Environmental Research of the
Federal Ministry for the
Environment, Nature Conservation,
Building and Nuclear Safety

Project number: (FKZ) 3713 64 417

Report number: [entered by the UBA library]

Necessity of testing biocidal products and their eluates within the regulatory authorization process aiming for an adequate environmental assessment of mixtures – extending the database for wood preservative products

by

Anja Coors [1], Pia Vollmar [1], Frank Sacher [2]

[1] ECT Oekotoxikologie GmbH, Böttgerstraße 2 – 14, 65439 Flörsheim am Main, Germany

[2] TZW: DVGW-Technologiezentrum Wasser, Karlsruher Straße 84, 76139 Karlsruhe, Germany

On behalf of the German Environment Agency

Completion date November 2016

Abstract

Biocidal products are formulated preparations that contain one or more active substances and additives added to serve various functions. They thereby represent intentional mixtures of chemical substances that may reach the environment in their initial or in a changed composition. The present project addressed three aspects in a mixture risk assessment of biocidal products, which is required during the regulatory authorisation. These aspects range from direct regulatory application (component-based aquatic risk assessment of products) to more science-oriented exploratory work (indication for synergistic interactions and prediction of mixture toxicity in terrestrial organisms). No indication for synergistic interaction was found for the effects of fungicides that inhibit different enzymes in the same biosynthesis pathway. The terrestrial experiments provided evidence that mixture effects on the reproduction of collembolans can be fairly well predicted by concentration addition, while avoidance behaviour of collembolans was strongly underestimated. Based on a theoretical evaluation of 30 marketed biocidal products and experimental aquatic testing of seven wood preservative products, a proposal was developed for the identification of additives as relevant for a component-based assessment of biocidal products that could supersede experimental testing of biocidal products in the regulatory assessment. The proposed criteria for identifying 'mixture-relevant' additives (to be considered in addition to the active substances) relate to a combination of their aquatic toxicity and their content in the product as detailed in the regulation for classification, labelling and packaging (Regulation (EC) No 1272/2008). The availability and correctness of toxicity data for the relevant additives in a biocidal product were identified as the key factors determining the reliability and protectiveness of a component-based environmental risk assessment.

Kurzbeschreibung

Biozidprodukte enthalten einen oder mehrere Wirkstoffe sowie Beistoffe, welche verschiedene Funktionen im formulierten Produkt erfüllen. Damit stellen Biozidprodukte beabsichtigte Mischungen aus chemischen Substanzen dar, die in ihrer ursprünglichen oder in einer veränderten Zusammensetzung in die Umwelt gelangen können. Das vorliegende Projekt befasste sich mit drei Aspekten einer Mischungs-Risikobewertung von Biozidprodukten, die im Zulassungsverfahren erforderlich ist. Diese Aspekte reichen von der direkten, regulatorischen Anwendung (Komponenten-basierte aquatische Risikobewertung von Produkten) bis hin zu mehr wissenschaftlichen Fragestellungen (Hinweise auf synergistische Interaktionen und Vorhersage von Mischungstoxizität bei Bodenorganismen). Für Mischungen von Fungiziden, die unterschiedliche Enzyme im selben Biosynthesepfad blockieren, wurden keine Anzeichen für synergistischen Effekte gefunden. Die terrestrischen Experimente zeigten, dass Mischungseffekte auf die Reproduktion von Collembolen durch das Konzept der Konzentrations-Additivität recht gut vorhergesagt werden können, während das Vermeidungsverhalten der Collembolen durch die Mischungsvorhersage stark unterschätzt wurde. Basierend auf einer theoretischen Bewertung von 30 zugelassenen Biozidprodukten und der experimentellen Untersuchung von sieben Holzschutzmitteln, wurde ein Vorschlag entwickelt, welche Beistoffe eines Produktes als relevant für eine komponenten-basierte Bewertung einzustufen sind. Durch eine komponenten-basierte Bewertung kann das experimentelle Testen von Biozidprodukten im Rahmen der Zulassung ersetzt werden. Die vorgeschlagenen Kriterien für die Einstufung von Beistoffen als „relevant für die Mischung" (zusätzlich zu den Wirkstoffen des Produktes) beruhen auf der Kombination ihrer aquatischen Toxizität und ihrem Gehalt im Produkt analog zur Verordnung über die Einstufung, Kennzeichnung und Verpackung von Stoffen und Gemischen (Verordnung (EC) No 1272/2008). Als Schlüsselfaktoren zur Gewährleistung einer verlässlichen und protektiven Komponenten-basierten Umweltrisikobewertung stellten sich die Verfügbarkeit und Richtigkeit der Toxizitätsdaten für die relevanten Beistoffe des Biozidprodukts heraus.

Table of Contents

List of Figures 8
List of Tables 9
List of Abbreviations 11
Summary 13
Zusammenfassung 16
1 Introduction 19
2 Formulation Additives in Biocidal Products 22
2.1 Set of criteria for the category 'CLP' 23
2.2 Set of criteria for the category 'HAZ' 24
2.3 Set of criteria for the category '>10% STU' 25
2.4 Composition of wood preservatives 26
2.4.1 Formulation additives categorized as 'CLP' 28
2.4.2 Formulation additives categorized as 'HAZ' 29
2.4.3 Formulation additives categorized as '>10% STU' 29
2.4.4 Overlap between the three categories tentatively identifying relevant additives 30
2.5 Rodenticides 33
2.6 Insecticides, acaricides and products to control other arthropods 34
3 Experimental Verification of Mixture Toxicity Predictions 35
3.1 Experimental testing of wood preservative products 35
3.1.1 Growth inhibition of green algae 35
3.1.2 Immobility of Daphnia magna 36
3.1.3 Mortality of fish embryos 37
3.1.4 Verification of exposure concentrations 37
3.1.5 Data analysis 37
3.1.6 Mixture toxicity prediction and comparison with observation 38
3.2 Products selected for experimental testing 39
3.3 Observed aquatic toxicity of tested biocidal products and comparison with predictions 43
3.3.1 Product 6 43
3.3.2 Product 10 44
3.3.3 Product 14 45
3.3.4 Product 16 45
3.3.5 Product 20 46
3.3.6 Product 31 47
3.3.7 Product 32 48

4 Compliance between mixture toxicity prediction and observation and reasons for toxicity overestimation 50
5 Proposal for identification of 'mixture relevant' product components 53
6 Synergistic interactions in biocidal products 56
6.1 Piperonylbutoxide as an example of a synergist 56
6.2 Mixtures of ergosterol biosynthesis inhibitors 59
7 Mixture toxicity of wood preservative products in the terrestrial compartment 62
7.1 Experimental methods 62
7.1.1 Performance of reproduction tests 62
7.1.2 Performance of avoidance tests 63
7.1.3 Data analysis 63
7.2 Predictability of reproduction inhibition and avoidance behaviour in collembolans and relationship between the two endpoints 63
8 Outlook 65
9 References 66
10 Confidential Annex I 71
10.1 A: Study Reports Green Algae 71
10.2 B: Study Reports Daphnia magna 71
10.3 C: Study Reports Fish Embryo Toxicity 71
10.4 D: Study Reports Collembola 72

List of Figures

Figure 1: Venn diagram of additives allocated to the three different categories 30

Figure 2: Venn diagram of additives allocated to the three different categories after considering ready biodegradability 32

List of Tables

Table 1: Summary of criteria used to derive a categorisation as 'CLP' in the present study 24

Table 2: Summary of criteria prescribed in the REACH regulation that determines whether a component of a mixture shall be labelled as 'hazardous to the environment' on the SDS 25

Table 3: Key information regarding the 21 wood preservative products 26

Table 4: Average composition of the 21 wood preservative products 27

Table 5: Additives in the 21 wood preservative products categorized as 'CLP' 28

Table 6: Additives in the 21 wood preservative products categorized as 'HAZ' 29

Table 7: Theoretical contribution of the active substances and additives in the 21 wood preservative to the joint toxicity and additives categorised as '>10% STU' 30

Table 8: Additives in the 21 wood preservative products labelled as hazardous to the environment 31

Table 9: Composition of the six rodenticides 33

Table 10: Additives in the 6 rodenticide products categorized as 'CLP', 'HAZ', or '>10% STU' 33

Table 11: Subsets of product components for which mixture toxicity calculations were conducted, if applicable 39

Table 12 Key characteristics of the seven wood preservative products selected for experimental testing 40

Table 13: Key characteristics of the active substances in the wood preservative products selected for testing 42

Table 14: Observed aquatic toxicity of product 6 and resulting MDR values 43

Table 15: Observed aquatic toxicity of product 10 and resulting MDR values 44

Table 16: Observed aquatic toxicity of product 14 and resulting MDR values 45

Table 17: Observed aquatic toxicity of product 16 and resulting MDR values 46

Table 18: Observed aquatic toxicity of product 20 and resulting MDR values 47

Table 19: Observed aquatic toxicity of product 31 and resulting MDR values 48

Table 20: Observed aquatic toxicity of product 32 and resulting MDR values 49

Table 21 Synergistic and antagonistic effects of PBO documented in the literature 57

Table 22: Toxicity estimates (mg/l) derived for tebuconazole, fenpropidin, and fenpropimorph with the green algae R. subcapitata 60

Table 23: Model deviation ratios (MDR) for binary equipotent mixtures of ergosterol biosynthesis inhibitors in the green algae R. subcapitata. 61

Table 24: Toxicity estimates derived in reproduction and avoidance tests with the collembolan F. candida and resulting model deviation ratios (MDR).....64

List of Abbreviations

ANOVA	Analysis of Variance
a.s.	Active substance
BPD	Biocidal Product Directive
BPR	Biocidal Product Regulation
CA	Concentration Addition
CAS	Chemical Abstracts Service
CBA	Component-Based Approach
CLP	Classification, Labelling and Packaging of Chemicals
DMI	Demethylase Inhibitor
EBI	Ergosterol Biosynthesis Inhibitor
EC	European Commission
ECHA	European Chemicals Agency
EC_x	Effect concentration causing x% effect
E_bC_x	Effect concentration causing x% reduction of biomass
E_rC_x	Effect concentration causing x% reduction of growth rate
EU	European Union
FET	Fish embryo toxicity
GLP	Good Laboratory Practise
HAZ	Classification group in present study for additives declared as hazardous for the environment on the product SDS
IA	Independent Action
IPBC	3-Iodo-2-propynylbutylcarbamate
LD/LC_{50}	Dose/concentration causing 50% lethality
LOEC	Lowest observed effect concentration
MAK	Maximale Arbeitsplatz Konzentration (work place safety limits)
MDR	Model Deviation Ratio
M-Factor	Multiplication factor according to CLP regulation, Annex I Part 4
MS	Member State
NOEC	No observed effect concentration
OC	Organic Carbon
OECD	Organization for Economic Co-Operation and Development
QSAR	Quantitative structure-activity relationships
PAH	Polycyclic aromatic hydrocarbon
PBO	Piperonylbutoxide

PBT	Persistent, Bioaccumulative, and Toxic
PT	Product Type
RBD	Ready biodegradability
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
SD	Standard Deviation
SDS	Safety Data Sheet
SoC	Substance of Concern
STU	Sum of toxic units
TU	Toxic Units
twa	Time-Weighted Average
U.S. EPA	United States Environment Protection Agency
$WHC_{(max)}$	(Maximum) Water Holding Capacity

Summary

Biocidal products are formulated preparations that contain one or more active substances (a.s.) and various intentionally added substances serving a broad range of functions (i.e., formulation additives). Biocidal products thereby represent intentional mixtures of chemical substances that may reach the environment in their initially formulated composition or in a changed composition due to differences in the properties of the components (e.g., differences in leaching behaviour or differences in biodegradability). The environmental risk assessment required to be conducted during the authorisation process of a biocidal product shall take into account also potential synergistic and additive joint effects of the components of this product. During the lifetime of the present project, the European Chemicals Agency (ECHA) released a transitional guidance document that provides detailed advice on how such mixture effects represented by biocidal products shall be taken into account in the environmental risk assessment. Together with results from a previous project funded by the German Environmental Agency, the present project informed the development of this transitional guidance and shall further support potentially necessary adaptations and the finalisation of open issues in this guideline.

The transitional guidance favours a component-based approach, while testing of the product (i.e. the whole-mixture approach) is foreseen as an option in case of limiting data availability or if higher tiers based on the theoretical assessment still indicate non-acceptable environmental risks. The component-based approach predicts the toxicity of the mixture (here: the biocidal product) using the toxicity estimates of the individual components that were identified as relevant for such an approach. The recommended default for this prediction is the concept of concentration addition (CA). The present project was set up to address three aspects that arise when applying CA for the environmental risk assessment of biocidal products as mixtures:

1. the identification of additives relevant for a component-based approach,
2. the problem of potential synergistic interactions, and
3. mixture toxicity with regard to terrestrial endpoints.

Regarding the first aspect, the transitional guidance states as relevant substances the a.s. of the product, a.s. from any other biocidal product types, any other substances of concern (SoC), and, on a case-by-case basis, synergists. The definition of the SoC in this context is currently under discussion among the competent authorities. The present project established different sets of criteria that tentatively identified product components as relevant within a component-based assessment approach. As a first step in the process of developing a proposal for the identification of 'relevant' components, a survey of formulation additives in 30 authorised biocidal products was conducted, and the additives were categorized according to the various, above mentioned sets of criteria. As a second step, seven selected wood preservative products were experimentally tested for their acute aquatic toxicity using three different test organisms (the water flea *Daphnia magna*, the green algae *Raphidocelis subcapitata*, and embryos of the freshwater fish *Danio rerio*). The results of this whole-mixture approach (i.e., the experimentally observed product toxicity) were compared to the results of a component-based approach (i.e. the theoretically predicted toxicity of the product). These comparisons were performed taking the previously defined different sets of criteria for 'relevant' product compounds into account in order to evaluate which components were indeed relevant for a correct (or at least protective) prediction of aquatic toxicity. The criteria applied to the product components were, hence, solely used to explore their usability in terms of a component-based assessment approach of biocidal products, but not with regard to a classification or an actual environmental risk assessment of the selected wood preservative products.

The results of this first and main aspect of the project can be summarized as follows. There was a large number of very diverse formulation additives contained in the 30 investigated biocidal products, and aquatic toxicity data could not be retrieved for all of them. The compiled acute aquatic toxicity data

used for predicting the toxicity of the products were heterogeneous with regard to endpoints and test organisms, thereby contributing to deviations between predicted and observed mixture toxicity. Product toxicity was frequently overestimated by the CA prediction (i.e., the product was less toxic than predicted), which is most likely due to the fact that several worst-case assumptions were made when using the compiled available data for the predictions. The toxic unit approach indicated that formulation additives dominated the aquatic toxicity of the most sensitive endpoint in almost half of the wood preservative products, which was confirmed by the experimental testing. This finding clearly underlines the importance of considering not only the a.s. but also additives in a component-based assessment. Including all additives in the mixture toxicity prediction would be the most precautious and conservative approach. Yet, there was no indication from the experimental testing of products that this approach would actually result in a more protective regulatory decision. Including all additives increased the degree of overestimation in some cases, but never reduced unexplained underestimation of toxicity. This finding demonstrates that a clear and reliable concept is needed to identify the essential additives to be considered in addition to the a.s. of a product in a component-based environmental risk assessment of a biocidal product. A need to consider by default all a.s. from other biocidal product types in the CBA was not supported by the present project, as preservatives (the only additives from other product types in the investigated products) were not found necessary to explain the experimentally observed toxicity. The results further demonstrated that a CBA including only the additives that lead to a labelling of the product as hazardous to the environment would ignore substantially contributing components and result in a (potentially non-protective) underestimation of product toxicity. This was indicated by the (theoretical) toxic unit approach and verified by experimental testing. Overall, the results support an approach for identifying additives (in addition to the a.s. of the product) as relevant for a component-based mixture assessment that is based on some criteria of the regulation for classification, labelling, and packaging (CLP), restricted to the combination of aquatic toxicity and the content of the respective substance(s) in the mixture. Specifically, any additive should be considered in the prediction where either i) the lowest available endpoint for aquatic toxicity is ≤1 mg/l and the content in the product ≥0.1% w/w after multiplication of the actual concentration with the respective multiplication-factor (as defined in the CLP regulation) or ii) the lowest available endpoint for aquatic toxicity is >1 mg/l and ≤100 mg/l, and the content in the product is ≥1% w/w. An additional criterion may be the listing of an additive as hazardous to the environment on the safety data sheet of the biocidal product (regardless of the classification of the product itself as hazardous or not) as this criterion may cover specific hazard properties that are not included in the CLP-based criteria stated above. For the here investigated set of products, these proposed criteria for identifying 'mixture-relevant' components include all additives that are expected to contribute at least 20% to the overall toxicity, based on the toxic unit approach. Based on the proposed criteria, in 48 out of 273 cases (i.e., 17.6%) an additive was identified as relevant in the 21 wood preservative products. Hence, 2-3 additives would have to be considered on average along with the a.s. in a prediction.

Extending this approach for selecting mixture-relevant additives to other product types and the actual environmental mixture that results from the usage of a biocidal product remains an open issue that was not experimentally investigated in the present project. However, there is no obvious reason why this approach could not be applied to these mixtures as well. For an environmental mixture resulting from the usage of a biocidal product (such as eluates from treated wood), fewer additives would often be identified as relevant for a mixture assessment if fate-related properties such as biodegradability were taken into account. If 'ready biodegradability' were used additionally as an exclusion criterion, the above stated number of 48 additives to be considered were reduced to 21 cases, i.e. on average about one additive per product.

With regard to the second aspect, the problem of potential synergists, the known synergist piperonyl-butoxide (PBO) was considered to serve as an example, and a comprehensive literature search on synergistic interactions of PBO with other chemicals was conducted. Yet, no marketed wood preservative

product with PBO was found, and PBO was classified lately as a.s instead of being an additive. The experimental focus of the synergistic interaction aspect of the project therefore switched to exploring one possible mechanism of synergistic interaction, the inhibition of different enzymes in the same biochemical pathway. Specifically, the joint effects of fungicides inhibiting different enzymes in the ergosterol synthesis was studied in green algae, as previous studies indicated synergistic interaction for this scenario. However, the results of algal growth inhibition tests conducted in the present project with mixtures of ergosterol biosynthesis inhibitors (tebuconazole & fenpropidin as well as tebuconazole & fenpropimorph) provided no evidence for a synergistic interaction. Hence, the hypothesis of synergistic interactions occurring generally between substances inhibiting different enzymes in the same biosynthesis pathway is contradicted by these two examples. The single-substance tests for the three fungicides determined toxicity estimates that differed (partly considerably) from the endpoints used in regulatory assessments and previous studies that reported indication for synergistic interaction, which may explain the discrepancy to earlier indication of synergistic interaction.

The third aspect, terrestrial mixture toxicity, was addressed using collembolans as non-target test organisms. The suitability of the CA concept for predicting the toxicity of a wood preservative product was experimentally tested with regard to a chronic endpoint (reproduction) and a behavioural endpoint (avoidance of treated soil). The apical endpoint (effects on reproduction) was fairly well predictable although with a greater degree of deviation than acute aquatic toxicity. The behavioural endpoint (avoidance of treated soil) was strongly underestimated by the CA prediction for the product. It remains open whether this underestimation points at the presence of additives that induce strong avoidance (but little toxicity) or whether it indicates that behavioural responses *per se* do not follow concentration-additivity.

Overall, the present study provided extensive evidence that the toxicity of biocidal products can reliably be predicted by the concept of concentration addition in order to support a component-based environmental risk assessment. Consideration of the relevant, but not all formulation additives was shown to be the key aspect for a reliable component-based assessment. The availability and correctness of toxicity data for the relevant additives is the key factor that determines the reliability of such a component-based assessment.

Zusammenfassung

Biozidprodukte sind Formulierungen, die einen oder mehrere Wirkstoffe enthalten sowie verschiedene Beistoffe, die eine große Bandbreite an Funktionen erfüllen. Biozidprodukte repräsentieren damit beabsichtigte Mischungen aus chemischen Substanzen, die in ihrer ursprünglichen Zusammensetzung oder – aufgrund von unterschiedlichen Eigenschafen der Komponenten (z.B. Unterschiede im Auswaschungsverhalten oder in der biologischen Abbaubarkeit) – in einer veränderten Zusammensetzung in die Umwelt gelangen können. Die im Rahmen des Zulassungsverfahrens erforderliche Umweltrisikobewertung eines Biozidproduktes soll auch mögliche synergistische und additive Effekte der Produktkomponenten berücksichtigen. Während der Laufzeit dieses Projektes veröffentlichte die Europäische Chemikalienagentur (ECHA) einen vorläufigen, detaillierten Leitfaden zur Berücksichtigung der Mischungstoxizität in der Umweltrisikobewertung. Zusammen mit den Ergebnissen eines bereits abgeschlossenen, durch das Umweltbundesamt geförderten Vorhabens, trug das vorliegende Projekt zur Entwicklung des Leitfadens bei und soll notwendige Änderungen und die endgültige Klärung noch offener Punkte unterstützen.

Der vorläufige Leitfaden bevorzugt eine Komponenten-basierte Vorgehensweise in der Bewertung, während das ökotoxikologische Testen des Produktes (d.h. ein Gesamt-Mischungs-Ansatz) eine Alternative z.B. bei unzureichender Datenlage oder bei einer ggf. notwendigen verfeinerten Risikobewertung darstellt. Mit der komponenten-basierten Vorgehensweise (CBA) wird die Toxizität einer Mischung (hier: eines Biozidprodukts) berechnet, basierend auf den Toxizitätskennwerten der einzelnen Komponenten, die als relevant identifiziert wurden. Als Standard für diese Berechnung wird das Konzept der Konzentrations-Additivität (CA) empfohlen. Das vorliegende Projekt behandelte drei Aspekte, die bei der Anwendung des CA Konzepts in der Umweltrisikobewertung von Biozidprodukten eine Rolle spielen:

1. Die Identifizierung von Beistoffen, die für eine Komponenten-basierte Bewertung relevant sind,
2. die Problematik möglicher synergistischer Interaktionen, und
3. die Mischungstoxizität in Bezug auf terrestrische Endpunkte.

Bezüglich des ersten Aspekts benennt der vorläufige Leitfaden die folgenden Substanzen als relevant für eine komponenten-basierte Bewertung: die Wirkstoffe des Produktes, Wirkstoffe aus anderen Biozid-Produkttypen, alle anderen bedenklichen Beistoffe (substances of concern, SoC) sowie fallweise zu berücksichtigende Synergisten. Die Definition der SoC in diesem Zusammenhang ist zurzeit noch im Prozess der Abstimmung zwischen den zuständigen Behörden. Im vorliegenden Projekt wurden mehrere unterschiedliche Vorschläge zu Kriterien erstellt, anhand derer die für eine komponenten-basierte Bewertung relevanten Produktkomponenten identifiziert werden könnten. Als ersten Schritt in der Entwicklung eines Vorschlags zur Identifizierung von „relevanten“ Komponenten wurden die Beistoffe in 30 zugelassenen Biozidprodukten anhand der o.g. vorläufig definierten Kriterien kategorisiert. Im zweiten Schritt wurden sieben ausgewählte Holzschutzmittel experimentell auf ihre akute aquatische Toxizität hin getestet. Für die Testung wurden drei unterschiedliche Testorganismen verwendet (der Wasserfloh *Daphnia magna*, die Grünalge *Raphidocelis subcapitata* und die Embryonen des Süßwasserfisches *Danio rerio*). Die Ergebnisse dieses Gesamt-Mischungs-Ansatzes (d.h. die experimentell bestimmte Toxizität des Produktes) wurden mit den Ergebnissen des komponenten-basierten Ansatzes (d.h. der theoretisch vorhergesagten Toxizität des Produktes) verglichen. Die Vergleiche wurden unter Berücksichtigung der vorher definierten unterschiedlichen Kriterien durchgeführt, um die Komponenten zu identifizieren, die tatsächlich relevant waren für eine korrekte (oder zumindest protektive) Vorhersage der aquatischen Toxizität des Produktes. Die Kriterien, die auf die Produkt-

komponenten angewendet wurden, dienten ausschließlich zur Anwendung innerhalb des komponenten-basierten Vorgehens. Eine Einstufung oder tatsächliche Umweltrisikobewertung der ausgewählten Holzschutzmittel war nicht das Ziel des Vorhabens.

