money, but they may nevertheless become relevant in the context of decisions regarding the development of new PPP or the support of well established pesticides.

Based on the currently proposed implementation options, no ecotoxicological testing beyond current regulations will be required for a standard consideration of mixture toxicity. However, as apparent from the PPP serving as examples in the present study, it is less likely that an environmental risk can be excluded in a first-tier risk assessment. This means that either higher-tier risk assessment, including relevant ecotoxicological studies, are required or stricter risk mitigation measures have to be accepted. The two options both carry costs for the applicants, but of different nature. Either costs for conducting additional studies that support the acceptability of effects or profit that cannot be made because larger risk-reducing buffer zones reduce the usability and thereby the absolute sale volume for a given PPP.

Reduced standard testing with combination products or active substances may be possible if component-based mixture toxicity considerations and decisions are accepted by the authorities. However, the standard studies that may be saved thereby are not very costly compared to higher-tier studies such as mesocosm or earthworm field studies that may be required in a refined risk assessment. Currently, it is not clear if standard testing for combination products may in fact be reduced. If the absence of synergistic interactions has to be proven for a given product before mixture toxicity concepts may be applied, testing with formulated PPP would rather be expected to increase than decrease.

Any firm conclusion on additional economic costs or savings invoked through mixture consideration in environmental risk assessment for biocide or plant protection products, however, at this stage seems premature.

8 Tool

A collection of excel tools was implemented in order to facilitate the component-based assessment of chemical mixtures, using the two reference concepts of Concentration Addition and Independent Action, which have been discussed in detail in chapter 3. In particular the tool collection allows to:

1. calculate the expected toxicity of a mixture with an arbitrary number of compounds, according to Concentration Addition and using various types of input data (PEC/PNEC ratios, relative fractions, masses, etc);
2. calculate the IA-expected effect of a mixture with an arbitrary number of compounds;
3. calculate the maximum possible difference between the CA and IA expected ECx;
4. visualize the toxic unit distribution for the analysed mixture (see Figure 8.1. for an example);
5. analyses the quantitative consequences if a particular component of the mixture is synergized (Figure 8.2) or a whole subset of compounds is subject to synergistic interactions (Figure 8.3).

If you are interested in receiving a copy of the tool, please get in touch with Thomas Backhaus at thomas.backhaus@gu.se.

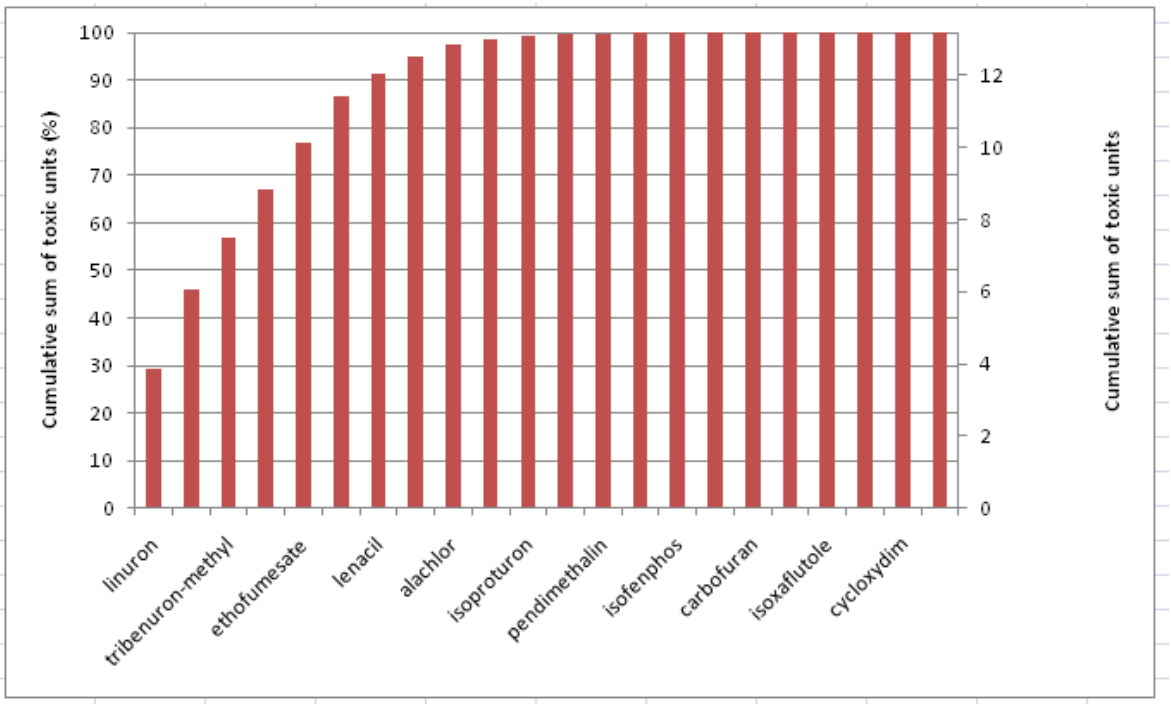


Figure 8.1 Toxic Unit Distribution of the pesticide mixture analysed by Junghans et al. (2006)

The study of Junghans et al (2006) investigated a toxicity of a mixture of 22 pesticides at concentrations that are expected to result from standard application in a typical agricultural setting. The toxicity was determined in an algal assay, the depicted toxic units are based on the ratio of PEC/NOEC.

The visualization provides the cumulative toxic unit distribution, expressed as percent of the total sum of toxic units. The figure gives a clear impression of the uneven distribution of the toxic units in the mixture. Few compounds contribute substantially to the mixture toxicity. Already the first 7 compounds contribute with more than 90% of the total sum of toxic units. The remaining 15 compounds contribute only to a minor extent (10% in total).

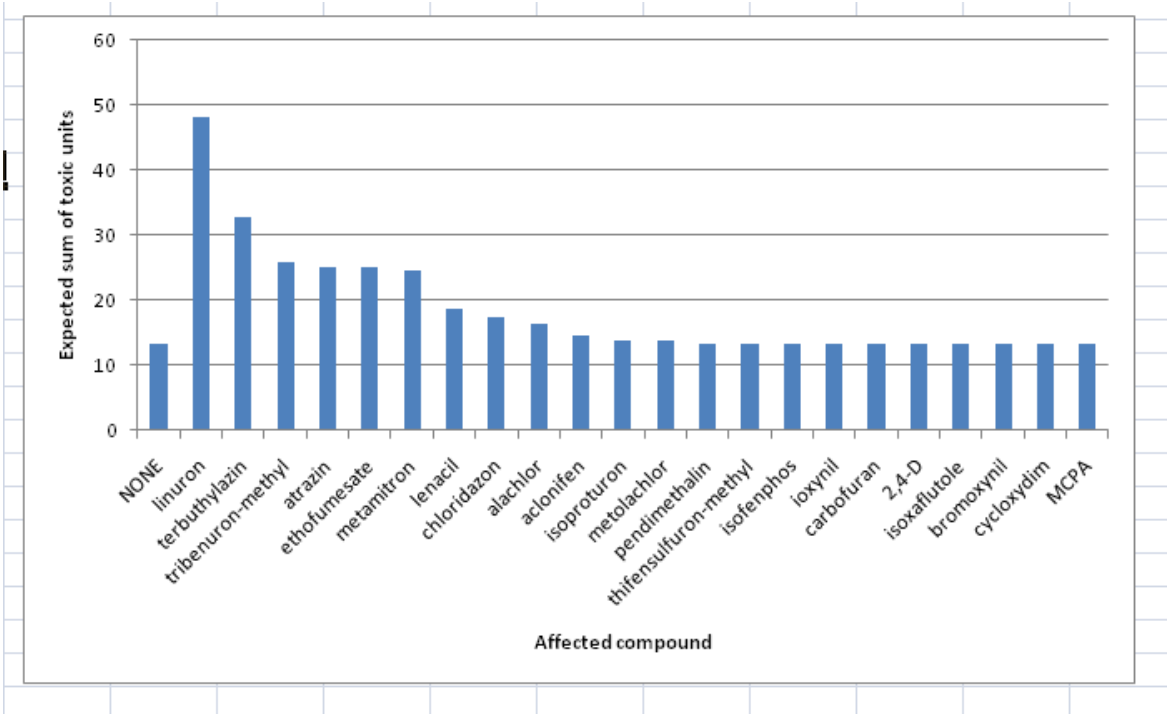


Figure 8.2 Impact of synergistic interactions on the expected sum of toxic units (STU) I

