Environmental Research

of the Federal Ministry for the Environment,

Nature Conservation and Nuclear Safety

Biocides / Plant Protection Products

Project No. (FKZ) 3709 65 404

# 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

by

Anja Coors[1], Ivonne Löffler[1], Priscila Noronha-Jänsch[1], Barbara Weisbrod[1], Ute Schoknecht[2], and Frank Sacher[3]

[1] ECT Oekotoxikologie GmbH, Flörsheim a.M.;
[2] BAM Bundesanstalt für Materialforschung und -prüfung, Berlin;
[3] DVGW-Technologiezentrum Wasser (TZW), Karlsruhe;

ON BEHALF OF THE

FEDERAL ENVIRONMENT AGENCY

May 2013

# Report Cover Sheet

| | |
|---|---|
| **Report No.** | UBA-FB 00 |
| **Report Title** | Ecotoxicological combined effects from chemical mixtures<br>Part 2: Development of ecotoxicological tests with biocidal products and eluates: investigating the suitability of biotests with algae and daphnids to estimate mixture toxicity |
| **Author(s)**<br>**(Family Name, First Name)** | Coors, Anja; Löffler, Ivonne; Noronha-Jänsch, Priscila; Weisbrod, Barbara; Schoknecht, Ute; Sacher, Frank |
| **Performing Organisation**<br>**(Name, Address)** | Helmholtz-Zentrum für Umweltforschung GmbH – UFZ<br>Dept. Bioanalytische Ökotoxikologie<br>Permoserstr. 15<br>Leipzig |
| **Funding Agency** | Umweltbundesamt<br>Postfach 14 06<br>06813 Dessau-Roßlau |
| **Report Date** | 22 May 2013 |
| **Project No. (FKZ)** | 3709 65 404 |
| **No. of Pages** | 95 |
| **Supplementary Notes** | In cooperation with:<br>ECT Oekotoxikologie GmbH, Flörsheim a.M.;<br>BAM Bundesanstalt für Materialforschung und -prüfung, Berlin;<br>DVGW-Technologiezentrum Wasser (TZW), Karlsruhe |
| **Keywords** | Biocides, wood preservatives, environmental risk assessment, mixture toxicity |

# Berichtskennblatt

| | |
|---|---|
| **Berichtsnummer** | UBA-FB 00 |
| **Titel des Berichts** | Ökotoxische Kombinationswirkungen von Stoffgemischen<br>Teil 2: Entwicklung von ökotoxikologischen Tests mit Biozid-Produkten und Eluaten: Prüfung der Anwendbarkeit von Tests mit Algen und Daphnien für die Erfassung der Mischungstoxizität |
| **Autor(en)**<br>**(Name, Vorname)** | Coors, Anja; Löffler, Ivonne; Noronha-Jänsch, Priscila; Weisbrod, Barbara; Schoknecht, Ute; Sacher, Frank |
| **Durchführende Institution**<br>**(Name, Anschrift)** | Helmholtz-Zentrum für Umweltforschung GmbH – UFZ<br>Dept. Bioanalytische Ökotoxikologie<br>Permoserstr. 15<br>Leipzig |
| **Fördernde Institution** | Umweltbundesamt<br>Postfach 14 06<br>06813 Dessau-Roßlau |
| **Abschlussdatum** | 22. Mai 2013 |
| **Forschungskennzahl (FKZ)** | 3709 65 404 |
| **Seitenzahl des Berichts** | 95 |
| **Zusätzliche Angaben** | In Kooperation mit:<br>ECT Oekotoxikologie GmbH, Flörsheim a.M.;<br>BAM Bundesanstalt für Materialforschung und -prüfung, Berlin;<br>DVGW-Technologiezentrum Wasser (TZW), Karlsruhe |
| **Schlagwörter** | Biozide, Holzschutzmittel, Umweltrisikobewertung, Mischungstoxizität |

## Abstract

Three different wood preservative products, their eluates produced by leaching tests, mixtures of some of their ingredients and some of their ingredients as single substances were tested for growth inhibition of green algae as well as acute and chronic toxicity to *Daphnia magna*. The tests were conducted according to OECD standard guidelines and supported by analytical chemistry. The model deviation ratio (MDR) was used as quantitative measure for the compliance between observed mixture toxicity and the toxicity predicted by concentration addition. An MDR considerably larger than 2 may indicate synergistic interactions or the necessity to include so-far neglected substances into the prediction. For the here investigated wood preservative products and their eluates, the importance of taking formulation additives and transformation products into account has been clearly demonstrated. Acute as well as chronic toxicity could be reliably predicted with less than 2fold deviation when all relevant ingredients were known and included in the prediction. Yet, there was a tendency to overestimate mixture toxicity for endpoints of sub-lethal toxicity at low effect levels.

## Kurzbeschreibung

Drei verschiedene Holzschutzmittelprodukte, ihre technisch hergestellten Eluate, Mischungen einiger ihrer Inhaltsstoffe sowie einige Inhaltsstoffe selber wurden im Hinblick auf die Wachstumshemmung von Grünalgen und die akute sowie chronische Toxizität gegenüber *Daphnia magna* getestet. Die Untersuchungen wurden gemäß OECD Standardrichtlinien durchgeführt, inklusive einer begleitenden chemischen Analytik. Die *model deviation ratio* (MDR) wurde genutzt als quantitatives Maß für die Güte der Übereinstimmung zwischen beobachteter und der nach dem Konzept der Konzentrationsadditivität vorhergesagten Mischungstoxizität. Eine MDR deutlich über 2 kann synergistische Interaktionen anzeigen oder die Notwendigkeit bisher nicht berücksichtigte Substanzen in die Mischungsvorhersage miteinzubeziehen. Für die hier untersuchten Holzschutzmittel und deren Eluate konnte die Relevanz von Formulierungsbeistoffen und Transformationsprodukten für die Toxizität der Mischung eindeutig belegt werden. Die akute und die chronische Toxizität der Mischungen konnte mit einer Abweichung kleiner als Faktor 2 zuverlässig vorhergesagt werden, sofern alle relevanten Substanzen bekannt waren und berücksichtigt wurden. Allerdings zeigte sich eine Tendenz zur Überschätzung der Mischungstoxizität bei der Verwendung von Endpunkten der subletalen Toxizität bei gering ausgeprägten Effekten.

# Table of Contents

## List of Figures

## List of Tables

## List of Abbreviations

| | |
|---|---|
| a.s. | active substance |
| BPD | Biocidal Product Directive |
| BPR | Biocidal Product Regulation |
| CA | Concentration Addition |
| *Dar*T | *Danio rerio* embryo toxicity test |
| $E_bC_{50}$ | Median Effective Concentration with regard to biomass |
| $EC_{50}$ | Median Effective Concentration |
| ECHA | European Chemicals Agency |
| $E_rC_{50}$ | Median Effective Concentration with regard to growth rate |
| FET | Fish Embryo Toxicity |
| HPLC | High-Performance Liquid Chromatography |
| IPBC | 3-Iodo-2-propynyl-N-butylcarbamate |
| $LC_{50}$ | Median Lethal Concentration |
| MDR | Model Deviation Ratio |
| MSDS | Material Safety Data Sheet |
| NOEC | No Observed Effect Concentration |
| OECD | Organisation for Economic Co-operation and Development |
| PBC | Prop-2-ynyl-N-butylcarbamate |
| REACH | Registration, Evaluation, Authorisation of Chemicals |
| SoC | Substance of Concern |
| TU | Toxic Unit |
| UBA | Umweltbundesamt (Federal Environment Agency) |

# 1 Summary[1]

## 1.1 Introduction

Biocides are generally marketed and used not as pure technical material, but as formulated products. Hence, one or more active substances (a.s.) are combined with other substances (i.e., formulation additives) to generate a formulated ready-to-use product. Often a wide variety and considerable number of different formulation additives are contained in biocidal products in addition to the active substances. These formulation additives can be of organic or inorganic nature, and they serve for a multitude of purposes. Among others, formulation additives can improve the solubility or stability of the active substances, prolong shelf life, or enhance uptake into the treated material (such as, for example, penetration aids in wood preservatives). The knowledge about how to formulate a given active substance in a way that optimises its usability is of monetary importance, which explains why the information about most formulation additives contained in biocidal products is kept confidential. However, information on the presence of hazardous and dangerous substances contained in biocidal products must be made available to users according to the rules of REACh (Registration, Evaluation, Authorisation and Restriction of Chemical substances) and CLP (Classification and Labelling).

According to the Biocidal Product Directive (BPD, EU 1998) relating to the marketing of biocidal products in Europe as well as to the upcoming Biocidal Product Regulation (BPR, EU 2012) that repeals the directive in September 2013, an environmental risk assessment must be performed not only for active substances, but also for any substance of concern contained in a biocidal product. These substances must not be seen isolated, but their combined effects must be considered as stated in the BPR: "*A risk assessment on the active substance present in the biocidal product shall always be carried out. If there are, in addition, any substances of concern present in the biocidal product then a risk assessment shall be carried out for each of these. In carrying out the assessment, the possibility of cumulative or synergistic effects shall also be taken into account.*" (EU 2012, Annex VI Common Principles, p. 110).

A substance of concern is defined in the BPR as a substance that "*has an inherent capacity to cause an adverse effect [...] and is present or is produced in a biocidal product in sufficient concentration to present risks of such an effect*" (EU 2012). This definition appears as a somewhat circular reasoning: a risk assessment shall be conducted for substances of concern, but it is only known that a substance is of concern after a risk assessment has been conducted (at least up to some degree). The exact identification of substances of concern is currently intensively discussed among European competent authorities, also in the context of how cumulative and synergistic effects shall be taken into account in the risk assessment.

In this context, the following question is addressed: Is it sufficient to consider hazardous and dangerous substances together with the active substances in a theoretical mixture toxicity model in order to obtain a toxicity estimate for the product? Or is it necessary to also consider

[1] This summary has been submitted as extended abstract: Coors A, Sacher F, Schoknecht U, Weisbrod B, Kehrer A (2013) Formulation additives in the environmental risk assessment of biocidal products. SETAC Europe 23rd Annual Meeting, 12-16 May 2013, Glasgow, UK.

confidential formulation additives and prove absence of synergism among active substances and/or substances of concern?

## 1.2 Materials and methods

Three wood preservative products were investigated here that all contained two active substances and a number of hazardous components. The information on the presence and, if available, concentration of hazardous components were taken from the material safety data sheets and the labelling of the commercially obtained products, i.e. no information on confidential formulation additives was used. Theoretical and experimental investigations were conducted with the products and technical eluates prepared from these products in order to verify if their toxicity can be correctly predicted by the concept of concentration addition (CA).

Technical eluates, which take differential leaching of product components from treated wood into account, were prepared from the three products according to the relevant OECD 313. Aquatic ecotoxicity tests with the green algae *Pseudokirchneriella subcapitata* and the crustacean *Daphnia magna* were conducted according to relevant OECD guidelines (i.e., OECD 201, 202, and 211), and were accompanied by analytical chemistry. The toxicity observed for the biocidal products and the eluates was compared with the respective toxicity predicted by CA based on the measured toxicity of the individual mixture components. The endpoints used for these comparisons covered typical regulatory endpoints such as the $EC_{50}$ for *D. magna* immobilisation, $EC_{50}$ for algal growth inhibition and NOEC for *D. magna* reproduction. Thereby, the investigation of the predictability of mixture toxicity was extended from acute to long-term and sub-lethal endpoints.

## 1.3 Results and discussion

The absence of synergistic interactions among combinations of the active substances was confirmed by testing generic mixtures in some of the biotests. The observed toxicity of product C and its eluates was predicted by CA with a deviation of less than factor 2 for algal growth inhibition, *Daphnia* immobilisation and *Daphnia* reproduction as long as the one hazardous component contained in the product was included in the prediction. This indicates that all relevant product components were considered and that an environmental risk assessment for this product could be based on theoretical mixture toxicity calculations. For algal growth inhibition, the observed toxicity of product B and its eluate was also predicted by CA with less than factor 2 deviation. In contrast, *Daphnia* immobilisation was underestimated by the CA prediction by more than factor 4 for the product, but not for the eluate. In the proven absence of synergism between active substances, this indicates that the product (but not the eluate) contains formulation additives that are toxic to *Daphnia* (but not to algae), or that synergistic interactions occurred with formulation additives, e.g. that organic solvents contained in this product in high amounts interacted with the toxicokinetics of the active substances in *Daphnia*, but not algae. In the case of product A, it turned out that the active substance IPBC was almost completely transformed to PBC. Mixture toxicity predictions based only on measured concentrations of the active substances (but not the transformation product) considerably underestimated the toxicity of the product and its eluate towards *Daphnia* and algae. It could be established that ionic cobalt as formulation additive contributed also to the overall toxicity in the eluate in addition to the transformation product of IPBC.

## 1.4 Conclusions

Theoretical considerations supported by experimental investigations demonstrated that formulation additives as well as transformation products can significantly contribute or even dominate the toxicity of wood preservatives, serving here as examples of biocidal products. The consideration of the labelled hazardous components in addition to the active substances was sufficient to predict theoretically by CA the toxicity of some products and eluates with a deviation of less than factor 2 from the observed toxicity for a number of typical regulatory endpoints, both acute and long-term. This supports for formulated products the reliability of a theoretical hazard assessment. However, as illustrated by other products and eluates this approach is only reliable if indeed all relevant product ingredients are considered.

# 2 Zusammenfassung

## 2.1 Einleitung

Biozide werden grundsätzlich nicht als reines technisches Material, sondern als formuliertes Produkt auf den Markt gebracht und durch den Verbraucher eingesetzt. Das bedeutet, dass zur Herstellung eines unmittelbar einsetzbaren, formulierten Biozidproduktes mindestens ein Wirkstoff mit anderen Substanzen (sogenannten Beistoffen oder Formulierhilfsmitteln) kombiniert wird. Zusätzlich zum Wirkstoff enthalten Biozidprodukte daher oftmals eine große Bandbreite sehr verschiedener Beistoffe in möglicherweise erheblichen Mengen. Diese Beistoffe können organischer oder anorganischer Natur sein und dienen einer Vielzahl verschiedener Zwecke, wie beispielsweise der Verbesserung der Stabilität und Löslichkeit des Wirkstoffs, der Verlängerung der Haltbarkeit des Produktes oder der Verbesserung der Aufnahme des Wirkstoffes in die zu behandelnden Materialien (Hilfsstoffe in Holzschutzmitteln, die die Aufnahme in das Holz erleichtern sind hier ein Beispiel). Das Wissen über die optimale Formulierung eines bestimmten Wirkstoffes ist von großer ökonomischer Bedeutung. Dadurch erklärt sich, warum die Identität der in Biozidprodukten enthaltenen Beistoffe vorzugsweise geheim gehalten wird. Gefährliche und besorgniserregende Inhaltsstoffe müssen allerdings nach geltendem Recht, konkret im Zusammenhang mit Regelungen auf europäischer Ebener wie REACh (Registration, Evaluation, Authorisation and Restriction of Chemical substances) und CLP (Classification and Labelling), dem Verbraucher bekannt gemacht werden.

Eine Umweltrisikobewertung muss nicht nur für die in Biozidprodukten enthaltenen Wirkstoffe, sondern auch für alle enthaltenen besorgniserregenden Beistoffe ("*substances of concern*") durchgeführt werden. Dies ist festgelegt in der entsprechenden europäischen Gesetzgebung, sowohl in der *Biocidal Product Directive* (BPD, EU 1998) als auch in der *Biocidal Product Regulation* (BPR, EU 2012), die die BPD im September 2013 ablöst. Nach BPR dürfen Wirkstoffe und besorgniserregende Beistoffe bei der Umweltrisikobewertung nicht isoliert, sondern sollen vielmehr in ihrer gemeinsamen Wirkung betrachtet werden: "*A risk assessment on the active substance present in the biocidal product shall always be carried out. If there are, in addition, any substances of concern present in the biocidal product then a risk assessment shall be carried out for each of these. In carrying out the assessment, the possibility of cumulative or synergistic effects shall also be taken into account.*" (EU 2012, Annex VI Common Principles, p. 110).