Die Ergebnisse dieses ersten und wichtigsten Teils des Vorhabens werden im Folgenden zusammengefasst. In den 30 ausgewählten Biozidprodukten war eine Vielzahl sehr unterschiedlicher Beistoffe enthalten, wobei nicht für alle aquatische Toxizitätsdaten verfügbar waren. Die ermittelten Daten zur akuten aquatischen Toxizität, die für die Vorhersage der Produkttoxizität verwendet wurden, waren sehr heterogen in Bezug auf Endpunkte und Testorganismen und trugen daher zu Abweichungen zwischen vorhergesagter und beobachteter Mischungstoxizität bei. Die Produkttoxizität wurde durch das CA-Konzept häufig überschätzt (d.h. das Produkt war weniger toxisch als berechnet). Dies kann mit großer Wahrscheinlichkeit auf die *Worst-Case* Annahmen zurückgeführt werden, die im Zuge der Berechnungen getroffen wurden. Das Konzept der Toxizitäts-Einheiten (*toxic unit approach*) zeigte, dass Beistoffe den sensitivsten Endpunkt der aquatische Toxizität in fast der Hälfte der Holzschutzmittel dominierten, was auch durch die Ergebnisse der experimentellen Untersuchungen bestätigt wurde. Dies belegt deutlich, dass nicht nur Wirkstoffe, sondern auch Beistoffe in einem Komponenten-basierten Vorgehen berücksichtigt werden sollten. Der konservativste Ansatz wäre es, alle Komponenten eines Produktes in die Mischungstoxizitäts-Vorhersage einzubeziehen. Die experimentellen Untersuchungen belegten allerdings nicht, dass dies tatsächlich zu einer protektiveren regulatorischen Entscheidung führen würde. Die Berücksichtigung aller Beistoffe erhöhte zwar in einigen Fällen den Grad der Überschätzung, reduzierte aber in keinen Fall eine Unterschätzung der Toxizität. Dieses Ergebnis zeigt, dass ein klares und verlässliches Konzept benötigt wird, um Beistoffe zu identifizieren, die neben den Wirkstoffen in einer komponenten-basierten Umweltrisikobewertung eines Biozidproduktes berücksichtigt werden sollten. Die Notwendigkeit einer standardmäßigen Berücksichtigung aller Wirkstoffe aus anderen Biozid-Produkttypen im komponenten-basierten Vorgehen wird durch die vorliegenden Ergebnisse ebenfalls nicht unterstützt. Konservierungsstoffe (als einzige Wirkstoffe anderer Produkttypen in den untersuchten Holzschutzmitteln) mussten nicht berücksichtigt werden, um die experimentell beobachtete Toxizität des Produktes zu erklären. Die Ergebnisse zeigten außerdem, dass eine Berücksichtigung nur der Beistoffe, die zur Einstufung des Produktes als „*hazardous*“ führen, zu einer Unterschätzung der Toxizität und möglicherweise zu einer nicht-protektiven Risikobewertung führen würde. Dies belegen sowohl die Berechnungen nach dem (theoretischen) Konzept der Toxizitäts-Einheiten als auch die experimentellen Untersuchungen. Insgesamt unterstützen die Ergebnisse ein Konzept, nach dem Beistoffe als relevant für eine komponenten-basierte Bewertung identifiziert werden, das analog ist zu einigen Kriterien der CLP-Verordnung. Die Kriterien zur Bestimmung von mischungsrelevanten Beistoffen beschränken sich damit auf die Kombination der aquatischen Toxizität und den Gehalt in der Mischung. Ein Beistoff sollte in der komponenten-basierten Bewertung berücksichtigt werden, wenn entweder i) sein niedrigster vorhandener Endpunkt der aquatischen Toxizität ≤1 mg/l ist und sein Gehalt im Produkt ≥0.1% w/w, nach der Multiplikation der tatsächlichen Konzentration mit dem entsprechenden Multiplikations-Faktor (wie in der CLP-Verordnung definiert) oder ii) sein niedrigster vorhandener Endpunkt der aquatischen Toxizität >1 mg/l und <100 mg/l und sein Gehalt im Produkt ≥1% w/w ist. Ein zusätzliches Kriterium könnte die Kennzeichnung des Beistoffes als „gefährlich für die Umwelt“ im Sicherheitsdatenblatt des Biozidproduktes sein (ungeachtet der Einstufung des Produktes selbst als gefährlich oder nicht). Dieses Kriterium deckt spezielle Eigenschaften ab, die nicht in den CLP-basierten Kriterien aufgeführt sind. Die vorgeschlagenen Kriterien zur Identifizierung mischungsrelevanter Komponenten umfassen für die hier untersuchten Produkte alle Beistoffe, die nach dem Konzept der Toxizitäts-Einheiten (*toxic units*) mindestens 20% zur Gesamttoxizität beitragen würden. Basierend auf den vorgeschlagenen Kriterien wurde in den 21 Holzschutzmitteln in 48 von 273 Fällen (17.6%) ein Beistoff als relevant identifiziert. Somit müssten für eine komponenten-basierte Bewertung durchschnittlich 2 bis 3 Beistoffe zusätzlich zu den Wirkstoffen berücksichtigt werden.

Die Übertragung dieser Kriterien für mischungsrelevante Beistoffe auf andere Produkttypen und die tatsächlich aus der Nutzung des Produktes in der Umwelt resultierende Mischung, bleibt eine offene Fragestellung, die in diesem Projekt nicht experimentell untersucht wurde. Es besteht jedoch kein Grund zur Annahme, dass diese Kriterien nicht auch auf andere Mischungen/Produkttypen angewendet werden könnten. Für eine Mischung in der Umwelt, die aus der Anwendung eines Biozidproduktes entsteht (wie zum Beispiel ein Eluat von behandeltem Holz), würden oftmals weniger Beistoffe als relevant für eine Mischungsbewertung erachtet werden, wenn Eigenschaften wie die biologische Abbaubarkeit in Betracht gezogen werden. Falls „leichte biologische Abbaubarkeit“ als zusätzliches Ausschlusskriterium verwendet wird, wird die oben genannte Anzahl von 48 zu berücksichtigenden Beistoffen auf 21 Fälle reduziert, was durchschnittlich einen relevanten Beistoff pro Produkt ergibt.

Bezüglich des zweiten Aspekts, der Problematik möglicher synergistischer Interaktionen, wurde der bekannte Synergist Piperonylbutoxid (PBO) als Beispiel ausgewählt und eine umfassende Literatursuche zu synergistischen Interaktionen von PBO mit anderen Chemikalien durchgeführt. Es stellte sich heraus, dass PBO in keinem zurzeit vermarkteten Holzschutzmittel enthalten ist. Der experimentelle Fokus für die Untersuchung synergistischer Interaktionen im Rahmen des Projektes wurde daher verlegt auf die Untersuchung eines möglichen Mechanismus von synergistischer Interaktion: die Blockierung verschiedener Enzyme im selben biochemischen Biosynthesepfad. Speziell die gemeinsamen Effekte von Fungiziden, die unterschiedliche Enzyme in der Ergosterol Biosynthese inhibieren, wurden in Grünalgen untersucht, da frühere Studien synergistische Interaktionen für dieses Szenario anzeigten. Die Ergebnisse der Wachstumshemmtests mit Grünalgen für Mischungen von Ergosterol Biosynthese-Inhibitoren (Tebuconazol & Fenpropidin, sowie Tebuconazol & Fenpropimorph) lieferten jedoch keinen Hinweis auf synergistische Interaktionen. Somit wurde die Hypothese von synergistischen Interaktionen, die zwischen Substanzen auftreten, welche verschiedene Enzyme im selben Biosyntheseweg inhibieren, mit diesen zwei Beispielen widerlegt. Die in Einzelsubstanz-Tests mit den drei Fungiziden ermittelten Toxizitätskennwerte, wichen (teilweise erheblich) von den Endpunkten ab, die in regulatorischen Bewertungen und früheren Studien verwendet wurden. Dies könnte möglicherweise die Diskrepanz zu früheren Hinweisen auf Synergismus erklären.

Zur Untersuchung des dritten Aspekts, der terrestrischen Mischungstoxizität, wurden Collembolen (Springschwänze) als Nicht-Zielorganismen verwendet. Die Eignung des CA-Konzepts zur Vorhersage der Toxizität von Holzschutzmitteln wurde experimentell in Bezug auf einen chronischen (Reproduktion) und auf einen verhaltensbezogenen Endpunkt (Vermeidung kontaminierten Bodens) getestet. Der apikale Endpunkt Reproduktion konnte gut vorhergesagt werden, wenn auch mit einer größeren Abweichung als bei der akuten aquatischen Toxizität. Der verhaltensbezogene Endpunkt wurde vom CA-Konzept für das ausgewählte Produkt stark unterschätzt. Es ist unklar, ob diese Unterschätzung auf die Anwesenheit von nicht-berücksichtigten Beistoffen hindeutet, die starkes Vermeidungsverhalten, aber geringe Toxizität provozieren, oder ob verhaltensbezogene Endpunkte grundsätzlich nicht dem Konzept der Konzentrations-Additivität folgen.

Insgesamt lieferte diese Studie einen umfassenden Beleg dafür, dass die Toxizität von Biozidprodukten mithilfe des Konzepts der Konzentrations-Additivität verlässlich vorhergesagt werden kann, um eine Komponenten-basierte Umweltrisikobewertung zu unterstützen. Die Berücksichtigung relevanter (aber nicht aller) Beistoffe der Formulierung wurde als Schlüssel-Element für ein verlässliches Komponenten-basiertes Vorgehen identifiziert. Der Hauptfaktor, der die Verlässlichkeit eines solchen Komponenten-basierten Vorgehens bestimmt, ist die Verfügbarkeit und Richtigkeit von Toxizitätsdaten für die relevanten Additive.

1 Introduction

The authorisation of biocidal products in the European Union (EU) follows a two-step process where an active substance (a.s.) is first authorised at the level of the EU and the products are then authorised at the level of the member states or directly union-wide. The legislative background for this process is the Biocidal Product Regulation (BPR, EC 2012), which replaced the Biocidal Product Directive (BPD, EC 1998) in September 2013. Biocidal products are formulated preparations that contain one or more a.s. and various intentionally added formulation additives serving a broad range of functions. Biocidal products thereby commonly represent intentional mixtures of chemical substances that may reach the environment in their initially formulated composition or in a changed composition due to differences in the properties of the components (e.g., differences in leaching behaviour or differences in biodegradability). Formulation additives of products (i.e., components in the product other than the a.s.) can exhibit toxicity on their own and may thereby increase the toxicity of the product as it has been shown for formulated plant protection products (Coors & Frische 2011), and as it has been critically discussed elsewhere (Cox & Surgan 2006, Weinhold 2010). The environmental risk assessment required to be conducted during the authorisation process of a biocidal product shall take into account also potential synergistic and additive joint effects of the components of this product. This is explicitly stated in Article 19, §2 (d) and (e) of the BPR (EC 2012). This statement relates to the product and thereby includes in principle the a.s. and all other contained substances (i.e., the additives) contained in the product. During the lifetime of the present project (which started in May 2013), the European Chemicals Agency (ECHA) released a transitional guidance document (ECHA 2014) that provides detailed advice on how mixture effects of biocidal products shall be taken into account in the environmental risk assessment. Together with results from a previous project funded by the German Environmental Agency (Altenburger et al. 2012, Coors et al. 2012 a,b, Backhaus et al. 2013), the present project informed the development of this transitional guidance and shall further support potentially necessary adaptations and the finalisation of open issues.

There are two fundamentally different ways to assess the effects of a multi-component product, including joint effects. That is 1) the so-called whole-mixture approach where the mixture (i.e., the formulated product or the environmental mixture resulting from its usage) is experimentally tested for its toxicity, and 2) the so-called component-based approach (CBA) where the toxicity of the mixture is theoretically predicted from the toxicity of the mixture components using established mixture concepts. There are two more possibilities to deal with the joint effects of biocidal products in a regulatory context (Backhaus et al. 2013, ECHA 2014): Applying a mixture assessment factor or making decisions based on available information for similar products (bridging). As these approaches do not aim to provide an estimate for the actual product toxicity but rather represent pragmatic regulatory solutions, they are not further considered in the present study. While the whole-mixture approach provides a straightforward effect estimate that will also cover any synergistic interaction among the components in the product, it involves a considerable amount of effort, costs and animal suffering (i.e. experimental testing with various non-target organisms, including fish). Therefore, the CBA is favoured in the transitional guidance, while the whole-mixture testing is foreseen as an alternative option e.g. in case of insufficient data availability or if higher tiers based on the theoretical assessment still indicate non-acceptable environmental risks (ECHA 2014). In this context, the present project was set up to address three aspects that arise when applying the CBA in the environmental risk assessment of biocidal products as mixtures:

1. the identification of additives relevant for a CBA,
2. the problem of potential synergistic interactions, and
3. mixture toxicity with regard to terrestrial endpoints.

One key aspect in any component-based mixture assessment of formulated products is the decision which components shall actually be considered in the calculations. The BPR refers to a.s. and substances of concern (SoC) as relevant for the risk assessment. The transitional guidance (ECHA 2014) states as substances relevant for a mixture assessment the a.s. of the product, a.s. from any other biocidal product type, SoC, and, on a case-by-case basis, synergists. It is explicitly stated in the transitional guidance that this definition of relevant substances may be revisited, particularly given that a definition of SoC within the context of the mixture risk assessment is currently under development. In the present project, different sets of criteria were defined that tentatively identified product components as relevant within a mixture assessment. Explored options included criteria based on the regulation on classification, labelling and packaging of substances and mixtures (CLP Regulation, EC 2008a) that defines in Annex II, Part 4 substances that are 'relevant for a mixture assessment' with regard to an assessment of this mixture as hazardous to the environment. The predicted relative contribution of each product component to the predicted joint toxicity based on a toxic unit approach served as another tentative criterion to identify a component as relevant for a mixture assessment. Other options were the inclusion of only the a.s. of the product and, on the other extreme, all product components for which aquatic toxicity data were available. As a first step in the process of developing a proposal for the identification of 'relevant' components, a survey of formulation additives in authorised biocidal products was conducted, and the additives were categorized according to the various, above mentioned sets of criteria. As a second step, seven selected wood preservative products were experimentally tested for their aquatic toxicity using three different test organisms (the water flea *Daphnia magna*, the green algae *Raphidocelis subcapitata*, and embryos of the freshwater fish *Danio rerio*). The results of this whole-mixture approach (i.e., the experimentally observed product toxicity) were compared to the results of a component-based approach (i.e. the theoretically predicted toxicity of the product). These comparisons were performed taking the previously defined different sets of product compounds into account in order to evaluate which components were indeed relevant for a correct prediction of aquatic toxicity. The criteria applied to the product components were, hence, solely used to explore their usability in terms of a component-based assessment approach of biocidal products, but not with regard to a classification or an actual environmental risk assessment of the selected wood preservative products. Extrapolation to predicted environmental mixtures resulting from the usage of biocidal products (e.g. eluates of wood treated with wood preservatives) and other product types were not experimentally verified, but only theoretically addressed.

Established models to predict the toxicity of mixtures such as the concept of concentration addition (CA, Loewe & Muischnek 1926), independent action (IA, Bliss 1939), and combinations thereof (e.g. De Zwart & Posthuma 2005) assume additive effects of the mixture components. There are no models established to quantitatively predict synergistic or antagonistic interactions, i.e. more-than-additive or less-than-additive effects, respectively. The transitional guidance (ECHA 2014) foresees a screening step for synergistic interactions of product components to safeguard against underestimation of product toxicity by an additive CBA. The screening step involves checking available information (such as the intended use of the additives, scientific literature, and potential synergists listed in the transitional guidance) for the presence of potential or known synergists in the product. Presence of such compounds would then trigger a case-by-case assessment, involving either additional safety factors (individually derived from the available information) or direct toxicity testing of the mixture. Piperonylbutoxide (PBO) is a known synergist that has been used for many years to enhance the insecticidal activity of pyrethroids in target insects (Bernard & Philogène 1993). It served as an example synergist in the present project, and the available literature has been extensively evaluated with regard to its synergistic effects in non-target organisms. However, it turned out that during the project lifetime, no wood preservative product marketed in the EU contained PBO as intended synergist. Because experimental verification of synergistic interactions toward non-target organisms caused by PBO was therefore not possible with a marketed product, the focus of the synergism aspect of the present project switched to the experimental verification of a different possible mechanism of synergistic interaction.

Inhibition of different enzymes within the same biochemical pathway has been proposed as an event with the potential to result in synergistic effects (SCHER/SCENIHR/SCCS 2011). Indeed, studies with plant protection products indicated substantial underestimation of algal toxicity that contained fungicides inhibiting different enzymes in the ergosterol biosynthesis of fungi (Coors & Frische 2011). These indications were solely based on theoretical calculations using single-substance and product toxicity data compiled from dossiers submitted within the authorisation process. In the present project, this potential mechanism of synergism was experimentally evaluated, because this class of fungicides is not only authorised for plant protection products, but also for wood preservative products.

The suitability of theoretical mixture toxicity calculations based on CA and IA has been well established by many experimental studies with regard to aquatic toxicity (Deneer 2000, Belden et al. 2007, Kortenkamp et al. 2009, Cedergreen 2014). Yet, there is far less knowledge with regard to the predictability of terrestrial toxicity. In the environmental risk assessment of wood preservatives, the terrestrial compartment shall be assessed if exposure is likely, i.e. depending on the use class of the product. Since insecticides are often contained in wood preservative products, collembolans as closely related taxonomic group may be particularly sensitive. Treated posts of fences and other wood buildings represent a point source to the terrestrial compartment within a rather small area. If non-target organisms such as collembolans would actively avoid such a contaminated area, such a behaviour could limit impacts on their natural populations. The collembolan avoidance test is not a data requirement in the authorisation process of biocides, and it is also explicitly not a laboratory test aiming to replace a collembolan reproduction test. However, the relationship between responses in the collembolan reproduction test and the collembolan avoidance test as well as their predictability by the CA concept were compared for an example wood preservative product and its individual active substances. These explorative investigations are not addressing current environmental risk assessment schemes, but may help to inform future developments and understand the advantages and limitations of applying mixture toxicity concepts to the terrestrial compartment.

Overall, the present project was conducted over 3.5 years (May 2013 to November 2016), and addressed three aspects in the mixture risk assessment of biocidal products by theoretical approaches as well as by experimental work. The objectives of the addressed aspects ranged from direct regulatory application (survey of formulation additives and component-based aquatic risk assessment of biocidal products) to more science-oriented investigations (indication for synergistic interactions and predictability of reproductive and behavioural mixture toxicity in collembolans). Parts of the results have already been presented at scientific conferences or prepared as master thesis (Coors 2015, Kehrer et al. 2015, Vollmar et al. 2016, Heim 2015), and publications in scientific journals are in preparation.

2 Formulation Additives in Biocidal Products

The first step in the present project was a survey of formulation additives in biocidal products. This step served to support the understanding of the general composition of biocidal products and the selection of products for the subsequent investigation by ecotoxicological testing. Given the complexity of the product compositions and the need to adapt the set of criteria based on regulatory guidance developed during the project lifetime, this step was more laborious than initially envisaged.

The survey was based on 30 biocidal products that were selected by the German Environment Agency and for which the Agency also provided confidential information on product components and basic data sets. These data were complemented by searching appropriate data bases for additional information on the a.s. as well as on the formulation additives. Compiled information was retrieved from the ECHA database (http://echa.europa.eu) for substances registered under REACH, from safety data sheets (SDS) obtained through internet search from producers, and from the ECOTOX database (http://cfpub.epa.gov/ecotox) provided by the U.S. EPA. With regard to aquatic toxicity, median effect concentrations (EC_{50}) for *Daphnia magna* (48 h EC_{50} for immobility), fish (96 h EC_{50} for mortality), and green algae (72 h EC_{50} for inhibition of growth rate) were preferably compiled. If the preferred endpoint was not available, other endpoints were taken as surrogate such as inhibition of yield in algae, effects in other algal taxa, immobility of other crustacean species, EC_{50} for longer or shorter exposure times, and no observed effect concentrations (NOEC). In addition, information on ready biodegradability according to OECD 301 and OECD 310 (OECD 1992 and OECD 2006b, respectively), water solubility and volatility was systematically compiled for the additives. The compiled data were not quality assessed but taken as they were provided, usually by the producers of the compounds.

The 30 biocidal products belonged to three different product types (PT): wood preservatives (PT 08), rodenticides (PT 14), and insecticides, acaricides and products to control other arthropods (PT 18). The products from these three types will be discussed in the following in separate sub-chapters.

It was one generally observed pattern that the formulated biocidal products often contained not only substances added individually, but also complex and only partly identified preparations of substances (i.e., mixtures) that were occasionally biocidal products themselves (e.g. preservatives). This fact contributes to the large variety of substances that a biocidal product is composed of. It also complicates the attempt to sort formulation additives by their function in the biocidal product, because the substance preparations (but not the individual components in this preparation) are added to serve a specified function. Hence, the very same substance can be added with a preparation labelled as 'thickener' to one product and with a preparation labelled as 'stabilizer' to another product, while in fact the substance may be a solvent. Therefore, no general list of formulation additives as individual substances with their intended functions in biocidal products could be derived.

Three different candidate sets of criteria were established to tentatively identify additives that should be considered in a component-based assessment of the product. All additives in all 30 products were allocated accordingly to one or more of the following categories. This assessment was conducted separately for each additive in each product, resulting in multiple counts for additives that were present in several products.

- No data for any of the three aquatic endpoints was available. Note that no search was conducted in the scientific literature and no estimates were derived by quantitative structure-activity relationships (QSAR) as this was beyond the scope of the present project.
- At least one aquatic toxicity endpoint was available, and based on that information the additive was not appointed to any of the three following categories.
- Category 'CLP': the additive was identified as being relevant for a mixture assessment based on criteria stated in the CLP regulation.
- Category 'HAZ': the additive was listed as a component in the SDS of a given product as being hazardous to the environment.

- Category '>10% STU': the calculated relative contribution of the additive accounted for more than 10% of the sum of toxic units (STU) predicted for the joint toxicity of the product for at least one of the three endpoints.

The criteria of the three categories 'CLP', 'HAZ', and '>10% STU' are explained in more detail in the following.

It is important to note that is was not the aim of the project to actually conduct a new or evaluate existing assessments of the products or their components with regard to their potential identification as being hazardous to the environment. The sets of criteria related to the assessment of environmental hazard are solely applied and used to explore their usability as selection criteria in terms of a protective, but at the same time manageable component-based assessment approach of biocidal products.

2.1 Set of criteria for the category 'CLP'

The CLP regulation uses aquatic toxicity data in combination with fate-related data to evaluate substances with regard to their environmental hazard, i.e. to assign them eventually to Acute Category 1, and/or to one of Chronic Category 1 to 4. The decisive aquatic toxicity endpoint is defined for this purpose as the lowest value of the EC_{50} for inhibition of growth rate in algae or aquatic plants, the EC_{50} for immobilisation or mortality in aquatic crustaceans, and the LC_{50} for fish. In Annex I 4.1.3.1, the CLP regulation defines the 'relevant components' of a mixture, which are those that shall be considered in the evaluation of an environmental hazard of a mixture (e.g. a preparation of various components). In addition to the criteria stated above for the assessment of single substances, the proportion of a component in the mixture of interest is taken into account. Substances can be deemed 'relevant for the mixture assessment' according to this definition without actually triggering a labelling of the preparation itself as hazardous to the environment.

It was beyond the scope of the present project to compile data on chronic aquatic toxicity, bioaccumulation potential, or potential for rapid degradation in the environment other than an assignment of 'ready biodegradability' based on OECD 301 (OECD 1992) and OECD 310 (OECD 2006b) tests. Therefore, only the key set of criteria according to the CLP regulation was applied in the present study to each of the known components contained in the selected biocidal products.