This example is also based on the study by Junghans et al (2006). It provides an analysis on how the expected sum of toxic units is influenced if one specific compound is more toxic in a mixture context than is expected from its toxicity as a single substance. The sum of toxic units (c/NOEC) according to CA equals 12.48 for this particular mixture of 22 pesticides (leftmost bar).

The bars to the right then provide the sum of toxic units that result from the indicated compound being by a factor of 10 more toxic in the mixture than expected from its analysis as an individual substance. It can be clearly seen that the sum of toxic units of the mixture is not visibly influenced if compounds with a small toxic unit are synergized (right hand side, see also Fig. 8.2 for the actual toxic unit distribution). For example, if the compound with the highest toxic unit is subject to a synergistic interaction that makes it 10 times more potent in a mixture context, the overall sum of toxic units increases from 12.5 to 48.5, i.e. by a factor of 3.9. If, however, isoproturon (the compound with the median toxic unit) is synergised by the same factor, the total sum of toxic units increases only by a mere factor of 1.1 to 13.7.

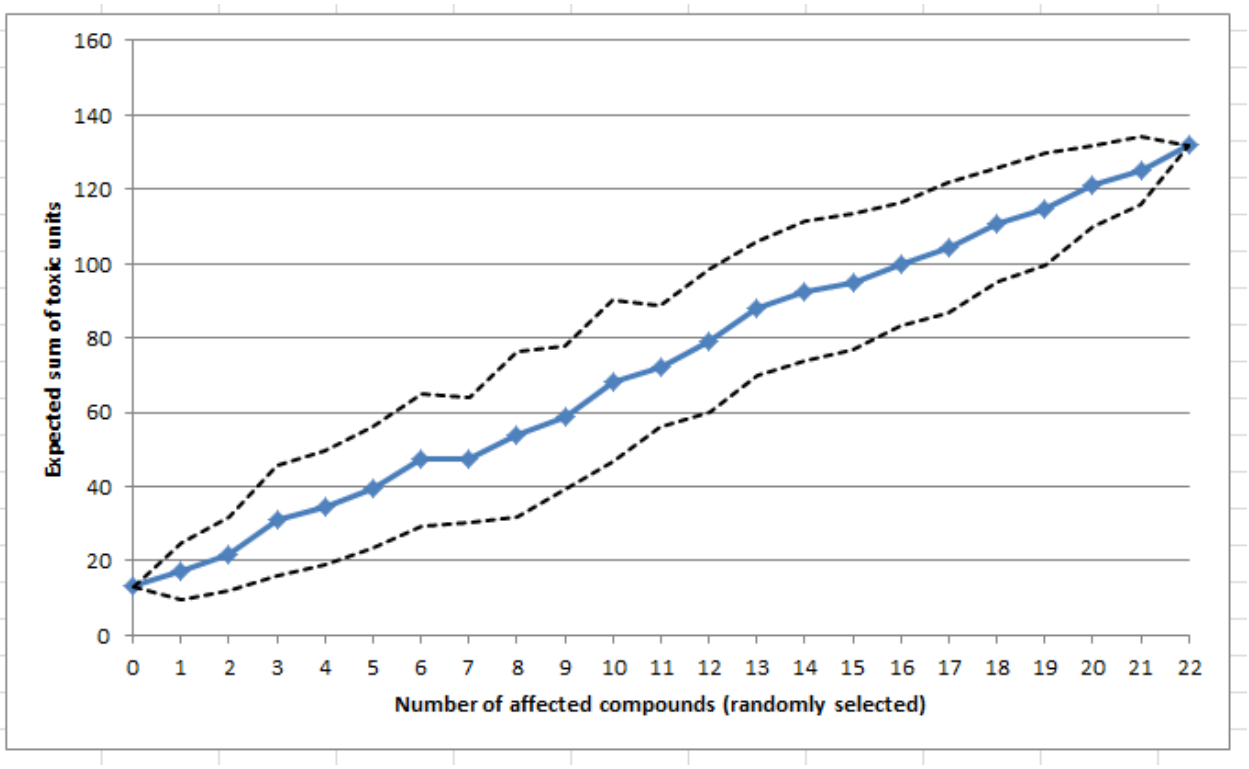


Figure 8.3 Impact of synergistic interactions on the expected sum of toxic units (STU) II
This example is also based on the study by Junghans et al (2006). It provides an analysis on how the expected sum of toxic units is influenced if a given fraction of the mixture components is subject to synergistic interactions.

The sum of toxic units (c/NOEC) according to CA equals 12.48 for this mixture of 22 pesticides (number of synergized compounds = 0). The blue line provides the expected sum of toxic units under the assumption that the indicated number of randomly selected mixture components are a factor of 10 more toxic in a mixture context than expected from their analysis as individual substances. It can be seen that, if all compounds are a factor of 10 more toxic in the mixture, the total sum of toxic units is also 10 times higher than expected from CA. If half the compounds are "synergized" (number of affected compounds=11), then the sum of toxic units increases from 12.5 for the CA case to 75.3, i.e. the toxicity of the total mixture is a factor of 6 more toxic than expected.

The dashed line provides the confidence belt for the predicted increase in toxicity, which comprises the range from a sum of toxic unit of 56.3 to 88.1 (i.e. an excess toxicity in comparison to CA between a factor of 4.5 and 7.0).

9 Conclusions and Outlook

Through review of the currently available evidence on combined effects from exposure of organisms against mixtures of compounds, consideration of regulatory options to deal with mixtures, study of the relevance of mixtures in biocide and plant protection products and development of specific tiered schemes for their assessment, the major conclusions from this project can be summarised as laid out in the following.

Component-based mixture toxicity assessment is theoretically and experimentally supported

One central challenge in dealing with combined effects for mixture exposure lies in the fact that anticipated mixture occurrence is too variable in composition and regime to allow generation of experimental evidence for every conceivable exposure situation that would require an environmental risk assessment. The available scientific evidence collated over the last decades mainly in ecotoxicology support earlier conceptual considerations that combined effects may in general be quantitatively reasonably well predicted based on the knowledge of their individual activities. While synergistic or antagonistic interactions do occur, i.e. the observed combined effects show larger or smaller effects than expected based on their individual activities the number of documented cases where these amount to magnitudes relevant in a regulatory setting (e.g. larger than a factor of 2) are small (cf. Chapt. 3 – State of the art in .mixture toxicity assessment)

Tiered approaches solve dead end discussions, such as the relevance of mode-of-action knowledge

Considerable debate has focused on questions of which concept is theoretically the most adequate to assess combined effects, how much mode of action information is necessary to select the most appropriate assessment model and how to deal with heterogeneous data sets. In a regulatory setting a tiered framework for mixture assessment is one way to overcome those situations that will otherwise lead to a state where no mixture assessment can be derived due to lack of required specific information.