Ein besorgniserregender Stoff ("*substance of concern*") ist in der BPR definiert als eine Substanz, die "die inhärente Eigenschaft hat, nachteilige Effekte hervorzurufen [...] und die in einer Konzentration im Produkt enthalten ist oder entsteht, die das Risiko des Auftretens solcher Effekte bedingt [*has an inherent capacity to cause an adverse effect [...] and is present or is produced in a biocidal product in sufficient concentration to present risks of such an effect]*" (EU 2012). Diese Definition ist in gewisser Weise zirkulär: eine Umweltrisikobewertung ist notwendig für besorgniserregende Substanzen, wobei aber eine Substanz erst durch die (zumindest ansatzweise) Durchführung einer Umweltrisikobewertung als besorgniserregend im Produkt erkannt wird. Die genaue Identifikation besorgniserregender Substanzen in Biozidprodukten wird zurzeit innerhalb der zuständigen europäischen Behörden intensiv diskutiert, auch im Zusammenhang mit der Frage wie kumulative und synergistische Effekte konkret in der Risikobewertung berücksichtigt werden sollen.

In diesem übergeordneten Zusammenhang wurde in der vorliegenden Arbeit die folgende Fragestellung bearbeitet: Ist die Berücksichtigung der Wirkstoffe und zusätzlich der gefährlichen und besorgniserregenden Beistoffe ausreichend, um anhand theoretischer Mischungsmodelle eine verlässliche Toxizitätsabschätzung für das Biozidprodukt zu erstellen? Oder ist es notwendig, auch die vertraulichen Beistoffe zu berücksichtigen sowie die Abwesenheit von synergistischen Interaktionen zwischen Wirkstoffen bzw. zwischen Wirkstoffen und Beistoffen zu belegen?

## 2.2 Material und Methoden

In dieser Arbeit wurden drei Holzschutzmittel untersucht, die alle jeweils zwei Wirkstoffe und mindestens einen gefährlichen Beistoffe enthalten. Die Information über die Identität und, falls vorhanden, enthaltene Mengen der Beistoffe wurden dem Datensicherheitsblatt bzw. den Beschriftungen der Produkte entnommen. Es wurden demnach keine vertraulichen Informationen über die Zusammensetzung der untersuchten Biozidprodukte genutzt. Mit den Holzschutzmittel und den daraus hergestellten technischen Eluaten wurden theoretische und experimentelle Untersuchungen durchgeführt, um zu überprüfen ob ihre Toxizität mit Hilfe des Konzeptes der Konzentrationsadditivität korrekt vorhergesagt werden kann.

Technische Eluate von Holzschutzmitteln spiegeln das unterschiedliche Auswaschverhalten der Inhaltsstoffe wieder; sie wurden nach der entsprechenden Richtlinie OECD 313 hergestellt. Die im Rahmen dieser Arbeit durchgeführten aquatischen Ökotoxizitätstests mit der Grünalge *Pseudokirchneriella subcapitata* und dem Kleinkrebs *Daphnia magna* folgten ebenfalls den entsprechenden Testrichtlinien (OECD 201, 202 und 211) und wurden durch eine begleitende chemische Analytik unterstützt. Die in den Tests bestimmte Toxizität der Holzschutzmittel und Eluate wurde mit der jeweiligen basierend auf Konzentrationsadditivität vorhergesagten Toxizität ins Verhältnis gesetzt. Die für diesen Vergleich herangezogen Endpunkte umfassten mit dem $EC_{50}$ für die Immobilisierung von Daphnien, dem $EC_{50}$ für die Wachstumshemmung von Algen und dem NOEC für die Reproduktion von Daphnien die typischerweise in der Regulatorik verwendeten Endpunkte. Gleichzeitig kamen damit nicht nur Endpunkte für akute sondern auch für langfristige Wirkungen zum Einsatz.

## 2.3 Ergebnisse und Diskussion

Durch das Testen von generischen Mischungen in einigen der Biotests konnte die Abwesenheit von synergistischen Interaktionen zwischen den Wirkstoffen nachgewiesen werden. Die im Test bestimmte Toxizität des Produkts C und seiner Eluate gegenüber Algen und Daphnien wich um weniger als Faktor 2 von der nach Konzentrationsadditivität vorhergesagten Toxizität ab, sofern der eine enthaltene gefährliche Beistoff in der Vorhersage berücksichtigt war. Dieses Ergebnis belegt, dass alle relevanten Substanzen berücksichtigt wurden und die Umweltrisikobewertung für dieses Produkt basierend auf theoretischen Mischungstoxizitätsberechnungen durchgeführt werden kann. Bei Produkt B und seinem Eluat lag die Abweichung zwischen beobachteter und vorhergesagter Toxizität ebenfalls unter Faktor 2 im Hinblick auf die Wachstumshemmung von Algen. Im Hinblick auf die Immobilisierung von Daphnien wurde die Toxizität des Produktes, nicht aber des Eluates, um mehr als das Vierfache durch die Vorhersage unterschätzt. Aufgrund der nachgewiesenen Abwesenheit von Synergismus zwischen den Wirkstoffen deutet dieses Ergebnis auf das Vorhandensein eines Daphnien- aber nicht algentoxischen Beistoffes im Produkt (nicht aber im Eluat) hin. Eine

andere Erklärungsmöglichkeit sind synergistische Interaktionen zwischen Wirkstoffen und Beistoffen, wie zum Beispiel eine Beeinflussung der Toxikokinetic der Wirkstoffe in Daphnien (nicht aber in Algen) durch die in erheblichen Mengen in diesem Produkt enthaltenen organischen Lösungsmittel. Im Fall von Produkt A zeigte sich, dass der Wirkstoff IPBC nahezu vollständig in PBC umgewandelt worden war. Die Vorhersage der Mischungstoxizität basierend allein auf den gemessenen Konzentrationen der Wirkstoffe (unter Ausschluss des Transformationsproduktes PBC) unterschätzte daher die im Test bestimmte Toxizität des Produktes und seines Eluates gegenüber Algen und Daphnien erheblich. Weitere Untersuchungen ergaben, dass neben dem Transformationsprodukt PBC auch ionisches Kobalt als enthaltener Beistoff zur Gesamttoxizität im Eluat beitrug.

## 2.4 Schlussfolgerungen

Theoretische Überlegungen unterstützt durch experimentelle Untersuchungen ergaben, dass sowohl Beistoffe als auch Transformationsprodukte von Wirkstoffen erheblich zur Gesamttoxizität eines Holzschutzmittels beitragen oder diese sogar überwiegend bestimmen können. Die Berücksichtigung der bereits bekannten gefährlichen Inhaltsstoffe zusammen mit den Wirkstoffen erlaubte für einige Produkte und Eluate eine theoretische Vorhersage der Gesamttoxizität nach Konzentrationsadditivität mit einer Abweichung kleiner Faktor 2 von der experimentell bestimmten Toxizität bei einer Reihe von typischerweise verwendeten regulatorischen Endpunkten (akuten und langfristigen). Dadurch wird die Verwendung von theoretischen Mischungstoxizitätsmodellen in der Risikobewertung unterstützt. Wie einige Beispiele hier aber auch zeigten, gilt diese Schlussfolgerung nur, wenn tatsächlich alle relevanten Inhaltsstoffe berücksichtigt werden.

# 3 Introduction

According to the directive regulating their placing on the market in the European Union (EC 1998) biocides compromise a diverse group of 23 product types, among them wood preservatives (product type 8). Wood preservatives aim to protect wood from insect attack and fungal decay and therefore usually contain insecticides or fungicides. Often they contain both insecticides and fungicides in combination, and thereby represent a mixture of active substances (a.s.) potentially released into the environment. In addition to the active substances, formulated products typically contain a broad range of formulation additives, which results in wood preservative products being typically a complex mixture of various chemicals. The composition of the environmentally relevant mixture of wood preservatives may considerably differ from the composition of the product, because the various substances in the product may elute from treated wood at different rates and may also differ in their environmental fate.

With regard to the environmental risk assessment of biocidal products, the relevant European directive states that the results obtained for one (or more) active substances and any substance of concern (SoC) present in the product shall be combined to assess the environmental risk of the biocidal product (BPD 98/8/EC, EC 1998). The technical guidance document supporting the biocide directive (ECB 2008) states that additivity shall be assumed for effects of active substances on the same target organ in the case of human health and states with regard to effects in the environment that combination effects shall be carefully considered by the competent authority. For biocidal products that are not released as such but undergo considerable changes in composition before they enter the environment (such as eluates of wood preservative products), the technical guidance document (ECB 2008) recommends a component-based approach following the concept of Concentration Addition (CA) to assess the environmental risk of the complex mixture. The assessment of mixture (eco-)toxicity is also foreseen by the new Biocidal Products Regulation (BPR 528/2012, EU 2012) that will replace the current Biocidal Products Directive (EC 1998) in September 2013. Article 19(2) of the Regulation states that "*the evaluation [...] shall take into account the following factors: [...] (d) cumulative effects, (e) synergistic effects*." This is further elaborated in Annex VI (common principles for the evaluation of biocidal products) stating that the risks associated with the relevant individual components of the biocidal product shall be assessed, taking into account any cumulative and synergistic effects.

Currently, there is a discussion going on in Europe among regulatory authorities and with industry how the component-based approach mentioned in the Directive and Regulation shall be applied to assess the environmental risks of the mixtures that biocidal products represent. The guidance on how mixture effects should be considered during the authorisation of a biocidal product as provided in the current technical guidance documents is rather limited and not specific enough for unambiguous implementation. Several research projects were funded by the German Federal Environment Agency (Umweltbundesamt, UBA) in this context. The present study is part of a larger project (with Part 1 being „Relevance and adequate consideration in environmental risk assessment of plant protection products and biocides", Altenburger et al. 2012) that developed, among other tasks, detailed implementation options for considering mixtures toxicity in the authorisation of biocidal products and plant protection products.

Another preceding project funded by the German Federal Environment Agency investigated the suitability of the fish embryo toxicity (FET) test, particularly the FET conducted with *Danio rerio* (i.e. the *Dar*T), as a screening method to check for concentration additive behaviour of biocidal products. CA predictions were compared with the experimentally observed toxicity of five different wood preservative products as well as with those of generic mixtures of their active substances. The results of this project (Coors et al. 2011, Coors et al. 2012) indicate that the investigated technical active substances interact in a concentration-additive way with each other. CA predictions based only on the active substances were also in agreement (less than factor 5 deviation) with the toxicity observed for four of the formulated products. Yet, the CA prediction based on the toxicity of only the active substances underestimated the toxicity of one product by about factor 65. This large deviation could be tracked back to the toxicity of one individual formulation additive in the product. Hence, this study provided clear evidence that CA predictions can be used for the environmental risk assessment of wood preservative products, but that the inclusion of relevant formulation additives must be ensured. The still open question is how such relevant formulation additives can reliably be identified without experimental testing of the mixture.

It has been shown that for a large majority of mixtures of pesticides the experimentally observed joint toxicity deviates by less than factor 2 from the CA-prediction (Deneer 2000, Belden et al. 2007). While a deviation by less than factor 2 has also been found for the majority of assessed plant protection products, the formulation additives contained in these products as well as the heterogeneity of data used for the mixture toxicity predictions resulted in a considerable number of cases with deviations greater than factor 2 (Coors & Frische 2011). Hence, the toxicity of formulated mixtures of pesticides as well as biocides may be considerably influenced by formulation additives as evidenced in these studies (Coors & Frische 2011, Coors et al. 2012).

The aim of the present study was to build on these preceding studies and extend the investigation to other non-target organisms and to endpoints of chronic toxicity. The selected organisms and endpoints comprise toxicity to green algae as well as acute and chronic toxicity to the freshwater crustacean *Daphnia magna*. Similar to the design of the preceding study, the toxicity of three wood preservative products (selected among the five products previously investigated), their eluates and relevant components were experimentally tested. Comparisons of CA-predicted and observed toxicity of the mixtures aimed to verify if the findings obtained previously in the *Dar*T can be extended to other organism groups and endpoints relevant for regulatory decisions.

# 4 Material and methods

Three different types of biotests were conducted within the project: (1) algal growth inhibition tests with the green algae *Pseudokirchneriella subcapitata* according to OECD 201 (OECD 2011), (2) acute immobilization tests with the waterflea *Daphnia magna* according to OECD 202 (OECD 2004), and (3) reproduction tests with *D. magna* according to OECD 211 (OECD 2008).

This chapter describes the tested substances, including wood preservative products and the preparation of their eluates, performance of the biotests, analytical methods for determination of test substances, and subsequent data analysis.

## 4.1 Test substances

Three biocidal active substances, generic mixtures of the single substances, three biocidal products, four eluates of the biocidal products, and one formulation additive were investigated in all or some of the three biotests. Detailed information on these test substances will be given in the following.

### 4.1.1 Single substances

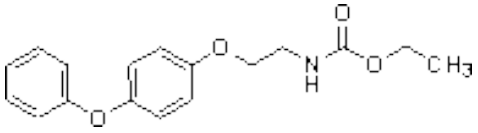
#### Fenoxycarb

Fenoxycarb (Table 1) is an insecticide of the chemical group of carbamates and is included in the Annex I of the respective EU directives (or regulation) for both plant protection products and biocides. According to the related draft evaluation report (Anonymous 2008), the target organisms of fenoxycarb as a biocidal a.s. are beetles on wood in services.

Fenoxycarb acts as an insect growth regulator by mimicking the insect juvenile hormone and belongs according to the Insecticide Resistance Action Committee (IRAC 2008) to the mode-of-action group 7B (juvenile hormone mimics). It interferes with moulting of insect larvae and inhibits finally the metamorphosis to adult insects. Fenoxycarb has no neurotoxic activity in insects (Anonymous 2008). The most sensitive aquatic organisms identified in the EU risk assessment were daphnids, which can be explained by the specific mode of action of fenoxycarb.

Fenoxycarb was found to be stable over the exposure period of 48 h in the fish embryo tests (Coors et al. 2011). Fenoxycarb PESTANAL® was obtained from Sigma-Aldrich, Germany, with a purity of 99.6 %.

Table 1: Properties of fenoxycarb

| Feature | Property of fenoxycarb |
|---|---|
| Molecular structure |  |
| IUPAC name | Ethyl-2-(4-phenoxyphenoxy)ethylcarbamate |
| CAS | 72490-01-8 |
| Molecular mass & formula | 301.3 g/mol; $C_{17}H_{19}NO_4$ |
| log $K_{ow}$ | 4.07 (25 °C) |
| log $pK_a$ | Not applicable |
| Solubility | In water: 7.9 mg/l (25 °C); in acetone: 770 g/l (25 °C) |
| Henry's law constant | $3.3 * 10^{-5}$ Pa $m^3$ $mol^{-1}$ (25 °C) |
| Acute toxicity to fish embryos ($LC_{50}$) | 3.14 mg/l |
| Acute toxicity to fish ($LC_{50}$) | 0.66 mg/l |
| Growth inhibition of algae ($E_bC_{50}$) | 0.54 mg/l |
| Acute toxicity to *D. magna* ($EC_{50}$) | 0.60 mg/l |
| Chronic toxicity to *D. magna* (NOEC) | 0.0000016 mg/l |

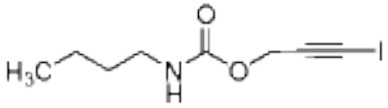
The compilation is based on Tomlin (2006), the draft evaluation report (Anonymous 2008), and previous results (Coors et al. 2012). LC50: median lethal concentration; EbC50: median effective concentration with regard to biomass; ErC50: median effective concentration with regard to growth rate; EC50: median effective concentration; NOEC: no observed effect concentration

## IPBC

IPBC (Table 2) is a carbamate fungicide (common name: iodocarb) that is included in Annex I of the EU directive concerning biocidal products, but is not registered as an active ingredient for plant protection products. The target organisms of IPBC are rotting and disfiguring fungi (SC 2008), among them particularly "Bläuepilze", i.e. sapstain and blue stain fungi such as for example *Cladosporium sp*. In the classification system of the Fungicide Resistance Action Committee, the proposed mode of action of IPBC are effects on fatty acids resulting in changes of cell membrane permeability (group F4, FRAC 2008). Green algae were identified as the most sensitive aquatic organisms among the tested trophic groups (SC 2008). The transformation of IPBC into PBC (prop-2-ynyl-N-butylcarbamate) is described in the literature (SC 2008). IPBC is known to be degraded to PBC (prop-2-ynyl-N-butylcarbamate) by biotic and abiotic mechanisms with PBC being the terminal and only metabolite in biotic degradation tests with bacteria (Cook et al. 2002). A complete loss of fungicidal activity by this transformation was observed (Cook et al. 2002).