A product component was classified as 'relevant for the mixture' according to CLP (i.e., categorized as 'CLP') based on the data compiled in the present study if

- its lowest available endpoint for aquatic toxicity was ≤1 mg/l and its mass proportion (% w/w) in the product ≥0.1 after multiplication of the actual concentration with the respective M-Factor (Multiplication-Factor according to Table 4.1.3 of Annex I Part 4 of the CLP regulation, EC 2008a),

or

- its lowest available endpoint for aquatic toxicity was >1 mg/l and ≤100 mg/l, and its mass proportion in the product ≥1% w/w.

The M-Factor is only relevant if the relevant aquatic toxicity endpoint is ≤1 mg/l. The set of criteria is summarized and illustrated in Table 1. Consideration of biodegradability in the environment is important for the classification of 'Chronic' categories and, hence, also for the identification as 'relevant for the mixture' according to CLP: a component that is rapidly degradable is only considered as relevant for the mixture if its lowest aquatic toxicity endpoint is equal to or below 1 mg/l. However, degradability (here: ready biodegradability) was not taken into account in the categorization as 'CLP' or 'not-CLP', because of the planned comparison with experimentally observed product toxicity. Yet, ready degradability as fate-related criterion was included in a second step and the rational and resulting influence will be evaluated and discussed later on as well.

Table 1: Summary of criteria used to derive a categorisation as 'CLP' in the present study

Lowest aquatic toxicity endpoint	Multiplication (M-) Factor	Proportion in product ≥0.1% w/w (after multiplication with M-Factor)	Proportion in product ≥1% w/w
no data	n.a.	no	no
≤0.001 mg/l	1000	yes	yes
≤0.01 mg/l	100	yes	yes
≤0.1 mg/l	10	yes	yes
≤1 mg/l	1	yes	yes
≤100 mg/l	n.a.	no	yes (unless readily degradable in the 2nd step)
>100 mg/l	n.a.	no	no

The M-Factor only applies for components with an aquatic toxicity endpoint below 1 mg/l (otherwise, n.a., not applicable). There were no additives with an aquatic toxicity endpoint below 0.001 mg/l in the present study

2.2 Set of criteria for the category 'HAZ'

For the purpose of this study, categorisation as 'HAZ' was solely based on the SDS prepared by the producers of the respective biocidal products. Hence, an additive was counted as 'HAZ' if it was mentioned in the SDS as hazardous to the environment with a related R- or H-sentence. In addition, additives labelled as hazardous to human health were recorded (but not categorized as 'HAZ' based on this criterion only), excluding those that were labelled only because of existing work place safety limits (*Maximale Arbeitsplatz Konzentrationen*, MAK). While this categorisation relied solely on the provided SDS, the criteria for the labelling of components as hazardous to the environment described here are a bit more in detail in order to illustrate how 'CLP' and 'HAZ' categorisation may differ for the same compound in the same product.

The requirements for SDS are set in Article 31 and Annex II of the REACH regulation (EC 2006) and a related Commission regulation (EU 2015). Practical guidance is provided in an ECHA guidance document (ECHA 2015). Table 2 summarizes some key criteria that trigger the listing of a compound as hazardous to the environment on the product label. In addition to the minimum criteria, producers are free to list more components, i.e. those that do not meet the criteria for labelling. In comparison to the 'CLP' categorisation described above, the 'HAZ' category appears less conservative because (i) components with high toxicity (≤1mg/l) and low content (<1% w/w) only need to be listed if the product also is hazardous to the environment, and (ii) components with low aquatic toxicity (between 1 and 100 mg/l) only need to be listed if they are not rapidly degradable. On the other hand, the 'CLP' category in the present study may be less conservative than the 'HAZ' category because components with specific hazard concerns, e.g. those identified as being persistent (P), bioaccumulative (B), and/ or toxic (T), are covered by 'HAZ' but not necessarily by 'CLP'.

Table 2: Summary of criteria prescribed in the REACH regulation that determines whether a component of a mixture shall be labelled as 'hazardous to the environment' on the SDS

Criteria	Proportion in product ≥0.1% w/w (after multiplication with M-Factor)	Proportion in product ≥1% w/w
no data	to be labelled as 'contains x% of components with unknown hazards to the environment' according to CLP regulation, Annex 1 4.1.3.6.1 (EC 2008)	
lowest aquatic toxicity endpoint ≤1 mg/l	yes, if product itself meets criteria for labelling as hazardous to the environment	yes
≤100 mg/l	no	yes, unless rapidly degradable
fulfilling PBT or vPvB criteria	yes	yes
fulfilling other reasons for concern	yes	yes

The M-Factor only applies for components with an aquatic toxicity endpoint below 1 mg/l

2.3 Set of criteria for the category '>10% STU'

The category '>10% STU' focusses on the relative contribution of each additive to the expected joint toxicity of the product. Based on the CA concept, the relative theoretical contribution was calculated in terms of Toxic Units (TU) as

$$\%\, STU_i = \frac{{}^{C_i}/_{EC_{50\,i}}}{\sum TU_i} * 100$$

with C_i being the concentration of component *i* in the product (mg/l), and $EC_{50\,i}$ being the median effect concentration of the component *i* (mg/l). This calculation was conducted separately for each trophic level, i.e. survival of *Daphnia* and fish and growth of green algae using the data compiled in the present study (see above). An additive was counted in the category '>10% STU' if its individual toxic unit (TU_i) was >10% of the sum of toxic units (STU) for at least one of the three aquatic toxicity endpoints.

While trying to favour usage of similar endpoints within each calculation (i.e, same test species, same exposure duration, and same response variable), this principle was traded-off in favour of including as many components as possible by also including other endpoints. Hence, there is a considerable amount of diversity in the input data for the calculations that may impact to an unknown degree the predicted STU. In addition, a number of worst-case assumptions were made to enable calculations:

- Censored $EC_{50\,i}$ values (e.g. EC_{50}>100 mg/l) were included in the calculation with the given value (i.e. 100 mg/l in this example)
- If a range was given as estimate for aquatic toxicity (e.g. EC_{50} between 1 and 10 mg/l), the lower value was used in the calculation
- The typical concentration of each component in the product was assumed, while in cases where the concentration was given as censored value (e.g. <10% w/w), this maximum concentration was used for the calculations

It is important to note that components for which no data on aquatic toxicity were available could not be included in these calculations; their theoretical relative proportion and contribution is therefore by definition zero.

2.4 Composition of wood preservatives

There were in total 21 wood preservatives evaluated in the present study (Table 3). Overall, they contained eight different a.s. with fungicidal and/or insecticidal activity in various combinations. Currently, there are 38 a.s. authorised in the EU for use in wood preservative products (PT8, ECHA July 2016) with four of them being based on boron and another five being based on copper. Hence, the selection of the 21 products is not fully covering the scope of a.s. and their possible combinations, but can be deemed representative enough (particularly for fungicides) to draw some general conclusions.

Table 3: Key information regarding the 21 wood preservative products

Product	Active substances [1]	Formulation type [2]	Product labelled as hazardous to the environment [3]	Predicted contribution of additives to joint toxicity (%STU) [4]
1	dichlofluanid (F)	S	no	11.5 (fish)
2	IPBC (F)	S	no	58.5 (algae)
3	IPBC (F), tebuconazole (F)	W	yes	37.1 (algae)
4	tebuconazole (F)	S	yes	99.8 (algae)
5	boric acid and tetraborate (F, I)	W	no	34.8 (algae)
6	boric acid and tetraborate (F, I)	W	no	91.6 (algae)
7	IPBC (F), propiconazole (F)	S	yes	81.9 (*Daphnia*)
8	IPBC (F), propiconazole (F)	W	yes	0.3 (algae)
9	IPBC (F), propiconazole (F)	S	yes	62.1 (algae)
10	IPBC (F), propiconazole (F)	W	yes	0.6 (algae)
11	IPBC (F), propiconazole (F)	W	yes	3.8 (algae)
12	IPBC (F), propiconazole (F)	W	no	0.3 (algae)
13	IPBC (F), propiconazole (F)	S	no	81.9 (*Daphnia*)
14	IPBC (F), tebuconazole (F)	W	yes	1.6 (algae)
15	IPBC (F), tebuconazole (F)	S	no	83.5 (*Daphnia*)
16	IPBC (F), tebuconazole (F), propiconazole (F)	W	yes	31.1 (algae)
17	IPBC (F), tebuconazole (F), propiconazole (F)	W	yes	1.3 (algae)
18	IPBC (F), tebuconazole (F), propiconazole (F)	W	yes	5.7 (algae)
19	IPBC (F), tebuconazole (F), propiconazole (F)	S	yes	90.5 (*Daphnia*)
20	IPBC (F), tebuconazole (F), propiconazole (F)	W	yes	1.0 (algae)
21	boric acid (F, I), fenoxycarb (I), propiconazole (F), fenpropimorph (F)	W	yes	97.4 (algae)

[1] F: fungicide, I: insecticide; [2] W: water-based, S: solvent-based; [3] labelling as hazardous to the environment according to the SDS of the product; [4] shown is only the joint toxicity predicted by concentration addition for the expected most sensitive taxa (in brackets) using all available data

There were 8 solvent- and 13 water-based products. For both formulation types, products containing two a.s. were the most frequent ones, while the maximum number of four a.s. was represented by only one product.

The contribution of the additives to the predicted joint toxicity of each product for the most sensitive taxa ranged from negligible (0.3%) to dominating (99.8%), which demonstrates the necessity of taking additives into account in the mixture assessment. There was a significant correlation between the toxicity contribution of additives and the formulation type (one-way analysis of variance, ANOVA, p=0.024) with additives contributing more to the joint toxicity in solvent-based products. This was particularly driven by *Daphnia*, which was the most sensitive organism only for solvent-based products. There was no correlation between the labelling of the product as hazardous to the environment and the relative toxicity contribution of all additives together to the joint toxicity (one-way ANOVA, p=0.548). This lack of correlation indicates that product toxicity may be dominated by additives without those additives triggering a labelling of the product. Hence, a component-based environmental risk assessment that includes only the additives that lead to a labelling of the product as hazardous to the environment would ignore substantially contributing components and result in a (potentially non-protective) underestimation of product toxicity.

The contribution of the a.s. to the overall product mass covered a large range from 0.2 to 77.3% w/w, which was related to two a.s. (boric acid and disodium tetraborate) typically being present at high concentrations in the respective products. In 50% of the products, the a.s. contributed 1% or less to the product mass (median of 1.0, Table 4).

Solvent-based formulations contained on average more than 75% organic solvents and at least 53.4% (w/w). Typically, one solvent was contained at a large proportion together with several organic co-solvents at much lower proportions. Water-based formulations did frequently also contain various organic solvents (all products except one), up to a maximum proportion of 7.1% (w/w). The most frequent solvents were naphtha (CAS 64742-48-9), contained in 9 products and often as main solvent, dipropyleneglycol monomethylether (CAS 34590-94-8) contained in 11 products, and propane-1,2-diol (CAS 57-55-6) contained in 7 products. Other organic solvents were present in two or three different products, such as glycol ether (CAS 112-34-5), methoxypropanol (CAS 107-98-2), white spirit (CAS 64742-82-1), and butoxyethanol (CAS 111-76-2).

Table 4: Average composition of the 21 wood preservative products

Product characteristic	Mean (SD)	Median	Range
Active substances (% w/w)	5.6 (16.8)	1.0	0.2 - 77.3
Organic solvents (% w/w) in solvent-based products	78.7 (14.4)	83.4	53.4 - 92.1
Organic solvents (% w/w) in water-based products	2.5 (2.2)	1.6	0.0 - 7.1
Number of additives (excluding water)	13.0 (8.4)	12	2 - 34
Number of additives at <0.1% (w/w)	5.6 (5.7)	5	0 - 23
Number of additives at 0.1% to <1% (w/w)	4.0 (3.4)	3	0 - 9
Number of additives at ≥1% (w/w)	3.4 (1.8)	3	1 - 8
Number of additives labelled as hazardous, but not with regard to the environment	1.0 (0.9)	1	0 - 3
Proportion of product mass supported by aquatic toxicity data for at least one endpoint (% w/w)	85.7 (12.3)	88.3	65.0 - 100.0

Shown are descriptive statistics (mean with standard deviation, SD, median, and minimum-maximum range)

The total number of additives in the 21 products amounted to 273, including repeated counting due to the presence of the same additive in several products. Based on CAS numbers, 122 different additives remained together with 50 'unknowns', i.e. those with confidential identity or lacking CAS number. For

88 of the 273 additives (30 of the 122 different ones), no data for any of the three aquatic toxicity endpoints were available. The total number of additives per product varied largely among the products, ranging from 2 to 34.

Half of the products contained 12 or more different substances added as additive (including organic solvents and excluding water). The function of these substances (or the preparations with which they were added) covered a large range and included anti-skinning agent, binder, buffer, defoamer, corrosion inhibitor, dispersant, dryer, emulsifier, filler, foaming agent, pigment, preservative, siccative, solvent, stabilizer, surfactant, thickener, and wetting agent.

The additives contained at proportions of more than 1% (w/w) comprised solvents, binders, pigments, emulsifiers, and wetting agents, but also some substances with unknown function and unclear identity. Most additives were contained at a mass proportion of less than 0.1% (w/w); there were up to 23 of those combined in a single product.

On average the products contained one additive labelled as hazardous for human health, but not with regard to the environment. These additives were not further considered in the present study due to the focus on environmental risks.

For at least 65% (and up to 100%) of the total product mass, there was at least one endpoint for aquatic toxicity available. Hence, up to about 35% (on average about 10%) of the total product mass consisted of one or more additives for which no aquatic toxicity data were available.

2.4.1 Formulation additives categorized as 'CLP'

There were in total 44 cases where an additive was categorised as 'CLP' in a product. This represents 16% of the total number of additives of 273 in the 21 products. These figures include double counting for additives being contained in different products and, hence, the number is reduced to 24 different individual additives categorised as 'CLP' based on CAS number, representing 19% of the total number of individual additives. Table 5 provides an overview on the average number of additives per product that were or were not categorised as 'CLP' in the present study.

Table 5: Additives in the 21 wood preservative products categorized as 'CLP'

Characteristic	Mean (SD)	Median	Range
Not 'CLP' (absolute number of additives)	6.7 (5.6)	5	0 - 21
Not 'CLP' (% of total number of additives)	44.7 (23.1)	50.0	0 – 83.3
Not 'CLP' in absence of data (absolute number)	4.2 (3.2)	4	0 - 12
'CLP' (absolute number)	2.1 (1.3)	2	0 - 5
'CLP' (% of total number of additives)	21.1 (13.4)	18.8	0 – 50.0
'CLP', after RBD* (absolute number)	0.8 (1.2)	0	0 - 4
'CLP', after RBD* (% of total number of additives)	4.8 (7.3)	0.0	0 – 25.0

Shown are descriptive statistics per product (mean with standard deviation, SD, median, and minimum-maximum range) for the components in the 21 products categorised based on the CLP regulation (EC 2008a). Categorisation is based on aquatic toxicity data if respective data were available. * additives that remained 'CLP' after consideration of ready biodegradability (RBD)

Available aquatic toxicity data indicated that, on average, 5 to 6 additives per product (equivalent to about 45% of the total average number of additives per product) were not classified as 'CLP'. Up to 12 additives were potentially relevant due to the absence of data. These additives comprised often substances that are most likely inert and not relevant for aquatic toxicity (such as iron oxide), but also some substances of unknown identity or otherwise possible concern. The average number of additives

categorised as 'CLP' based on data was relatively low with 2 per product, equivalent to about 20% of the additives, with the absolute number of 'CLP'-categorised additives ranging from 0 to 12 per product. When ready biodegradability (RBD) was used as additional criterion (only for components with the lowest aquatic toxicity endpoint >1 mg/l), the number of additives categorised as 'CLP' was reduced to ranging from 0 to 4 with at least 50% of the products containing no 'CLP' additive.

2.4.2 Formulation additives categorized as 'HAZ'

There were in total 10 cases where an additive was categorised as 'HAZ' in a product and these were indeed 10 different individual additives, based on CAS numbers. Hence, about 8% of the total number of additives were labelled on their product SDS as hazardous to the environment. Between none and up to three additives were labelled on average for one product as being hazardous to the environment (Table 6). This represents about 3% of the total number of known additives in an average product. At least half of the products contained no additives that were categorised as 'HAZ'.

Table 6: Additives in the 21 wood preservative products categorized as 'HAZ'

Characteristic	Mean (SD)	Median	Range
'HAZ' (absolute number of additives)	0.5 (0.9)	0	0 - 3
'HAZ' (% of total number of additives)	3.1 (6.8)	0	0 - 25.0
Hazardous to human health only (absolute number)	1.0 (0.9)	1	0 - 3

Shown are descriptive statistics per product (mean with standard deviation, SD, median, and minimum-maximum range) for the components in the 21 products categorised based on the product SDS

2.4.3 Formulation additives categorized as '>10% STU'

The results of the mixture toxicity analysis based on toxic units are compiled in Table 7 as descriptive statistics over the 21 products. The a.s. contained in the products explained on average about 50 to 70% of the expected overall joint toxicity toward the three aquatic organism algae, *Daphnia,* and fish. This proportion varied widely among the products with the range spanning almost the full scale from 0.2 to 99.7% STU.

Additives categorised as 'CLP' explained on average a clearly smaller proportion of the overall joint toxicity than the a.s. (except for *Daphnia*). Yet, the range was similarly large as there were products were 'CLP' additives explained more than 90% of the STU in all three endpoints. The toxicity contribution of 'CLP' additives was greatest with regard to *Daphnia*, and similarly low for algae and fish (about 10% STU in half of the products by 'CLP' additives). This clearly demonstrates that additives identified as relevant for the mixture according to the CLP regulation can indeed be expected to contribute significantly to the overall mixture toxicity. Other additives, i.e. those not categorised as 'CLP' based on available data contributed on average less than 5% (less than 1% in at least 50% of the products) to the overall expected toxicity. Yet, there were cases where 'non-CLP' additives contributed up to almost 24% of the toxicity.

There were in total 28 cases where an additive was categorised as '>10% STU' in a product. This represents 10% of the total number of additives of 273 in the 21 products. Excluding double counting, the number is reduced to 13 different individual additives categorised as '>10% STU', representing 10.5% of the total number of individual additives. There was on average one additive per product categorised as '>10% STU' and up to three of them per product.

It is important to note here that additives for which aquatic toxicity data were not available for a specific endpoint had not been included in the mixture calculation for this endpoint. Hence, the above stated statistics could change if data were available for all product components and all endpoints.

Table 7: Theoretical contribution of the active substances and additives in the 21 wood preservative to the joint toxicity and additives categorised as '>10% STU'

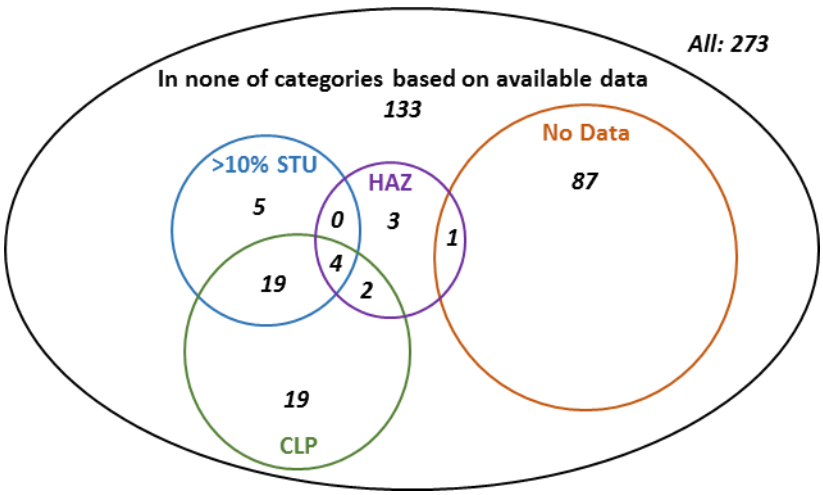
Proportions (%)	Mean (SD)	Median	Range
Proportion of STU_{algae} explained by active substances	68.0 (37.0)	87.4	0.2 - 99.7
Proportion of $STU_{Daphnia}$ explained by active substances	47.7 (38.1)	32.0	0.4 - 99.9
Proportion of STU_{fish} explained by active substances	69.9 (32.8)	88.5	0.2 - 99.7
Proportion of STU_{algae} explained by 'CLP' additives	31.2 (37.0)	12.3	0 - 99.1
Proportion of $STU_{Daphnia}$ explained by 'CLP' additives	48.0 (41.2)	53.2	0 - 99.4
Proportion of STU_{fish} explained by 'CLP' additives	29.3 (33.0)	11.4	0 - 99.6
Proportion of STU_{algae} explained by other additives	0.7 (1.1)	0.4	0 - 4.6
Proportion of $STU_{Daphnia}$ explained by other additives	4.3 (7.3)	0.2	0 - 23.7
Proportion of STU_{fish} explained by other additives	0.8 (1.4)	0.3	0 - 4.0
Number of additives categorised as '>10% STU'	1.3 (0.9)	1.0	0 - 3.0
Proportion of additives categorised as '>10% STU'	13.6 (13.0)	8.3	0 - 50.0

Shown are descriptive statistics per product (mean with standard deviation, SD, median, and minimum-maximum range) for the proportions that active substances and additives contribute to the predicted toxicity of the mixture (calculated as % of STU) with regard to algae, *Daphnia* and fish. Only additives for which toxicity data were available are included in the calculations.

2.4.4 Overlap between the three categories tentatively identifying relevant additives

Based on at least one available aquatic toxicity endpoint the additive was not allocated to any of the three categories (Figure 1) with regard to its presence in the specific product in 133 cases (124 individual additives based on CAS numbers). There were in total 53 cases where an additive was allocated to one or more of the categories of potentially relevant components. The overlaps between the three categories were relatively small with 25 cases (i.e. less than 50%) being in any of the four intersections.

Figure 1: Venn diagram of additives allocated to the three different categories



The 10 additives that were labelled as hazardous to the environment on the product SDS are summarized in Table 8. All of them were contained at very low proportions. They included several organic metals (particularly organic cobalt compounds) and amines.

Table 8: Additives in the 21 wood preservative products labelled as hazardous to the environment

Additive	CAS	Content in product (% w/w)	Categorised as 'CLP'	Relative TU (% STU)
3-(2H-Benzotriazolyl)-5-(1,1-di-methyl-ethyl)-4-hydroxy-benzenepropanoic acid octyl esters	127519-17-9	<1	no	0.1 - 0.5
Cobalt 2-ethylhexanoate	13586-82-8	<0.5	yes	0 - 0.4
Cobalt bis(2-ethylhexanoate)	136-52-7	<1	yes	0 - 8.0
Cobalt borate neodecanoate	68457-13-6	<0.5	yes	0 - 29.1
Cocodimethylamine	61788-93-0	<0.1	yes	n.d. - 17.4
4,5-Dichloro-2-octyl-isothiazolone	64359-81-5	<0.5	yes	56.5 - 88.7
Nonoxynol-10 phosphate	51609-41-7	<0.5	n.d.	n.d.
Solvent naphtha	64742-94-5	<0.5	no	0.1
N-(Tallowalkyl)trimethylenediamine, ethoxylated	61790-85-0	<0.1	no	n.d. - 2.3
N,N',N'-Tris(2-hydroxyethyl)-N-tallow-1,3-diaminopropane	90367-27-4	<0.5	yes	26.5 - 53.2

Relative TU given as range (min/max) across the three endpoints; n.d.: not possible to determine

Four of the 'HAZ' additives were neither categorised as 'CLP' nor as '>10% STU'. In one of these cases, nonoxynol-10 phosphate, no data on aquatic toxicity were available to enable such categorisation. Yet, the primary degradation product of this substance (nonylphenol) is listed as substance of very high concern by ECHA (www.echa.europa.eu/addressing-chemicals-of-concern/authorisation/substances-of-very-high-concern-identification/candidate-list-of-substances-of-very-high-concern-for-authorisation) on the so-called candidate list (branched and linear 4-nonylphenol), which may have caused the listing of this additive as hazardous to the environment on the SDS. None of the other additives categorized as 'HAZ' was listed on this candidate list (status as of July 2016). In another one of these cases, ethyxylated N-(tallowalkyl)trimethylenediamine, data were not available for all endpoints, particularly not for algae. Available data may have resulted in a categorisation as 'CLP' and/or '>10% STU' in addition to the categorisation as 'HAZ' because ethoxylated alkylamines and tallowalkylamines are known for high acute toxicity (Giesy et al. 2000, Krogh et al. 2003, data compiled in the present study for other alkylamines). In the last two of these cases, the labelling as hazardous to the environment may actually not have been necessary based on the relatively low toxicity in combination with the low content. Hence, they may have been listed on the SDS due to other reasons that were not further evaluated within this project.