Transformation of current knowledge into regulatory assessment schemes is not self-evident

Translation of the evidence-based mixture toxicity approaches into regulatory schemes requires additional criteria beyond scientifically indicated knowledge. In particular, with respect to aggregation of the available data basis or the definition of default factors questions emerge, that cannot be based on purely scientific reasoning. The gain in performing this lies in the fact that with the type of data currently available in environmental risk assessment mixture assessment would become possible. Specific schemes for biocide and plant protection products are proposed in this work. The impact of additional mixture risk assessment in the suggested setting lies in a more comprehensive assessment while at the same time hardly any need for provision of additional data is anticipated.

Impact of mixture assessment in PPP authorization

Any firm conclusion on additional economic costs or savings invoked through mixture consideration in environmental risk assessment for biocide or plant protection products, however, at this stage seems premature.

Future information and research needs can be foccused

From a risk perspective the open questions regarding information and knowledge gaps for performing mixture assessment are the following:

- While a product assessment can be based on the composition of components as present in the original product, the assessment of mixture exposures in the environment is limited by the availability of adequate exposure data for the relevant components. To overcome this bottleneck, supplementary exposure modeling for product components or experimental investigation of 'typical' mixtures, e.g. elutriates, would be required.
- A major unresolved question on mixture toxicity is that of interactive combined effects and in particular synergistic effects. Currently, only anecdotal evidence for the existence of so-called synergistic effects is prevalent. Generating indicators for interactive behaviour e.g. through modern toxicogenomics techniques is a vision but first steps could comprise of more systematic review of the existing evidence for potentially emerging patterns.
- While much of the evidence referred in this work and others relates to the study of active substances for plant protection products, in the arena of biocide mixture toxicity evidence for other than antifoulant products is scare[25] but urgently required due to their often environmentally open applications.
- Finally, the refinement in current risk assessment for PPPs may extend for several reasons into higher tier studies. At this level of biological response, however, again very little knowledge is available as to the prediction and assessment of mixture toxicity.

[25] A recent experimental study to this end can be found in part 2 to this report.

10 References

Altenburger R, Boedeker W, Faust M, Grimme LH. 1996. Regulations for combined effects of pollutants: Consequences from risk assessment in aquatic toxicology. *Food and Chemical Toxicology* 34:1155-1157.

Altenburger R, Nendza M, Schüürmann G. 2003. Mixture toxicity and its modeling by quantitative structure-activity relationships. *Environmental Toxicology and Chemistry* 22:1900–1915.

Altenburger R, Walter H, Grote M. 2004. What Contributes to the Combined Effect of a Complex Mixture? *Environmental Science & Technology* 38:6353-6362.

Altenburger R, Greco WR. 2009. Extrapolation Concepts for Dealing with Multiple Contamination in Environmental Risk Assessment. *Integrated Environmental Assessment and Management* 5:62-68.

Altenburger R, Scholz S, Schmitt-Jansen M, Busch W, Escher BI. 2012. Mixture Toxicity Revisited from a Toxicogenomic Perspective. *Environmental Science & Technology* 46:2508-2522.

Andersen ME, Dennison JE. 2004. Mechanistic approaches for mixture risk assessments--present capabilities with simple mixtures and future directions. *Environmental Toxicology and Pharmacology* 16:1-11.

Arai T, Harino H, Ohji M, Langston W. 2009. Ecotoxicology of Antifouling Biocides. Springer.

Arrhenius Å, Grönvall F, Scholze M, Backhaus T, Blanck H. 2004. Predictability of the mixture toxicity of 12 similarly acting congeneric inhibitors of photosystem II in marine periphyton and epipsammon communities. *Aquatic Toxicology* 68:351-367.

Arrhenius Å, Backhaus T, Grönvall F, Junghans M, Scholze M, Blanck H. 2006. Effects of Three Antifouling Agents on Algal Communities and Algal Reproduction: Mixture Toxicity Studies with TBT, Irgarol, and Sea-Nine. *Archives of Environmental Contamination and Toxicology* 50:335-345.

ATSDR. 2001. Guidance for the Preparation of an Interaction Profile. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Division of Toxicology.

ATSDR. 2004. Guidance manual for the assessment of joint toxic actions of chemical mixtures. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Division of Toxicology.Backhaus T, Arrhenius Å, Blanck H. 2004a. Toxicity of a Mixture of Dissimilarly Acting Substances to Natural Algal Communities: Predictive Power and Limitations of Independent Action and Concentration Addition. *Environmental Science & Technology* 38:6363-6370.

Backhaus T, Blanck H, Faust M. 2010. Hazard and risk assessment of chemical mixtures under REACH – State of the art, gaps and options for improvement. Report prepared for KEMI, the Swedish Chemicals Agency. PM 3/10, Swedish Chemicals Agency, Order No 510 968, Sundbyberg

Backhaus T, Faust M, Scholze M, Gramatica P, Vighi M, Grimme LH. 2004b. Joint algal toxicity of phenylurea herbicides is equally predictable by concentration addition and independent action. *Environmental Toxicology and Chemistry* 23:258-264.

Backhaus T, Faust M. 2012. Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework. *Environmental Science & Technology* 46:2564-2573.

Bakker JF, Belzunce-Segarra MJ, Castro R, van de Heuvel-Greve M, Klamer HJC, Brack W, Altenburger R, Poulsen V, Thomas KV, Leonards PEG. 2007. Effect Directed Analysis and Toxicity Identification Evaluation. In Damià B, Mira P, eds, *Sustainable Management of Sediment Resources*. Vol Volume 1. Elsevier, pp 163-214.

Bao VW, Leung KM, Kwok KW, Zhang AQ, Lui GC. 2008. Synergistic toxic effects of zinc pyrithione and copper to three marine species: Implications on setting appropriate water quality criteria. *Marine Pollution Bulletin* 57:616-623.

Belden JB, Gilliom RJ, Lydy MJ. 2007. How Well Can We Predict the Toxicity of Pesticide Mixtures to Aquatic Life? *Integrated Environmental Assessment and Management* 3:364–372.

Bellas J. 2008. Prediction and assessment of mixture toxicity of compounds in antifouling paints using the sea-urchin embryo-larval bioassay. *Aquatic Toxicology* 88:308-315.

Berenbaum MC. 1981. Criteria for analyzing interactions between biologically active agents. *Advances in cancer research* 35:269-335.

Bingham G, Gunning RV, Delogu G, Borzatta V, Field LM, Moores GD. 2008. Temporal synergism can enhance carbamate and neonicotinoid insecticidal activity against resistant crop pests. *Pest Management Science* 64:81-85.

Bödeker W, Altenburger R, Faust M, Grimme LH. 1990. Methods for the assessment of mixtures of pesticides: Mathematical analysis of combination effects in phytopharmacology and ecotoxicology. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes (Braunschweig)* 42:70-78.

Bödeker W, Altenburger R, Faust M, Grimme LH. 1992. Synopsis of concepts and models of the quantitative analysis of combination effects: from biometric to ecotoxicology. *Archives of Complex Environmental Studies* 4:45-53.

Bödeker W, Drescher K, Altenburger R, Faust M, Grimme LH. 1993. Combined effects of toxicants: the need and soundness of assessment approaches in ecotoxicology. *Science of The Total Environment* 134, Supplement 2:931-939.