IPBC was found to be instable over the exposure period of 48 h in the fish embryo tests (Coors et al. 2012), with an average loss of more than 40 % of the initial concentration. IPBC was obtained from Dr. Ehrenstorfer, Germany, with a purity of 99.0 %.

Table 2: Properties of IPBC

| Feature | Property of IPBC |
|---|---|
| Molecular structure |  |
| IUPAC name | 3-Iodo-2-propynyl-N-butylcarbamate |
| CAS | 55406-53-6 |
| Molecular mass & formula | 281.1 g/mol; $C_8H_{12}INO_2$ |
| log $K_{ow}$ | 2.81 (25 °C) |
| log $pK_a$ | Not applicable |
| Solubility | In water: 168 mg/l (pH 7; 25 °C); in acetone: 720 g/l (20 °C) |
| Henry's law constant | 3.3-6.5 * $10^{-3}$ Pa $m^3$ $mol^{-1}$ (25 °C) |
| Acute toxicity to fish embryos ($LC_{50}$) | 0.349 mg/l |
| Acute toxicity to fish ($LC_{50}$) | 0.067 mg/l |
| Growth inhibition of algae ($E_bC_{50}$) | 0.022 mg/l |
| Growth inhibition of algae ($E_rC_{50}$) | 0.053 mg/l |
| Acute toxicity to *D. magna* ($EC_{50}$) | 0.160 mg/l |
| Chronic toxicity to *D. magna* (NOEC) | 0.050 mg/l |

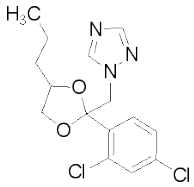
The compilation is based on SC (2008) and previous results (Coors et al. 2012). LC50: median lethal concentration; EbC50: median effective concentration with regard to biomass; ErC50: median effective concentration with regard to growth rate; EC50: median effective concentration; NOEC: no observed effect concentration

## Propiconazole

Propiconazole (Table 3) is a fungicide of the chemical group of triazoles and is included in the Annex I of the respective EU directives (or regulation) for both plant protection products and biocides. The intended use of propiconazole as biocide is preventive and curative treatment of freshly felled wood and wood in service (SC 2007). Target organisms are various fungi, among them sapstain and blue stain fungi. According to the assessment report, propiconazole is usually used in mixtures with other fungicides for wood preservation (SC 2007). As other triazoles, propiconazole inhibits the C14-demethylase and thereby the (ergo)sterol biosynthesis (de-methylation inhibitor, DMI-fungicide). It belongs to the group G1 in the classification system of the Fungicide Resistance Action Committee (FRAC 2008).

Propiconazole was found to be stable over the exposure period of 48 h in the fish embryo tests (Coors et al. 2012). It is moderately water soluble but completely miscible with many organic solvents, e.g. acetone. Propiconazole PESTANAL® was obtained from Sigma-Aldrich, Germany, as a mixture of stereo isomers with a purity of 98.9 %.

Table 3: Properties of propiconazole

| Feature | Property of propiconazole |
|---|---|
| Molecular structure |  |
| IUPAC name | (±)-1-[2-(2,4-Dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole |
| CAS | 60207-90-1 |
| Molecular mass & formula | 342.2 g/mol; $C_{15}H_{17}Cl_2N_3O_2$ |
| log $K_{ow}$ | 3.72 (pH 6.6, 25 °C) |
| log $pK_a$ | 1.09 (very weak base) |
| Solubility | In water: 100 mg/l (20 °C); in acetone: completely miscible |
| Henry's law constant | $9.2 * 10^{-5}$ Pa $m^3$ $mol^{-1}$ (20 °C) |
| Acute toxicity to fish embryos ($LC_{50}$) | 20.4 mg/l |
| Acute toxicity to fish ($LC_{50}$) | 4.3 mg/l |
| Growth inhibition of algae ($E_rC_{50}$) | 0.058 mg/l *; 9.0 mg/l ** |
| Acute toxicity to *D. magna* ($EC_{50}$) | 10.2 mg/l |
| Chronic toxicity to *D. magna* (NOEC) | 0.31 mg/l |

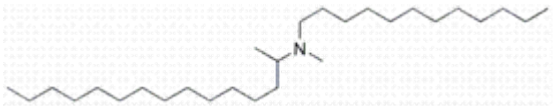
The compilation is based on Tomlin (2006) and previous results (Coors et al. 2012). * recalculated from a test with a formulation (emulsion concentrate, EC); ** endpoint from new study with technical substance as communicated by UBA LC50: median lethal concentration; ErC50: median effective concentration with regard to growth rate; EC50: median effective concentration; NOEC: no observed effect concentration

### Dimethylalkylamine

The formulation additive N-alkyl(C12-C16)-N,N-dimethylamine, a tertiary amine (short: dimethylalkylamine), is added to the biocidal product C (see below) as a penetration aid and wetting agent at 10 % w/w. Besides, it is used in industrial processes as an intermediate for the manufacturing of quaternary ammonium compounds, amine oxides and betaine surfactants (according to supplier information). The toxicity to water organisms is reported as below 1 mg/l (Lonza 2009, Table 4). A batch of dimethylalkylamine was obtained from the producer of product C in 2011 and stored since then at room temperature in the dark.

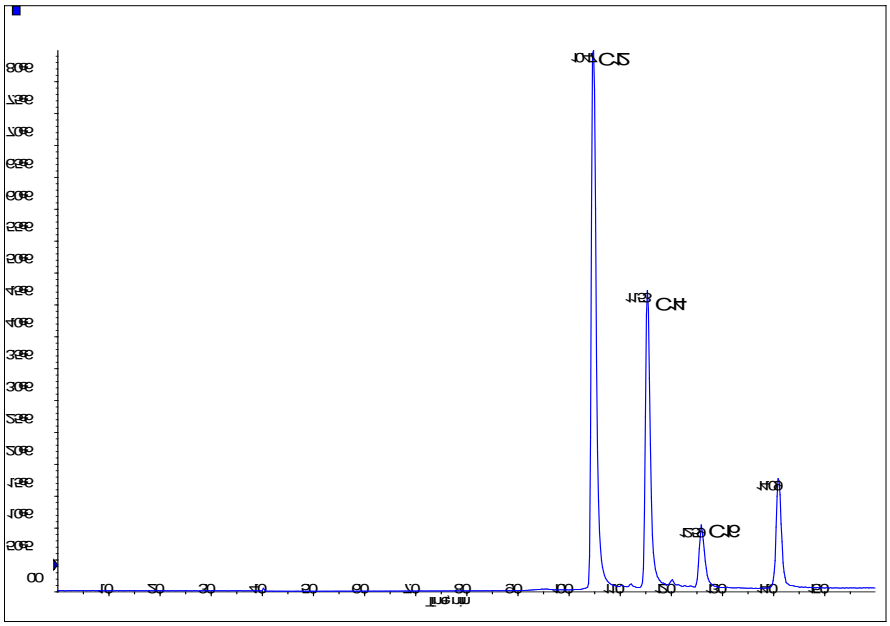
By high-performance liquid chromatography (HPLC) analysis of the standard it was demonstrated that the dimethylalkylamine with an alkylchain of 12 carbon atoms dominates the mixture, followed by the 14 carbon atoms alkyl-chain and small amounts of the 16 carbon atoms alkyl-chain (Figure 1).

Table 4: Properties of (C12-C16)dimethylalkylamine

| Feature | Property of dimethylalkylamine |
|---|---|
| Molecular structure |  |
| IUPAC name | N-Alkyl(C12-16)-N,N-dimethylamine |
| CAS | 68439-70-3 |
| Molecular mass & formula | 213.3 g/mol ($C_{14}H_{31}N$); 241.4 g/mol ($C_{16}H_{35}N$); 269.5 g/mol ($C_{18}H_{39}N$) |
| log $K_{ow}$ | 5.47 (calculated) |
| log $pK_a$ | Not applicable |
| Solubility | Insoluble in water |
| Henry's law constant | Not available |
| Acute toxicity to fish embryos ($LC_{50}$) | 1.28 mg/l |
| Acute toxicity to fish ($LC_{50}$) | 0.1 – 1.0 mg/l |
| Growth inhibition of algae ($EC_{50}$) | < 1.0 mg/l |
| Acute toxicity to *D. magna* ($EC_{50}$) | < 1.0 mg/l |
| Chronic toxicity to *D. magna* (NOEC) | Not available |

The compilation is based on the material safety data sheet (Lonza 2009) and previous results (Coors et al. 2012). LC50: median lethal concentration; EC50: median effective concentration; NOEC: no observed effect concentration

Fig. 1: Chromatogram of a 0.1 mg/l standard of a technical mixture of (C12-C16)alkyldimethylamine



The Figure illustrates the relative amounts of the amines with an alkylchain of 12 carbons (C12), 14 carbons (C14), and 16 carbons (C16), respectively.

### Other single substances

In the course of the project, two more substances were analysed by chemical measurement, but not by biological testing. These two substances were: (1) PBC (prop-2-ynyl-N-butylcarbamate, CAS 864493-71-0), the main metabolite of IPBC (Cook et al. 2002) where the iodine is replaced by hydrogen; (2) cobalt, which was contained as formulation additive in product A. No ecotoxicological data could be retrieved from the literature for PBC. For cobalt, acute toxicity in *Daphnia magna* ($EC_{50}$ 48 h) is reported with 1.67 mg/l (Khangarot & Das 2009). For the green algae *P. subcapitata*, a growth inhibition test with cobalt sulphate according to the relevant guideline and conducted under GLP was reported to the European Chemicals Agency (ECHA) under REACH obligations. The endpoints determined in this study were an $EC_{50}$ (72 h, growth rate) of 0.09 mg Co/l and a NOEC of 0.04 mg Co/l (www.echa.europe.eu; accessed 5 September 2012). For acute toxicity to *Daphnia magna*, an $EC_{50}$ (96 h) of 0.71 mg Co/l and for *D. magna* reproduction a 21-day NOEC of 0.068 mg/l can be found in this database.

### 4.1.2 Biocidal products

Three wood preservative products were studied in the present project. They had been selected among the five products investigated in a previous project (Coors et al. 2011, 2012). In contrast to the two non-selected products the three selected all contain formulation additives labelled as hazardous. Two of the products were solvent-based formulations, i.e. contained large amounts of organic solvents, while one product was a water-based formulation. In order to keep the identity of these products anonymous, they are referred to as product A, B, and C here. The basic features of the three products are given in Table 5.

Table 5: Properties of the investigated biocidal products (product type 8, wood preservative products)

| Product | Active ingredients | Formulation | Hazardous components |
|---|---|---|---|
| A | IPBC: 0.0028 mg/mg product<br>propiconazole: 0.0087 mg/mg product | solvent-based | mixture of aliphatic carbohydrates C8-C10<br>methoxy-propoxy-propanol |
| B | fenoxycarb: 0.00005 mg/mg product<br>propiconazole: 0.0095 mg/mg product | solvent-based | 2-butanonoxim<br>dipropylene glycol methyl ether (DGME);<br>naphtha (petroleum)* |
| C | fenoxycarb: 0.0004 mg/mg product<br>propiconazole: 0.025 mg/mg product | water-based | N-alkyl(C12-16)-N,N-dimethylamine;<br>(S)-2-hydroxypropionic acid;<br>diethylene glycol monobutyl ether |

The compilation is based on the material safety data sheet and registration information. *Testbenzin 180/210

All products contained propiconazole as fungicidal active substance and either IPBC or fenoxycarb as second active substance. In addition to the active substances, all of the products contained at least one more substance listed as hazardous components in their Material Safety Data Sheets (MSDS). Beyond the product components listed in Table 5, all products contained a number of further substances of confidential nature, which are considered inert based on current regulations. In addition, product A contained cobalt(II) carboxylates at a non-specified concentration according to the labelling on the product container.

The products were either bought in commercial stores or directly obtained from the producer in 2010 and stored since then at room temperature in the dark in accordance with producers'

recommendations. No maximum storage periods were stated for these products by the producers.

### 4.1.3 Eluates of biocidal products

Products A and B can be applied by painting in commercial usage, while product C is only for dipping application and industrial usage. Therefore, two eluates were prepared from product C: one from a painting application to enable comparison to the eluates from products A and B and one eluate resulting from dipping treatment in order to study a more realistic eluate composition (based on the intended usage). Hence, in total four eluates were produced and tested in the present study.

The treatment of wood and preparation of eluates from treated wood was performed at the BAM Federal Institute for Materials Research and Testing (BAM Bundesanstalt für Materialforschung und –prüfung), Division 4.1. ('Biodeterioration and Reference Organisms').

The preparation of eluates was performed on the basis of OECD 313 (OECD 2007). Briefly, pine sapwood (*Pinus sylvestris*) was cut to test specimens of dimensions of 110 mm x 40 mm x 10 mm (for painting) and 15 mm x 25 mm x 50 mm (for dipping treatment). The test specimens were stored at 20 +/- 2 °C and 65 +/- 5 % rel. humidity to obtain the wood moisture content of 10 %. The wood specimens were stored for about 1.5 years before they were used in the study. The angles of the test specimens for painting were rounded, and the ends of all test specimens were sealed with a mixture of Sigillon I + II to avoid uptake and leaching of the preservative via these cross sections.

The painting treatment consisted of one to several brush applications of the product to the wood specimens, which were placed on a balance during this procedure to measure the actually applied amount. For the dipping treatment, the test specimens were placed in a product-in-water solution (5.3 % v/v) for 24 h. After application, treated specimens were stored for 24 h at 20 +/- 2 °C and 65 +/- 5 % rel. humidity before the beginning of the leaching procedure. The applied amount of product resembled the respective recommended usages of the products.

As eluent a solution of the *Daphnia* test medium was used instead of the deionised water prescribed in the OECD 313 guideline for leaching of treated wood specimens in order to allow direct testing (without further dilution with test medium) of the eluate with aquatic organisms. Concentrates of the test medium (provided by ECT) were added to deionised water (pH 5.84, conductivity 0.4 µS/cm) on the day before the leaching procedure was started. The pH of this solution was 8.35, and the conductivity increased to 220 µS/cm. The solution was stored in the testing room to adjust its temperature to 20 +/- 2 °C.

Fig. 2: Glass beaker with treated test specimen (pine sapwood) prepared for the addition of the eluent

The treated wood specimens were arranged in beaker glasses (Figure 2), and the eluent was added to obtain 2.5 ml/cm$^2$ (equal to 25 l/m$^2$) treated surface area. After 6 h of water contact the eluent was replaced, and the specimens were exposed to fresh eluent for another 18 h. The eluates of both leaching periods and all replicates of each of the four product eluates were pooled into one sample and then distributed to glass bottles at volumes of 200-600 ml. The eluates were frozen at -18 °C and thereafter shipped to ECT in cool boxes. They were still frozen upon receipt and directly stored at -20 °C until further usage. Shortly after they were received, one bottle of each eluate was collected by the partner TZW and analysed for the test substances (see below). In contrast to all other samples taken later in the biotests, these eluates (referred to later as “measured shortly after preparation”) were not adjusted to pH 2 before chemical analysis.