Two of the organic cobalt compounds were located in the intersection 'HAZ' & 'CLP' but did hardly contribute to the joint toxicity in these products (<10% in all endpoints).

Four of the additives categorised as 'HAZ' were also categorised as 'CLP' and '>10% STU' (Table 8), i.e. they were placed in the intersection of the three categories (Figure 1). These additives can be deemed relevant for a CBA-based product assessment based on all three tentative identification schemes.

There were 19 additives in the intersection 'CLP' and '>10% STU'. Of those, 17 were readily biodegradable and had an aquatic toxicity endpoint above 1 mg/l. They would be removed from the cate-

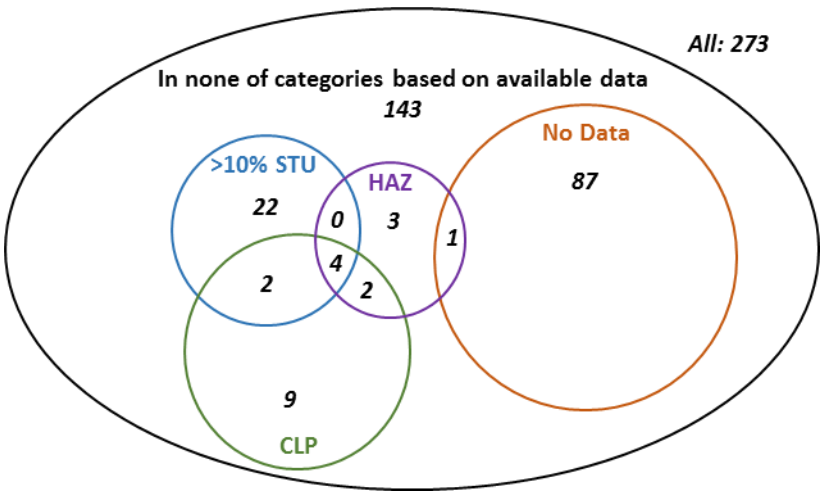
gory 'CLP' if rapid degradation in the environment would be employed as additional criterion as foreseen by the CLP regulation. As illustrated in Figure 2 they all remained then solely in the category '>10% STU'. These were all organic solvents such as naphtha or 2-(methoxymethylethoxy)propanol that may in addition to being readily biodegradable also be relatively volatile. Hence, they appear relevant to predict the aquatic toxicity of the product, particularly if experimentally determined toxicity is to be compared with the theoretically predicted one as it is the aim in the present project. Excluding these solvents from the component-based mixture calculation in such a scenario would lead to erroneous conclusions with regard to the reliability of the mixture prediction for the product. They are therefore included in the present study in the predictions. Yet, in an environmental risk assessment of mixtures resulting from the usage of the products, they appear to be of much lower relevance.

The remaining two additives in the intersection of 'CLP' and '>10% STU' were a not readily degradable organic solvent and an alkylamine that was structurally related, but not identical to the dimethylalkylamine contained in a wood preservative product investigated in previous projects (Coors et al. 2012a, b, 2014). The alkylamine was not labelled as hazardous and not readily degradable, but dominated the toxicity of the product in which it was contained (more than 90% of STU at all three endpoints). With regard to a reliable mixture assessment, this additive clearly appears relevant.

Among the 19 additives solely categorised as 'CLP' (Figure 1), there were several organic solvents, some metal compounds, two active substances from another product type (PT06, in-can preservatives) and other organic substances. Ten of them were readily biodegradable with the lowest aquatic toxicity endpoint above 1 mg/l and therefore removed when ready biodegradability was considered (Figure 2). The remaining 'CLP' additives were basically metal organic and inorganic compounds and several preservatives. The same preservatives were categorised in some other products as 'not-CLP' due to a slightly lower content. In all cases, the expected contribution of in-can preservatives in the wood preservative products was negligible (<10% of the total STU).

There were five cases where an additive was expected to contribute more than 10% of the joint product toxicity, but was neither categorized as 'HAZ' nor as 'CLP' (Figure 1). These cases involved three different additives (based on CAS numbers) that all contributed less than 20% to the expected toxicity of the respective product. Two of the additives were readily degradable and volatile organic solvents, and the third one was a surfactant

Figure 2: Venn diagram of additives allocated to the three different categories after considering ready biodegradability



2.5 Rodenticides

There were in total six rodenticides evaluated in the present study. All of them were solid materials, i.e. pastes or waxes. They contained overall three different active substances (bromadiolon, difenacoum, and difethialon) either singly (four products) or in combination (bromodiolon and difenacoum, two products). The rodenticidal products contained between 8 and 14 additives (median of 11.5 additives, Table 9). These additives covered edible baits such as wheat, seeds, milk powder, vegetable oils, and flours that composed large proportions of the products (>1% up to 95% per substance), and for which no aquatic toxicity data were available. Few additives were contained with up to 1% (w/w); these were anti-oxidants, preservatives, bittering agents (human taste deterrents), dyes, emulsifiers, stabilizers, and palatable solvents.

For the mixture calculation based on the TU approach, it was assumed that all substances in the solid rodenticides would dissolve in water equally, i.e. assuming that the aqueous solution resulting from environmental exposure would have the same relative proportions of product components as the solid product itself. This is of course an erroneous assumption as the products contain easily soluble compounds (such as sugar) as well as insoluble compounds. However, there was no other way to calculate the aquatic toxicity of the mixture. The resulting TUs have to be seen therefore with caution and rather indicate the potential of the relative contribution to the overall mixture toxicity.

Table 9: Composition of the six rodenticides

Product characteristic	Mean (SD)	Median	Range
Active substances (% w/w)	0.0046 (0.001)	0.005	0.0025 - 0.005
Total number of additives	11.3 (2.7)	11.5	8 - 14
Number of additives at <0.1% (w/w)	3.8 (1.5)	3.5	2 - 6
Number of additives between 0.1% and <1% (w/w)	3.0 (2.0)	4.0	0 - 5
Number of additives at ≥1% (w/w)	4.5 (1.9)	4.5	2 - 7
Number of additives labelled as hazardous, but not with regard to the environment	0.2 (0.4)	0	0 - 1
Proportion of product mass supported by aquatic toxicity data for at least one endpoint (% w/w)	2.1 (1.9)	1.2	0.6 - 5.0

Shown are descriptive statistics (mean with standard deviation, SD, median, and minimum-maximum range)

Table 10: Additives in the 6 rodenticide products categorized as 'CLP', 'HAZ', or '>10% STU'

Characteristic	Mean (SD)	Median	Range
'CLP' (absolute number)	0.2 (0.4)	0	0 - 1
'CLP' (% of total number of additives)	1.2 (2.9)	0.0	0 - 7.1
'HAZ' (absolute number)	0.2 (0.4)	0	0 - 1
'>10% STU' (absolute number of additives)	1.5 (0.8)	2	0 - 2
Proportion of STU_{algae} explained by active substances	24.6 (33.5)	11.5	2.1 - 90.1
Proportion of $STU_{Daphnia}$ explained by active substances	34.9 (45.7)	6.5	3.1 - 97.9
Proportion of STU_{fish} explained by active substances	29.6 (37.4)	16.2	1.6 - 99.7

Shown are descriptive statistics per product (mean with standard deviation, SD, median, and minimum-maximum range)

None of the rodenticide products was classified as hazardous to the environment (Table 10). In one of them one additive was listed as hazardous to the environment in the SDS. This additive (denatonium benzoate) is a bittering agent that shall prevent accidental human consumption. It was contained in all of the products, but in none of them at a concentration leading to categorisation as 'CLP' or '>10% STU' in the present study. There was actually only one additive in all seven products categorized as 'CLP'. This was an *in-can* preservative (identical to those in the wood preservative products). Preservative and anti-oxidants were the only additives in these products expected to contribute more than 10% to the joint toxicity. Overall, these results indicate that very few, if any, additives would need to be taken into account in a component-based environmental risk assessment of rodenticides for the aquatic environment. Note that potential secondary poisoning was not addressed here.

2.6 Insecticides, acaricides and products to control other arthropods

Three products from this product type were included in the present study. They were formulated as either gel or suspension. The three products each contained only one a.s., the insecticides indoxacarb or spinosad. Similar to the rodenticides, these products contained edible baits and attractants such as sugars, honey, starch, plant oil, and yeast at relatively large proportions (>1% and up to 60% w/w). Preservatives, thickeners, and pH regulators were also contained at >1% or at least at >0.1%. There were two preservatives (in the same products) that contributed less than 0.1% of the mass of the product.

There were no additives listed as hazardous in the product SDS, while two of them were classified as hazardous to the environment in their SDS. None of the known additives was categorized as 'CLP' or '>10% STU' based on available data. Overall, this suggests that the toxicity of these products is expected to be predictable solely on the basis of the one contained active substance.

3 Experimental Verification of Mixture Toxicity Predictions

Seven wood preservative products were selected and investigated in experimental tests for their acute toxicity toward *D. magna* and fish embryos as well as for their toxicity toward green algae. The experimentally observed toxicity was then compared to the theoretically predicted mixture toxicity. The predicted toxicity was calculated taking different sets of product components into account (see above) in order to explore which components were actually necessary for a correct (or at least protective) toxicity prediction. Only water-based products were selected for the experimental testing.

Experimental methods and results of the tests are described in the following in a brief and summarized form. Detailed study reports are provided as confidential annexes to the present final report, due to the confidential identity and composition of the investigated commercial products.

3.1 Experimental testing of wood preservative products

All experimental tests were conducted at ECT Oekotoxikologie GmbH according to the relevant OECD guidelines (eventually with some slight deviations), and generally deemed valid (see below). While the tests were not conducted formally under the premises of Good Laboratory Practice (GLP), they were conducted in a GLP-certified laboratory. Within this GLP framework, regular reference tests were conducted that proved the required sensitivity of the test organisms (results not reported here). Because of the water-based formulation, all products were fully miscible with water and no solvents were used in any of the tests. Controls consisted therefore of the respective test medium without addition of the product.

3.1.1 Growth inhibition of green algae

Growth inhibition of algae was tested according to the OECD guideline 201 (OECD 2006a) over an exposure period of 72 h. The used test organism was the unicellular freshwater green algae *Raphidocelis subcapitata* (SAG 61.81), formerly known as *Pseudokrichneriella subcapitata*, and supplied by the Georg-August-University Göttingen.

The medium used for the pre-culture and the growth inhibitions tests consisted of trace elements and macronutrients in the final concentrations according to OECD guideline 201, except that concentrations of Na_2EDTA and $FeCl_3$ were increased by a factor of 10 based on the experience over many years. The growth medium was filter-sterilised (at 0.2 µm) and vessels used for pre-culturing and in the test were sterilised by heating before use. Pre-culture and test conditions were constant temperature (23°C ± 2°C) in a climate-controlled chamber (confirmed by temperature records), and permanent light provided by fluorescent tubes of universal white type (Osram Lumilux 58W/865) at a light intensity between 60 and 120 µE m^{-2} s^{-1} as required by the guideline (confirmed by at least three measurements per test). The vessels containing algae were constantly shaken with 100 ± 5 oscillations/min and placed randomly on the shaker.

All definitive tests were conducted with six replicate vessels for the control and three replicate vessels for each test concentration level. Each replicate glass vessel contained 100 ml algal growth medium and was inoculated with a nominal cell concentration of 0.5×10^4 cells/ml from a pre-culture of *R. subcapitata* in its exponential growth phase (after 3 to 4 days of incubation). The pH of the test solutions was recorded at the beginning and the end of each test. The pH of the test solutions was 8.0 ± 0.3 at test start, and increased during the test due to the depletion of CO_2 by growing algae. In the controls, the change in pH was with up to 2.0 units slightly above the 1.5 units allowed by the guideline in three of the algae tests. However, this was not deemed to invalidate the results as no inhibition of growth in the controls compared to the other treatments was observed. The validity criterion of an at least 16-fold induction of yield was clearly fulfilled in all tests (at least 261-fold induction reached in the tests). Similarly, the validity criterion of a coefficient of variation of average specific growth rate in the con-

trol equal to or below 7% was fulfilled in all tests. The coefficient of variation for the section-by-section specific growth rate was not determined in the tests as no cell density measurements were made on day 1 and 2. However, from other algae tests run in parallel it is known that the way of pre-culturing *R. subcapitata* at ECT reliably results in an exponentially growing pre-culture that will, hence, not cause a lag-period in growth in the actual test.

All products were tested as geometric dilution series with a spacing factor of 2.24 to 3.16, i.e. not exceeding the maximum spacing factor of 3.2 prescribed by the guideline. Concentration levels used in the definitive tests were selected based on range finding tests run with a reduced number of replicates.

Algal cell density was determined by measuring fluorescence using a fluorometer (Multiple Plate Reader Tecan ULTRA). The results (relative fluorescence units, RFU, corrected for fluorescence of blank measurements) were converted into biomass concentration (cells/ml) based on a calibration curve that was generated individually for each test from a dilution series of the pre-culture at the day of the test start. For this calibration curve, cell density in the dilution series determined microscopically by counting cells in a Thoma chamber was correlated with measured RFU.

The two response variables yield and growth rate were calculated according to the guideline for each replicate vessel. Yield is the biomass (cell density) at the end of the test minus the starting biomass (nominal inoculum of 0.5×10^4 cells/ml). Average specific growth rate μ (slope of the growth curve) is the logarithmic increase in the biomass.

3.1.2 Immobility of *Daphnia magna*

Immobility of *D. magna* was tested according to the OECD guideline 202 (OECD 2004) over an exposure period of 48 h. The used test organism was the freshwater crustacean *Daphnia magna* Straus (Crustacea: Cladocera, clone M 10).

The M4 medium used for the haltering of the *Daphnia* stock culture and for the tests consisted of trace elements, vitamins and macronutrients according to Elendt (1990). Freshly prepared aerated M4 medium has a pH of 7.5 to 8.2, a conductivity of 600 to 680 µs/cm, and a hardness of 14.0 ± 0.2 °dH.

Conditions for the *D. magna* stock culture and all tests were a temperature of 20 ± 2°C under a light regime of 16h/8h light/dark with a light intensity of 50 to 1000 lx (culture) or in the dark (tests with photosensitive items such as IPBC). In two of the tests, the temperature range was slightly lower (16 to 20°C) than prescribed by the guideline. However, this small exceedance is not deemed to invalidate the results. The validity criterion of at least 10% survival in the control was fulfilled in all tests. Similarly, changes in pH as well as oxygen saturation levels during the exposure period fulfilled the requirements of the guideline.

All definitive tests were conducted with four replicate vessels for the control and four replicate vessels for each test concentration level. Each replicate glass vessel contained 50 ml test solution. Tests were started by adding five *D. magna* new-born offspring (less than 24 h old, second or third brood offspring from stock cultures) to each replicate vessel in a random order. The test animals were not fed during the 48 h exposure period, and the medium was not aerated and not renewed.

All products were tested as geometric dilution series with a spacing factor of 1.41 to 3.16, i.e. in some cases slightly exceeding the maximum spacing factor of 2.2 prescribed by the OECD guideline 202. Despite this exceedance, the number of observed intermediate response was sufficient for concentration-response modelling. Concentration levels used in the definitive tests were selected based on range finding tests run with a reduced number of replicates.

Immobility of *D. magna* determined after 48 h of exposure as described in the guideline was recorded as response variable.

3.1.3 Mortality of fish embryos

Mortality of fish embryos was tested according to the OECD guideline 236 (OECD 2013). Because the related animal protection law changed during the lifetime of the project, the first fish embryo toxicity (FET) tests were conducted over an exposure period of 48 h and the last three ones over an exposure period of 96 h (in addition to an evaluation after 48 h). The used test organism was the freshwater zebrafish *Danio rerio* (Chordata: Cypriniformes). The eggs used for the test were obtained from an in-house culture of *D. rerio* maintained at conditions as prescribed by the guideline.

The maintenance medium was prepared from reconstituted water (diluted 1:1 with deionised water) as recommended by the guideline. Freshly prepared aerated maintenance medium has a pH of 7.2 to 7.8, a conductivity of 750 to 810 µS/cm, and a hardness between 9 and 12 °dH.

Conditions during exposure were a constant temperature of 26 ± 1°C in a climate-controlled incubator with light conditions of approximately 300 to 700 lx and a light/dark cycle of 12/12 h. The test vessels were not aerated and the medium was not renewed during the test. All tests fulfilled the applicable validity criteria of the guideline, except in one case where the oxygen saturation was 77% in the highest test concentration instead of the required 80%. It is unlikely that the slightly lower oxygen saturation caused the observed mortality; it was rather the decay of the dead embryos that caused oxygen depletion.

All definitive tests were conducted with four replicate vessels for the control and four replicate vessels for each test concentration level. Each replicate vessel (glass dishes) contained 100 ml test solution. Tests were started within one hour of fertilization by transferring the eggs to pre-test vessels containing the respective test solutions. Eggs were checked for fertilization using a microscope, and 10 fertilized eggs were transferred to each final test vessel.

All products were tested as geometric dilution series with a spacing factor of 2.0 to 2.2, i.e. not exceeding the maximum spacing factor of 2.2 prescribed by the guideline. Concentration levels used in the definitive tests were selected based on range finding tests run with a reduced number of replicates.

Embryos were checked daily for mortality. According to OECD 236, the following parameters were used as indicator of mortality: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. An embryo was scored as dead if one of these four criteria was fulfilled. If scored dead, the embryo was removed to prevent deterioration and impact on remaining embryos.

3.1.4 Verification of exposure concentrations

The samples for the chemical analysis were taken from freshly prepared test solutions and in most tests also from test solutions at the end of the exposure period (i.e., aged test solutions). Analytical samples were taken from the lowest, a medium, and the highest test concentration, and stored in brown glass flasks at ≤-18°C until analysis. Chemical analysis was conducted at the Technologiezentrum Wasser, Karlsruhe.

As a rule, the active substances and relevant additives (as far as methods were available or could be established within the scope of the project) were analysed by direct injection of the samples into a liquid chromatographic system with tandem mass spectrometer (HPLC-MS-MS). Limits of quantification were sufficient for a reliable detection and quantification of the amounts added to the test items. Details of the analytical procedures are provided in the study reports (confidential annexes).

3.1.5 Data analysis

The key response variables growth rate, immobilisation and mortality were evaluated statistically. LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) were determined by hypothesis testing with the software ToxRat Professional, version 2.10, release 20.02.2010 (ToxRat Solutions GmbH, Alsdorf, Germany). Applied statistical tests were selected based

on the assessment of the assumptions for parametric tests (i.e., normal distribution of errors and variance homogeneity). They included the parametric William's multiple sequential t-test, the non-parametric Welch-t test for inhomogeneous variances with Bonferroni-Holm adjustment, and Fisher's exact binomial test with Bonferroni correction (all tests conducted one-sided with alpha=0.05).

Median effect concentrations (EC_{50}), i.e., the estimated concentration causing 50% effect were estimated by means of concentration response modelling based on the nominal concentrations of the test item (i.e. the product) and using individual replicates. Concentration response modelling was done in the free software R, version 3.1.3 (R Development Core Team 2013) using the most recent version of the package "drc" (Ritz & Streibig 2005, Ritz et al. 2015). A three parameter log-logistic model was used for growth rate and yield with the lower limit fixed at 0, according to the function LL.3 given as

$$f(x) = 0 + \frac{d - 0}{1 + e^{(b*(\log(x) - \log(EC_{50})))}}$$

The parameter *b* describes the steepness of the regression curve, the parameter *d* is the upper limit and the EC_{50} is directly modelled as parameter.

A two parameter log-logistic model was used for the immobilisation test with *D. magna* and for the fish embryo toxicity test with the lower limit fixed at 0 and the upper limit fixed at 1, according to the function LL.2 given as

$$f(x) = 0 + \frac{1}{1 + e^{(b*(\log(x) - \log(EC_{50})))}}$$

Confidence intervals (95%) for all EC_{50} values were obtained with the implemented function "ED" of the "drc" package using the delta method and the t-distribution.

3.1.6 Mixture toxicity prediction and comparison with observation

The relative proportion P_i of each mixture component is defined by its individual concentration in the mixture (C_i) and the total concentration of all actually considered components in the mixture as

$$P_i = \frac{C_i}{\sum C_i}$$

Based on the relative proportions, which are constant across the dilution series of a mixture, and the individual toxicity estimate of each component ($EC_{50,i}$) the predicted toxicity estimates for the mixture ($EC_{50,mix}$), at the identical effect level of 50%, were calculated according to the CA concept as

$$EC_{50,mix} = \frac{1}{\sum \frac{P_i}{EC_{50,i}}}$$

As measure for the agreement between predicted and observed toxicity, the Model Deviation Ratio (MDR) was calculated for each toxicity estimate as

$$MDR = \frac{predicted\ EC_{50,mix}}{observed\ EC_{50,mix}}$$

An MDR above 1 indicates that the toxicity of the mixture is underestimated by the CA prediction, while an MDR below 1 indicates that it is overestimated.

Predicted and observed EC_{50} as well as the resulting MDR values for the various subsets (Table 11) were calculated based on nominal concentrations, i.e., the typical (maximal) component concentrations in the product as declared by the producer.

Table 11: Subsets of product components for which mixture toxicity calculations were conducted, if applicable

Subset
Only the active substances (from PT08)
Active substances and all additives categorized as 'HAZ'
Active substances and all additives categorized as 'HAZ' or 'CLP'
Active substances and additives from other PTs
All components in the product with toxicity data for the assessed endpoint

The observed EC_{50} values in mg product/l were transferred to EC_{50} values in mg sum substances/l taking into account the different subsets of product components (Table 11) and their nominal concentrations in the products. The predicted EC_{50} values were accordingly re-calculated for the same subsets to obtain values in the same unit (mg sum substances/l) and relating to the same set of product components. Therefore, the EC_{50} values and the resulting MDR will vary with the number of considered product components, thereby indicating which subset results in the greatest agreement.

In addition, the mean recovery rate of each of the analytically determined components was calculated by relating measured concentrations in freshly prepared medium (measured initial concentration) to nominal concentrations in the product. This recovery rate was used to re-calculate the proportion P_i of each of the considered components and subsequently to correct the predicted $EC_{50\ mix}$ as well as the observed $EC_{50\ mix}$ for each subset of components. Note that the observed EC_{50} in mg product/l test medium translates to different figures in terms of mg sum substances/l depending on which substances are included in this sum. A third calculation was based on concentrations averaged over the exposure period. The calculation of an average mixture composition over time takes to some degree into account the change in the composition of the tested mixture due to instability of some mixture components. To this end, the time-weighted average (*twa*) concentration was calculated for each component *i* at each concentration level according to OECD guideline 211 (OECD 2012) as

$$twa_i = \frac{conc_{i,t0} - conc_{i,t2}}{\ln(conc_{i,t0}) - \ln(conc_{i,t2})}$$

with $conc_{i,\,t0}$ and $conc_{i,\,t2}$ being the measured concentrations of component *i* at the start and after two to four days of exposure, respectively. The *twa*-recovery rate over time was then calculated by relating twa_i to the nominal concentration $conc_{i,\,t0}$. For each component, the mean *twa*-recovery rate (calculated as arithmetic mean of the three concentration level-related *twa*-recovery rates) was then used to calculate the corrected P_i and correct the predicted and observed $EC_{50\ mix}$ for each subset of components as described above. In addition, the *twa*-recovery rate was calculated by relating twa_i to the initial measured concentration $conc_{i,\,t0}$ in order to quantify dissipation during the period of exposure.