Bonnemain H, Dive D. 1990. Studies on synergistic toxic effects of copper and dithiocarbamate pesticides with the ciliate protozoan Colpidium campylum (stokes). *Ecotoxicology and Environmental Safety* 19:320-326.

Bonvin F, Rutler R, Chèvre N, Halder J, Kohn T. 2011. Spatial and Temporal Presence of a Wastewater-Derived Micropollutant Plume in Lake Geneva. *Environmental Science & Technology* 45:4702-4709.

Boobis A, Budinsky R, Collie S, Crofton K, Embry M, Felter S, Hertzberg R, Kopp D, Mihlan G, Mumtaz M, Price P, Solomon K, Teuschler L, Yang R, Zaleski R. 2011. Critical analysis of literature on low-dose synergy for use in screening chemical mixtures for risk assessment. *Critical Reviews in Toxicology* 41:369-383.

Borgert CJ, Quill TF, McCarty LS, Mason AM. 2004. Can mode of action predict mixture toxicity for risk assessment? *Toxicology and Applied Pharmacology* 201:85-96.

Boxall ABA, Sinclair CJ, Fenner K, Kolpin D, Maund SJ. 2004. When Synthetic Chemicals Degrade in the Environment. What are the absolute fate, effects, and potential risks to humans and the ecosystem? *Environmental Science & Technology* 38:368A-375A.

Bruna F, Pavlovic I, Celis R, Barriga C, Cornejo J, Ulibarri MA. 2008. Organohydrotalcites as novel supports for the slow release of the herbicide terbuthylazine. *Applied Clay Science* 42:194-200.

Calamari D, Vighi M. 1992. A proposal to define quality objectives for aquatic life for mixtures of chemical substances. *Chemosphere* 25:531-542.

Campbell PGC, Tessier A. 1996. Ecotoxicology of metals in the aquatic environment: geochemical aspects. In Newman MC, C.H. Jagoe, eds, *Ecotoxicology - a hierarchical treatment*. CRC Press, Inc., pp 11–58.

CDR. Web Page. The Applicant Guide: Tank-mixes. Online available at: http://www.pesticides.gov.uk/guidance/industries/pesticides/topics/pesticide-approvals/pesticides-registration/applicant-guide/the-applicant-guide-tank-mixes. Health & Safety Executive (HSE) / Chemicals Regulation Directorate (CRD).

Cedergreen N, Streibig JC. 2005. The toxicity of herbicides to non-target aquatic plants and algae: assessment of predictive factors and hazard. *Pest Management Science* 61:1152-1160.

Chesworth JC, Donkin ME, Brown MT. 2004. The interactive effects of the antifouling herbicides Irgarol 1051 and Diuron on the seagrass Zostera marina (L.). *Aquatic Toxicology* 66:293-305.

Chévre N, Loepfe C, Singer H, Stamm C, Fenner K, Escher BI. 2005. Including Mixtures in the Determination of Water Quality Criteria for Herbicides in Surface Water. *Environmental Science & Technology* 40:426-435.

Cima F, Bragadin M, Ballarin L. 2008. Toxic effects of new antifouling compounds on tunicate haemocytes: I. Sea-Nine 211™ and chlorothalonil. *Aquatic Toxicology* 86:299-312.

Commission Regulation (EU) No 545/2011 of 10 June 2011. Commission Regulation (EU) No 545/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards the data requirements for plant protection products. *Official Journal of the European Union*,L 155:54:67-126.

Commission Regulation (EU) No 546/2011 of 10 June 2011. Commission Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products. *Official Journal of the European Union*,L 155:54:127-175.

Coors A, Dobrick J, Möder M, Kehrer A. 2012. Mixture toxicity of wood preservative products in the fish embryo toxicity test. *Environ Toxicol Chem* 31:1239-1248

Coors A, Moltmann JF, Knacker T. 2008. Pestizid-Mischungen in der Umwelt: Beschreibung der Relevanz von Kombinationswirkungen für die ökotoxikologische Risikobewertung vor dem Hintergrund der üblichen Anwendungspraxis von Pflanzenschutzmitteln. FKZ 360 03 041. Umweltbundesamt (UBA).

Coors A. 2009. Die Eignung von Konzentrations Additivität zur Vorhersage der toxischen Kombinationswirkung technischer Stoffgemische für Wasserorganismen – analysiert am Beispiel von Pflanzenschutzmittel Kombinationspräparaten. FKZ 360 03 046. UBA.

Coors A, Frische T. 2011. Predicting the aquatic toxicity of commercial pesticide mixtures. *Environmental Sciences Europe* 23.

Council conclusions 17820/09 Combination effects of chemicals - Council conclusions. Adopted by the Council on 22 December 2009 (Available online at: http://register.consilium.europa.eu/pdf/en/09/st17/st17820.en09.pdf).

Council Directive 91/414/EEC of 15 July 1991. Council Directive of 15 July 1991 concerning the placing of plant protection products on the market (91/414/EEC). *Official Journal of the European Communities* L 230.

Cox C, Surgan M. 2006. Unidentified Inert Ingredients in Pesticides: Implications for Human and Environmental Health. *Environ Health Perspect* 114.

Creton S, Douglas M, Wheeler JR, Hutchinson TH. 2010. Challenging the requirement for chronic fish toxicity studies on formulated plant protection products. *Toxicology Letters* 199:111-114.

De Silva PMCS, Pathiratne A, van Gestel CAM. 2010. Toxicity of chlorpyrifos, carbofuran, mancozeb and their formulations to the tropical earthworm Perionyx excavatus. *Applied Soil Ecology* 44:56-60.

de Zwart D, Posthuma L. 2005. Complex mixture toxicity for single and multiple species: Proposed methodologies. *Environmental Toxicology and Chemistry* 24:2665-2676.

DeLorenzo ME, Serrano L. 2003. Individual and Mixture Toxicity of Three Pesticides; Atrazine, Chlorpyrifos, and Chlorothalonil to the Marine Phytoplankton Species Dunaliella tertiolecta. *Journal of Environmental Science and Health, Part B* 38:529-538.

DeLorenzo ME, Serrano L. 2006. Mixture Toxicity of the Antifouling Compound Irgarol to the Marine Phytoplankton Species Dunaliella tertiolecta. *Journal of Environmental Science and Health, Part B* 41:1349-1360.

Deneer JW. 2000. Toxicity of mixtures of pesticides in aquatic systems. *Pest Management Science* 56:516-520.

Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998. Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. *Official Journal of the European Communities* L 123:1-63.

Drescher K, Boedeker W. 1995. Assessment of the Combined Effects of Substances: The Relationship between Concentration Addition and Independent Action. *Biometrics* 51:716-730.

EC (European Commission). 2003. Technical Guidance Document on Risk Assessment in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. Part I-IV. European Commission, Joint Research Centre (JRC), Institute for Health and Consumer Protection (IHCP), European Communities, EUR 20418 EN/1-4.

EC DG-SANCO. 2001. Working Document Guidance Document on Aquatic Ecotoxicology. Sanco/3268/2001 rev.4 (final), 17 October 2002. European Commission, Health & Consumer Protection Directorate-General, Directorate E - Food Safety: plant health, animal health and welfare, international questions, E1 - Plant health.

EC DG-SANCO. 2002a. Draft Working Document Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC. SANCO/10329/2002 rev 2 final, 17 October 2002. European Commission, Health & Consumer Protection Directorate-General, Directorate E - Food Safety: plant health, animal health and welfare, international questions, E1 - Plant health.