The total treated wood surface area, the applied amounts of products, the final volumes of eluates and the maximum possible active substance concentrations in each of the eluate are summarized in Table 6.

Table 6: Characteristics of the preparation of the four eluates

| Product | Treatment | Treated surface ($m^2$) | Applied product ($g/m^2$) | Total volume of eluate (l) | Maximum possible concentration of substances in the eluate (mg/l) |
|---|---|---|---|---|---|
| A | painting | 0.0636 | 14.246 | 3.18 | propiconazole: 12.544<br>IPBC: 38.976 |
| B | painting | 0.0636 | 10.494 | 3.18 | propiconazole: 1.90<br>fenoxycarb: 0.0086 |
| C | painting | 0.2544 | 10.176 | 12.72 | propiconazole: 20.00<br>fenoxycarb: 0.320<br>dimethylalkylamine: 80.00 |
| C | dipping | 0.0318 | 1.081 | 1.59 | propiconazole: 17.00<br>fenoxycarb: 0.272<br>dimethylalkylamine: 68.00 |

Given is the treated surface area of wood test specimens (3 to 24 specimens in groups of 3 per replicate), the amount of product applied to the specimens and the total volume of eluate, pooled from two leaching periods and all replicates. The maximum possible concentration of the tests substances in the eluates is based on the assumption of 100 % emission of the applied amount

## 4.2 Preparation of test media

The test media for the biological tests were all prepared according to the same procedure. Exceptions were test media with fenoxycarb and product B (see below). Generally, a stock solution of the test substance in the respective test medium (for algae or *Daphnia*, respectively) was prepared, treated several times by ultrasonication and left stirring overnight in the dark at room temperature. On the day of the test, a geometric dilution series of the stock solution in the respective test medium was prepared freshly for each test or exchange of test medium (in the *Daphnia* reproduction test).

In the case of fenoxycarb (and the three component generic mixture, which contained fenoxycarb), the stock solution and the geometric dilution series were prepared in acetone (CAS 67-64-1; VWR international) once for each test and stored at -20 °C for up to four weeks. Test media were then freshly prepared by adding 0.050 ml of the respective acetone dilution to 1 liter of test medium while stirring. Solvent controls received accordingly 0.05 ml/l pure acetone.

Because product B is insoluble in water it was tested based on the water-accommodated fraction (WAF) approach (Singer et al. 2000) as it was done in a previous project (Coors et al. 2012). A loading rate of 2000 mg product/l test medium (*Daphnia* acute test) or 1000 mg product/l test medium (algae test) was stirred over a period of 4 days at 20 °C in the dark. In the case of the *Daphnia* test, the preparation was additionally treated by ultrasonication. Afterwards, the suspensions were left to separate over a period of 3 days (at 20 °C in the dark). The lower phase (the water phase) was then collected and used to prepare a series of dilutions, which were in turn stirred overnight again before use.

Eluates were thawed in a 20 °C water bath and tested after diluting them with test medium at appropriate volumes. In the case of the algal growth inhibition test, 10-fold concentrated algae

test medium was added to the eluate to ensure nutrient concentrations in the highest eluate test proportions similar to control treatments. Further dilutions were then prepared from this highest dilution. Since the normally used algal growth medium according to OECD 201 tended to precipitation at 10fold concentration, an alternative algae medium (ISO medium, ISO 2004) was used in these tests.

In all three test systems, seven concentration levels were usually tested with the single substances and products, while between three and seven dilutions were tested with the eluates, depending on the predicted toxicity. Geometric dilution series were prepared using spacing factors ranging from 1.26 to 2.24. The only exception was fenoxycarb and the mixture with fenoxycarb in the *Daphnia* reproduction test, where spacing factors of 3.16 and 3.0, respectively, were used. These somewhat greater spacing factors are still in accordance with the recommendation of the OECD 211 guideline.

## 4.3 Algal growth inhibition test

All algal growth inhibition tests were performed with the same species of green algae, i.e., *Pseudokirchneriella subcapitata*. The algae culture was obtained from SAG Collection of Algal Cultures (Sammlung Algenkulturen Göttingen, Deutschland). For most tests and pre-cultures, filter sterilized test medium according to Kuhl & Lorenzen (1964) was used. Only for tests with the eluates, ISO medium was used. All tests and pre-cultures were haltered in the same climate-controlled chamber at 21-24 °C, constant light (60-120 µE/$m^{2}$*s, Osram Lumilux 58W/865) and constant shaking (about 100 cycles/min). All tests were started with 5000 cells/ml added as an inoculum from a pre-culture in its exponential growth phase. The exposure in the tests was always in glass vessels over 72 h with a test volume of at least 20 ml (mostly 100 ml). The pH was measured at test start and at test end in all treatments and in the control. Only data for the controls and highest test concentrations will be reported here. After 72 h exposure, fluorescence was measured as surrogate for algal biomass in 96-well plates using a Multiplate Reader Tecan ULTRA (Tecan). Fluorescence was measured in four replicate wells for each test vessel using an excitation wavelength of 440 nm and an emission wavelength of 670 nm. For each test evaluation, an individual calibration curve was produced that related fluorescence as measured in the test with the concentration of *P. subcapitata* cells as determined by manual cell counting. Based on these correlations, the algae cell concentration ("biomass", according to OECD guideline 201) was calculated for each vessel in a test. All tests were conducted following the standard operation procedure for the algal growth inhibition test that has been established according to Good Laboratory Practice at ECT for many years.

In all algal growth inhibition tests, six replicates were run for the control and three replicates for each test concentration level. In tests where acetone was used as solvent, the solvent control was run with six replicates and used for comparisons, while the blank controls were run in triplicate. Fluorescence measurements were made daily in the tests with the four single substances, but only once after 72 h exposure in all other tests.

## 4.4 *Daphnia* tests

All *Daphnia* acute immobilisation tests and the first series of *Daphnia* reproduction tests were conducted with the same clone of *Daphnia magna* Straus, i.e., clone 5 obtained from U. Ensenbach, Aventis, Frankfurt/Main in 2000. The second series of *Daphnia* reproduction tests

was conducted with *Daphnia magna* Straus, clone M10 (Cousyn et al. 2001) obtained from K. Pauwels, KU Leuven, Belgium, in 2011.

All tests and the culture of both *Daphnia* clones used Elendt M4 medium (Elendt 1990). All animals were haltered under climate-controlled conditions (20 ± 2 °C) and a light/dark cycle of 18/6 h (50-1000 lux). In the *Daphnia* cultures, the medium was exchanged twice per week and offspring was removed at least twice per week. *Daphnia* cultures received two to three times per week algae (*Desmodesmus subspicatus*) at a level between 0.1 and 0.2 mg C/*Daphnia* and day. In addition, they received dried baker yeast once per week. All tests were conducted following the standard operation procedure for the two different *Daphnia* tests that has been established according to Good Laboratory Practice at ECT for many years.

### 4.4.1 Acute immobilisation test

*Daphnia* acute immobilisation tests were started with 2nd or 3rd brood offspring, less than 24 h old, from the *Daphnia* culture. The test animals were not fed during the test. All tests were performed in glass vessels with 25 ml of test media with five animals per vessel. There were four replicates for each treatment, including the control and solvent control, resulting in 20 test animals per treatment. Oxygen and pH were measured in each test at least in the control and the highest test concentration at the start and at the end of the test. Immobilisation was assessed after 48 h of exposure by slightly moving the test vessel and scoring all test daphnids as immobile that did not respond to this trigger by showing swimming behaviour within 15 s.

### 4.4.2 Reproduction test

*Daphnia* reproduction tests were started with 2nd or 3rd brood offspring, less than 24 h old, from the *Daphnia* cultures. All tests were performed in glass vessels with 70 ml of test media with one animal per vessel. There were up to 15 replicates for the (solvent) control and 10 replicates for each treatment level and the blank control (if applicable). Oxygen and pH were measured in each test at least in the control and the highest test concentration in freshly prepared and aged test media. The test duration was always 21 days. Test media were exchanged three times per week and daphnids were always fed when being transferred to fresh medium. The food level aimed at following the prescriptions of the OECD guideline 211, i.e., starting with 0.1 mg C/daphnid/day and increasing to 0.2 mg C/daphnid/day from day 5 onwards. This was achieved in the second series of *Daphnia* reproduction tests. In the first series, however, problems in the culture of *D. subspicatus* and particularly in producing a nomograph relating algae cell number to content of organic carbon (TOC) in the algae cells lead to lower-than-planned food levels and thereby caused food-limiting conditions. Re-calculations of fed algae indicated a food level of 0.03-0.06 mg C/daphnid/day throughout the test. Mortality and number of living offspring were recorded daily for each individual *Daphnia*. In addition, the body size (from the top of the head to the base of the spina) was measured at the end of the test.

Only one eluate could be tested in the reproduction test. Since investigating the relevance of formulation additive not only for acute but also for chronic toxicity was in the focus of this study, the painting eluate of product C, which contained dimethylalkylamine, was selected for this purpose.

## 4.5 Analytical determination of concentrations in test media and eluates

Samples were usually taken for the supporting chemical analysis from the highest, a medium and the lowest test concentrations as well as from the controls (or solvent controls). Samples taken at day 0 (begin of exposure) were collected from the freshly prepared test media before addition of test organisms. Samples taken at day 2 (after 48 h exposure) were collected from pooled replicates after removal of *Daphnia*. Algae were not removed from aged test media before chemical analysis. However, test substances adsorbed to or incorporated into the algae cells were not available to the analytical measurement. In all samples taken from test media the pH was adjusted to about pH 2 by adding concentrated hydrochloric acid. Thereafter, the samples were stored in brown glass flasks in a freezer at -20 °C. Samples were collected by the partner TZW, thawed overnight in a refrigerator and analysed the next day. In some cases, thawed samples were stored up to three days in the refrigerator before analysis. In addition to the test media, the four eluates were analysed within a period of four weeks after preparation and storage at -20 °C. They were frozen directly after preparation without adjusting the pH to 2. All analyses were performed by the partner TZW.

For the analytical determination of the target analytes several procedures were used. Dimethylalkylamine was analysed separately from the other compounds. Propiconazole, fenoxycarb and IPBC were analysed with one method. Depending on the expected concentration levels, however, two different procedures were applied for these three compounds. For higher concentrations, a method without pre-concentration step was used and for lower concentrations one which included a pre-concentration of the analytes by a vacuum concentrator. Few samples were additionally analysed for PBC, a transformation product of IPBC. For these analyses PBC was included in the direct injection method, which was used for the analysis of propiconazole, fenoxycarb and IPBC.

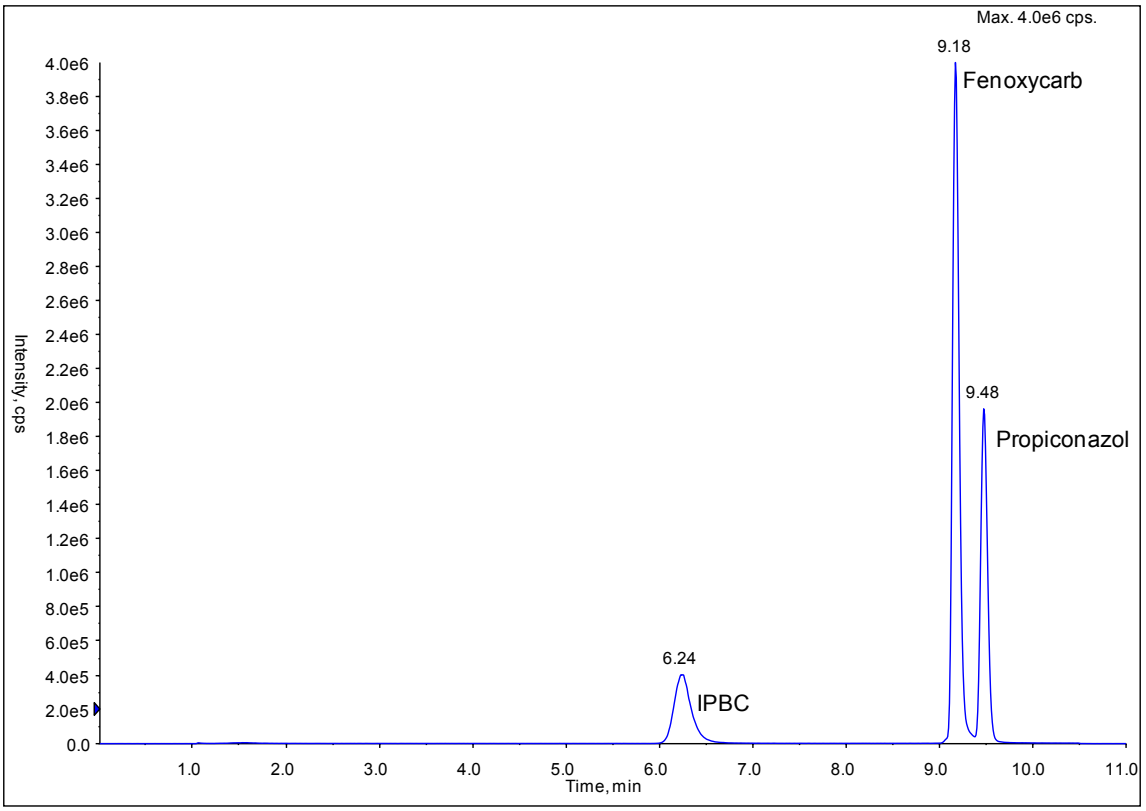
### 4.5.1 Analysis of (C12-C16) dimethylalkylamine

Analysis of (C12-C16) dimethylalkylamine was done by direct injection into a HPLC-MS-MS system. 500 µl of methanol were added to 500 µl of sample and an aliquot of 20 µL was injected into a liquid chromatograph 1260 Infinity from Agilent Technologies (Waldbronn, Germany) which was coupled via an electrospray interface to an API 5000 tandem mass spectrometer (AB Sciex, Langen, Germany). Chromatographic separation was done on a Zorbax Eclipse XDB-C18 analytical column (250 mm x 2 mm, 5 µm; Agilent Technologies) using a 10 mM aqueous ammonium acetate solution (eluent A) and a 10 mM ammonium acetate solution in methanol (eluent B) as elution solvents. The elution gradient started at 40 % of eluent A, changed to 100 % of eluent B until minute 2, stayed constant until minute 11 and was adjusted back to 40 % of eluent A between minute 11 and minute 12. After 5 minutes equilibration time, the next run started. Flow rate of the eluent was 0.25 ml/min and temperature of the column oven was adjusted to 40 °C. Detection of (C12-C16) dimethylalkylamine was done in the positive mode applying an ionisation voltage of 5.5 kV. Before and after each series of samples, a control sample and a blank sample were run. As the available (C12-C16) dimethylalkylamine standard was a mixture of several homologues, quantification was based on the sum of C12, C14 and C16 dimethylalkylamine peak. Transitions used for quantification were 214.3 to 46.1 and 214.3 to 57.1 for C12 dimethylalkylamine, 242.3 to 46.1 and 242.3 to 57.1 for C14 dimethylalkylamine, and 270.3 to 46.1 and 270.3 to 57.1 for C16 dimethylalkylamine. Quantification was done against a calibration of (C12-C16) dimethylalkylamine in tap water.