3.2 Products selected for experimental testing

For the experimental verification of mixture toxicity predictions in aquatic toxicity tests, wood preservative products were selected that represent various critical aspects based on the analysis of their composition (Chapter 2.4). In addition to the five products selected among the 30 products analysed in the present study, two more were tested (product 31 and 32). For these two, no confidential data on the composition were available, and the predictions had therefore to rely solely on the information given in the SDS of these products. Characteristics of the seven wood preservative products tested in the present study are summarized in Table 12. All products were water-based formulations in order to

reduce any interference of organic solvents with the findings regarding compliance with mixture toxicity expectation. Testing solvent-based formulations and thereby evaluating the possible impact of organic solvents on the reliability of mixture toxicity predictions (such as potential toxico-kinetic synergistic interactions as pointed out in Coors et al. 2014 and Spurgeon et al. 2010) was beyond the scope of the present study. The tested products were either provided by the producers or obtained from commercial suppliers via web-based shops. SDS obtained along with the products were compared with confidential dossier information (if available) to ensure consistency of information.

Table 12 Key characteristics of the seven wood preservative products selected for experimental testing

Product	6	10	14	16	20	31	32
Active substances	boric acid, tetraborate	IPBC, propiconazole	IPBC, tebuconazole	IPBC, tebuconazole, propiconazole	IPBC, tebuconazole, propiconazole	cypermethrin	permethrin
Product labelled as hazardous to the environment	no	yes	yes	yes	yes	no	yes
Number of additives (among them those without any aquatic toxicity data)	2 (0)	10 (5)	9 (5)	12 (1)	16 (3)	1 at least *	2 at least *
Number 'HAZ' additives	0	0	0	3	0	0	0
Number 'CLP' additives (among them those readily biodegradable and aquatic toxicity >1 mg/l)	1 (1)	0 (0)	1 (1)	3 (0)	2 (2)	no known ones	1 at least *
Number '>10% STU' additives	1	2	0	2	1	no known ones	1 at least *
Predicted most sensitive taxon	algae	algae	algae	algae	algae	fish	daphnia

* labelled as hazardous for human health on SDS

Product 6 contained inorganic a.s. and very few (and only organic) additives for which a complete base set of aquatic toxicity data was available. It further represented the case of a product with one additive categorised as 'CLP' that was a readily biodegradable organic solvent expected to contribute a relevant proportion to the joint toxicity of the product (i.e., located in the intersection of 'CLP' & '>10% STU'). The predicted toxicity contribution of this individual additive ranged from 38.5% (fish) to 91.6% (algae). It thereby represents a case where the expected toxicity contribution of additives is high or even higher than that of the a.s.

Product 10 contained no additives categorised in the present study as 'HAZ' or 'CLP', but two that were categorised at '>10% STU'. Those substances were predicted to contribute 10.1 and 12.3% to the joint toxicity in one of the three endpoints (*Daphnia*). Hence, this product represents a case where additives

would not need to be considered in a component-based mixture assessment unless a toxic unit approach with a rather low threshold of 10% STU was applied to identify relevant components for the mixture assessment. Yet, it clearly represents a border case to this assumption because (i) it contained a number of (partly unknown) additives without aquatic toxicity data, (ii) two additives contributed together 20% to the joint toxicity (but each of them alone under a tentative threshold of 20% STU), and (iii) it contained two *in-can* preservatives. Such actives from another PT are proposed to be relevant for a mixture risk assessment in the transitional guidance (ECHA 2014). Both preservatives exhibit high aquatic toxicity (E_rC_{50} algae ≤0.1 mg/l), but were not categorised as 'CLP' due to a product content <0.1% after multiplication with the relevant M-factor. They were expected to contribute less than 1% to the overall toxicity for any of the three endpoints.

Similar to product 10, no aquatic toxicity data were available in product 14 for about half of the contained additives. None of the additives was labelled as 'HAZ' to the environment on the product SDS, while one was categorised as 'CLP'. This was a readily degradable organic solvent with an aquatic toxicity >1 mg/l that was not expected to contribute more than 10% STU. In addition, product 14 contained the same two *in-can* preservative as product 10. Hence, product 14 represented similar to product 10 the borderline case where a number of unknown additives or additives with unknown aquatic toxicity, and additives from other product types are contained. In contrast to product 10, the toxicity of product 14 is predicted to be dominated by the active substances solely (>92% STU in all three endpoints) although one additive is categorised as 'CLP'.

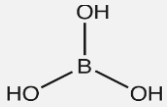
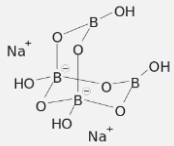
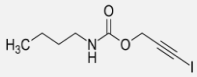
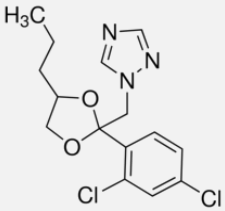
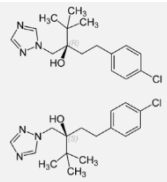
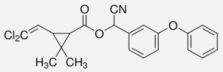
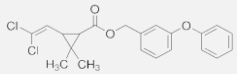
Product 16 represents in terms of number of additives (12) the median of the evaluated products. There were no active substances from other product types present in product 16. In terms of additives categorised as 'HAZ' it represents the maximum with 3 such additives. Only one of them (ethoxylated N-(tallowalkyl)trimethylenediamine,) was not categorised as 'CLP' or '>10% STU', based on the available data (fish and *Daphnia* acute toxicity only). Hence, two additives were located in the intersection of all three categories. One more additive was categorised as 'CLP' only, but not predicted to contribute to the joint toxicity by more than 0.1% (relatively low toxicity and content just above 1%). Overall, additives were expected to contribute significantly to the joint toxicity in product 16 in all three endpoints. For two of these potentially relevant additives, data for algal toxicity and for one data for *Daphnia* were lacking. Hence, product 16 was selected as the most likely example to demonstrate the importance of considering additives in a protective component-based assessment of biocidal products.

Product 20 represents a case where many additives are contained, but few of them were categorised as potentially relevant for the mixture assessment. The two 'CLP' additives are both readily degradable organic solvents and only one of them is expected to contribute to the joint toxicity, although only with regard to *Daphnia* (29% STU).

Product 31 and 32 were selected to add products with insecticidal a.s., and to explore the predictability of the toxicity of the product without complete knowledge on its composition. Both products contained an insecticidal pyrethroid as single a.s. and were bought in online shops. All information is based on the accompanying SDS of these products. They contained one or two, respectively, additives listed as hazardous for human health on the SDS, but none listed as hazardous to the environment. One of the two thereby known additives in product 32 was categorised as 'CLP' and was expected to contribute significantly (79.5% STU) to algal toxicity, the only available aquatic toxicity endpoint.

The key characteristics of the a.s. in the products selected for testing are summarized in Table 13. The data, including aquatic toxicity endpoints, were taken from the dossiers prepared and reviewed by the competent authorities in the course of the regulatory authorisation process of the a.s. at the level of the EU . Data for other endpoints potentially available in the literature (which may have fitted better to the endpoints actually assessed in the experimental testing) were not systematically searched, because this would not reflect the typical regulatory setting in which a component-based risk assessment of a biocidal product would be conducted.

Table 13: Key characteristics of the active substances in the wood preservative products selected for testing

	Boric Acid	Borax	IPBC	Propiconazole	Tebuconazole	Cypermethrin	Permethrin
IUPAC Name	Ortho-boric acid	Sodium tetraborate pentahydrate	3-Iodo-2-propynylbutylcarbamate (IPBC)	1-((2-(2,4-Dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl)methyl)-1H-1,2,4-triazole	1-(4-Chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentane-3-ol	(*R*,*S*)-α-Cyano-3-phenoxybenzyl-(1*RS*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate	(±)-3-Phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
CAS number	10043-35-3	12179-04-3	55406-53-6	60207-90-1	107534-96-3	52315-07-8	52645-53-1
Molecular structure							
Declaration according to 67/548/EWG and 1272/2008 (GHS)*	-	-	R50 H400, Aqu. Acute 1	R50/53 H400, Aqu. Acute1 H410, Aqu. chron. 1	R51/53 H411, Aqu. chron. 2	R50/53 H400, Aqu. Acute1 H410, Aqu. chron. 1	R50/53 H400, Aqu. Acute1 H410, Aqu. chron. 1
Green algae (72 h E_rC_{50} in mg/l)	17.5 (related to boron) * *R. subcapitata*	17.5 (related to boron) * *R. subcapitata*	0.053 *Desmodesmus subspicatus*	0.058 *Desmodesmus subspicatus*	3.8 *R. subcapitata*	> 0.033 ** *R. subcapitata*	>1.13 *R. subcapitata*
***Daphnia* (48 h EC_{50} in mg/l)**	141 (related to boron)	141 (related to boron)	0.16	10.2	2.8	0.00471	0.00127
Fish (96 h EC_{50} in mg/l) with fish species indicated	125 (related to boron) *Catostomus latipinnis*	125 (related to boron) *Catostomus latipinnis*	0.067 *Oncorhynchus mykiss*	4.3 *Oncorhynchus mykiss*	4.4 *Oncorhynchus mykiss*	0.00293 *Oncorhynchus mykiss*	0.0051 *Oncorhynchus mykiss*

Aquatic toxicity data compiled from competent authority reports (EC 2007a,b; EC 2008b, EC 2009a,b; EC 2013; EC 2014); * NOEC instead of $EC_{50;}$ ** above water solubility

3.3 Observed aquatic toxicity of tested biocidal products and comparison with predictions

All conducted tests were valid according to the respective OECD guidelines (201, 202 or 236) except for the fish embryo toxicity test with product 31, where the hatching rate in the negative control was too low. However, the hatching rate was above the validity criterion in all treatments, and it became apparent after the test that there were problems with the control medium preparation. Therefore, the result of the test (no mortality caused by the test item) is deemed reliable.

In the following, the results of the experimental tests and the mixture toxicity comparison are presented and discussed individually for each product. Toxicity estimates and related MDR values are based on nominal concentrations, i.e. concentrations of a.s. and additives in the products as stated by the producer. In addition, estimates and MDR values are provided for the measured *twa* concentrations (or initial measured concentrations, if mentioned) when the calculation was based on the a.s. only. This is because not all potentially relevant additives could be measured due to limitations in the availability of analytical methods. Where possible and available, estimates and MDR values based on measured concentrations of all potentially relevant components are presented and discussed in the text.

3.3.1 Product 6

Product 6 was found to be most toxic to algae (Table 14), in agreement with the expectation from the CA prediction. The observed toxicity exceeded the CA-predicted toxicity by factor 2 to 2.6 for the three endpoints when the calculation solely included the nominal concentrations of the a.s. This deviation was hardly changed when measured concentrations were used in the calculation due to the fact that nominal and measured concentrations of boron agreed very well (100 ± 5% recovery at test start and test end in all three tests).

Table 14: Observed aquatic toxicity of product 6 and resulting MDR values

Endpoint	Observed aquatic toxicity (mg product/l) with 95% confidence interval	MDR – only a.s., measured concentrations	MDR – only a.s., nominal concentrations	MDR – a.s. and 'CLP' additives, nominal concentrations	MDR – a.s. and 'CLP' additives, measured concentrations	MDR – a.s. and all additives with available toxicity data, nominal concentrations
Algal growth inhibition (72 h E_rC_{50})	269.3 (171.9-366.8)	2.41	2.51	0.21	0.37	0.21
Daphnia immobilisation (48 h EC_{50})	2,652.1 (727-4,578)	2.10	2.05	0.43	0.48	0.42
Fish embryo toxicity (96 h EC_{50})	1,841.3 (1,320-2,570)	2.47	2.62	1.61	1.64	1.60

Calculations of MDR was based either on measured or nominal concentrations. 'Measured concentration' means time-weighted average concentrations calculated from analytical determinations in the test solutions at test start and end. 'Nominal concentration' means assumed concentrations in test solution based on labelled concentrations of the components in the product.

The slight underestimation of joint toxicity was changed for two endpoints (among them the most relevant algae toxicity) into an almost 5-fold overestimation of toxicity when additives were also taken into account. This was solely due to the one additive categorized as 'CLP' & '>10% STU' as the MDR hardly changed when the non-categorised additive was also taken into account. The tentatively relevant additive was a readily degradable organic solvent, which was also reflected by its measured concentrations during the test. The loss of this additive over the exposure duration in the test ranged from 29% (48 h *Daphnia* test) to 54% (72 h algae test). Based on *twa*-concentrations of the a.s. and this additive, the MDR values ranged from 0.37 (algae) to 1.64 (fish), indicating generally a good agreement between CA prediction and observed product toxicity (less than factor 3 deviation).

Product 6 was not declared as hazardous to the environment, and it also did not contain components listed as hazardous to the environment on the SDS. Based on the proposal of the transitional guidance (ECHA 2014), a component-based approach would therefore only consider the two a.s. Based on the experimental data of the present study, such a theoretical assessment would lead to a slight underestimation of product toxicity (between factor 2 and 3). Considering specifically the most sensitive endpoint, algal growth inhibition, a component-based approach including the additive categorized as 'CLP' would support a protective environmental risk assessment that would be over-protective by about factor 5 if the ready biodegradability of the additive would be ignored.

3.3.2 Product 10

In agreement with the CA prediction, algal growth inhibition was the most sensitive endpoint in the tests with product 10 (Table 15). The predicted algal toxicity was about 7-fold higher than the observed one. This was independent of measured a.s. concentrations and of consideration of any additives in the calculation.

Table 15: Observed aquatic toxicity of product 10 and resulting MDR values

Endpoint	Observed aquatic toxicity (mg product/l) with 95% confidence interval	MDR – only a.s., measured concentrations	MDR – only a.s., nominal concentrations	MDR – a.s. and additives of other product type (preservatives), nominal concentrations	MDR – a.s. and all additives with available toxicity data, nominal concentrations
Algal growth inhibition (72 h E_rC_{50})	37.5 (32.1-42.9)	0.14	0.13	0.13	0.13
Daphnia immobilisation (48 h EC_{50})	119.9 (0-364.4)	0.40	0.33	0.33	0.25
Fish embryo toxicity (48 h EC_{50})	96.1 (82.1-110.2)	0.21	0.17	0.17	0.17

Calculations of MDR was based either on measured or nominal concentrations. 'Measured concentration' means time-weighted average concentrations calculated from analytical determinations in the test solutions at test start and end. 'Nominal concentration' means assumed concentrations in test solution based on labelled concentrations of the components in the product.

With regard to the less sensitive endpoints, toxicity was also overestimated by the CA prediction, although to a lesser degree. Calculations based on measured concentrations increased the overestimation slightly in all three taxa. Consideration of preservatives in the mixture calculation had no impact on

the MDR in any of the three endpoints, which is in agreement with the very low expected contribution of these additives to the overall toxicity (<0.1% STU). The consideration of all additives with available data slightly increased the overestimation of product toxicity by the CA prediction, but only in the case of *Daphnia* toxicity. This is in agreement with the predicted toxicity contribution of two of the additives (categorized as '>10% STU' based only on *Daphnia* toxicity). Hence, a component-based mixture risk assessment for product 10 based only on the a.s. would support an environmental risk assessment that can be deemed protective, if not over-protective by up to factor 7.

3.3.3 Product 14

Algae were also the most sensitive species for product 14 (Table 16), again in agreement with the CA prediction. Considering only a.s. at their measured concentrations, aquatic toxicity was overestimated by up to factor 4. The deviation between prediction and observation hardly changed in the case of algae, but became greater for the other two endpoints when considering nominal a.s. concentrations. Taking additives (regardless which of them) into account for the calculation did not change the MDR, which fits with the expectation that none of the additives with known individual toxicity estimates would measurably contribute to the joint toxicity. Hence, no additives (including active substances from other product types and one additive categorized as 'CLP') would need to be included in a component-based assessment of product 14 to reach at a protective environmental risk assessment. Their inclusion, on the other hand, did not increase overestimation of mixture toxicity. Based on the most sensitive endpoint, the component-based assessment would be slightly over-protective (by factor 3).

Table 16: Observed aquatic toxicity of product 14 and resulting MDR values

Endpoint	Observed aquatic toxicity (mg product/l) with 95% confidence interval	MDR – only a.s., measured concentrations	MDR – only a.s., nominal concentrations	MDR – a.s. and additives of other product type (preservatives), nominal concentrations	MDR – a.s. and all additives with available toxicity data, nominal concentrations
Algal growth inhibition (72 h E_rC_{50})	22.6 (17.9-27.3)	0.32	0.33	0.33	0.33
Daphnia immobilisation (48 h EC_{50})	40.8 (37.2-44.4)	0.72	0.54	0.52	0.52
Fish embryo toxicity (48 h EC_{50})	62.5 (55.5-69.4)	0.24	0.15	0.15	0.15

Calculations of MDR was based either on measured or nominal concentrations. 'Measured concentration' means time-weighted average concentrations calculated from analytical determinations in the test solutions at test start and end. In the case of the algae test, measured concentrations relate only to test start, i.e. represent initial measured concentrations. 'Nominal concentration' means assumed concentrations in test solution based on labelled concentrations of the components in the product.

3.3.4 Product 16

Algae were by at least factor 100 more sensitive than the other two test species toward product 16 (Table 17). This is not fully in agreement with the CA concept that predicted a difference of about factor 1.2 in toxicity between algae and the next sensitive taxon, fish. Considering only the a.s. at their measured concentrations, toxicity in algae was underestimated by about factor 12, while toxicity for *Daphnia* was predicted with a deviation of less than factor 2 and that for fish embryos was about 4-fold

overestimated. The MDR for fish and *Daphnia* became smaller (indicating more overestimation of toxicity) when nominal concentrations were used and when additives were included in the calculation. The MDR values for algae, in contrast, indicated stronger underestimation of toxicity based on nominal concentrations of only the a.s. This underestimation was reduced when additives were included. Yet, overall no protective assessment of product 16 would be derived for the most sensitive endpoint, algae, in a component-based approach. That was most likely due to the lack of algal toxicity data for two of the three additives categorised as 'HAZ' (one of them was also 'CLP'), while for two or all three of them data were available for *Daphnia* and fish, respectively. The two additives with lacking algal data were both amines (cocodimethylamine and ethoxylated N-(tallowalkyl)trimethylenediamine), a group of compounds for which high algal toxicity is known and also reflected in the present data set. Unfortunately, these two additives were not commercially available. No algal growth inhibition tests could therefore be conducted with them as individual substances to definitively verify the conclusion of their relevant contribution to the joint toxicity. Overall, the case of product 16 clearly demonstrates the importance of including all relevant additives in the mixture toxicity prediction, and the necessity of respective toxicity data, particularly for the likely most sensitive endpoint.

Table 17: Observed aquatic toxicity of product 16 and resulting MDR values

Endpoint	Observed aquatic toxicity (mg product/l) with 95% confidence interval	MDR – only a.s., measured concentrations	MDR – only a.s., nominal concentrations	MDR – a.s. and additives of category 'HAZ', nominal concentrations	MDR – a.s. and additives of category 'CLP' and 'HAZ', nominal concentrations	MDR – a.s. and all additives with available toxicity data, nominal concentrations
Algal growth inhibition (72 h E_rC_{50})	1.33 (0.74-1.90)	11.89	22.98	16.61	16.60	15.84
Daphnia immobilisation (48 h EC_{50})	186.6 (47.4-325.4)	0.84	0.71	0.26	0.27	0.23
Fish embryo toxicity (48 h EC_{50})	306.9 (273.2-340.5)	0.24	0.18	0.09	0.09	0.08

Calculations of MDR was based either on measured or nominal concentrations. 'Measured concentration' means time-weighted average concentrations calculated from analytical determinations in the test solutions at test start and end. In the case of the algae test, measured concentrations relate only to test start, i.e. represent initial measured concentrations. 'Nominal concentration' means assumed concentrations in test solution based on labelled concentrations of the components in the product.

3.3.5 Product 20

Algae were also to most sensitive species for product 20 (Table 18) as predicted by CA. Considering only a.s. at their measured concentrations, predicted and observed toxicity agreed very well for *Daphnia,* while slight underestimation was found for algae and slight overestimation for fish embryos. MDR values for *Daphnia* remained within factor 2 deviation when based on nominal concentrations as well as when considering additives. For algae, underestimation increased when the MDR was based on nominal concentrations due to higher-than-nominal concentrations of one a.s. being measured in the test solutions. The additional consideration of additives hardly changed the MDR. For fish embryos,

overestimation of toxicity increased to about factor 7 when the additives were included. Hence, a component-based assessment for product 20 based on available toxicity data of the components would lead to an underestimation of product toxicity between factor 2 and 3 for the most sensitive endpoint.

Table 18: Observed aquatic toxicity of product 20 and resulting MDR values

Endpoint	Observed aquatic toxicity (mg product/l) with 95% confidence interval	MDR – only a.s., measured concentrations	MDR – only a.s., nominal concentrations	MDR – a.s. and additives of other product type (preservatives), nominal concentrations	MDR – a.s. and all additives with available toxicity data, nominal concentrations
Algal growth inhibition (72 h E_rC_{50})	1.53 (1.00-2.07)	2.73	3.10	3.10	3.07
Daphnia immobilisation (48 h EC_{50})	48.1 (0-197.9)	1.29	1.05	0.73	0.72
Fish embryo toxicity (48 h EC_{50})	153.5 (136.8-170.3)	0.22	0.14	0.14	0.14

Calculations of MDR was based either on measured or nominal concentrations. 'Measured concentration' means time-weighted average concentrations calculated from analytical determinations in the test solutions at test start and end. 'Nominal concentration' means assumed concentrations in test solution based on labelled concentrations of the components in the product.

3.3.6 Product 31

Daphnia was found to be the most sensitive species for product 31 (Table 19), while CA predicted fish survival as more sensitive. *Daphnia* toxicity was overestimated about 8-fold when only the a.s. at its measured concentration was considered. Since the measured concentration was lower than the nominal, overestimation increased to about factor 33 when the MDR was based on nominal a.s. concentration. Additional consideration of the known additive had no impact on the MDR. Differences in the ratio between cis and trans isomers in the product (cis:trans/80:20) and the technical material (cis:trans/40:60) for which the used single substance estimate was derived may explain this deviation when assuming that toxicity to *D. magna* is stereoisomer-specific. Indeed, the various forms of the cis- and trans isomers of cypermethrin differ in toxicity and degradability (EC 2013), and the 1R-cis-αS and 1R-trans-αS stereoisomers were reported to dominate the toxicity of cypermethrin toward *Ceriodaphnia*, with the trans-isomer being more rapidly degraded (Liu et al. 2004).

Toxicity toward fish embryos and algae was strongly overestimated by CA, by at least factor 500 independent of the consideration of additives. In the case of algae, this was due to using a greater-than NOEC value, i.e. a concentration at the solubility limit where no algal toxicity was observed. In the case of fish embryos, this finding of strong overestimation likely points at the much lower sensitivity of fish embryos for pyrethroids compared to adult fish. The fish embryo toxicity test has recently been discussed as being far less sensitive for neurotoxic agents (such as pyrethroids) than the acute toxicity test with adult fish (Klüver et al. 2015). This discrepancy would explain the over-estimation of fish embryo toxicity by the CA concept using adult fish acute toxicity data as input values. Hence, the observed low MDR does not indicate (over)protectiveness of the CA prediction for product 31 with regard to adult fish mortality.