EC DG-SANCO. 2002b. Working Document Guidance Document on Risk Assessment for Birds and Mammals Under Council Directive 91/414/EEC. SANCO/4145/2000 - final, 25 September 2002. European Commission, Health & Consumer Protection Directorate-General, Directorate E - Food Safety: plant health, animal health and welfare, international questions, E1 - Plant health.

EC DG-SANCO. 2005. Guidance Document on the Assessment of the Equivalence of Technical Materials of Substancees Regulated Under Council Directive 91/414/EEC.Sanco/10597/2003 –rev. 7 final2 (14 December 2005). European Commission - Health & Consumer Protection Directorate-General, Directorate D - Food Safety: production and distribution chain D3 - Chemicals, Contaminants and Pesticides.

EC DG-SANCO. 2009. Guidance Document on the Assessment of the Equivalence of Technical Materials of Substancees Regulated Under Council Directive 91/414/EEC. Sanco/10597/2003 –rev. 8.1, May 2009. European Commission, Health & Consumer Protection Directorate-General, Directorate D - Food Safety: production and distribution chain D3 - Chemicals, Contaminants and Pesticides.

EC DG-SANCO, SCHER, SCENIHR, SCCS. 2012. Opinion concerning Toxicity and Assessment of Chemical Mixtures. By Scientific Committees on Health and Environmental Risks (SCHER), Emerging and Newly Identified Health Risks (SCENIHR), and Consumer Safety (SCCS).

EC DG-SANCO. 2011. Guidance Document on the assessment of the equivalence of technical materials of substances regulated under Regulation (EC) No 1107/2009. Sanco/10597/2003 –rev. 9 (17 June 2011). European Commission, Health & Consumer Protection Directorate-General, Directorate E – Safety of the Food Chain E3 - Chemicals, Contaminants and Pesticides.

EC DG-SANCO, SCHER, SCENIHR, SCCS. 2011a. Public consultation on the preliminary opinion concerning Toxicity and Assessment of Chemical Mixtures. By Scientific Committees on Health and Environmental Risks (SCHER), Emerging and Newly Identified Health Risks (SCENIHR), and Consumer Safety (SCCS).

EC DG-SANCO, SCHER, SCENIHR, SCCS. 2011b. Public consultation on the preliminary opinion concerning Toxicity and Assessment of Chemical Mixtures (Final approved Opinion). By Scientific Committees on Health and Environmental Risks (SCHER), Emerging and Newly Identified Health Risks (SCENIHR), and Consumer Safety (SCCS).

EC DG-SANCO, SCHER, SCENIHR, SCCS. 2011c. Public consultation on the preliminary opinion concerning Toxicity and Assessment of Chemical Mixtures (Preliminary Opinion approved for Public Consultation). By Scientific Committees on Health and Environmental Risks (SCHER), Emerging and Newly Identified Health Risks (SCENIHR), and Consumer Safety (SCCS).

ECB (European Chemicals Bureau). 2008. TNsG Product evaluation. Technical Notes for Guidance in Support of Annex VI of Directive 98/8/EC of the European Parliament and the Council Concerning the Placing of Biocidal Products on the Market. Common Principles and Practical Procedures for the Authorisation and Registration of Products.

ECHA (European Chemicals Agency). 2008. Guidance on information requirements and chemical safety assessment Chapter R.10: Characterisation of dose [concentration]-response for environment. Guidance for the implementation of REACH.

EFSA (European Food Safety Authority). 2009a. Updating the opinion related to the revision of Annexes II and III to Council Directive 91/414/EEC concerning the placing of plant protection products on the market – Ecotoxicological studies.Scientific Opinion of the Panel on Plant Protection Products and their Residues (Question No EFSA-Q-2009-00556), Adopted on 18 June 2009. *The EFSA Journal* 1165:1-24.

EFSA (European Food Safety Authority). 2009b. Guidance document on risk assessment for birds and mammals. On request from EFSA, Question No EFSA-Q-2009-00223 First published on 17 December 2009. *EFSA Journal* 7:1438 [1358 pp.].

EFSA (European Food Safety Authority). 2010. Scientific Opinion on the development of specific protection goal options for environmental risk assessment of pesticides, in particular in relation to the revision of the Guidance Documents on Aquatic and Terrestrial Ecotoxicology (SANCO/3268/2001 and SANCO/10329/2002). *EFSA Journal* 8:1821 [1855pp.].

EIFAC (European Inland Fisheries Advisory Commission). 1987. Water quality criteria for European freshwater fish.

Eullaffroy P, Frankart C, Biagianti S. 2007. Toxic effect assessment of pollutant mixtures in Lemna minor by using polyphasic fluorescence kinetics. *Toxicological and Environmental Chemistry* 89:683-696.

Faust M, Altenburger R, Boedeker W, Grimme LH. 1993. Additive effects of herbicide combinations on aquatic non-target organisms. *Science of The Total Environment* 134:941-952.

Faust M, Altenburger R, Boedeker W, Grimme LH. 1994. Algal toxicity of binary combinations of pesticides. *Bulletin of Environmental Contamination and Toxicology* 53:134-141.

Faust M. 1999. Kombinationseffekte von Schadstoffen auf aquatische Organismen: Prüfung der Vorhersagbarkeit am Beispiel einzelliger Grünalgen. GCA-Verlag.

Faust M, Altenburger R, Backhaus T, Blanck H, Boedeker W, Gramatica P, Hamer V, Scholze M, Vighi M, Grimme LH. 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. *Aquatic Toxicology* 63:43-63.

Faust M, Scholze M. 2003. Competing Concepts for the Prediction of Mixture Toxicity: Do the Differences Matter for Regulatory Purposes? Technical Report: Deliverable 13. EU-Project BEAM (Bridging Effect Assessment of Mixtures to Ecosystem Situations and Regulation) - EVK1-CT1999-00012, Workpackage 7 - Options for Predictive Mixture Toxicity Assessment, Final Report to project partners, consultants, and European Commission services.

Fernández-Alba AR, Hernando MD, Piedra L, Chisti Y. 2002. Toxicity evaluation of single and mixed antifouling biocides measured with acute toxicity bioassays. *Analytica Chimica Acta* 456:303-312.

Feron VJ, Groten JP. 2002. Toxicological evaluation of chemical mixtures. *Food and Chemical Toxicology* 40:825-839.

FRAC. 2008. Mode of Action of Fungicides. FRAC classification on mode of action 2007 (www.frac.info). Fungicide Resistance Action Committee.

Freier B, Pallut B, Jahn M, Sellmann J, Gutsche V, Zornbach W, Komm R, Schlage B, Herzer A, Günther A. 2008. Netz Vergleichsbetriebe Pflanzenschutz - Jahresbericht 2007. Network of reference farms for plant protection - Annual Report 2007. Nr. 144 (2008). Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Braunschweig, Germany.

Freier B, Pallutt B, Jahn M, Sellmann J, Gutsche V, Zornbach W, Moll E, Herzer A, Komm R, Ullrich C, Schlage B. 2009. Netz Vergleichsbetriebe Pfl anzenschutz Jahresbericht 2008. Network of

reference farms for plant protection Annual Report 2008. Nr. 149 (2009). Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Braunschweig, Germany.

Frische T, Matezki T, Backhaus T. 2007. Analysing the risk of tank mixtures of plant protection products to aquatic organisms. *SETAC - Europe (Society for Environmental Toxicology and Chemistry), Annual Conference 2007*. SETAC, Porto, Portugal.