### 4.5.2 Analysis of propiconazole, fenoxycarb, IPBC and PBC at higher concentration levels

Analysis of propiconazole, fenoxycarb, IPBC and PBC at concentrations above 0.01 µg/l was done by direct injection into a HPLC-MS-MS system. If necessary, the samples were diluted with tap water prior to injection. Before injection 25 µL of a 1 mg/l solution of propiconazole-d5 in acetone were added as internal standard. Depending on the expected concentration, sample volumes of either 5 µl (for concentrations above 1 µg/l) or 100 µl (for concentrations between 0.01 and 1 µg/l) were injected into an liquid chromatograph 1200 Series from Agilent Technologies (Waldbronn, Germany) which was coupled via an electrospray interface to an API 5000 tandem mass spectrometer (AB Sciex, Langen, Germany). Chromatographic separation was done on a Zorbax Eclipse XDB-C18 analytical column (250 mm x 2 mm, 5 µm; Agilent Technologies) using a 10 mM aqueous ammonium acetate solution (eluent A) and a 10 mM ammonium acetate solution in methanol (eluent B) as elution solvents. The elution gradient started at 50 % of eluent A, changed to 100 % of eluent B until minute 4, stayed constant until minute 10 and was adjusted back to 50 % of eluent A between minute 10 and minute 11. After 5 minutes equilibration time, the next run started. Flow rate of the eluent was 0.2 ml/min and temperature of the column oven was adjusted to 40 °C. Detection of the three analytes was done in the positive mode applying an ionisation voltage of 5.5 kV. Before and after each series of samples, a control sample and a blank sample were run. Transitions used for quantification were 342.0 to 159.0 and 342.0 to 69.1 for propiconazole, 302.0 to 116.0 and 302.0 to 87.9 for fenoxycarb, 282.0 to 164.8 and 282.0 to 57.0 for IPBC, and 156.1 to 99.9 and 156.1 to 56.9 for PBC. The internal standard propiconazole-d5 was detected by the transitions 347.0 to 159.0 and 347.0 to 74.1. Quantification was done against a calibration of the target compounds in tap water. Propiconazole-d5 was used for quantification of propiconazole while the other analytes were quantified without internal standard. As illustrated by Figure 3, the peaks for the different analytes could be clearly distinguished.

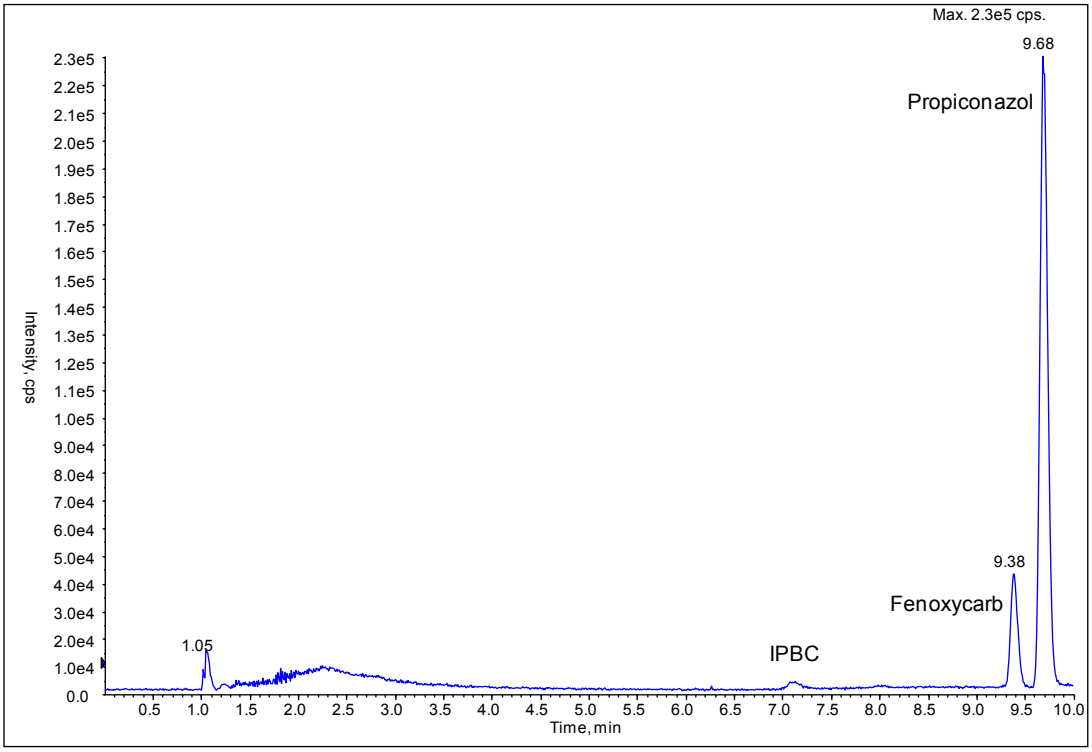
Fig. 3: Chromatogram of a standard solution of propiconazole, fenoxycarb and IPBC, each at 0.1 mg/l




### 4.5.3 Analysis of propiconazole, fenoxycarb and IPBC at lower concentration levels

If the expected concentration levels of propiconazole, fenoxycarb and IPBC were below 0.01 µg/l a sample pre-treatment by a vacuum concentrator was introduced prior to HPLC-MS-MS analysis. A sample volume of 10 ml was spiked with 20 µl of a 0.01 mg/l solution of propiconazole-d5 in acetone as internal standard. Then sample volume was evaporated to dryness in a vacuum concentrator SpeedDry 2-33IR from Martin Christ Gefriertrocknungsanlagen GmbH (Osterode, Germany) applying a temperature of 30 °C and a pressure of 16 mbar. After 14 h the dry residue was reconstituted with 40 µl methanol and 160 µl HPLC grade water. Then 80 µl were injected into the HPLC-MS-MS system using the conditions described for the direct injection of propiconazole, fenoxycarb and IPBC. Quantification was done against a calibration of the three target compounds in tap water. The calibration samples were treated equally to the real samples (i.e. also pre-concentrated by a vacuum concentrator). The peaks of all three analytes were still clearly separated and quantifiable (Figure 4).

Fig. 4: Chromatogram of a standard solution of propiconazole, fenoxycarb and IPBC (each at 1 ng/l) after pre-concentration




The propiconazole peak is interfered by the peak of the internal standard, but can be easily separated for quantification by evaluating mass transitions

### 4.5.4 Method validation

For (C12-C16) dimethylalkylamine, propiconazole, fenoxycarb and IPBC, method validation was done according to the German standard procedure described in DIN 32645. A calibration with ten concentration levels was established in tap water and from the linear calibration curve the validation parameters limit of detection (LOD, “Nachweisgrenze”) and limit of quantification (LOQ, “Bestimmungsgrenze”) were calculated as well as a variation coefficient Vx0 describing the scattering of the data points. Calculation of these parameters was done by using the commercial software SQS 2000 (“Software zur statistischen Qualitätskontrolle analytischer Daten”), version 2.01. For the pre-concentration procedure the recovery was determined in addition to the other validation parameters by comparing the results of the pre-concentrated calibration samples to the data of a direct injection of a calibration at the same concentration range.

The results of the validation procedure for propiconazole, fenoxycarb, IPBC and (C12-C16) dimethylalkylamine (Amine) are summarised in Table 7. The data prove that the developed methods are well suited for analysing the target compounds at the required concentration levels.

Table 7: Results of the validation procedure for the four analytes regarding the direct injection method and the pre-concentration method

| | Propiconazole | Fenoxycarb | IPBC | Dimethylalkylamine |
|---|---|---|---|---|
| Direct injection | | | | |
| LOD (µg/l) | 0.17 | 0.29 | 0.20 | 0.46 |
| LOQ (µg/l) | 0.62 | 1.1 | 0.74 | 1.7 |
| $V_{x0}$ (%) | 0.36 | 0.62 | 0.43 | 0.80 |
| Pre-concentration | | | | |
| Recovery (%) | 104 | 108 | 43 | - |
| LOD (µg/l) | 0.22 | 0.05 | 0.03 | - |
| LOQ (µg/l) | 0.80 | 0.20 | 0.11 | - |
| $V_{x0}$ (%) | 0.75 | 0.79 | 0.46 | - |

LOD, limit of detection; LOQ, limit of quantification; VxO, coefficient of variation of the calibration curve

### 4.5.5 Analysis of cobalt

Few samples were additionally analysed for cobalt. This was done by inductively coupled plasma mass spectrometry (ICP-MS) according to EN ISO 17294-2 (2004). Samples were injected without further pre-treatment into the ICP-MS (7500ce from Agilent Technologies).

## 4.6 Data analysis and mixture toxicity predictions

Cell numbers of algae measured in the algal growth inhibition tests as described above were analysed for the response variables yield and growth rate according to the prescriptions of the relevant OECD guideline 201 using the software program ToxRat Professional, version 2.10, release 22.02.2010.

The data on immobilisation measured in the *Daphnia* acute toxicity tests were analysed directly using the number of immobile daphnids after 48 h in relation to the number of introduced daphnids.

Data observed in the *Daphnia* reproduction test were analysed according to OECD 211 for the cumulative number of living offspring per surviving female within 21 days and body size at the end of the test.

In addition, the intrinsic rate of population increase, r, was calculated iteratively according to the Euler-Lotka equation (Lotka 1913) given as

$$1 = \int e^{-rx} l_x m_x dx$$

where $l_x$ represents the proportion of survivors at age x, and $m_x$ the number of offspring released at day x. We calculated r for each treatment level considering the 10 to 15 replicates

(individual daphnids) as population. There was no within-treatment variance estimate calculated for r, since the Monte-Carlo simulations necessary for this purpose were beyond the scope of the present study. Negative or non-defined values obtained for r resulting from mortality in case of little or no reproduction were set to zero for concentration-response modelling.

### 4.6.1 Concentration-response modelling

Effective concentrations ($EC_x$), i.e., the estimated concentration causing x % effect were estimated by means of concentration response modelling. For *Daphnia* acute immobilisation, only median effect concentrations ($EC_{50}$) were calculated, while for all other response variables $EC_{50}$, $EC_{20}$ and $EC_{10}$ were estimated. Since a 50 % reduction in body size after 21 days makes no biological sense, only $EC_{20}$ and $EC_{10}$ were estimated for this response variable.

Concentration response modelling was always based on nominal concentrations or, in the case of eluates, on the proportions of eluate volume in the test medium. Individual replicates were always used for the response modelling, but mean response values for each treatment level will be shown in graphical representations for the sake of clarity.

Concentration response modelling was done in the free software R version 2.12.2 (R Development Core Team 2011) using the most recent version of the package “drc” (Ritz & Streibig 2005). This software was used as it allows fitting a non-linear model function, a feature that is not available in ToxRat. Yet, non-linear regression analysis is the method recommended for continuous data, as explained for example in Annex 5 of the updated OECD 201 (OECD 2011). A three parameter log-logistic model was used for all sub-lethal response variables (such as reproduction, yield, and growth rates), with the lower limit fixed at 0, according to the function LL.3 given as

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

The parameter b describes the steepness of the regression curve, the parameter d is the upper limit and the parameter e is equal to the $EC_{50}$.

Since immobilisation are binomial data, a 2-parameter log-logistic model (LL.2) was used, which is identical to the 3-parameter above with the difference that the upper limit is fixed at 1 (not more than 100 % survival or immobilisation). This reduces the model function to

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

with the parameter b relating to the slope of the curve and the $EC_{50}$ being directly iteratively modelled as the second parameter.

Confidence intervals (95 %) for all $EC_x$ values were obtained with the implemented function “ED” of the “drc” package using the delta method and the t-distribution.

### 4.6.2 Statistical hypothesis testing

Hypothesis testing was applied to statistically derive no observed effect concentrations (NOEC) for several response variables (algal yield and growth rate as well as *Daphnia* reproduction and body size at test end) using the software program ToxRat Professional, version 2.10, release 22.02.2010. Since all four response variables showed overall normal distribution, parametric tests were used for determination of NOEC. Hence, in case of variance homogeneity, the William's test was used and if this requirement was not fulfilled, the Bonferroni test with correction for inhomogeneous variances (Welch t-test) was applied. The level of significance $\alpha = 0.05$ was chosen for all analyses.

### 4.6.3 Prediction of mixture toxicity and comparison with observed toxicity

The prediction of mixture toxicity was based on the concept of Concentration Addition (CA) first described by Loewe & Muischnek (1926). Based on the CA concept, the $EC_x$ of a mixture ($EC_{x\,mix}$) is calculated from the $EC_x$ values (with x denoting the same x % of effect) of all considered individual substances i ($EC_{x\,i}$) in the mixture by the equation

$$EC_{x\,mix} = \frac{1}{\sum_i \frac{P_i}{EC_{x\,i}}}$$

with the proportion of each substance i ($P_i$) in the mixture calculated from their concentrations as

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

The summed proportions of all substances that are considered in this prediction is equal to 1, while the unit of the $EC_{x\,mix}$ (either on a mass or a molar basis) relates to the sum of all substances considered in the mixture. These were at most three, i.e. propiconazole, fenoxycarb and the additive dimethylalkylamine. Hence, other additives in the products were not considered.

In order to quantify the compliance between the predicted and the observed toxicity of the mixtures, the model deviation ration (MDR) introduced by Belden et al. (2007) and used in previous studies (Coors & Frische 2011, Coors et al. 2011) was calculated as

$$MDR = \frac{prediction}{observation} = \frac{predicted\ EC_{x\,mix}}{observed\ EC_{x\,mix}}$$

A MDR of 1 characterizes perfect compliance between predicted and observed toxicity of the mixture. An MDR greater than 1 denotes that the mixture is more toxic than predicted (i.e. an

underestimation of mixture toxicity by the CA concept), while an MDR smaller than 1 indicates that the mixture is less toxic than predicted (i.e. an overestimation of mixture toxicity by the CA concept).

The $EC_x$ mix and respective MDR values were calculated for all effect levels (10 %, 20 % and 50 %) for which the respective estimates for the single substances had been determined.

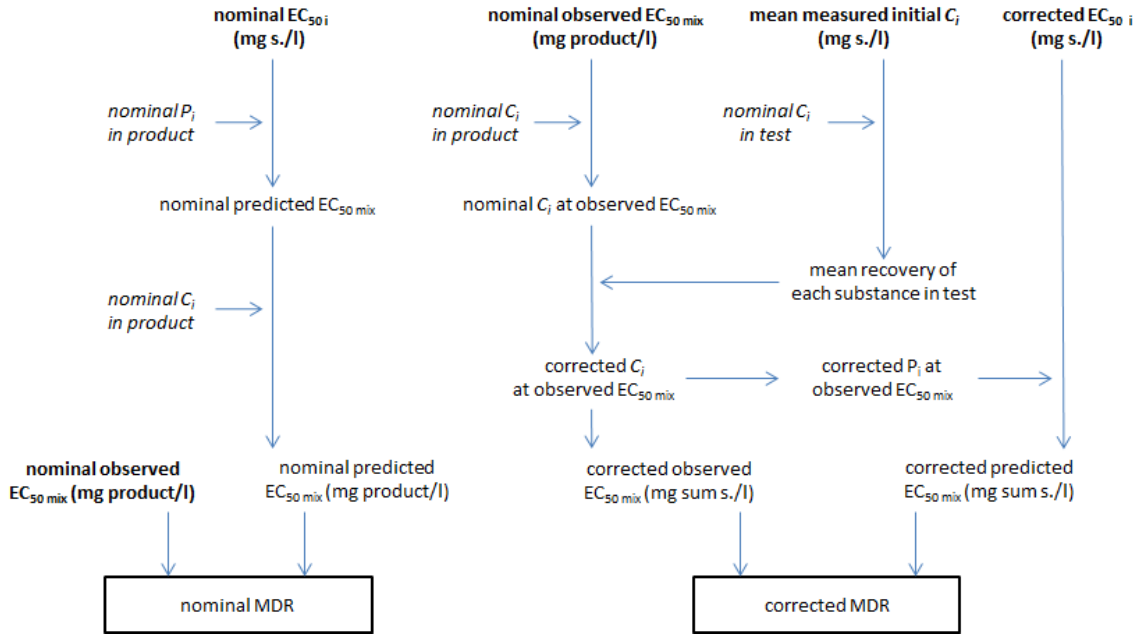
The same formulae were also used to predict a NOEC for the mixture, simply by replacing $EC_x$ with the respective NOEC values.