Table 19: Observed aquatic toxicity of product 31 and resulting MDR values

Endpoint	Observed aquatic toxicity (mg product/l) with 95% confidence interval	MDR – only a.s., based on measured concentrations	MDR – only a.s., based on nominal concentrations	MDR – a.s. and additives of other product type (preservatives), based on nominal concentrations	MDR – a.s. and all additives with available toxicity data, based on nominal concentrations
Algal growth inhibition (72 h E_rC_{50})	14,042 (extrapolated)	<0.004	<0.002	n.a.	<0.002
Daphnia immobilisation (48 h EC_{50})	161.7 (0-332.2)	0.12	0.03	n.a.	0.03
Fish embryo toxicity (96 h EC_{50})	>10,000 (n.a.)	<0.0004	<0.0003	n.a.	<0.0003

Calculations of MDR was based either on measured or nominal concentrations. 'Measured concentration' means time-weighted average concentrations calculated from analytical determinations in the test solutions at test start and end. 'Nominal concentration' means assumed concentrations in test solution based on labelled concentrations of the components in the product. The toxicity estimate for algae was extrapolated beyond the highest tested concentration of 12,500 mg/l. n.a.: not applicable.

3.3.7 Product 32

Daphnia was also found to be the most sensitive species for product 32 (Table 20), which was in agreement with the CA prediction. Predicted and observed *Daphnia* as well as algal toxicity was in good agreement, even without considering the known additives. Toxicity toward fish embryos was similar to that of product 31 strongly overestimated. This finding further supports the explanation of lower (neuro)toxicity of pyrethroids in fish embryos compared to adult fish as discussed above. Yet, for the most sensitive endpoint, *Daphnia*, the component-based approach considering only the a.s. reached already at an estimate that deviated less than 2-fold from observed product toxicity.

Table 20: Observed aquatic toxicity of product 32 and resulting MDR values

Endpoint	Observed aquatic toxicity (mg product/l) with 95% confidence interval	MDR – only a.s., measured concentrations	MDR – only a.s., nominal concentrations	MDR – a.s. and additives of other product type (preservatives), nominal concentrations	MDR – a.s. and all additives with available toxicity data, nominal concentrations
Algal growth inhibition (72 h E_rC_{50})	395.0 (0-713.9)	1.43	0.99	0.98	0.98
Daphnia immobilisation (48 h EC_{50})	0.397 (0-1.45)	1.69	1.10	1.10	1.10
Fish embryo toxicity (96 h EC_{50})	217.9 (178.9-256.9)	0.013	0.01	0.01	0.01

Calculations of MDR was based either on measured or nominal concentrations. 'Measured concentration' means time-weighted average concentrations calculated from analytical determinations in the test solutions at test start and end. 'Nominal concentration' means assumed concentrations in test solution based on labelled concentrations of the components in the product. The toxicity estimate for algae was extrapolated beyond the highest tested concentration of 12,500 mg/l. n.a.: not applicable.

4 Compliance between mixture toxicity prediction and observation and reasons for toxicity overestimation

The question which deviation is deemed acceptable (i.e., still indicating compliance) is essential for the comparison of predicted and observed mixture toxicity and subsequent conclusions on the reliability of the mixture prediction. The studies of Belden et al. (2007) and Deneer (2000) provided the first systematic overviews on such comparisons, and reported a less than two-fold deviation between CA prediction and observed aquatic toxicity for the majority of the mixtures (>80%). Yet, the mixtures investigated in these studies consisted exclusively of active substances (pesticides) where single-substance as well as mixture tests had been performed in similar settings (i.e., same laboratory, identical test species, and identical recorded endpoints). Based on these studies, an up to two-fold difference between predicted and observed mixture toxicity can be seen as 'good agreement', which is why factor 2 was used or suggested in subsequent studies as a threshold above that synergism or antagonism is indicated (Coors & Frische 2011, Coors et al. 2012a, 2014, Cedergreen at al. 2008, Cedergreen 2014). Various approaches have been suggested that enable a formal statistical comparison between predicted and observed mixture toxicity responses (Jonker et al. 2005, Iwasaki & Brinkman 2015, Takeshita et al. 2016), either for complete concentration-response curves or for point estimates such as an EC_x. However, the application of these methods is only possible if complete information on the single-substance and the mixture toxicity is available (e.g., observed responses in all replicates). This is clearly not the case for the comparison of predictions and observations for the biocidal products, neither in the present study nor in usual regulatory praxis. Apart from this, a statistically significant difference between predicted and observed responses does not necessarily imply a biological significance or any relevance in a regulatory context. Therefore, the derivation of quantitative measures for the degree of deviation (such as the MDR) and an established threshold for this measure indicating non-additive interaction must be preferred in the context of pragmatic regulatory decision making.

Compared to research-oriented investigations, a higher diversity in the single-substance input data (e.g. different test species within a taxa group) is unavoidable within the context of component-based mixture assessments in the regulatory context as the input data come from different sources. A threshold of factor 2 deviation for compliance may be too restrictive in such a case. A study by Coors & Frische (2011) compared predicted and observed aquatic toxicity of plant protection products, using the regulatory endpoints of the a.s. as laid down in the authorisation dossiers of the EU. When combining the most similar available endpoints for the single active substances and products (i.e., identical test species, test duration, response variable etc.), a deviation of less than factor 2 was found in more than 50% of the cases. Note that formulation additives were never included in the predictions of aquatic toxicity of the formulated products in this study. When the most sensitive endpoints for each taxa group were combined (i.e., the endpoints actually used in the environmental risk assessments), the distribution of the MDR values became clearly skewed towards a systematic overestimation of product toxicity (Coors & Frische 2011).

The data compiled in the present study represent a case of similarly large diversity in the type of input data as the study of Coors & Frische (2011). Hence, deviations between prediction and observation may be greater than 2 simply due to data diversity and related uncertainty (De Laender et al. 2009), i.e., they do not necessarily indicate synergism, antagonism or the need to include other mixture components in the prediction. There were several aspects in the present study that were expected to result in a tendency for overestimating toxicity, thereby in an MDR being skewed towards values below 1:

- The application of the CA concept instead of the IA concept, which is assumed to fit better the joint action of the various components with presumed dissimilar modes of action in the products. However, CA is not only less data demanding than IA, but the degree of toxicity overestimation by CA compared to IA is relatively small in most cases. Together, this is why CA is consistently proposed

as default model (Junghans et al. 2006, Cedergreen et al. 2008, Kortenkamp et al. 2009, Backhaus & Faust 2012, ECHA 2014)
- Assumptions on component concentrations in the products (nominal concentrations) that could not always be corrected by analytical measurements due to lack of analytical methods
- Use of NOEC or EC_{10} values instead of EC_{50} values for some additives
- Use of censored values for some additives (100 mg/l in case of a toxicity estimate >100 mg/l)
- Use of the lowest toxicity estimate for some additives (1 mg/l in case a range is given for toxicity as >1 mg/l and <100 mg/l)
- Toxicity estimates for a.s. were compiled from the competent authority reports, not considering other, possibly more similar endpoints available in the literature. These regulatory assessments contain usually the endpoint for the most sensitive species within a given taxa group, while a different species may have been used in the present study for testing.

The algal toxicity of propiconazole appears to be an example illustrating the last aspect. The endpoint used for the predictions was an E_rC_{50} (72h) of 0.058 mg/l derived with the green algae *Desmodesmus subspicatus* and laid down in the competent authority report (EC 2007a). A previous research project reported with an E_rC_{10} of 1.0 mg/l, an E_bC_{50} of 1.23 mg/l, and an extrapolated E_rC_{50} of 26.4 mg/l (Coors et al. 2012b, Coors et al. 2014) a considerably lower sensitivity of the green algae *R. subcapitata*, i.e. the species used for testing the products in the present study. Propiconazole was contained in three of the products (10, 16, and 20). The algal toxicity of product 10 was overestimated by factor 7 when considering only the a.s. (propiconazole and IPBC). Using the E_rC_{10} of 1.0 mg/l reported for *R. subcapitata* would reduce this overestimation of product toxicity to less than factor 3. In the other two products with propiconazole, algal toxicity was underestimated when the CA prediction included only the a.s. The observed degree of underestimation would be considerably larger if the toxicity estimate for *R. subcapitata* was used as input for the prediction.

The case of cypermethrin in product 31 represents an example where overestimation of toxicity was most likely due to isomer-specific toxicity to crustaceans (Liu et al. 2004) and the difference in isomeric composition of the a.s. in the product compared to that investigated for regulatory purposes. Such a case cannot easily be foreseen. It does not necessarily lead to overestimation, but potentially also to underestimation of product toxicity.

Life-stage specific toxicity most likely explains the overestimation of toxicity towards fish embryos for products that contained pyrethroids. Pyrethroids are neurotoxic agents, a mode-of-action group that were found to exhibit less or no toxicity toward fish embryos in contrast to that observed in adult fish (Klüver et al. 2015). While results were not conclusive for permethrin in that study (Klüver et al. 2015), the present study does support in line with Knöbel et al. (2012) the lack of correlation between the two fish life stages for permethrin. Apart from the case of pyrethroids, fish embryo toxicity was consistently overestimated for the investigated products, except for product 6. This may indicate a generally lower sensitivity of the fish embryo toxicity test than the adult fish mortality test according to OECD guideline 203 (OECD 2008), which has been critically mentioned and addressed before (Klüver et al. 2015). While this finding may be due to the often shorter exposure period in the fish embryo test compared to the 96 h fish adult test (see also Knöbel et al. 2012), this explanation could not be systematically investigated within the scope of the present project. Reported correlations between acute toxicity in fish embryos and adult fish are based on a considerable pool of data, convincingly strong and highly significant (Lammer et al. 2009, Embry et al. 2010, Klüver et al. 2015). Yet, the toxicity can easily differ for an individual substance by one (Knöbel et al. 2012) to three orders of magnitude (Lammer et al. 2009). Hence, a deviation between predicted and observed fish toxicity by about factor 10 (excluding the products with pyrethroids) appears as within the range of predictability. It remains open, however, if the CA prediction would not only be protective for product toxicity towards fish embryos, but also protective for product toxicity towards adult fish. While this question cannot be

answered, there is no evidence from the present or former projects (e.g. from the results for algae and *Daphnia* product tests) that CA would not provide protective estimates for adult fish mortality as well.

Overall, the various aspects discussed above will cause a tendency for mixture toxicity overestimation in the data set produced here for biocidal products. It can therefore be argued that in the present study MDR values above 2 occurring despite of this tendency indicate insufficient environmental protectiveness of a component-based assessment of the product. In the following, the threshold of an MDR of 2 is therefore applied as a sign of greater-than-predicted toxicity pointing at the need to consider more product components to explain the observed toxicity, and thereby exclude the occurrence of synergism.

5 Proposal for identification of 'mixture relevant' product components

Considering in a component-based assessment approach *per se* only the active substances of a biocidal product is not in agreement with the relevant BPR (EC 2012) and the related transitional guidance (ECHA 2014). This is supported by the findings of the present project that indicate a dominating contribution of additives to the expected joint toxicity (>50% STU) for the most sensitive endpoint in nine out of the 21 wood preservative products, four out of the six rodenticides and none of the three products from PT 18. In only 7 out of 21 wood preservative products, the summed toxicity contribution of additives was below 10% STU. Experimental testing verified in 3 of the tested 7 products more than 2-fold underestimated toxicity in the most sensitive endpoint (always algae) when only the a.s. were taken into account, which points at the need to consider additives in the prediction.

The transitional guidance (ECHA 2014) proposes to consider in addition to the a.s. of the product also a.s. from other PTs (pending a final agreed definition of SoC with regard to the mixture assessment). This requirement was not supported by the results of the present study. At least for the case of *in-can* preservatives (PT06), it was found that such preservatives were in most cases not expected to contribute more than 10% to the joint toxicity, and that their consideration was not necessary to explain the observed toxicity in the tested products containing them. Products 10 and 14 provided clear experimental support for this finding. Hence, no specific need is indicated to deal with active substances from other PTs in any different way than with additives in general when it comes to identifying product components as relevant for a mixture assessment.

The transitional guidance (ECHA 2014) furthermore proposes , in accordance with the BPR, additives as 'mixture relevant' that are classified as dangerous or hazardous to the environment (EC 1967 or EC 2008a, respectively) if their presence in the product leads to a classification of the product as dangerous and/or hazardous to the environment. The here evaluated set of 21 wood preservative products included 7 that were not classified as dangerous or hazardous to the environment according to their SDS. Hence, by definition these products do not contain additives that would lead to such a classification of the product and that should therefore be deemed relevant for the mixture assessment. Nevertheless, the expected summed contribution of additives ranged in these products from 0.3% to 91.6% STU in the most sensitive endpoint. In all except one of these 7 products, the additives were actually expected to contribute more than 10% of the joint toxicity. This is also reflected in the observed lack of correlation between the classification of a product as hazardous to the environment and the relative toxicity contribution of the additives to the joint toxicity of the product. A component-based environmental risk assessment that includes only the additives that lead to a labelling of the product as hazardous to the environment would therefore ignore substantially contributing components and result in a (potentially non-protective) underestimation of product toxicity. Product 6 was the only one that was subject to experimental testing among the 7 evaluated products which were not classified as hazardous to the environment. The test results demonstrated that the product toxicity was underestimated (by factor 2 to 3) when only the a.s. were considered, which confirms the need to include additives in the prediction. Hence, both the theoretical survey of wood preservative products and the experimental testing of one product point out that the current proposal of the transitional guidance for the identification of relevant additives may not be sufficient for a protective component-based environmental risk assessment.

The criteria of the CLP regulation (EC 2008a) result in a more conservative selection of additives that should be considered in a component-based assessment than the current proposal of the transitional guidance (ECHA 2014). The CLP criteria have the advantage to be already established regulatory and required to be applied in the process of product classification and labelling anyway. Experimental testing with product 6 and 32 verified that consideration of 'CLP' categorised additives reduced underestimation of product toxicity without leading to considerable overestimation of the toxicity on the other

hand. The results obtained with product 16 re-iterate that the consideration of 'CLP' categorized additives improves the toxicity prediction, but demonstrated at the same time that this does not necessarily always results in a protective mixture assessment. Thereby product 16 illustrates the key problem with the component-based approach: the requirement of available data for all relevant compounds with regard to the most sensitive endpoint. Yet, this is a requirement that is repeatedly mentioned in the transitional guidance; it cannot be solved by adjusting criteria for identification of relevant components. Product 20 also points at a lack of available data as the (slight) underestimation of toxicity was not even reduced when all additives with aquatic toxicity data were included in the calculation. One or more of the numerous additives without aquatic toxicity data in this product may be responsible for the unexplained part of the observed joint toxicity. While there were indications which additive in product 16 may be responsible for the unexplained part of toxicity (weight-of-evidence based on high toxicity in the available endpoints and read-across from structurally similar compounds, i.e. alkylamines) no such hints were identified for product 20. Based on the here evaluated products, it appears particularly important to ensure data completeness with regard to algal toxicity (the relevant endpoint for most products) and for metal organic compounds and amines since representatives from these groups were often identified as mixture relevant.

Product 16 further demonstrated that the category 'HAZ' helped identifying relevant components that had not been covered by the 'CLP' category (due to lack of toxicity data or specific hazard information). Hence, including all additives that are listed as hazardous to the environment on the product SDS, additionally to those that fall into the 'CLP' category, would safeguard against missing out on some relevant components. The category 'HAZ' reflects the statement in the transitional guidance to include additives that are classified because of specific hazard concerns, i.e. long-range transport potential, persistence, bioaccumulation potential and toxicity. These criteria relate basically to the candidate list set up and published by ECHA (https://echa.europa.eu/candidate-list-table). However, it can be argued that including an additive in a mixture toxicity prediction only because of its potential for bioaccumulation or long-range transport does not make much sense, because it will solely be the aquatic toxicity of an additive that is relevant to the prediction of aquatic mixture toxicity of the product. Degradability properties (persistence) are similarly of little relevance for the calculation of product toxicity, but do inform decisions about which environmental mixture resulting from the usage of the product shall actually be assessed.

The question which mixture should actually be assessed is also relevant with regard to additives that are rapidly degradable as defined in the CLP regulation (EC 2008a). They were included in the present calculations in order to allow the comparison with the test results for the products. Within the authorisation process, however, the criteria for additives deemed relevant in a component-based assessment may need to be adapted. One possibility would be to apply these criteria to the components predicted to be in the environmental mixture resulting from the use of the product, predicted according to the usage-specific emission scenario documents.

The additives categorized as 'CLP' covered most of the additives categorized as '>10% STU', and all additives that were expected to contribute more than 20% to the joint product toxicity. The experimental testing with the products demonstrated that including additives with more than 10% STU but less than 20% STU did not notably improve the mixture prediction. Hence, this tentative selection criterion was not found to provide any additional advantages, while it would provoke on the other side a substantial workload, i.e. the calculation of relative toxic units for all additives in a product. Therefore, it is not deemed a helpful tool for identifying additives that are relevant to a component-based assessment of biocidal products.

Including all additives in a component-based assessment would be the most precautious and conservative approach. Yet, there was no indication from the experimental testing of products that this

approach would actually result in a more protective regulatory decision. Including all additives increased the degree of overestimation in some cases, but never reduced unexplained underestimation of toxicity.

Overall, the results of the present study support an approach for identifying additives as relevant for a component-based mixture assessment that is based on some criteria of the CLP regulation, restricted to the combination of aquatic toxicity and content in the mixture. An additional criterion may be the listing of an additive as hazardous to the environment on the SDS of the biocidal product. It is not deemed necessary and suitable to include all additives, to deal specifically with a.s. from other product types, or to apply the expected toxicity contribution (% STU) as criterion when identifying additives as 'mixture relevant'. Based on these proposed criteria, in 48 out of 273 cases (i.e., 17.6%) an additive was identified as relevant in the 21 wood preservative products. On average, 2-3 additives would have to be considered along with the a.s. in a CBA for the environmental risk assessment. If 'ready biodegradability' was used additionally as an exclusion criterion, the number of additives to be considered was reduced to 21 cases, i.e. on average about one additive per product.

The extrapolation of this proposal to other product types remains open, as no experimental verification was conducted with other products than wood preservatives. However, there is no reason to assume that an extrapolation would not be meaningful or appropriate. The key difference would most likely be the definition of the mixture that should actually be assessed (i.e. the product itself or the environmental mixture resulting from the usage and, hence, its composition), which will largely differ among product types based on the emission scenarios. The same holds true for extrapolation to eluates of wood preservative products, which represent the environmentally relevant mixtures.

6 Synergistic interactions in biocidal products

The definition of 'synergism' is not unambiguous in the literature (Berenbaum 1989), particularly among different scientific fields. According to Bernard & Philogène (1993) there are (true) synergists, which are non-toxic chemicals that enhance the toxicity of pesticides. Often the term 'synergist' is restricted to metabolic inhibitors, while those that enhanced the toxicity by other mechanisms (such as enhanced bioavailability, uptake, or transport) are called quasi-synergists (Bernard & Philogène 1993). Potentiation on the other hand refers to more-than-additive effects of two or more chemicals that are all toxic at the applied level. In the field of ecotoxicology, these different underlying mechanisms of action are all seen as synergistic interaction (Spurgeon et al. 2010). In the context of the present project, a 'synergist' is defined as any substance that interacts with another chemical in a way that their joined action is greater than that predicted by assuming additive action.

A key question in a component-based environmental risk assessment of biocidal products relates to the uncertainty of synergistic interactions. Synergistic interactions would lead to an underestimation of risks if the assessment uses classic theoretical mixture toxicity concepts for additivity such as CA and IA. Theoretical assessments and experimental testing aim to help answer the question whether the environmental risk assessment of a biocidal product containing a synergist can still be based on theoretical mixture concepts (eventually modified by using a synergy factor) or if product testing is generally warranted instead.

6.1 Piperonylbutoxide as an example of a synergist

Synergists may intentionally be added to biocidal products to enhance the effect of the a.s. on target organisms. These as well as other additives contained in a product may also unintentionally enhance effects on non-target organisms. Piperonylbutoxide (PBO) was selected in the present project to represent intentionally added synergists. During the lifetime of the project, however, PBO changed its status as from being added as additive to being considered as biocidal a.s. on its own.

PBO has long been known for synergistically enhancing the effects of pyrethroids in target insects (Bernard & Philogène 1993). As mirrored by the currently marketed insecticidal biocides (see below), this feature of PBO is still made use of. The mechanism by which PBO acts as synergist is the inhibition of cytochrom P450 mono-oxygenase I, an enzyme that detoxifies pyrethroids in many insect species (Bernard & Philogène 1993, Ishaaya 1993). PBO is particularly useful and widely applied in the treatment of target insects that have developed insecticide resistance by up-regulating this specific detoxification mechanism.

Since mono-oxygenase I also detoxifies insecticides from other structural groups such as organophosphates (dimethoate, malathion, and parathion) and carbamates (carbaryl and methomyl), PBO can synergize their effects as well (Bernard & Philogène 1993). Recently, Darriet & Chandre (2013) described the synergistic interaction of PBO with insecticides from still another group, the neonicotinoids, in the target organisms *Aedes aegypti* and *Anopheles gambiae*. The synergy was found to be greatest when PBO was combined with a pyrethroid and a neonicotinoid. Mono-oxygenase I also appears to play a role in the resistance of target insects against the juvenile hormone-mimic pyriproxyfen, as resistance towards this insecticide could be suppressed by PBO (Karatolos et al. 2012).

Non-target species may use similar enzymes for detoxification of insecticides, which can lead to the occurrence of synergistic PBO effects in these organisms. Examples have been described for the amphipod *Hyalella azteca* (Amweg et al. 2006, Brander et al. 2009). Since mono-oxygenase I also detoxifies other xenobiotics, PBO may accordingly also synergize the effects of these substances. This has been described for the herbicidal effects of atrazine and terbutryn in maize (Varsano et al. 1992). In addition, the ability of PBO to synergize toxic effects is not limited to pesticides. Weinstein & Garner

(2008) reported synergistic effects of PBO in combination with the polycyclic aromatic hydrocarbons (PAH) fluoranthene and benz[a]pyrene in the shrimp *Palaemonetes pugio*. Wang et al. (2013) found synergistic effects of PBO in combination with methanol in the fruit fly *Drosophila melanogaster*. In cockroaches, PBO showed synergistic interaction with linear alcohol ethoxylates (Sims & Appel 2007).

In the freshwater micro-crustacean *Ceriodaphnia dubia*, however, the toxicity of the organophosphates dichlorvos, chlorfenvinphos, and mevinphos was not synergistically enhanced by PBO (Ankley et al. 1991). Yet, PBO reduced the toxicity of other organophosphates that require metabolic activation, which indicates the involvement of mono-oxygenase I in the metabolism of organophosphates in *Ceriodaphnia dubia* and *Daphnia pulex* (Ankley et al 1991). The occurrence of reduced toxicity due to PBO inhibiting the metabolic activation of an organophosphate (here: fenitrothion) was confirmed for *Daphnia magna* (Damasio et al. 2007). The involvement of mono-oxygenases in the detoxification of xenobiotics in *Daphnia magna* was supported by the results of Akkanen & Kukkonen (2003) who reported on PBO inhibiting the extensive biotransformation of pyrene in this species. Brausch & Smith (2009) investigated the effect of PBO on the acute toxicity of the pyrethroid cyfluthrin and the PAH naphthalene in *Daphnia magna* and found that PBO increased the toxicity of both compounds in tolerant but not in susceptible clones. Overall, the available literature on effects of PBO in *Daphnia magna* supports the involvement of mono-oxygenases in detoxification processes, but also indicates limited enhancement of toxicity in susceptible clones. This may be due to a low level of expression or involvement of these enzymes in non-tolerant *Daphnia*, meaning that the inhibition of the enzymes by PBO cannot further enhance the toxicity of substances that could be detoxified by them. The acute toxicity of PBO toward *D. magna* has been reported as a 48 h EC_{50} of 2.83 mg/l (Ankley et al. 1991).

Table 21 summarizes the compiled literature data that allowed calculating a synergy ratio as a measure of the degree of synergisms in test organism-substance combinations. The synergy ratio (Bernard & Philogène 1993) is calculated from the observed toxicity estimate without presence of synergistic divided by the observed toxicity estimate in the presence of synergistic. A synergy ratio below 1 indicates accordingly antagonism.