Gatidou G, Thomaidis NS. 2007. Evaluation of single and joint toxic effects of two antifouling biocides, their main metabolites and copper using phytoplankton bioassays. *Aquatic Toxicology* 85:184-191.

Giesy JP, Dobson S, Solomon kR. 2000. Ecotoxicological risk assessment for Roundup® herbicide. *Reviews of Environmental Contamination and Toxicology* 167:35-120.

Gramatica P, Vighi M, Consolaro F, Todeschini R, Finizio A, Faust M. 2001. QSAR approach for the selection of congeneric compounds with a similar toxicological mode of action. *Chemosphere* 42:873-883.

Granmo Å, Ekelund R, Sneli JA, Berggren M, Svavarsson J. 2002. Effects of antifouling paint components (TBTO, copper and triazine) on the early development of embryos in cod (Gadus morhua L.). *Marine Pollution Bulletin* 44:1142-1148.

Gressel J. 1993. Synergizing Pesticides To Reduce Use Rates. *Pest Control with Enhanced Environmental Safety*. Vol 524-ACS Symposium Series. American Chemical Society, pp 48-61.

Grimme LH, Altenburger R, Bödeker W, M. F. 1994. Kombinationswirkungen von Schadstoffen: Toxizität binärer Kombinationen von Pestiziden und Tensiden im Algenbiotest.

Haller WT, Stocker RK. 2003. Toxicity of 19 adjuvants to juvenile Lepomis macrochirus (bluegill sunfish). *Environmental Toxicology and Chemistry* 22:615-619.

Hazen JL. 2000. Adjuvants: Terminology, Classification, and Chemistry. *Weed Technology* 14:773-784.

Hellio C, Yebra D. 2009. Advances in marine antifouling coatings and technologies. Taylor & Francis.

Hernando MD, Ejerhoon M, Fernández-Alba AR, Chisti Y. 2003. Combined toxicity effects of MTBE and pesticides measured with Vibrio fischeri and Daphnia magna bioassays. *Water Research* 37:4091-4098.

Hill GK, E. Müller, B. Altmayr 2008. Rebschutz 2008 Rheinhessen. Verein Ehemaliger Rheinhessischer Fachschüler Oppenheim in Zusammenarbeit mit dem DLR Rheinhessen Nahe - Hunsrück, Wormser Str. 111, 55276 Oppenheim.

HRAC. 2008. Classification of Herbicides According to Mode of Action. Available online at: http://www.hracglobal.com/.

Junghans M, Backhaus T, Faust M, Scholze M, Grimme LH. 2006. Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. *Aquatic Toxicology* 76:93-110.

Kitulagodage M, Astheimer LB, Buttemer WA. 2008. Diacetone alcohol, a dispersant solvent, contributes to acute toxicity of a fipronil-based insecticide in a passerine bird. *Ecotoxicology and Environmental Safety* 71:597-600.

Knauer K, Leimgruber A, Hommen U, Knauert S. 2010. Co-tolerance of phytoplankton communities to photosynthesis II inhibitors. *Aquatic Toxicology* 96:256-263.

Knauert S, Escher B, Singer H, Hollender J, Knauer K. 2008. Mixture Toxicity of Three Photosystem II Inhibitors (Atrazine, Isoproturon, and Diuron) Toward Photosynthesis of Freshwater Phytoplankton Studied in Outdoor Mesocosms. *Environmental Science & Technology* 42:6424-6430.

Knauert S, Dawo U, Hollender J, Hommen U, Knauer K. 2009. Effects of photosystem II inhibitors and their mixture on freshwater phytoplankton succession in outdoor mesocosms. *Environmental Toxicology and Chemistry* 28:836-845.

Knowles A. 2008. Recent developments of safer formulations of agrochemicals. *The Environmentalist* 28:35-44.

Könemann H. 1981. Fish toxicity tests with mixtures of more than two chemicals: a proposal for a quantitative approach and experimental results. *Toxicology* 19:229-238.

Konstantinou IK. 2006. Antifouling Paint Biocides. Springer.

Kortenkamp A, Faust M, Scholze M, Backhaus T. 2007. Low-Level Exposure to Multiple Chemicals: Reason for Human Health Concerns? *Environmental Health Perspectives* 115:106–114.

Kortenkamp A, Backhaus T, Faust M. 2009. State of the Art Report on Mixture Toxicity. Final Report (22 December 2009), Available online at: http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf.

Kortenkamp A, Faust M. 2010. Combined exposures to anti-androgenic chemicals: steps towards cumulative risk assessment. *International Journal of Andrology* 33:463-474.

Koutsaftis A, Aoyama I. 2006. The interactive effects of binary mixtures of three antifouling biocides and three heavy metals against the marine algae Chaetoceros gracilis. *Environmental Toxicology* 21:432-439.

Koutsaftis A, Aoyama I. 2007. Toxicity of four antifouling biocides and their mixtures on the brine shrimp Artemia salina. *Science of The Total Environment* 387:166-174.

Krogh KA, Halling-Sørensen B, Mogensen BB, Vejrup KV. 2003. Environmental properties and effects of nonionic surfactant adjuvants in pesticides: a review. *Chemosphere* 50:871-901.

Kudsk P. 2008. Optimising herbicide dose: a straightforward approach to reduce the risk of side effects of herbicides. *The Environmentalist* 28:49-55.

Laetz CA, Baldwin DH, Collier TK, Hebert V, Stark JD, Scholz NL. 2009. The Synergistic Toxicity of Pesticide Mixtures: Implications for Risk Assessment and the Conservation of Endangered Pacific Salmon. *Environ Health Perspect* 117.

Lipok J, Studnik H, Gruyaert S. 2010. The toxicity of Roundup® 360 SL formulation and its main constituents: Glyphosate and isopropylamine towards non-target water photoautotrophs. *Ecotoxicology and Environmental Safety* 73:1681-1688.

Manzo S, Buono S, Cremisini C. 2008. Predictability of Copper, Irgarol, and Diuron Combined Effects on Sea Urchin Paracentrotus lividus. *Archives of Environmental Contamination and Toxicology* 54:57-68.

Meek M.E. (Bette), Boobis AR, Crofton KM, Heinemeyer G, Raaij MV, Vickers C. 2011. Risk assessment of combined exposure to multiple chemicals: A WHO/IPCS framework. *Regulatory Toxicology and Pharmacology* 60:S1-S14.

Mochida K, Ito K, Harino H, Kakuno A, Fujii K. 2006. Acute toxicity of pyrithione antifouling biocides and joint toxicity with copper to red sea bream (Pagrus major) and toy shrimp (Heptacarpus futilirostris). *Environmental Toxicology and Chemistry* 25:3058-3064.

Molander S, Dahl B, Blanck H, Jonsson J, Sjöström M. 1992. Combined effects of tri-*n*-butyl tin (TBT) and diuron on marine periphyton communities detected as pollution-induced community tolerance. *Archives of Environmental Contamination and Toxicology* 22:419-427.

Moore DRJ, Caux P-Y. 1997. Estimating low toxic effects. *Environmental Toxicology and Chemistry* 16:794-801.

Moreno J, Falcó JV, Oltra MT, Jiménez R. 2008. The requirement for the inclusion of formulation efficacy trials in pesticide preregistration evaluations. *Pest Management Science* 64:527-535.