Finally, toxic units (TU) and relative toxic units (TU in % of sum of TU) were calculated for the products and the eluates. To this end the concentration of each individual substance in the pure product or undiluted eluate was divided by its effect estimate ($EC_{50}$ or NOEC). Note that the reciprocal of the sum of TU calculated at the $EC_{50}$ of a mixture is identical to the MDR of this mixture (Coors et al. 2012).

### 4.6.4 Correction of nominal concentrations for measured test concentrations

The $EC_{50}$ values derived based on nominal concentrations were corrected for measured test substance concentrations by using the mean of the recovery rates at two to three concentration levels at day 0, i.e. the mean initial measured concentration in relation to the nominal concentration.

Fig. 5: Scheme for the calculation of the Model Deviation Ratio (MDR) for the products (and similarly for generic mixtures)

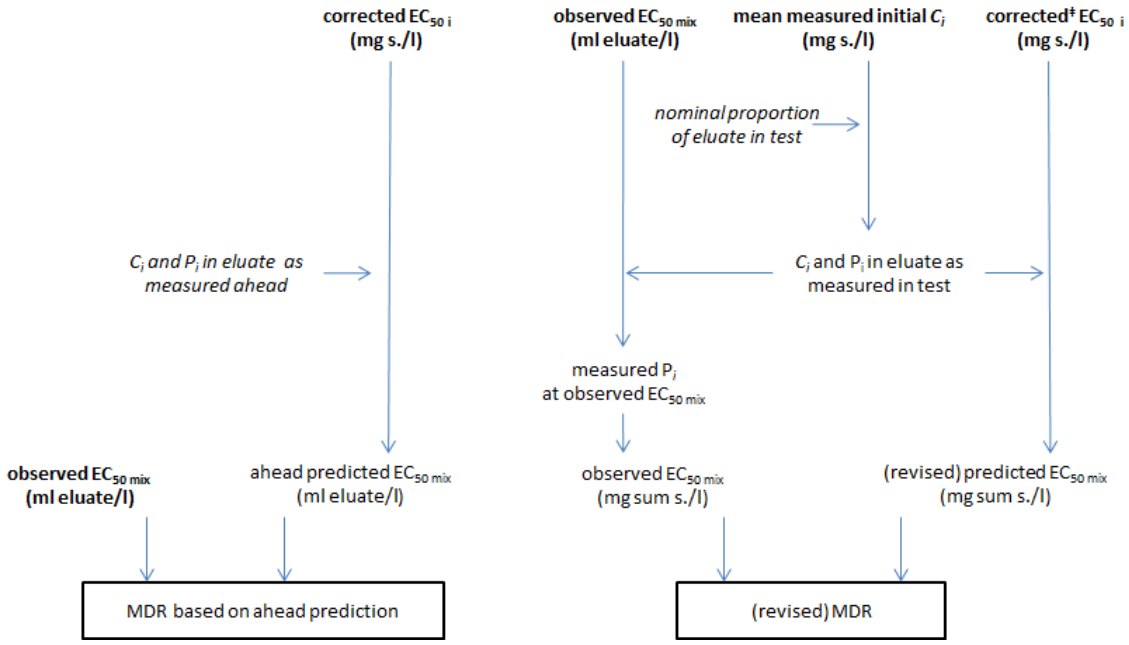



MDR values are based either on nominal concentrations or corrected for measured test concentrations. Ci denotes concentrations of the individual substances (s.) and Pi their respective proportions in the mixture. Values indicated in bold were experimentally determined and those in italics indicated information needed for the calculations. Only nominal MDR values were calculated for EC10, EC20 and NOEC endpoints

For the wood preservative products two MDR values were calculated for each endpoint: one based on nominal concentrations and one based on concentrations corrected for mean initial measured concentrations. In order not to compare apples with pears, the predicted mixture toxicity had to be revised as well for the measured concentrations of the test substances to derive a corrected MDR. This corrected MDR was only calculated for $EC_{50}$ endpoints because

correction for mean measured concentrations was not considered appropriate for lower $EC_x$ or NOEC values, which were generally at the lower end of the tested concentration range. For these endpoints only nominal MDR values were derived. Figure 5 provides a general scheme of how nominal and corrected MDR values were calculated for the tested products. The calculation of the corrected MDR values makes full use of all measured test substance concentrations, i.e., in the product test as well as in the single substance tests. The nominal MDR values were derived relying only on nominal concentrations, i.e., they were fully independent from chemical analysis.

Fig. 6: Scheme for the calculation of the Model Deviation Ratio (MDR) for the eluates,




MDR values are based either on concentrations measured ahead of the test or within the test. Ci denotes concentrations of the individual substances (s.) and Pi their respective proportions in the eluate. Values indicated in bold were experimentally determined and those in italics indicated information needed for the calculations. MDR based on ahead predictions were only calculated for EC50 endpoints. ‡calculations for EC20, EC10 and NOEC endpoints were based on nominal concentrations

The calculation of MDR values slightly differed for the eluates since no nominal concentrations were available. Instead test substance concentrations in the eluates measured shortly after their preparation ("ahead of the test") and those re-calculated from measured test concentrations ("within the test") were considered. Generally, MDR values were calculated based on the concentrations as measured in the respective biotests (after re-calculating the eluate concentration from measured test concentrations and nominal eluate proportions in the test). For comparison, MDR values based on ahead measured concentrations were derived for $EC_{50}$ endpoints only. The general scheme for calculations is shown in Figure 6.

# 5 Results

The results will be presented and discussed separately for the three biotests. Preceding the biological results, the results for the leaching tests of single substances from wood treated with the biocidal products will be reported.

## 5.1 Leaching of preservatives from treated wood

Within one month after preparation, the concentrations of the test substances were measured in the eluates (which had been stored frozen meanwhile). The results are presented in Table 8. All test substances were detected in the eluates. IPBC showed the lowest leaching rate with 0.01 % at a measured concentration of 0.0053 mg/l. Propiconazole and fenoxycarb showed the highest and about similar leaching rates, while the rate for the additive dimethylalkylamine was slightly lower. Regarding the products, the highest leaching rate was observed for the product with a water-based formulation. Here, the leaching rate after painting exceeded the leaching rate after dipping treatment by factor 2.3 to 2.5 for the three substances. Leaching rates for solvent-based products were considerably lower with less than 10 % for all substances.

Table 8: Concentration of single substances in the eluates of the wood preservative products

| Product, treatment | Substance | Maximum possible concentration (mg/l) | Measured concentration (mg/l) | Leaching (%) in present study | Leaching (%) observed previously |
|---|---|---|---|---|---|
| A, painting | propiconazole<br>IPBC | 12.5<br>39.0 | 0.42<br>0.0053 | 3.35<br>0.01 | 5<br>14 |
| B, painting | propiconazole<br>fenoxycarb | 31.35<br>0.165 | 1.90<br>0.0086 | 6.06<br>5.21 | 13<br>13 |
| C, painting | propiconazole<br>fenoxycarb<br>dimethylalkylamine | 20.00<br>0.320<br>80.00 | 3.8<br>0.058<br>10.4 | 19.0<br>18.1<br>13.0 | 40<br>30<br>n.d. |
| C, dipping | propiconazole<br>fenoxycarb<br>dimethylalkylamine | 17.0<br>0.272<br>68.0 | 7.8<br>0.12<br>20.4 | 45.9<br>44.1<br>30.0 | n.d. |

In the case of product C, two different methods of application (painting and dipping) were conducted. Given are the maximum possible concentrations for each substance assuming a leaching rate of 100 % of the applied amount and the concentration measured in the eluates within less than one month of storage at -20 °C. The eluates were not stabilized by pH adjustment. The leaching rate for the present study is calculated as the measured concentration in percentage of the maximum possible concentration. As comparison, leaching rates measured for the same products in a previous study are shown in the last column (Schoknecht 2010). n.d.: not determined

It is important to note here that the estimated leaching rates do not consider the fate of the test substances. Hence, namely IPBC may have leached indeed at a very low rate or may have dissipated quickly. This issue will be addressed later with regard to the presence of the metabolite PBC.

The leaching rates observed in the present study were about half of the rates observed earlier for the same products (Schoknecht 2010, Table 8).

## 5.2 Algal Growth Inhibition Tests

In total 13 definitive tests were conducted that will be reported here. Ahead of the definitive tests a number of range finder tests were conducted that will not be reported here. Only 2 of the 13 tests were not valid according to the validity criteria stated in the OECD guideline 201 (Table 9). These two tests (eluates of products A and B) were conducted in parallel and shared a control for which the coefficient of variation (CV) was slightly above the validity criterion of 7 %. These tests were successfully repeated for the eluate of product A, but not for the eluate of product B. The coefficient of variation of the section-by-section growth rate could only be analysed in the tests with daily measurements (i.e., the single substance tests). These CV always met the validity criterion.

Table 9: Validity criteria and respective measurements for the 13 conducted algal growth inhibition tests

| Test substance | pH at start | | pH at end | | Fold increase of biomass in control | CV of section-by-section growth rate in control | CV of growth rate in control |
|---|---|---|---|---|---|---|---|
| | Control | Highest test concentration | Control | Highest test concentration | | | |
| *Validity criterion* | *none* | | | | *≥ 16* | *≤ 35 %* | *≤ 7 %* |
| Fenoxycarb | | | | | 263 | 15.2 % | 1.7 % |
| Propiconazole | | | | | 249 | 21.3 % | 5.7 % |
| IPBC | | | | | 249 | 21.3 % | 5.7 % |
| Dimethylalkylamine | | | | | 229 | 20.7 % | 2.1 % |
| Product C – Eluate painting | 5.9 | 5.9 | 6.1 | 5.9 | 205 | n.a. | 2.6 % |
| Product C – Eluate dipping | 6.0 | 6.0 | 6.1 | 5.9 | 171 | n.a. | 2.1 % |
| Product B – Eluate | 7.7 | 7.0 | 7.7 | 7.9 | 74.4 | n.a. | 13.8 % |
| Product A – Eluate | 7.7 | 7.8 | 7.8 | 8.0 | 74.4 | n.a. | 13.8 % |
| Product A – Eluate | 7.9 | 7.8 | 7.2 | 7.8 | 358.6 | n.a. | 2.7 % |
| Product A – Eluate | 7.8 | 8.0 | 7.6 | 7.8 | 174.8 | n.a. | 3.9 % |
| Product C | 5.9 | 6.1 | 5.9 | 5.9 | 223 | n.a. | 1.3 % |
| Product B | 6.0 | 6.0 | 6.0 | 6.0 | 39.2 | n.a. | 2.7 % |
| Product A | 6.0 | 6.0 | 6.1 | 6.0 | 220 | n.a. | 1.2 % |

Given are oxygen content and pH at the start and the end of the test in control and highest test concentration (lower test concentrations were measured but are not reported here). In addition the coefficients of variation (CV) of the section-by-section growth rate is provided

### 5.2.1 Single Substances

The measured concentrations of the test substances in the four single-substance algal growth inhibition tests are shown in Table 10. Mean initial test concentrations between 80 and 120 % of the nominal concentrations were obtained in the tests with fenoxycarb and propiconazole. For dimethylalkylamine, the nominal concentration was strongly exceeded at the lowest measured concentration level. This may be a result of a pipetting mistake when preparing the dilution series. For IPBC, the measured initial concentrations were for some concentration levels below 80 % of the nominal concentrations.

Almost 80 % of the IPBC was lost during the exposure period, while the loss of the other substances was below 25 % or even below 10 % (propiconazole).

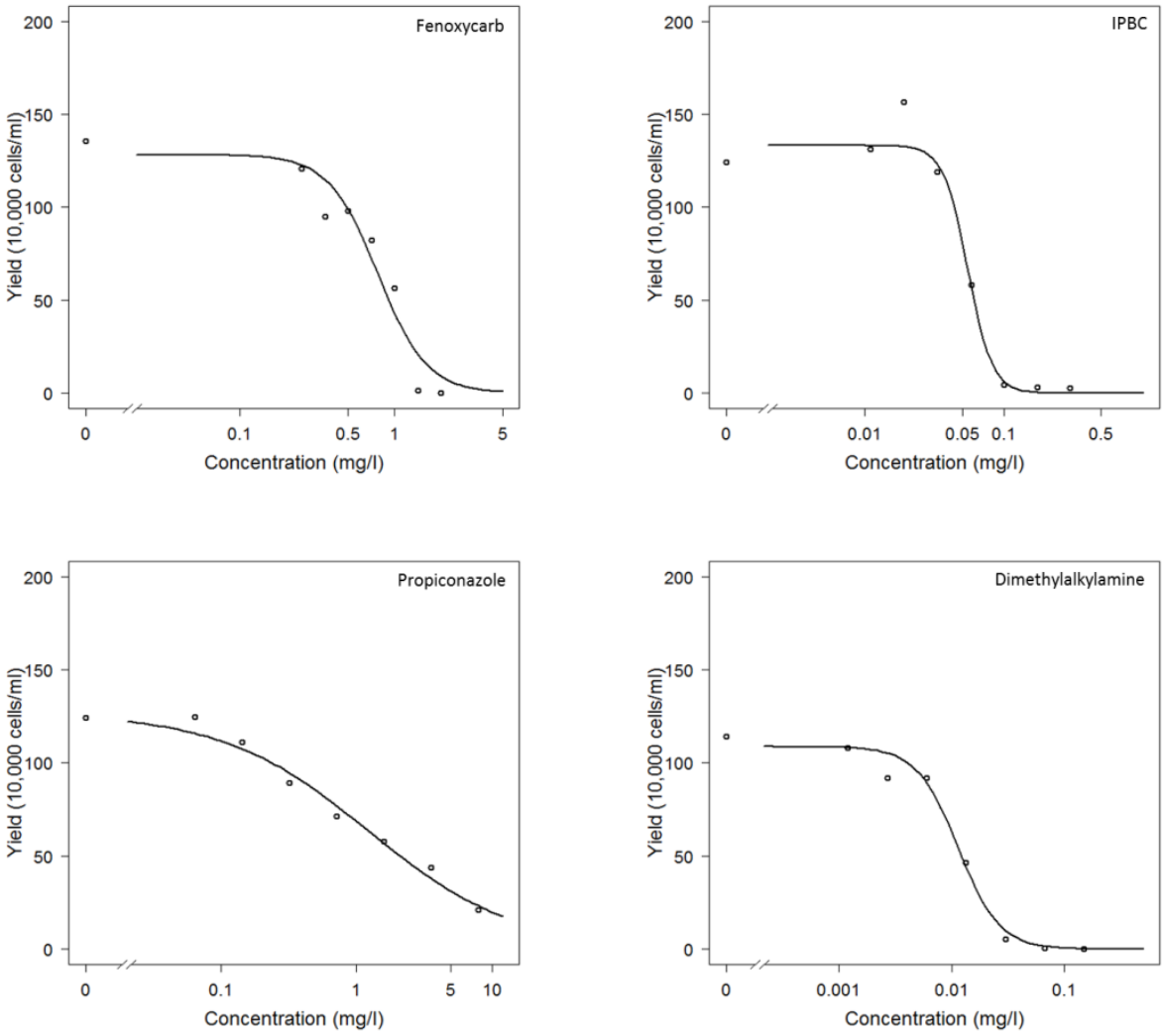
Table 10: Nominal and measured concentrations of the test substances in the single-substance algal growth inhibition tests

| Substance | Nominal test concentration (mg/l) | Measured test concentration (mg/l) day 0 / day 3 | Recovery (%) | Loss within test (%) |
|---|---|---|---|---|
| fenoxycarb | 0.250<br>2.00 | 0.260 / 0.240<br>1.70 / 1.30 | 104.0<br>85.0 | 7.7<br>23.5 |
| IPBC | 0.0111<br>0.0577<br>0.3000 | 0.0073 / n.d.<br>0.0550 / n.d.<br>0.160 / 0.033 | 65.8<br>95.3<br>53.3 | n.d.<br>n.d.<br>79.4 |
| propiconazole | 0.064<br>0.716<br>8.000 | 0.047 / n.d.<br>0.630 / n.d.<br>6.90 / 6.40 | 73.4<br>88.0<br>86.3 | n.d.<br>n.d.<br>7.2 |
| dimethylalkylamine | 1.20<br>13.42<br>150.00 | 3.20 / n.d.<br>11.0 / n.d.<br>130.0 / 100.0 | 266.7<br>82.0<br>86.7 | n.d.<br>n.d.<br>23.1 |