Table 21 Synergistic and antagonistic effects of PBO documented in the literature

Chemical (chemical group)	Test organism	LD50 or LC50 without synergist	Synergy Ratio	Source
Cyhalothrin (pyrethroid)	housefly (susceptible strain)	0.007 µg/fly	3.5	(Bernard & Philogène 1993)
Cyhalothrin (pyrethroid)	housefly (resistant strain)	33 µg/fly	300	(Bernard & Philogène 1993)
Fenfluthrin (pyrethroid)	housefly (susceptible strain)	0.048 µg/fly	12.0	(Bernard & Philogène 1993)
Fenfluthrin (pyrethroid)	housefly (resistant strain)	2 µg/fly	5.3	(Bernard & Philogène 1993)
RU 38702 (pyrethroid)	housefly (susceptible strain	0.013 µg/fly	4.3	(Bernard & Philogène 1993)
RU 38702 (pyrethroid)	housefly (resistant strain)	120 µg/fly	923	(Bernard & Philogène 1993)
Permethrin (pyrethroid)	housefly (susceptible strain)	0.022 µg/fly	4.4	(Bernard & Philogène 1993)
Permethrin (pyrethroid)	housefly (resistant strain)	130 µg/fly	812	(Bernard & Philogène 1993)

Dichlorvos (organophosphate)	housefly (susceptible strain)	0.032 µg/fly	1.4	(Bernard & Philogène 1993)
Dichlorvos (organophosphate)	housefly (resistant strain)	0.51 µg/fly	0.9	(Bernard & Philogène 1993)
Methomyl (carbamate)	housefly (susceptible strain)	0.23 µg/fly	24.7	(Bernard & Philogène 1993)
Methomyl (carbamate)	housefly (resistant strain)	2.7 µg/fly	5.0	(Bernard & Philogène 1993)
Permethrin (pyrethroid)	*Hyalella azteca* (amphipod)	14.2 mg/kg OC	7	Amweg et al. 2006
Cyfluthrin (pyrethroid)	*Daphnia magna* (susceptible clone)	0.62 µg/l	1.3	Brausch & Smith 2009
Cyfluthrin (pyrethroid)	*Daphnia magna* (resistant clone)	2.8 µg/l	5.6	Brausch & Smith 2009
Naphthalene (PAH)	*Daphnia magna* (susceptible clone)	7.8 mg/l	1.7	Brausch & Smith 2009
Naphthalene (PAH)	*Daphnia magna* (resistant clone)	> 20 mg/l	> 4.3	Brausch & Smith 2009
Fenitrothion (organophosphate)	*Daphnia magna* (susceptible clone)	0.8 µg/l	0.05	Damasio et al. 2007
Fenitrothion (organophosphate)	*Daphnia magna* (resistant clone)	4.9 µg/l	0.06	Damasio et al. 2007
Deltamethrin (pyrethroid)	*Culex* larvae (2 species)	0.8 and 1.5 µg/l	4.0 and 4.1	Fakoorziba et al. (2008)
Deltamethrin (pyrethroid)	*Anopheles* larvae (4 species)	0.2-4.2 µg/l	5.8-21.0	Fakoorziba et al. (2008)
Deltamethrin (pyrethroid)	*Chilo suppressalis* (3 field populations)	28.3-97.6 ng/larvae	2.4-9.6	He et al. (2012)
Pyriproxyfen (juvenile hormone mimic)	*Trialeurodes vaporariorum* (susceptible strain)	0.014 mg/l	1.4	Karatolos et al. (2012)
Pyriproxyfen (juvenile hormone mimic)	*Trialeurodes vaporariorum* (resistant strain)	63.9 mg/l	290	Karatolos et al. (2012)
Pyrethrins	*Hyalella azteca* (amphipod)	0.75 µg/l	0.7-3.2	Giddings et al. 2016

Synergy ratio (Bernard & Philogène 1993) is LD/LC50 determined without synergistic divided by LD/LC50 determined in presence of synergistic (synergist usually at plateau concentration, i.e. with maximum synergistic effect). Synergy ratio below 1 indicates antagonism.

Bernard & Philogène (1993) list a number of commonly used insecticide synergists such as sesamex (CAS 51-14-9), sulfoxide (as structural group), dillapiol (CAS 484-31-1), sesamine (CAS 607-80-7),

MGK-264 (N-(2-ethylhexyl)-8,9,10-trinorborn-5-ene-2,3-dicarboxamide, CAS 113-48-4), N-decylimidazole (CAS unknown), WARF-antiresistant (*N,N*-dibuthyl-4-chlorobenzene-sulfonamide, CAS 127-59-3), DEF (S,S,S-tributyl phosphorotrithioate, CAS 78-48-8), TPP (*O,O,O*-triphenylphosphate, CAS 115-86-6), IBP (5-benzyl-O,O-diisopropyl phosphorothioate, CAS 260-87-47-8), PSCP (phenylsaligenin cyclic phosphate, CAS 4081-23-6), iodomethane (CAS 74-88-4), t-phenylbutenone (CAS 122-57-6), DEM (diethylmaleate, CAS 141-05-9), DMC (chlorfenethol, CAS 80-06-8), ETP (1,1,1-trichloro-2,3-epoxypropane, CAS 3083-232-6), and ETN (1,2-epoxy-1,2,3,4-tetrahydronaphtalene, CAS 2461-34-9). None of these compounds was contained in any of the 30 biocidal products.

None of the 30 products selected by the German Environment Agency for this project contained PBO. An internet search revealed that there are several biocidal products on the European market that contain PBO. The list of currently registered products in the United Kingdom, for example, covers 10 products (all PT 18) that contain PBO together with one or two pyrethroids such as permethrin, tetramethrin, or d-phenothrin. PBO is contained in these products at a mass concentration of about factor 1.5 to 5 higher than the sum of pyrethroids. Various such products can be found in the internet for sale. Yet, no wood preservative products could be found for sale that contained PBO. Since the original approach of testing a marketed wood preservative with PBO failed due to unavailability of such a product, alternative options for experimentally addressing the topic of synergistic interactions were explored.

The options for investigating the potential of synergistic interactions in wood preservative products in the present project were prioritized in consultation with the German Environment Agency as:

1. Study the interaction of a.s. that are suspected to interact synergistically with each other (Coors & Frische 2011, SCHER/SCENIHR/SCCS 2011). One of the selected products (P21) contains such an example, i.e. two fungicides that inhibit different enzymes in the same biochemical pathway, the ergosterol biosynthesis.
2. Study the interaction of organic solvents with a.s. and other relevant additives. In previous projects, the *Daphnia* and fish toxicity of wood preservative products with naphtha as organic solvent could not be reliably predicted by CA but appeared to exhibit synergistic interaction (Coors et al. 2012a,b, 2014). This option was not prioritized highest, because not enough solvent-based products could be tested within the scope of the present project to derive meaningful and sound conclusions on this potential mechanisms of synergistic interaction in biocidal products. Yet, exploring the influence of organic solvents on the predictability of product mixture toxicity remains a relevant research topic as such potential interactions will impact the reliability of component-based assessments for solvent-based biocidal products.
3. Study the synergizing interaction of PBO with insecticides in generic mixtures. This approach was deemed of limited applied interest only, because it is not clear with which insecticides PBO may be combined in biocidal products in the future.

6.2 Mixtures of ergosterol biosynthesis inhibitors

Studying the interaction of suspected synergistic combinations was chosen as alternative to investigating PBO in the present project. Based on previous investigations (Coors & Frische 2011), the combination of fungicides that inhibit different enzymes in the biosynthesis of ergosterol (ergosterol biosynthesis inhibitors, EBI) was selected for this purpose as potential synergist interactions were found for algae (but not for other organisms). Particularly the combination of tebuconazole as inhibitor of C14-demethylase (DMI fungicide, demethylase inhibitor) and fenpropimorph and fenpropidin as inhibitors of Δ^{14} reductase was selected for experimental investigation.

The single substances tebuconazole, fenpropidin and fenpropimorph were tested in algal growth inhibition tests according to OECD guideline 201 (OECD 2006a). Subsequently, binary mixtures

(fenpropidin & tebuconazole, fenpropimorph & tebuconazole) were tested for algal growth inhibition as fixed-ratio mixture dilution series at an equipotent ratio. The algal growth inhibition tests were conducted as those described in Chapter 3.1, and were all valid based on the criteria of the guideline. Detailed study reports are attached as confidential annex to the present report. When testing fenpropimorph and fenpropidin, problems were encountered in deriving a complete concentration-response curve with regard to growth rate. In the case of fenpropidin, no inhibition of growth rate by 50% or more could be achieved within the limits of water solubility in several independent tests. Therefore, the evaluation of synergistic effects was based on the response variable yield instead of growth rate for this binary mixture. In the case of fenpropimorph, the problem of little water solubility and substantial adsorption could be solved by pre-conditioning all vessels with respective test solutions, both in the single-substance and the mixture test. In contrast to the product tests, all concentration levels were analysed in freshly prepared and aged test solutions in these tests. All toxicity estimates and resulting MDR values are based on mean measured concentrations (geometric mean of concentrations measured in freshly prepared test solutions and after three days of exposure).

The toxicity estimates for the single substances are summarized in Table 22. The comparison of the here determined algal toxicity with available regulatory data indicates for tebuconazole good agreement: the E_rC_{50} is given as 5.3 mg/l for a 72 h static test with *Desmodesmus subspicatus* (EC 2007b). For fenpropimorph, the here determined 72 h EC_{50} for yield inhibition of *R. subcapitata* was about 46-fold lower than the 72 h E_bC_{50} of 0.327 mg/l reported as endpoint in the environmental risk assessment of fenpropimorph (EC 2005, 2009c), while the NOEC for yield (0.005 mg/l) was similar. The 72 h E_rC_{50} determined as 1.818 does not contradict the value being reported as >1 mg/l in the regulatory dossiers (EC 2005, 2009c). The NOEC for growth rate was here determined as being at least factor 10 lower than the value of 0.058 mg/l reported in regulatory dossiers (EC 2005, 2009c). For fenpropidin, the here determined 72 h E_bC_{50} for *R. subcapitata* of 0.000225 mg/l was about 25-fold lower than the nominal 96 h E_bC_{50} for *D. subspicatus* of 0.0057 mg/l reported for the technical material in the environmental risk assessment of fenpropidin as pesticide (EFSA 2007). This report (EFSA 2007) also lists a 72 h E_bC_{50} for *R. subcapitata* (formerly: *S. capricornutum*) of 0.00026 mg/l derived with a formulated product, which was used in the risk assessment. The here derived 72 h E_bC_{50} for *R. subcapitata* is very similar to this value, and was obtained with technical material directly dissolved in aqueous test medium.

Table 22: Toxicity estimates (mg/l) derived for tebuconazole, fenpropidin, and fenpropimorph with the green algae *R. subcapitata*

Toxicity estimate	Tebuconazole	Fenpropidin	Fenpropimorph
Growth Rate			
EC_{50}	4.54	>0.005	1.8181
EC_{20}	3.56	0.00494	0.5830
EC_{10}	3.08	0.000066	0.2745
NOEC	0.43	0.000045	<0.005
Yield			
EC_{50}	2.56	0.000225	0.0071
EC_{20}	1.41	0.000012	0.0002 *
EC_{10}	0.10	0.000006	0.00002 *
NOEC	0.43	0.000045	<0.005

Shown are toxicity estimates based on measured concentrations and the No Observed Effect Concentration (NOEC) for the two response variables yield and growth rate; *: extrapolated beyond tested concentrations

Table 23 summarizes the MDRs determined for the two mixtures of EBI fungicides tested in the present project. All determined MDR values, regardless of effect level, response variable and mixture components indicated a less than 2.5-fold deviation between the CA-predicted and the observed mixture toxicity. Only MDR values based on extrapolated toxicity estimates indicated a greater than 2.5-fold overestimation of toxicity. Hence, there was no indication of synergistic interaction between tebuconazole and the DMI fungicides fenpropidin and fenpropimorph. Indications for synergism in earlier studies (Coors & Frische 2011) may therefore be traced back either to different input data for the individual mixture components (as the single substance tests in the present project provided estimates that differed from regulatory endpoints) or to formulation additives of the investigated pesticidal products. With regard to biocidal products, the lack of evidence for synergistic interaction among EBI fungicides reduces the concern about consideration of unknown synergisms (e.g. via a synergy factor) to some degree.

Table 23: Model deviation ratios (MDR) for binary equipotent mixtures of ergosterol biosynthesis inhibitors in the green algae *R. subcapitata*

Toxicity estimate	Tebuconazole & Fenpropidin	Tebuconazole & Fenpropimorph
Growth Rate		
EC_{50}	1.13 *	2.01
EC_{20}	2.10	1.59
EC_{10}	0.60	1.24
NOEC	0.91	n.d.
Yield		
EC_{50}	1.84	0.41
EC_{20}	0.60	0.24 *
EC_{10}	0.47	0.24 *
NOEC	0.91	n.d.

*: based on toxicity estimates extrapolated beyond tested concentrations; n.d.: not possible to determine

7 Mixture toxicity of wood preservative products in the terrestrial compartment

Product 14 was selected to serve as an example wood preservative product regarding the predictability of mixture toxicity toward collembolans as representative terrestrial organisms. Product 14 is registered in use class 2 and 3, i.e. without direct contact to soil but may be used outdoors on weather-exposed wood. It contains the two fungicides IPBC and tebuconazole together with a number of additives, among them two preservatives. There is one additive contained, an organic solvent, that is labelled as hazardous, but not with regard to the environment. Toxicity data regarding reproduction of collembolans are only available for tebuconazole with a NOEC of 250 mg a.s./kg soil d.w. reported in the dossier (EC 2007b). To enable mixture predictions based on EC_x values, single-substance tests for avoidance behaviour and reproduction were conducted with tebuconazole, IPBC, and the product. In addition, an avoidance test was conducted with the organic solvent contained in product 14 as single substance.

7.1 Experimental methods

All experiments with the test organism *Folsomia candida* (collembola) were conducted at ECT Oekotoxikologie GmbH according to OECD guideline 232 (OECD 2009, reproduction test) and ISO 17512-2 (ISO 2011, avoidance test), and generally deemed valid (see below). While the tests were not conducted formally under the premises of Good Laboratory Practice (GLP), they were conducted in a GLP-certified laboratory. Within this GLP framework, regular reference tests were conducted that proved the required sensitivity of the test organisms (not shown here). No solvents were used in the avoidance and reproduction tests with the product, while acetone was used as solvent for the tests with the single substances. Controls in the product tests were therefore spiked with deionised water without the addition of the test item, whereas additional solvent controls were used in the tests with the single substances, spiked with the solvent in similar concentration as in the treatments.

All reproduction and avoidance tests were conducted using artificial soil with an organic matter content of 5% and a composition according to OECD guideline 232 in order to enable comparisons.

7.1.1 Performance of reproduction tests

The reproduction tests were performed according to OECD guideline 232 (OECD 2009) over an exposure period of 28 days. All definitive tests were conducted with four replicate vessels for the water control (without solvent and test item), eight replicates for the solvent control (if applicable) and four replicate vessels for each test concentration level. Each replicate vessel (Ø 5.5 cm, 250 ml volume) contained 30 g soil and 7-10 mg of granulated dry yeast as food. The tests were started with juvenile *F. candida* (9 – 12 days old) obtained from synchronised breeding cultures. Ten individuals were added to the soil surface of each replicate test vessel at test start. The test conditions were temperature between 14.6 and 21.9°C, light intensity between 416 and 796 lux, and a light:dark cycle of 16:8 h. These conditions were according to the guideline (required are 20±2°C, 400-800 lux, and between 12:12 and 16:8 h light:dark cycle), except temperature. However, in the individual conducted tests the temperature varied between not more than 7.3°C from the mean temperature. As all validity criteria have been fulfilled in the conducted tests, the temperature deviations are not deemed to invalidate the results. The pH of the soil in the test vessels was between 5.4 and 6.8 during the tests, the soil moisture ranged from 38.9 to 58.7% of the maximum water holding capacity (WHC_{max}). The OECD guideline 232 prescribes a pH of 6±0.5 and a soil moisture between 40% and 60% of the WHC_{max}, which is fulfilled with slight deviation in some tests. After 28 days, the number of adults and juveniles was determined by counting under a binocular using ink as dye.

The following validity criteria apply to the (solvent) control at the end of the reproduction test according to the guideline: the mean adult mortality of collembolans should not exceed 20%, the mean number of juveniles should be at least 100, and the coefficient of variation (CV) of juvenile number should not exceed 30%. All conducted tests were valid, because the mean mortality (including missing individuals) of collembolans in the controls was at maximum 11.3%, the number of juveniles per vessel was at least 250, and the CV of numbers of juvenile ranged from 23.6% to 27.1%.

7.1.2 Performance of avoidance tests

The avoidance tests with the collembolan *F. candida* were performed according to ISO guideline 17512-2 (ISO 2011) over an exposure period of 48 h. All definitive tests were conducted with five replicate vessels for the (solvent) controls and five replicate vessels for each test concentration level. Each replicate contained 30 g test soil (fresh weight) in each half of the test vessel (Ø 5.5 cm, 250 ml volume). The other half of each test vessel was filled with 30 g soil (fresh weight) representing the (solvent) control. The tests were started by putting 20 juvenile *F. candida* (9 – 12 days old) obtained from synchronised breeding cultures onto the soil surface of each test vessel. Test conditions were temperature between 18.2-21.5°C, light intensity between 470 and 736 lux, and a light:dark cycle of 16:8 h. The test conditions according to the guideline are 20±2°C, 400-800 lux, and a light:dark regime between 12:12 and 16:8 h, which were hence met by the conducted tests. The pH of the soil in the test vessels was between 5.6 and 6.8 during the tests, the soil moisture ranged from 44.9 to 54.8% of the WHC_{max}. The ISO guideline 17512-2 prescribes a pH of 6±0.5 and a soil moisture between 40% and 60% of the WHC_{max}, which is thereby fulfilled in the conducted tests except a slightly too low pH in one of them. After 48 h of exposure, living and dead (=missing) collembolans were counted separately on each side of each test vessel.

All conducted avoidance tests were valid, because the mean mortality (including missing individuals) of collembolans did not exceed 20% and the mean number of collembolans in each section of the control combination treatments was between 40 to 60%. There were exceptions as in some of the highest test concentrations more than 20% mortality was observed; these treatments had to be excluded from the evaluation of avoidance then.

7.1.3 Data analysis

The response variables mortality (dead or missing in relation to introduced collembolans), reproduction (number of juveniles after 28 days) and avoidance were evaluated statistically. Avoidance (%) was calculated for each replicate vessel according to the guideline (ISO 2011) as

$$Avoidance\ (\%) = \frac{n_C - n_t}{N} * 100$$

With n_c and n_t being the counted live collembolans on the treated and the control soil, respectively, and N being the total number of counted live collembolans.

Statistical evaluations (i.e. hypothesis testing and concentration-response modelling) as well as calculation of model deviation ratios (MDR) were performed as described in Chapter 3.1. More details are provided in the individual study reports of the tests as part of the confidential annex.

7.2 Predictability of reproduction inhibition and avoidance behaviour in collembolans and relationship between the two endpoints

Both single substances as well as the biocidal product exhibited chronic toxicity toward collembolans (Table 24). The determined NOEC for tebuconazole was 5-fold lower than the values reported in the regulatory dossier for tebuconazole as biocide (EC 2007b). The determined value of 50 mg/kg soil d.w. was supported by two independent, formally valid tests. The chronic effects of the biocidal product on the reproduction of *F. candida* was predictable by CA with deviation of less than factor 5 in the case of

the NOEC and less than factor 4 with regard to effect estimates. The slight underestimation of product toxicity may indicate chronic collembolan toxicity of one or more additives in the product, which had not been considered in the prediction.

Both active substances and the product were also significantly avoided by collembolans. Considering only the active substances, the avoidance response was strongly underestimated by the CA prediction. In order to verify if one key additive, an organic solvent, was responsible for this underestimation, the solvent was tested as single substance as well and induced no avoidance response up to the highest tested concentration of 10 mg/kg soil d.w. Using this value in the CA prediction resulting in an MDR that still indicated underestimation of the behavioural response by at least factor 7.5.

The results for the three test items did not provide evidence for a correlation between the apical toxic response (reduction of offspring) and the behavioural response (avoidance) in collembolans. Tebuconazole for example induced a stronger avoidance response than IPBC, but exhibited lower chronic toxicity with regard to reproduction. Hence, it appears not possible to extrapolate from one test result to the other for a given substance.

Overall, it can be concluded that the chronic toxicity of terrestrial organisms can be fairly well predicted by CA, while the behavioural response is strongly underestimated. Whether this underestimation points at the presence of additives that induce strong avoidance (but little toxicity – given the MDR values for chronic toxicity) or whether it indicates that behavioural responses *per se* do not follow concentration-additive interaction remains open.

Table 24: Toxicity estimates derived in reproduction and avoidance tests with the collembolan *F. candida* and resulting model deviation ratios (MDR).

Endpoint	Tebuconazole	IPBC	Additive (Solvent)	Product 14	MDR – only a.s.	MDR – a.s. and one additive
Reproduction						
EC_{50}	217.4	17.87	n.d.	1057.7	2.31	n.d.
EC_{20}	79.9	16.47	n.d.	692.1	3.02	n.d.
EC_{10}	44.5	15.70	n.d.	544.9	3.43	n.d.
NOEC	50.0	10.00	n.d.	316.0	4.06	n.d.
Avoidance						
EC_{50}	14.2	146.4	>10.0	67.6	44.89	>7.51

All toxicity estimates are given in mg/kg soil d.w. and relate to the test item, i.e. the single substance or the product;
*: based on toxicity estimates extrapolated beyond tested concentrations; n.d.: not determined

8 Outlook

The theoretical approach combined with experimental verification allowed deriving a proposal for the identification of product additives that should be considered along with the active substances in a component-based environmental risk assessment for biocidal products. The proposed identification criteria appear practicable as they relate to criteria that need to be assessed anyway in the context of classification and labelling. The number of additives that would have to be considered also appears practicable with on average 2 to 3 per product. The workload for both applicants and regulatory authorities is thereby only increased to a presumably acceptable level. An open question is whether the criteria for 'mixture relevance' should apply to the composition of the biocidal product or to the environmental mixture resulting from the usage of the biocidal product (such as those resulting from leaching of treated wood). In the latter case, (ready) biodegradation of some additives would reduce the number of additives that need to be considered to about one per wood preservation product. The role of biodegradability and the question which mixture should actually be assessed appears therefore to require clarifying statements in the guidance for a mixture assessment of biocidal products.

Regarding the reliability of a component-based assessment, the present study provides extensive evidence that such an assessment would be protective, if not over-protective in some cases. From a regulatory perspective, this is clearly preferred over remaining concern about potentially under-protective assessments. An open question remains with regard to acute fish toxicity as the prediction based on adult fish toxicity was protective for fish embryo toxicity, while it could be questioned whether it was also protective for acute toxicity toward adult fish (which was not experimentally determined in the present project). Yet, there is no evidence from the present study or indication otherwise that CA would provide protective estimates only for two trophic levels, but not for fish as the third trophic level.

Concern about synergistic interactions between the different components of a biocidal products could be reduced by the present study. There was no evidence from the experimental testing of the wood preservative products that synergistic interactions occurred. The known synergist PBO is apparently far less frequently used in biocidal products than initially assumed. In addition, one mechanism of synergistic interaction, inhibition of different enzymes in the same biosynthesis pathway, was shown to be at least not generally present. The role of organic solvents in facilitating the uptake of other product components and thereby resulting in an apparent synergistic interaction could not be experimentally addressed in the present project. It remains an open question. However, most organic solvents in the here investigated biocidal products were readily biodegradable, which may considerably reduce the likelihood of actually occurring synergistic interaction in environmentally relevant mixtures. With regard to terrestrial mixture toxicity and its predictability, it remained open if the strong underestimation of avoidance of the product related to one or more additives that were not considered in the prediction. An alternative explanation would be that behavioural responses *per se* cannot be predicted by concentration addition. The slightly higher degree of uncertainty in the CA prediction of chronic effects on the reproduction of terrestrial organisms may likewise relate to not-considered additives or indicate a general difference in the predictability of mixture toxicity between aquatic and terrestrial compartments and their organisms.