Nordic Council of Ministers. 2012. Chemical cocktails – a serious matter of concern. Copenhagen. Available online at: http://www.norden.org/en/publications/publikationer/2012-711

Norwood WP, Borgmann U, Dixon DG, Wallace A. 2003. Effects of Metal Mixtures on Aquatic Biota: A Review of Observations and Methods. *Human and Ecological Risk Assessment: An International Journal* 9:795 - 811.

OECD. 2002. Avian Acute oral Toxicity Test. Draft Guideline 223. Organisation for Economic Co-operation and Development - OECD.

Olmstead A, LeBlanc G. 2005. Toxicity assessment of environmentally relevant pollutant mixtures using a heuristic model. *Integrated Environmental Assessment and Management* 1:114-122.

Padrós J, Pelletier É, Reader S, Denizeau F. 2000. Mutual in vivo interactions between benzo[a]pyrene and tributyltin in brook trout (Salvelinus fontinalis). *Environmental Toxicology and Chemistry* 19:1019-1027.

Padrós J, Pelletier É, Ribeiro CO. 2003. Metabolic interactions between low doses of benzo[a]pyrene and tributyltin in arctic charr (salvelinus alpinus): a long-term in vivo study. *Toxicology and Applied Pharmacology* 192:45-55.

Paquin PR, Gorsuch JW, Apte S, Batley GE, Bowles KC, Campbell PGC, Delos CG, Di Toro DM, Dwyer RL, Galvez F, Gensemer RW, Goss GG, Hogstrand C, Janssen CR, McGeer JC, Naddy RB, Playle RC, Santore RC, Schneider U, Stubblefield WA, Wood CM, Wu KB. 2002. The biotic ligand model: a historical overview. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 133:3-35.

Paul EA, Simonin HA, Tomajer TM. 2005. A Comparison of the Toxicity of Synergized and Technical Formulations of Permethrin, Sumithrin, and Resmethrin to Trout. *Archives of Environmental Contamination and Toxicology* 48:251-259.

Pereira J, Antunes S, Castro B, Marques C, Gonçalves A, Gonçalves F, Pereira R. 2009. Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient. *Ecotoxicology* 18:455-463.

PflSchAnwV. 1992. Verordnung über Anwendungsverbote für Pflanzenschutzmittel (Pflanzenschutz-Anwendungsverordnung) vom 10. November 1992 (BGBl. I S. 1887), die zuletzt durch Artikel 2 der Verordnung vom 20. Dezember 2011 (BGBl. I S. 2927) geändert worden ist.

PflSchG. 2012. Gesetz zum Schutz der Kulturpflanzen (Pflanzenschutzgesetz - PflSchG) vom 6. Februar 2012 (BGBl. I S. 148, 1281).

PflSchMGV. 1987. Verordnung über Pflanzenschutzmittel und Pflanzenschutzgeräte (Pflanzenschutzmittelverordnung). Pflanzenschutzmittelverordnung in der Fassung der Bekanntmachung vom 9. März 2005 (BGBl. I S. 734), zuletzt geändert durch Artikel 3 Abschnitt 2 § 7 des Gesetzes vom 13. Dezember 2007 (BGBl. I S. 2930).

Posthuma L, de Zwart D. 2006. Predicted effects of toxicant mixtures are confirmed by changes in fish species assemblages in Ohio, USA, rivers. *Environmental Toxicology and Chemistry* 25:1094-1105.

PPR. 2010. Scientific Opinion on the development of specific protection goal options for environmental risk assessment of pesticides, in particular in relation to the revision of the Guidance Documents on Aquatic and Terrestrial Ecotoxicology (SANCO/3268/2001 and SANCO/10329/2002) *EFSA Journal* 8:1821 [1855 pp.].

Price PS, Han X. 2011. Maximum Cumulative Ratio (MCR) as a Tool for Assessing the Value of Performing a Cumulative Risk Assessment. *International Journal of Environmental Research and Public Health* 8:2212-2225.

PSD. 2009. Ecotoxicology Guideline.Formulation studies in ecotoxicology:Guidance on the need for studies and their use in risk assessment. March 2009. HSE - Health and Safety Executive / Pesticide Safety Directorate.

Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. *Official Journal of the European Union* L309:52:1-50.

Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008. 2008. Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006 (Text with EEA relevance). *Official Journal of the European Union* 51:1-1355.

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. *Official Journal of the European Union* 49:1-849.

Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products (Text with EEA relevance). *Official Journal of the European Union* 55:1-123.

Reinert KH, Giddings JM, Judd L. 2002. Effects analysis of time-varying or repeated exposures in aquatic ecological risk assessment of agrochemicals. *Environmental Toxicology and Chemistry* 21:1977-1992.

Ren Q, Paulsen IT. 2005. Comparative Analyses of Fundamental Differences in Membrane Transport Capabilities in Prokaryotes and Eukaryotes. *PLoS Computational Biology* 1:e27.

Rodin VB, Zhigletsova SK, Kobelev VS, Akimova NA, Kholodenko VP. 2005. Efficacy of individual biocides and synergistic combinations. *International Biodeterioration & Biodegradation* 55:253-25Ryckaert B, Spanoghe P, Haesaert G, Heremans B, Isebaert S, Steurbaut W. 2007. Quantitative determination of the influence of adjuvants on foliar fungicide residues. *Crop Protection* 26:1589-1594.

Ryckaert B, Spanoghe P, Heremans B, Haesaert G, Steurbaut W. 2008. Possibilities To Use Tank-Mix Adjuvants for Better Fungicide Spreading on Triticale Ears. *Journal of Agricultural and Food Chemistry* 56:8041-8044.

Santos MM, Armanda Reis-Henriques M, Natividade Vieira M, Solé M. 2006. Triphenyltin and tributyltin, single and in combination, promote imposex in the gastropod Bolinus brandaris. *Ecotoxicology and Environmental Safety* 64:155-162.

Schäfer RB, von der Ohe PC, Kühne R, Schüürmann G, Liess M. 2011. Occurrence and Toxicity of 331 Organic Pollutants in Large Rivers of North Germany over a Decade (1994 to 2004). *Environmental Science & Technology* 45:6167-6174.

Schmidt K, Staaks GBO, Pflugmacher S, Steinberg CEW. 2005. Impact of PCB mixture (Aroclor 1254) and TBT and a mixture of both on swimming behavior, body growth and enzymatic biotransformation activities (GST) of young carp (Cyprinus carpio). *Aquatic Toxicology* 71:49-59.

Scholze M, Backhaus T, Faust M, Junghans M, Grimme LH. 2003. Framework of minimmum biometrical demands in terms of data quality and quantity for the predictive assessment of mixture toxicity. EU-Project BEAM (Bridging Effect Assessment of Mixtures to Ecosystem Situations and Regulation) - EVK1-CT1999-00012, Workpackage 6, Deliverable 11, Final Report to project partners, consultants, and European Commission services.

Sopeña F, Maqueda C, Morillo E. 2009. Formulation affecting alachlor efficacy and persistence in sandy soils. *Pest Management Science* 65:761-768.

Sprague JB. 1970. Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. *Water Research* 4:3-32.

Streffer C, Bucker J, Cansier A, Cansier D, Gethmann CF, Guderian R, Hanekamp G, Henschler D, Pach G, Rehbinder E. 2000. Umweltstandards: Kombinierte Expositionen Und Ihre Auswirkungen Auf Den Menschen Und Seine Umwelt. Springer.

Teather K, Jardine C, Gormley K. 2005. Behavioral and sex ratio modification of Japanese medaka (Oryzias latipes) in response to environmentally relevant mixtures of three pesticides. *Environmental Toxicology* 20:110-117.