Initial concentrations are measured on day 0 before adding the algae and starting the exposure, while concentrations measured at day 3 were taken from exposure vessels after 72 h and thereby included algae. The recovery is calculated as the measured initial concentration in percentage of the nominal concentration. The percentage loss within the test is the difference of the measured concentration at day 0 and 3 in relation to the initial concentration. n.d.: not determined

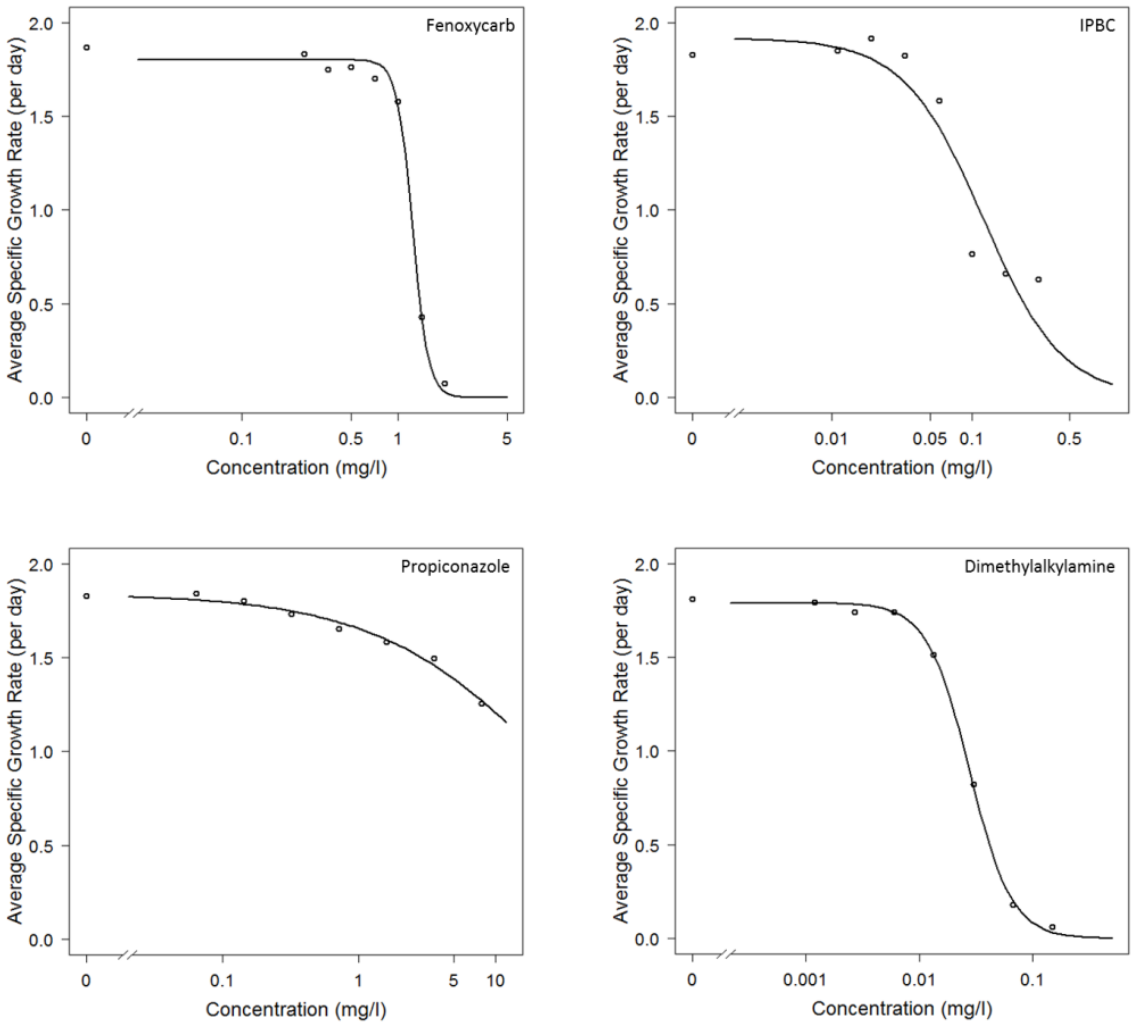
For all four test substances, clear concentration-response curves were obtained for both response variables (Figures 7 and 8). All $EC_x$ values could therefore be estimated with tight confidence intervals (Tables 11-14). The only exception was the response variable growth rate in the case of propiconazole, where the $EC_{50}$ (but not the $EC_{20}$ and $EC_{10}$) was extrapolated beyond the range of tested concentrations and can therefore not be considered very reliable.

Fig. 7: Concentration-response plots of the four tested single substances for the response variable yield in the algal growth inhibition tests




Shown are the mean values at each concentration level after 72 h exposure together with the 3-parameter log-logistic fit. The analysis was based on nominal concentrations

Fig. 8: Concentration-response plots of the four tested single substances for the response variable growth rate in the algal growth inhibition tests




Shown are the mean values at each concentration level after 72 h exposure together with the 3-parameter log-logistic fit. The analysis was based on nominal concentrations

Dimethylalkylamine and IPBC showed the highest toxicity towards algae, followed by fenoxycarb and then propiconazole. All estimated $EC_{50}$ values deviated by less than factor 2 from the effect concentrations used in the EU dossiers for regulatory decisions. The only exception was the growth rate $EC_{50}$ for propiconazole. However, the regulatory value was derived from a test with a formulated propiconazole product and has been corrected in the meantime based on new studies (personal communication D. Frein, UBA). The deviation of the (extrapolated) growth rate $EC_{50}$ in the present study from the "new" regulatory endpoint was only 2.4-fold. The NOEC values determined here for the four substances were below or in the range of the $EC_{10}$ values. The NOEC for a given substance did not differ between the two response variables.

Table 11: Toxicity of fenoxycarb in the algal growth inhibition test for the two response variables yield and average specific growth rate after 72 h exposure

| | Yield | Growth Rate |
|---|---|---|
| Parameter b (SE) | 2.73 (0.56) | 8.63 (0.72) |
| Parameter d (SE) | 128.3 (6.34) | 1.80 (0.02) |
| Nominal $EC_{50}$ (mg/l) and CI | 0.78 (0.63-0.92) | 1.24 (1.21-1.27) |
| Nominal $EC_{20}$ (mg/l) and CI | 0.47 (0.30-0.64) | 1.06 (1.01-1.10) |
| Nominal $EC_{10}$ (mg/l) and CI | 0.35 (0.18-0.52) | 0.96 (0.91-1.02) |
| Nominal NOEC (mg/l) | < 0.250 | < 0.250 |
| Recovery (%) | 94.5 | |
| Corrected $EC_{50}$ (mg/l) | 0.74 | 1.17 |
| Deviation from regulatory $EC_{50}$ | 1.36 | n.a. |

Given are the parameters b and d (standard error, SE) from the fitted log-logistic model and the ECx values with their 95 % confidence intervals (CI) at three effects levels. In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the ECx corrected for this recovery. The last row indicates the fold deviation of the observed corrected EC50 from the EC50 used in the regulatory risk assessment. n.a.: not available

Table 12: Toxicity of IPBC in the algal growth inhibition test for the two response variables yield and average specific growth rate after 72 h exposure

| | Yield | Growth Rate |
|---|---|---|
| Parameter b (SE) | 4.99 (1.85) | 1.54 (0.21) |
| Parameter d (SE) | 133.2 (5.97) | 1.91 (0.06) |
| Nominal $EC_{50}$ (mg/l) and CI | 0.055 (0.046-0.064) | 0.120 (0.094-0.145) |
| Nominal $EC_{20}$ (mg/l) and CI | 0.042 (0.029-0.054) | 0.048 (0.033-0.064) |
| Nominal $EC_{10}$ (mg/l) and CI | 0.035 (0.020-0.050) | 0.029 (0.016-0.041) |
| Nominal NOEC (mg/l) | 0.033 | 0.033 |
| Recovery (%) | 71.5 | |
| Corrected $EC_{50}$ (mg/l) | 0.039 | 0.086 |
| Deviation from regulatory $EC_{50}$ | 1.79 | 1.62 |

Given are the parameters b and d (standard error, SE) from the fitted log-logistic model and the ECx values with their 95 % confidence intervals (CI) at three effects levels. In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the ECx corrected for this recovery. The last row indicates the fold deviation of the observed corrected EC50 from the EC50 used in the regulatory risk assessment

Table 13: Toxicity of propiconazole in the algal growth inhibition test for the two response variables yield and average specific growth rate after 72 h exposure

| | Yield | Growth Rate |
|---|---|---|
| Parameter b (SE) | 0.80 (0.13) | 0.67 (0.08) |
| Parameter d (SE) | 126.7 (5.9) | 1.84 (0.02) |
| Nominal $EC_{50}$ (mg/l) and CI | 1.23 (0.63-1.84) | 26.4 * (15.43-37.37) |
| Nominal $EC_{20}$ (mg/l) and CI | 0.22 (0.04-0.40) | 3.37 (2.44-4.31) |
| Nominal $EC_{10}$ (mg/l) and CI | 0.08 (0.00-0.17) | 1.01 (0.49-1.53) |
| Nominal NOEC (mg/l) | 0.143 | 0.143 |
| Recovery (%) | 82.6 | |
| Corrected $EC_{50}$ (mg/l) | 1.02 | 21.80 |
| Deviation from regulatory $EC_{50}$ | n.a. | 2.4 (375.8) ** |

Given are the parameters b and d (standard error, SE) from the fitted log-logistic model and the ECx values with their 95 % confidence intervals (CI) at three effects levels. In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the ECx corrected for this recovery. The last row indicates the fold deviation of the observed corrected EC50 from the EC50 used in the regulatory risk assessment. * extrapolated beyond tested concentrations; ** value in brackets based on regulatory endpoint obtained from testing formulated propiconazole; n.a.: not available

Table 14: Toxicity of dimethylalkylamine in the algal growth inhibition test for the two response variables yield and average specific growth rate after 72 h exposure

| | Yield | Growth Rate |
|---|---|---|
| Parameter b (SE) | 2.37 (0.45) | 2.34 (0.12) |
| Parameter d (SE) | 108.7 (3.10) | 1.79 (0.01) |
| Nominal $EC_{50}$ (mg/l) and CI | 0.0115 (0.0095-0.0134) | 0.0277 (0.0264-0.0291) |
| Nominal $EC_{20}$ (mg/l) and CI | 0.0064 (0.0042-0.0086) | 0.0153 (0.0141-0.0166) |
| Nominal $EC_{10}$ (mg/l) and CI | 0.0045 (0.0024-0.0066) | 0.0109 (0.0096-0.0121) |
| Nominal NOEC (mg/l) | 0.001 | 0.001 |
| Recovery (%) | 145.1 | |
| Corrected $EC_{50}$ (mg/l) | 0.0167 | 0.0402 |
| Deviation from regulatory $EC_{50}$ | n.a. | n.a. |

Given are the parameters b and d (standard error, SE) from the fitted log-logistic model and the ECx values with their 95 % confidence intervals (CI) at three effects levels. In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the ECx corrected for this recovery. A comparison to the EC50 used in the regulatory risk assessment was not possible due to unavailability of data (n.a.)

### 5.2.2 Biocidal Products

The measured concentration of propiconazole in the test of product A deviated by less than 20 % from the nominal concentration, while IPBC was below the detection limit at all three tested concentration levels (Table 15). In the test of the water-accommodated fraction of product B propiconazole and fenoxycarb were detected at concentrations well below the nominal concentrations. The transfer of the two substances from the product into the test medium differed somewhat as indicated by the differing recovery rates.

Table 15: Nominal and measured concentrations of the test substances in the algal growth inhibition tests with the three products

| Product | Substance | Nominal test concentration (mg/l) | Measured test concentration (mg/l) day 0 / day 3 | Recovery (%) | Loss within test (%) |
|---|---|---|---|---|---|
| A | propiconazole | 0.0044<br>0.0124<br>0.0350 | 0.0052 / n.d.<br>0.010 / n.d.<br>0.028 / 0.023 | 118.9<br>80.8<br>80.0 | n.d.<br>n.d.<br>17.9 |
| | IPBC | 0.0136<br>0.0384<br>0.1088 | <0.0005 / n.d.<br><0.0005 / n.d.<br><0.0005 / <0.0005 | < 0.5 | n.d. |
| B | propiconazole | 0.2969<br>2.375<br>9.500 | 0.138 / n.d.<br>1.090 / n.d.<br>4.30 / 4.60 | 46.5<br>45.9<br>45.3 | n.d.<br>n.d.<br>-7.0 |
| | fenoxycarb | 0.0016<br>0.0125<br>0.0500 | 0.00049 / n.d.<br>0.0035 / n.d.<br>0.0125 / 0.0124 | 31.4<br>28.0<br>25.0 | n.d.<br>n.d.<br>0.8 |
| C | propiconazole | 0.0015<br>0.0042<br>0.0120 | 0.0015 / n.d.<br>0.0060 / n.d.<br>0.0150 / 0.0120 | 100.0<br>150.8<br>125.0 | n.d.<br>n.d.<br>20.0 |
| | fenoxycarb | 0.000024<br>0.000068<br>0.000192 | 0.000021 / n.d.<br>0.000062 / n.d.<br>0.000180 / 0.000097 | 87.5<br>91.3<br>93.8 | n.d.<br>n.d.<br>46.1 |
| | dimethylalkylamine | 0.006<br>0.017<br>0.048 | 0.0038 / n.d.<br>0.0120 / n.d.<br>0.0420 / 0.0230 | 63.3<br>70.7<br>87.5 | n.d.<br>n.d.<br>45.2 |

Given are the nominal concentrations of the test substances at three concentration levels in each of the three product tests, the concentrations in the respective concentration levels as determined by analytical measurements of samples at day 0 (initial concentration) and day 3 (aged test solution), and the resulting recovery as the measured initial concentration in percentage of the nominal concentration. The percentage loss within the test is the difference of the measured concentration at day 0 and 3 in relation to the initial concentration. n.d.: not determined

The measured initial concentration of propiconazole in the test of product C exceeded at two concentration levels the nominal concentrations, while a loss of the substance during the test of about 20 % was determined. Recovery of fenoxycarb was within 80-100 %, but slightly below 80 % for dimethylalkylamine. Assuming correct information on the concentrations of the substances in the product, these different recovery rates for the three substances in the product must be related to their different properties (e.g. different water solubility) or to analytical issues, but cannot be related to the preparation of the test medium. The loss of fenoxycarb and dimethylalkylamine during the test was with about 45 % rather high. Overall, the loss of the

test substances during the exposure period of three days differed among substances and, to some degree, among tests for a given substance. Propiconazole appeared to be the most stable compound.

For confirmation of the non-detection of IPBC, product A was again dissolved in algae test medium at a concentration of 12.8 mg/l and subjected to analytical measurement in two sub-samples: one was adjusted to pH 2 and one was not adjusted before both were frozen. The re-calculated concentration of propiconazole from this measurement deviated only slightly from the nominal propiconazole concentration in product A (2.8 mg/g product, Table 5). The results (Table 16) further confirmed the absence of IPBC from this solution and the irrelevance of pH adjustment for the determination. In addition to IPBC, the main metabolite PBC was measured in these samples. When assuming a complete and 1:1 stoichiometric transformation of IPBC (281.1 g/mol) to PBC (155.2 g/mol), the nominal concentration of IPBC would be equivalent to a PBC concentration of 0.061 mg/l, which was close to the determined concentration. This indicated indeed a considerable transformation of IPBC into PBC, i.e. about 70.5 %.