Overall, the present study provided extensive evidence that the toxicity of biocidal products can reliably be predicted by the CA concept in order to support a component-based environmental risk assessment. This holds true for acute aquatic toxicity and was also demonstrated for one endpoint of chronic terrestrial toxicity. It does not hold true, however, for behavioural endpoints such as avoidance behaviour. Consideration of the relevant, but not all formulation additives was shown to be crucial for a reliable component-based assessment. The availability of toxicity data for the relevant additives is the key factor that determines the reliability of such a component-based assessment.

9 References

Akkanen J, Kukkonen JVK (2003) Biotransformation and bioconcentration of pyrene in *Daphnia magna*. Aquat. Toxicol. 64, 53-61.

Altenburger R, Arrhenius A, Backhaus T, Coors A, Faust M, Zitzkat D (2012) Ecotoxicological combined effects from chemical mixtures - Part 1: Relevance and adequate consideration in environmental risk assessment of plant protection products and biocides. Report on behalf on the Federal Environment Agency (UBA), Germany. FKZ 3709 65 404.

Amweg EL, Weston DP, Johnson CS, You J, Lydy MJ (2006) Effect of piperonyl butoxide on permethrin toxicity in the amphipod *Hyalella azteca*. Environ. Toxicol. Chem. 25, 1817-1825.

Ankley GT, Dierkes JR, Jensen DA, Peterson GS (1991) Piperonyl butoxide as a tool in aquatic toxicological research with organophosphate insecticides. Ecotox. Environ. Safe. 21, 266-274.

Backhaus T, Altenburger R, Faust M, Frein D, Frische T, Johansson P, Kehrer A, Porsbring T (2013) Proposal for environmental mixture risk assessment in the context of the biocidal product authorization in the EU. Env. Sci. Eur. 25, 4.

Backhaus T, Faust M (2012) Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environ. Sci. Technol. 46, 2564-2573.

Belden JB, Gilliom RJ, Lydy M (2007) How well can we predict the toxicity of pesticides mixtures to aquatic life? Integr. Environ. Assess. Manag. 3, 364-372.

Berenbaum MC (1989) What is synergy? Pharmacol. Rev. 41, 93-141.

Bernard CB, Philogène BJR (1993) Insecticide synergists: role, importance, and perspectives. J. Toxicol. Environ. Health 38, 199-223.

Bliss CI (1939) The toxicity of poisons applied jointly. Ann. Appl. Biol. 26, 585-615.

Brander SM, Werner I, White JW, Deanovic LA (2009) Toxicity of a dissolved pyrethroid mixture to *Hyalella azteca* at environmentally relevant concentrations. Environ. Toxicol. Chem. 28, 1493-1499.

Brausch JM, Smith PN (2009) Development of resistance to cyfluthrin and naphthalene among *Daphnia magna*. Ecotoxicology 18, 600–609.

Cedergreen N (2014) Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. PLoS ONE 9(5), e96580.

Cedergreen N, Christensen AM, Kamper A, Kudsk P, Mathiassen SK, Streibig J, Sorensen H (2008) A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. Environ. Toxicol. Chem. 27, 1621-1632.

Coors A, Dobrick J, Möder M, Kehrer A (2012a) Mixture toxicity of wood preservative products in the fish embryo toxicity test. Environ. Toxicol. Chem. 31(6), 1239-1248.

Coors A, Frische T (2011) Predicting the aquatic toxicity of commercial pesticide mixtures. Env. Sci. Eur. 23, 22.

Coors A, Löffler I, Noronha-Jänsch P, Weisbrod B, Schoknecht U, Sacher F (2012b) Ecotoxicological combined effects from chemical mixtures. Part 2: Development of ecotoxicological tests with biocidal products and eluates: investigating the suitability of biotests with algae and daphnids to estimate mixture toxicity. Report on behalf on the Federal Environment Agency (UBA), Germany. FKZ 3709 65 404.

Coors A, Weisbrod B, Schoknecht U, Sacher F, Kehrer A (2014) Predicting acute and chronic effects of wood preservative products in *Daphnia magna* and *Pseudokirchneriella subcapitata* based on the concept of concentration addition. Environ. Toxicol. Chem. 33, 382-393.

Coors A. (2015) Umweltrisikobewertung von Chemikalienmischungen in verschiedenen regulatorischen Zusammenhängen. Oral presentation. SETAC-GLB, Zürich, Switzerland. 7.-10. September 2015.

Cox C, Surgan M (2006) Unidentified inert substances in pesticides: Implications for human and environmental health. Environ. Health Perspect. 114, 1803-1806.

Damasio J, Guilhermino L, Soares AMVM, Riva MC, Barata C (2007) Biochemical mechanisms of resistance in *Daphnia magna* exposed to the insecticide fenitrothion. Chemosphere 70, 74–82.

Darriet F, Chandre F (2013) Efficacy of six neonicotinoid insecticides alone and in combination with deltamethrin and piperonyl butoxide against pyrethroid-resistant *Aedes aegypti* and *Anopheles gambiae* (Diptera: Culicidae). Pest. Manag. Sci. 69, 905–910.

De Laender F, Janssen CR, De Schamphelaere KAC (2009) Non-simultaneous ecotoxicity testing of single chemicals and their mixture results in erroneous conclusions about the joint action of the mixture. Chemosphere 76, 428-432.

De Zwart D, Posthuma L (2005) Complex mixture toxicity for single and multiple species: proposed methodologies. Environ. Toxicol. Chem. 24, 2665-2676.

Deneer JW (2000) Toxicity of mixtures of pesticides in aquatic systems. Pest. Manag. Sci. 56, 516-520.

EC (1967) Directive 67/548/EWG of the Council of the European Economic Community of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. OJ 196, 16.8.1967, p. 1–98.

EC (1998) Biocidal Product Directive (BPD). 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.

EC (2005) Draft Assessment Report Fenpropimorph in view of its inclusion in Annex I of Council Directive 91/414/EEC.

EC (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.

EC (2007a) Assessment report for inclusion of the active substances propiconazole in Annex I or IA to Directive 98/8/EC. Product type 8 (wood preservatives). Finalised in the Standing Committee on Biocidal Products at its meeting on 29 November 2007 in view of its inclusion in Annex I to Directive 98/8/EC.

EC (2007b) Assessment report for inclusion of the active substances tebuconazole in Annex I or IA to Directive 98/8/EC. Product type 8 (wood preservatives). Finalised in the Standing Committee on Biocidal Products at its meeting on 29 November 2007 in view of its inclusion in Annex I to Directive 98/8/EC.

EC (2008a) 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. (OJ L 353, 31.12.2008).

EC (2008b) Assessment report for inclusion of the active substances IPBC in Annex I or IA to Directive 98/8/EC. Product type 8 (wood preservatives). Finalised in the Standing Committee on Biocidal Products at its meeting on 22 February 2008 in view of its inclusion in Annex I to Directive 98/8/EC.

EC (2009a) Assessment report for inclusion of the active substance boric acid in Annex I or IA to Directive 98/8/EC. Product type 8 (wood preservatives). Finalised in the Standing Committee on Biocidal Products at its meeting on 20 February 2009 in view of its inclusion in Annex I to Directive 98/8/EC.

EC (2009b) Assessment report for inclusion of the active substance disodium tetraborate in Annex I or IA to Directive 98/8/EC. Product type 8 (wood preservatives). Finalised in the Standing Committee on Biocidal Products at its meeting on 20 February 2009 in view of its inclusion in Annex I to Directive 98/8/EC.

EC (2009c) Assessment report for inclusion of the active substance fenpropimorph in Annex I or IA to Directive 98/8/EC. Product Type PT8 (wood preservatives). Finalised in the Standing Committee on Biocidal Products at its meeting on 20 February 2009 in view of its inclusion in Annex I to Directive 98/8/EC.

EC (2012) Biocidal Product Regulation (BPR). 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.

EC (2013) Assessment report for inclusion of the active substances cypermethrin cis:trans/40:60 in Annex I or IA to Directive 98/8/EC. Product type 8 (wood preservatives). Finalised in the Standing Committee on Biocidal Products at its meeting on 12 July 2013 in view of its inclusion in Annex I to Directive 98/8/EC.

EC (2014) Assessment report for inclusion of the active substance permethrin in Annex I or IA to Directive 98/8/EC. Product type 8 (wood preservatives). Finalised in the Standing Committee on Biocidal Products at its meeting on April 2014 in view of its inclusion in Annex I to Directive 98/8/EC.

ECHA (2014) Transitional Guidance on the Biocidal Products Regulation – Transitional Guidance on mixture toxicity assessment for biocidal products for the in environment. European Chemicals Agency, Helsinki, May 2014.

ECHA (2015) Guidance on the compilation of safety data sheets. Version 3.1, November 2015. European Chemicals Agency, Helsinki.

ECHA (2016) webpage accessed July 2016. http://www.reach-clp-biozid-helpdesk.de/de/Biozide/Wirkstoffe/Genehmigte-Wirkstoffe/Genehmigte-Wirkstoffe.html

EFSA (2007) Conclusion regarding the peer review of the pesticide risk assessment of the active substance fenpropidin. Finalised 17 December 2007. European Food Safety Authority Scientific Report 124, 1-84.

Elendt BP (1990) Selenium deficiency in crustacea: an ultrastructural approach to antennae damage in *Daphnia magna* Straus. Protoplasma 154, 25-33.

Embry MR, Belanger SE, Braunbeck TA, Galay-Burgos M, Halder M, Hinton DE, Leonard MA, Lillicrap A, Norberg-King T, Whale G (2010) The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. Aquat. Toxicol. 97, 79–87.

EU (2015) Commission Regulation EU 2015/830 of 28 May 2015 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal of the European Union. 28 May 2015.

Fakoorziba MR, Eghbal F, Vijayan VA (2008) Synergist efficacy of piperonyl butoxide with deltamethrin as pyrethroid insecticide on *Culex tritaeniorhynchus* (Diptera: Culicidae) and other mosquitoe species. Environ. Toxicol. 24, 19-24.

Giddings J, Gagne J, Sharp J (2016) Synergistic effect of piperonyl butoxide on acute toxicity of pyrethrins to Hyallela Azteca. Environ. Toxicol. Chem. 35, 2111-2116.

Giesy JP, Dobson S, Solomon KR (2000). Ecotoxicological risk assessment for roundup® herbicide. Reviews of Environmental Contamination and Toxicology 167, 35-120.

He Y, Zhang J, Chen J, Shen J (2012) Using synergists to detect multiple insecticide resistance in field populations of rice stem borer. Pestic. Biochem. Phys. 103, 121–126.

Heim J (2015) Vorhersagbarkeit der Mischungstoxizität von Biozidprodukten im Fischeitest. Masterarbeit zur Erlangung des Grades Master of Engineering, vorgelegt 1. September 2015, Hochschule RheinMain, Wiesbaden, Fachbereich Ingenieurwissenschaften, Studiengang Bio- und Umweltwissenschaften.

Ishaaya I (1993) Insect detoxifying enzymes: their importance in pesticide synergism and resistance. Arch. Insect Biochem. 22, 263-276.

ISO (2011) Soil quality — Avoidance test for determining the quality of soils and effects of chemicals on behaviour. Part 2: Test with collembolans (*Folsomia candida*). European Standard EN ISO 17512-2.

Iwasaki Y, Brinkman SF (2015) Application of a generalized linear mixed model to analyse mixture toxicity: survival of brown trout affected by copper and zinc. Environ. Toxicol. Chem. 34, 816-820.

Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE (2005) Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ. Toxicol. Chem. 24, 2701-2713.

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. Aquat. Toxicol. 76, 93-110.

Karatolos N, Williamson MS, Denholm I, Gorman K, Ffrench-Constant RH, Bass C (2012) Over-expression of a cytochrome P450 is associated with resistance to pyriproxyfen in the greenhouse whitefly *Trialeurodes vaporariorum*. PLoS ONE 7(2), e31077.

Kehrer A, Coors A, Sacher F, Schwarz-Schulz B (2015) Mixture toxicity assessment of biocides – from theory to practice: state of play and analyses for 30 products. Oral presentation. 25thAnnual Meeting of SETAC Europe, Barcelona, Spain, 3.-7. May 2015.

Klüver N, König M, Ortmann J, Massei R, Paschke A, Kühne R, Scholz S (2015) Fish embryo toxicity test: identification of compounds with weak toxicity and analysis of behavioral effects to improve prediction of acute toxicity for neurotoxic compounds. Environ. Sci. Technol. 49, 7002-7011.

Knöbel M, Busser FJ, Rico-Rico A, Kramer NI, Hermens JLM, Hafner C, Tanneberger K, Schirmer K, Scholz S (2012) Predicting adult fish acute lethality with the zebrafish embryo: Relevance of test duration, endpoints, compound properties, and exposure concentration analysis. Environ. Sci. Technol. 46 (17), 9690−9700.

Kortenkamp A, Backhaus T, Faust M (2009) State of the art report on mixture toxicity. Final Report to the European Commission under Contract Number 070307/2007/485103/ETU/D.1.

Krogh KA, Halling-Sorensen B, Mogensen BB, Vejrup KV (2003) Environmental properties and effects of non-ionic surfactant adjuvants in pesticides: a review. Chemosphere 50, 871-901.

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T (2009) Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp. Biochem. Physiol. C 149, 196–209.

Liu W, Gan JJ, Lee S, Werner I (2004) Isomer selectivity in aquatic toxicity and biodegradation of cypermethrin. J Agr. Food Chem. 52, 6233-6238.

Loewe S, Muischnek H (1926) Über Kombinationswirkungen: 1. Mitteilung: Hilfsmittel der Fragestellung. Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmacol. 114:313–326.

OECD (1992) OECD Guideline for the Testing of Chemicals, No. 301: “Ready Biodegradability”. Adopted 17 July, 1992, Organisation for Economic Co-Operation and Development, Paris.

OECD (2004) OECD Guideline for the Testing of Chemicals, No. 202: "*Daphnia* sp., Acute Immobilisation Test”. Adopted 13 April, 2004. Organisation for Economic Co-Operation and Development, Paris.

OECD (2006a) OECD Guideline for the Testing of Chemicals, No. 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test”. Adopted 23 March 2006 and annex 5 corrected 28 July 2011. Organisation for Economic Co-Operation and Development, Paris.

OECD (2006b) OECD Guideline for the Testing of Chemicals, No. 310: "Ready Biodegradability – CO_2 in sealed vessels (Headspace Test)”. Adopted 23 March 2006 and corrected 26 September 2014. Organisation for Economic Co-Operation and Development, Paris.

OECD (2008) OECD Guideline for Testing of Chemicals No. 203: "Fish, Acute Toxicity Test". Adopted 17 July 1992, Organisation for Economic Co-Operation and Development, Paris.

OECD (2009) OECD Guideline for Testing of Chemicals No. 232: "Collembolan Reproduction Test in Soil". Adopted 7 September 2009, Organisation for Economic Co-Operation and Development, Paris.

OECD (2012) OECD Guideline for Testing of Chemicals No. 211: “*Daphnia magna* Reproduction Test”. Adopted 02 October 2012, Organisation for Economic Co-Operation and Development, Paris.

OECD (2013) OECD Guideline for the Testing of Chemicals, No. 236: "Fish Embryo Acute Toxicity (FET) Test”. Adopted 26 July, 2013. Organisation for Economic Co-Operation and Development, Paris.

R Development Core Team (2013) R: A language and environment for statistical computing. Version 3.1.3. R Foundation for Statistical Computing, Vienna, Austria. www.R-project.org.

Ritz C, Streibig JC (2005) Bioassay Analysis using R. J. Statist. Software 12. Package drc, Version 2.5-12. Obtained April 2015.

Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-response analysis using R. PLoS ONE 10(12): e0146021. doi:10.1371/journal.pone.0146021.

SCHER/SCENIHR/SCCS (2011) Toxicity and assessment of chemical mixtures. Preliminary opinion approved for public consultation. Scientific Committee on Consumer Safety, Scientific Committee on Health and Environmental Risks, Scientific Committee on Emerging and Newly Identified Health Risks. Directorate-General Health and Consumer Protection, European Commission, Brüssel.

Sims SR, Appel AG (2007) Linear alcohol ethoxylates: insecticidal and synergistic effects on German cockroaches (Blattodea: Blattellidae) and other insects. J. Econ. Entomol. 100, 871-879.

Spurgeon DJ, Jones OAH, Dorne JLCM, Svendsen C, Swain S, Stürzenbaum S (2010) Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. Sci. Total. Environ. 408, 3725-3734.

Takeshita J-I, Seki M, Kamo M (2016) Criteria for deviation from predictions by the concentration addition model. Environ. Toxicol. Chem. 35, 1806-1814.

Varsano R, Rabinowitch HD, Rubin B (1992) Mode of action of piperonyl butoxide as herbicide synergist of atrazine and terbutryn in maize. Pestic. Biochem. Phys. 44, 174-182.

Vollmar P, Heim J, Kehrer A, Sacher F, Coors A (2016) Mixture toxicity assessment of biocides: experimental verification of the relevance of additives in wood preservative products. Oral presentation. 26th Annual Meeting of SETAC Europe, Nantes, France, 22.-26. May 2016.

Wang S-P, Hu X-X, Meng Q-W, Muhammad SA, Chen R-R, Li F, Li G-Q (2013) The involvement of several enzymes in methanol detoxification in *Drosophila melanogaster* adults. Comp. Biochem. Physiol. Part B 166, 7–14.

Weinhold B (2010) Mystery in a bottle: will the EPA require public disclosure of inert pesticide ingredients? Environ. Health Perspect. 118, A168-171.

Weinstein JE, Garner TR (2008) Piperonyl butoxide enhances the bioconcentration and photoinduced toxicity of fluoranthene and benzo[a]pyrene to larvae of the grass shrimp (*Palaemonetes pugio*). Aquat. Toxicol. 87, 28–36.

10 Confidential Annex I

10.1 A: Study Reports Green Algae

A1. Vollmar P, Sacher F, Coors A. Product 10: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, January 2016. 24 pages.

A2. Vollmar P, Sacher F, Coors A. Product 14: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, January 2016. 23 pages.

A3. Vollmar P, Sacher F, Coors A. Product 16: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, April 2016. 23 pages.

A4. Vollmar P, Sacher F, Coors A. Product 20: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, February 2016. 24 pages.

A5. Vollmar P, Sacher F, Coors A. Product 6: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, June 2016. 23 pages.

A6. Vollmar P, Sacher F, Coors A. Product 31: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, June 2016. 21 pages.

A7. Vollmar P, Sacher F, Coors A. Product 32: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, June 2016. 19 pages.

A8. Coors A, Volovei T, Heusner E, Sacher F. Tebuconazole: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, October 2014. 16 pages.

A9. Coors A, Vollmar P, Volovei T, Heusner E, Sacher F. Fenpropimorph: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, August 2016. 26 pages.

A10. Vollmar P, Scheiermann N, Sacher F, Coors A. Fenpropidin: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, September 2016. 20 pages.

A11. Vollmar P, Sacher F, Coors A. Mixture of Fenpropimorph and Tebuconazole: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, August 2016. 17 pages.

A12. Vollmar P, Sacher F, Coors A. Mixture of Fenpropidin and Tebuconazole: A Study on the Toxicity to Algae. ECT Oekotoxikologie GmbH, September 2016, 17 pages.

10.2 B: Study Reports *Daphnia magna*

B1. Vollmar P, Werschke C, Sacher F, Coors A. Product 10: A Study on the Acute Toxicity to *Daphnia magna*. ECT Oekotoxikologie GmbH, January 2016. 20 pages.

B2. Vollmar P, Werschke C, Sacher F, Coors A. Product 14: A Study on the Acute Toxicity to *Daphnia magna*. ECT Oekotoxikologie GmbH, January 2016. 20 pages.

B3. Vollmar P, Werschke C, Sacher F, Coors A. Product 16: A Study on the Acute Toxicity to *Daphnia magna*. ECT Oekotoxikologie GmbH, January 2016. 20 pages.

B4. Vollmar P, Werschke C, Sacher F, Coors A. Product 20: A Study on the Acute Toxicity to *Daphnia magna*. ECT Oekotoxikologie GmbH, October 2015. 22 pages.

B5. Vollmar P, Werschke C, Sacher F, Coors A. Product 6: A Study on the Acute Toxicity to *Daphnia magna*. ECT Oekotoxikologie GmbH, June 2016. 20 pages.

B6. Vollmar P, Werschke C, Sacher F, Coors A. Product 31: A Study on the Acute Toxicity to *Daphnia magna*. ECT Oekotoxikologie GmbH, June 2016. 18 pages.

B7. Vollmar P, Werschke C, Sacher F, Coors A. Product 32: A Study on the Acute Toxicity to *Daphnia magna*. ECT Oekotoxikologie GmbH, June 2016. 16 pages.

10.3 C: Study Reports Fish Embryo Toxicity

C1. Coors A, Heim J, Vollmar P, Sacher F. Product 10: A Study on the Acute Toxicity to Embryos of *Danio rerio*. ECT Oekotoxikologie GmbH, January 2016. 21 pages.

C2. Vollmar P, Heim J, Sacher F, Coors A. Product 14: A Study on the Acute Toxicity to Embryos of *Danio rerio*. ECT Oekotoxikologie GmbH, January 2016. 20 pages.

C3. Coors A, Heim J, Vollmar P, Sacher F. Product 16: A Study on the Acute Toxicity to Embryos of *Danio rerio*. ECT Oekotoxikologie GmbH, January 2016. 19 pages.

C4. Vollmar P, Heim J, Sacher F, Coors A. A Study on the Acute Toxicity to Embryos of *Danio rerio*. ECT Oekotoxikologie GmbH, February 2016. 20 pages.

C5. Vollmar P, Wanner N, Sacher F, Coors A. Product 6: A Study on the Acute Toxicity to Embryos of *Danio rerio*. ECT Oekotoxikologie GmbH, June 2016. 20 pages.

C6. Coors A, Wanner N, Sacher F, Vollmar P. Product 31: A Study on the Acute Toxicity to Embryos of *Danio rerio*. ECT Oekotoxikologie GmbH, June 2016. 13 pages.

C7. Vollmar P, Wanner N, Sacher F, Coors A. Product 32: A Study on the Acute Toxicity to Embryos of *Danio rerio*. ECT Oekotoxikologie GmbH, June 2016. 16 pages.

10.4 D: Study Reports Collembola

D1. Bandow C, Vollmar P, Guimaraes B, Coors A. Product 14: A Study on the Chronic Toxicity to *Folsomia candida.* ECT Oekotoxikologie GmbH, September 2016. 14 pages.

D2. Bandow C, Vollmar P, Guimaraes B, Coors A. Tebuconazole: A Study on the Chronic Toxicity to *Folsomia candida*. ECT Oektoxikologie GmbH, September 2016. 18 pages.

D3. Bandow C, Vollmar P, Guimaraes B, Coors A. IPBC: A Study on the Chronic Toxicity to *Folsomia candida*. ECT Oekotoxikologie GmbH, June 2016. 12 pages.

D4. Bandow C, Vollmar P, Guimaraes B, Coors A. Product 14: A Study on the Avoidance Behaviour of *Folsomia candida*. ECT Oekotoxikologie GmbH, August 2016. 12 pages.

D5. Bandow C, Guimaraes B, Coors A. Tebuconazole: A Study on the Avoidance Behaviour of *Folsomia candida*. ECT Oekotoxikologie GmbH, June 2016. 9 pages.

D6. Bandow C, Coors A. IPBC: A Study on the Avoidance Behaviour of *Folsomia candida*. ECT Oekotoxikologie GmbH, June 2016. 9 pages.

D7. Vollmar P, Bandow C, Coors A. Dimethylcapramide: A Study on the Avoidance Behaviour of *Folsomia candida*. ECT Oekotoxikologie GmbH, August 2016. 9 pages.