Teisseire H, Couderchet M, Vernet G. 1999. Phytotoxicity of diuron alone and in combination with copper or folpet on duckweed (Lemna minor). *Environmental Pollution* 106:39-45.

Teuschler LK, Hertzberg RC. 1995. Current and future risk assessment guidelines, policy, and methods development for chemical mixtures. *Toxicology* 105:137-144.

Teuschler LK. 2007. Deciding which chemical mixtures risk assessment methods work best for what mixtures. *Toxicology and Applied Pharmacology* 223:139-147.

Thomas KV. 2001. The environmental fate and behaviour of antifouling paint booster biocides: A review. *Biofouling* 17:73-86.

Thompson HM. 1996. Interactions between pesticides; a review of reported effects and their implications for wildlife risk assessment. *Ecotoxicology* 5:59-81.

Tišler T, Jemec A, Mozetič B, Trebše P. 2009. Hazard identification of imidacloprid to aquatic environment. *Chemosphere* 76:907-914.

U.S. EPA. 1986. Guidelines for the Health Risk Assessment of Chemical Mixtures. EPA/630/R-98/002. September 1986. Published on September 24, 1986, Federal Register 51(185):34014-34025. Risk Assessment Forum, U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA. 2002. Guidance on Cumulative Risk Assessment of Pesticide Chemicals That Have a Common Mechanism of Toxicity.

Vaj C, Barmaz S, Sørensen PB, Spurgeon D, Vighi M. 2011. Assessing, mapping and validating site-specific ecotoxicological risk for pesticide mixtures: A case study for small scale hot spots in aquatic and terrestrial environments. *Ecotoxicology and Environmental Safety* 74:2156-2166.

Valko M, Morris H, Cronin MTD. 2005. Metals, Toxicity and Oxidative Stress. *Current Medicinal Chemistry* 12:1161-1208.

Van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE. 2006. The 2005 World Health Organization Reevaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-Like Compounds. *Toxicological Sciences* 93:223-241.

van Vlaardingen PLA, Verbruggen EMJ. 2007. Guidance for the derivation of environmental risk limits within the framework of 'International and national environmental quality standards for substances in the Netherlands' (INS), Revision 2007. RIVM report 601782001/2007, Bilthoven, The Netherlands.

van Vliet P. 2010. Evaluation manual for the authorisation of plant protection products. NL part. Plant protection products. Chapter 7 Ecotoxicology; aquatic. Appendix C: Combination toxicity. version 1.0; January 2010, Available online at : http://www.ctb.agro.nl/portal/page?_pageid=33,132770&_dad=portal&_schema=PORTAL. College voor de toelating van gewasbeschermingsmiddelen en biociden - (ctgb).

Vasseur P, Dive D, Sokar Z, Bonnemain H. 1988. Interactions between copper and some carbamates used in phytosanitary treatments. *Chemosphere* 17:767-782.

Verbruggen EMJ, Van den Brink PJ. 2010. RIVM Report 601400001/2010. Review of recent literature concerning mixture toxicity of pesticides to aquatic organisms. RIVM (National Institute for Public Health and the Environment)

Vighi M, Altenburger R, Arrhenius Å, Backhaus T, Bödeker W, Blanck H, Consolaro F, Faust M, Finizio A, Froehner K, Gramatica P, Grimme LH, Grönvall F, Hamer V, Scholze M, Walter H. 2003. Water quality objectives for mixtures of toxic chemicals: problems and perspectives. *Ecotoxicology and Environmental Safety* 54:139-150.

Villaverde J, Pérez-Martínez JI, Maqueda C, Ginés JM, Morillo E. 2005. Inclusion complexes of α- and γ-cyclodextrins and the herbicide norflurazon: I. Preparation and characterisation. II. Enhanced solubilisation and removal from soils. *Chemosphere* 60:656-664.

Voulvoulis N, Scrimshaw MD, Lester JN. 1999. Alternative antifouling biocides. *Applied Organometallic Chemistry* 13:135-143.

Wang C, Zhao Y, Zheng R, Ding X, Wei W, Zuo Z, Chen Y. 2006. Effects of tributyltin, benzo[a]pyrene, and their mixture on antioxidant defense systems in Sebastiscus marmoratus. *Ecotoxicology and Environmental Safety* 65:381-387.

Wang W, Lampi MA, Huang X-D, Gerhardt K, Dixon DG, Greenberg BM. 2009. Assessment of mixture toxicity of copper, cadmium, and phenanthrenequinone to the marine bacterium Vibrio fischeri. *Environmental Toxicology* 24:166-177.

Wilkinson CF, Christoph GR, Julien E, Kelley JM, Kronenberg J, McCarthy J, Reiss R. 2000. Assessing the Risks of Exposures to Multiple Chemicals with a Common Mechanism of Toxicity: How to Cumulate? *Regulatory Toxicology and Pharmacology* 31:30-43.

Wu Y-q, Wang C-g, Wang Y, Zhao Y, Chen Y-x, Zuo Z-h. 2007. Antioxidant responses to benzo[a]pyrene, tributyltin and their mixture in the spleen of Sebasticus marmoratus. *Journal of Environmental Sciences* 19:1129-1135.

Zheng R, Wang C, Zhao Y, Zuo Z, Chen Y. 2005. Effect of tributyltin, benzo(a)pyrene and their mixture exposure on the sex hormone levels in gonads of cuvier (Sebastiscus marmoratus). *Environmental Toxicology and Pharmacology* 20:361-367.

Zhou S, Wang L, Zhang A, Lin K, Liu W. 2008. Preparation, Stabilization, and Bioefficacy of β-Cyclodextrin Inclusion Compounds of Chloramidophos. *Journal of Agricultural and Food Chemistry* 56:2708-2713.

Zhou S, Zhang D, Yang H, Zhang Y, Liu W. 2009. Mechanisms of Composition Change and Toxic Potentiation of Chloramidophos Emulsifiable Concentrate during Storage. *Journal of Agricultural and Food Chemistry* 57:930-937.

Zhou X, Hideo O, Shinichi N. 2006. Remarkable Synergistic Effects in Antifouling Chemicals against Vibrio fischeri in a Bioluminescent Assay. *Journal of Health Science* 52:243-251.

11 List of Articles

List of articles by participants of the project in context of the project.
Available in electronic format in the attached Annexes.

1. Altenburger R. 2011. Combined effects for metal co-exposure in ecotoxicology. Accepted manuscript published in: Sigel, A., Sigel, H., Sigel, R.K.O. (eds.): Metal Ions in toxicology: effects, Interactions, Interdependencies. *Metal ions in life sciences Vol. 8*. Royal Society of Chemistry, Cambridge, 1-26.
2. Altenburger, R, Scholz, S, Schmitt-Jansen, M, Busch, W, Escher, BI. 2012. Mixture toxicity revisited from a toxicogenomic perspective. Accepted manuscript published in: Environ. Sci. Technol. 46 (5), 2508 - 2522
3. Backhaus, T, Faust, M. 2012. Predictive environmental assessment of chemical mixtures: A conceptual framework. Environ Sci Technol. Doi 10.1021/es2034125
4. Coors, A, Frische, T. 2011. Predicting the aquatic toxicity of commercial pesticide mixtures. Env Sci Europe 23:22.
5. Backhaus T, Altenburger, R, Faust, M, Frein D, Frische T, Johansson P, Kehrer A, Porsbring T. Proposal for environmental mixture risk assessment in the context of the biocidal product authorization in the EU. submitted to Env Sci Europe