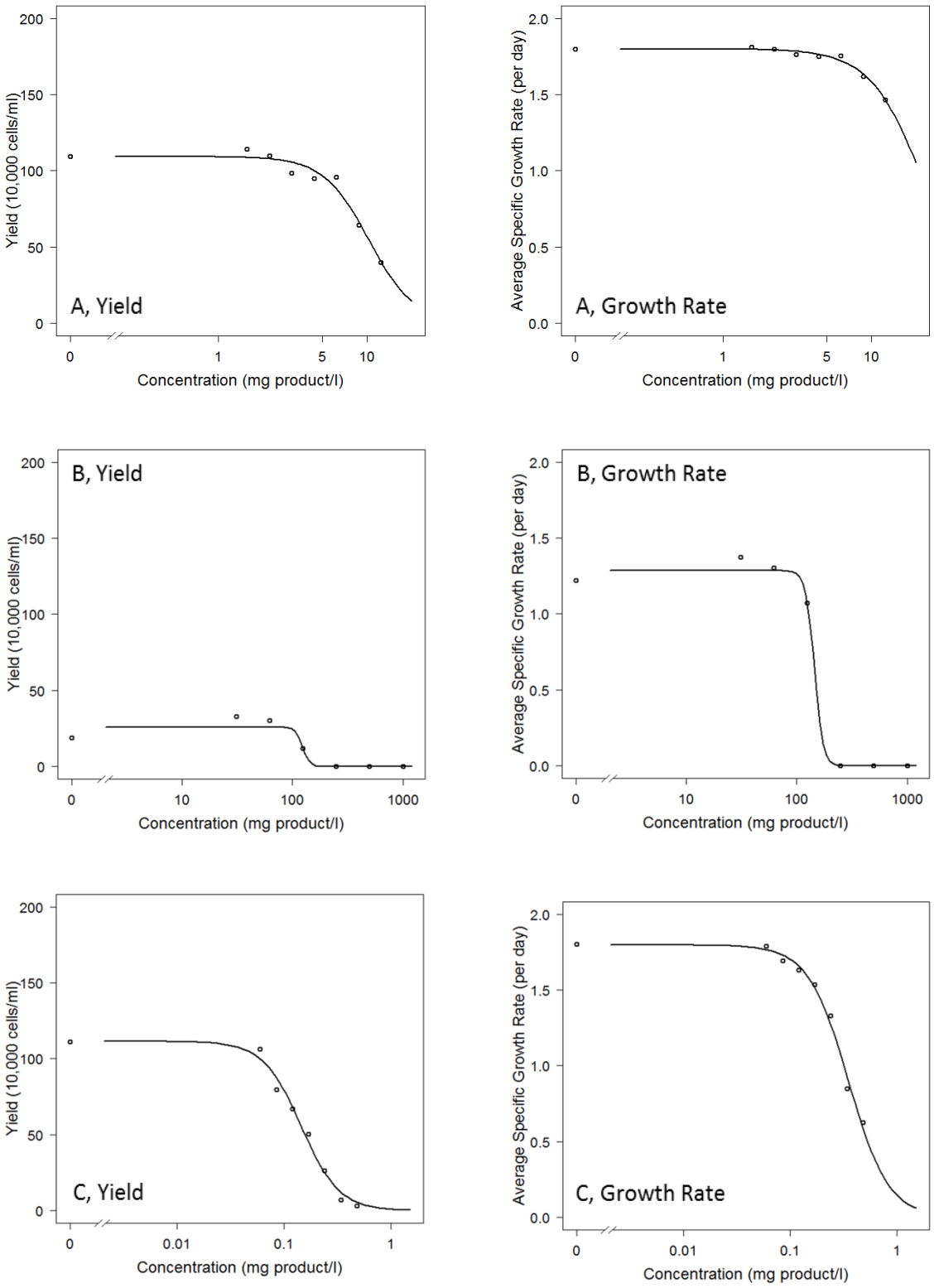
Cobalt was also measured in these samples in order to verify if the concentration of this additive would be high enough to contribute to overall toxicity. The measured concentration was independent from the pH, and transferred to a cobalt concentration of about 0.117 mg/g product.

Table 16: Nominal, measured and re-calculated concentrations of substances in product A dissolved at 12.8 mg/l in the algae test medium with and without adjustment of pH before freezing

| Substance | Nominal concentration (mg/l) | Measured concentration (mg/l) pH 2 / pH not adjusted | Re-calculated concentration in product A (mg/g) |
|---|---|---|---|
| propiconazole | 0.036 | 0.029 / 0.028 | 2.23 |
| IPBC | 0.111 | < 0.001 / < 0.001 | < 0.078 |
| PBC | unknown | 0.043 / 0.043 | 3.36 |
| cobalt | unknown | 0.001 / 0.002 | 0.117 |

Clear concentration response curves were obtained for the response variables yield and growth rate in the tests of all three products (Figure 9).

Fig. 9: Concentration-response plots of the three tested wood preservative products (A, B, and C) for the two response variables yield (left) and average specific growth rate (right) in the algal growth inhibition tests




Shown are the mean values at each concentration level after 72 h exposure together with the 3-parameter log-logistic fit. The analysis was based on nominal product concentrations

In the case of product A, the value for the detection limit of IPBC (0.0005 mg/l) was used for correcting $EC_{50}$ values and related predictions, resulting in censored corrected MDR values. Prediction and observation based on nominal concentrations were in agreement with a deviation of less than factor 2 or, in case of the extrapolated value for the response variable growth rate, with a deviation of less than factor 3 (Table 17). This was true for comparisons on three different effect levels (50 %, 20 % and 10 %) as well as for comparisons based on statistically derived NOEC values. Correcting the $EC_{50}$ values for the actual measured concentrations of the substances in the test resulted in MDR values above 27, indicating considerable underestimation of the observed product toxicity in both response variables. The difference between the nominal and corrected MDR values is due to the non-detection of IPBC in the test medium. As stated above, PBC was found at a concentration that indicated an almost complete transformation of IPBC into PBC. The corrected MDR of 27 is identical to an explained toxic unit (TU) of 0.037. If PBC fully accounted for the underestimated toxicity, its toxic unit in the product test at the observed $EC_{50}$ would consequently be 0.963 (=1-0.037). Extrapolating the measured concentration of PBC reported in Table 16 for product A to the algal growth inhibition test results in an estimated concentration of 0.035 mg PBC/l at the observed product $EC_{50}$ (yield), which translates to 0.063 mg IPBC/l assuming a 1:1 stoichiometric transformation. Based on the $EC_{50}$ of IPBC, a TU of 1.1 is calculated for this PBC concentration (hence assuming similar toxicity for the metabolite as for the parent compound). Given that only 0.963 TU are unexplained, the toxicity of PBC is presumably somewhat lower than that of IPBC based on these calculations. The concentration of cobalt at the $EC_{50}$ (yield) of the product (about 1.2 µg/l) is well below the reported $EC_{50}$ of 90 µg/l; the resulting TU of 0.01 for cobalt thus cannot explain the underestimation of product A toxicity based on measured concentrations.

Table 17: Predicted and observed $EC_x$ and NOEC values estimated for product A tested in the algal growth inhibition test

| Estimates | Yield | Growth Rate |
|---|---|---|
| Nominal predicted $EC_{50}$ (mg product/l) | 6.2 | *13.8* |
| Nominal observed $EC_{50}$ (mg product/l) and [CI] | 10.3 [9.3-11.4] | *23.2* [19.0-27.3] |
| Mean recovery (%) | 93.2 (propiconazole)<br>< 0.46 (IPBC) | |
| Corrected observed $EC_{50}$ (mg sum substances/l) | 0.027 | *0.062* |
| Corrected predicted $EC_{50}$ (mg sum substances/l) | 0.739 | *4.531* |
| Nominal MDR ($EC_{50}$) | 0.60 | 0.59 |
| Corrected MDR ($EC_{50}$) | > 27.0 | > 73.7 |
| Nominal predicted $EC_{20}$ (mg product/l) | 4.5 | 5.5 |
| Nominal observed $EC_{20}$ (mg product/l) and [CI] | 6.3 [5.0-7.6] | 12.9 [12.2-13.7] |
| Nominal MDR ($EC_{20}$) | 0.72 | 0.42 |
| Nominal predicted $EC_{10}$ (mg product/l) | 3.5 | 3.3 |
| Nominal observed $EC_{10}$ (mg product/l) and [CI] | 4.7 [3.3-6.2] | 9.2 [8.4-10.0] |
| Nominal MDR ($EC_{10}$) | 0.74 | 0.36 |
| Nominal predicted NOEC (mg product/l) | 3.53 | 3.53 |
| Nominal observed NOEC (mg product/l) | 2.21 | 3.13 |
| Nominal MDR (NOEC) | 1.60 | 1.13 |

Given are the nominal observed ECx values for the two response variables yield and growth rate with their 95 % confidence intervals [CI] estimated from the fitted 3-parameter log-logistic model. In addition, the nominal observed NOEC is provided. Corrections for measured test concentrations by the mean recovery were only applied in case of the EC50 values. IPBC was below the detection limit of 0.0005 mg/l in the highest test concentration (recovery of <0.46 %), resulting in censored corrected MDR values. EC50 values given in italics are based on extrapolations beyond tested concentrations

In the case of product B, the nominal and the corrected MDR based on the $EC_{50}$ (yield) is close to 1, and thereby indicates good compliance between CA prediction and observation. Nominal MDR values for lower effect levels tended to overestimate the toxicity of the product by factor 5 to 10 (Table 18). This was at least partly due to the fact that the actual concentration of the test substances in the water accommodated fraction was less than 50 % of the nominal concentrations. The MDR values based on the extrapolated $EC_{50}$ (growth rate) of propiconazole indicated underestimation of product toxicity, which was not supported by the other MDR values.

Table 18: Predicted and observed $EC_x$ and NOEC values estimated for product B tested in the algal growth inhibition test

| Estimates | Yield | Growth Rate |
|---|---|---|
| Nominal predicted $EC_{50}$ (mg product/l) | 128.4 | *2498.9* |
| Nominal observed $EC_{50}$ (mg product/l) and [CI] | 123.6 [96.2-150.8] | 144.4 [61.9-226.7] |
| Mean recovery (%) | 45.9 (propiconazole)<br>28.1 (fenoxycarb) | |
| Corrected observed $EC_{50}$ (mg sum substances/l) | 0.540 | 0.631 |
| Corrected predicted $EC_{50}$ (mg sum substances/l) | 1.019 | *20.630* |
| Nominal MDR ($EC_{50}$) | 1.04 | 17.3 |
| Corrected MDR ($EC_{50}$) | 1.89 | 32.7 |
| Nominal predicted $EC_{20}$ (mg product/l) | 23.1 | 348.9 |
| Nominal observed $EC_{20}$ (mg product/l) and [CI] | 111.8 [0-295.9] | 127.4 [114.3-140.6] |
| Nominal MDR ($EC_{20}$) | 0.21 | 2.74 |
| Nominal predicted $EC_{10}$ (mg product/l) | 8.4 | 105.7 |
| Nominal observed $EC_{10}$ (mg product/l) and [CI] | 105.5 [0-369.6] | 118.5 [91.9-145.0] |
| Nominal MDR ($EC_{10}$) | 0.08 | 0.89 |
| Nominal predicted NOEC (mg product/l) | < 15.0 | < 15.0 |
| Nominal observed NOEC (mg product/l) | 62.5 | 62.5 |
| Nominal MDR (NOEC) | < 0.24 | < 0.24 |

Given are the nominal observed ECx values for the two response variables yield and growth rate with their 95 % confidence intervals [CI] estimated from the fitted 3-parameter log-logistic model. In addition, the nominal observed NOEC is provided. Corrections for measured test concentrations by the mean recovery were only applied in case of the EC50 values. EC50 values given in italics are based on extrapolations beyond tested concentrations (i.e., growth rate EC50 of propiconazole)

For product C, the MDR values derived from nominal concentrations for the $EC_x$ values all indicated a slight (but less than factor 2) overestimation of product toxicity (Table 19). Correcting the $EC_{50}$ values for measured substance concentrations resulted in a slight (but less than factor 2) underestimation of product toxicity. Hence, overall the CA-predicted and the observed product toxicity were in very good agreement, independently from the assessed effect level and whether nominal or measured concentrations were used. Only when nominal NOEC values were used for the MDR calculation, a considerable (almost six-fold) overestimation of product toxicity was found for product C. All this hold only when dimethylalkylamine is considered in the calculations. Without taking this formulation additive into account, all MD values indicate large underestimation of product toxicity.

Table 19: Predicted and observed $EC_x$ and NOEC values estimated for product C tested in the algal growth inhibition test

| Estimates | Yield | Growth Rate |
|---|---|---|
| Nominal predicted $EC_{50}$ (mg product/l) | 0.115 (48.0) | 0.277 (787.7) |
| Nominal observed $EC_{50}$ (mg product/l) and [CI] | 0.144 [0.128-0.161] | 0.351 [0.320-0.382] |
| Mean recovery (%) | 125.3 (propiconazole)<br>90.9 (fenoxycarb)<br>73.8 (dimethylalkylamine) | |
| Corrected observed $EC_{50}$ (mg sum substances/l) | 0.015 (0.005) | 0.037 (0.011) |
| Corrected predicted $EC_{50}$ (mg sum substances/l) | 0.024 (1.016) | 0.057 (18.13) |
| Nominal MDR ($EC_{50}$) | 0.79 (332) | 0.79 (2244) |
| Corrected MDR ($EC_{50}$) | 1.56 (222) | 1.55 (1630) |
| Nominal predicted $EC_{20}$ (mg product/l) | 0.064 (8.7) | 0.153 (128.3) |
| Nominal observed $EC_{20}$ (mg product/l) and [CI] | 0.082 [0.066-0.098] | 0.192 [0.160-0.225] |
| Nominal MDR ($EC_{20}$) | 0.78 (106) | 0.80 (668) |
| Nominal predicted $EC_{10}$ (mg product/l) | 0.044 (3.2) | 0.109 (39.7) |
| Nominal observed $EC_{10}$ (mg product/l) and [CI] | 0.059 [0.044-0.074] | 0.136 [0.103-0.169] |
| Nominal MDR ($EC_{10}$) | 0.75 (54.1) | 0.80 (292) |
| Nominal predicted NOEC (mg product/l) | 0.01 (5.7) | 0.01 (5.7) |
| Nominal observed NOEC (mg product/l) | 0.06 | 0.06 |
| Nominal MDR (NOEC) | 0.17 (94.5) | 0.17 (94.5) |

Given are the nominal observed ECx values for the two response variables yield and growth rate with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model. The observed EC50 value for growth rate was extrapolated beyond the range of tested concentrations. In addition, the nominal observed NOEC is provided. Corrections for measured test concentrations by the mean recovery were only applied in case of the EC50 values. Values in brackets () were derived without consideration of dimethylalkylamine

### 5.2.3 Eluates

Both propiconazole and IPBC were detected in the tests conducted with the eluate of product A. The average IPBC concentration in the eluate re-calculated from measurements of test media was 0.006 µg/l (Table 20) and thereby similar to the concentration determined shortly after eluate preparation (0.0053 µg/l, see Table 8). The propiconazole concentration, in contrast, was about twice as high in the eluate based on the analytical measurement in the test compared to the previous measurement. Propiconazole was stable during the test, while more than 90 % of the IPBC dissipated (data from test 1, not shown here).

Table 20: The concentrations of the test substances in the four eluates of the three wood preservative products as measured in the algal growth inhibition tests

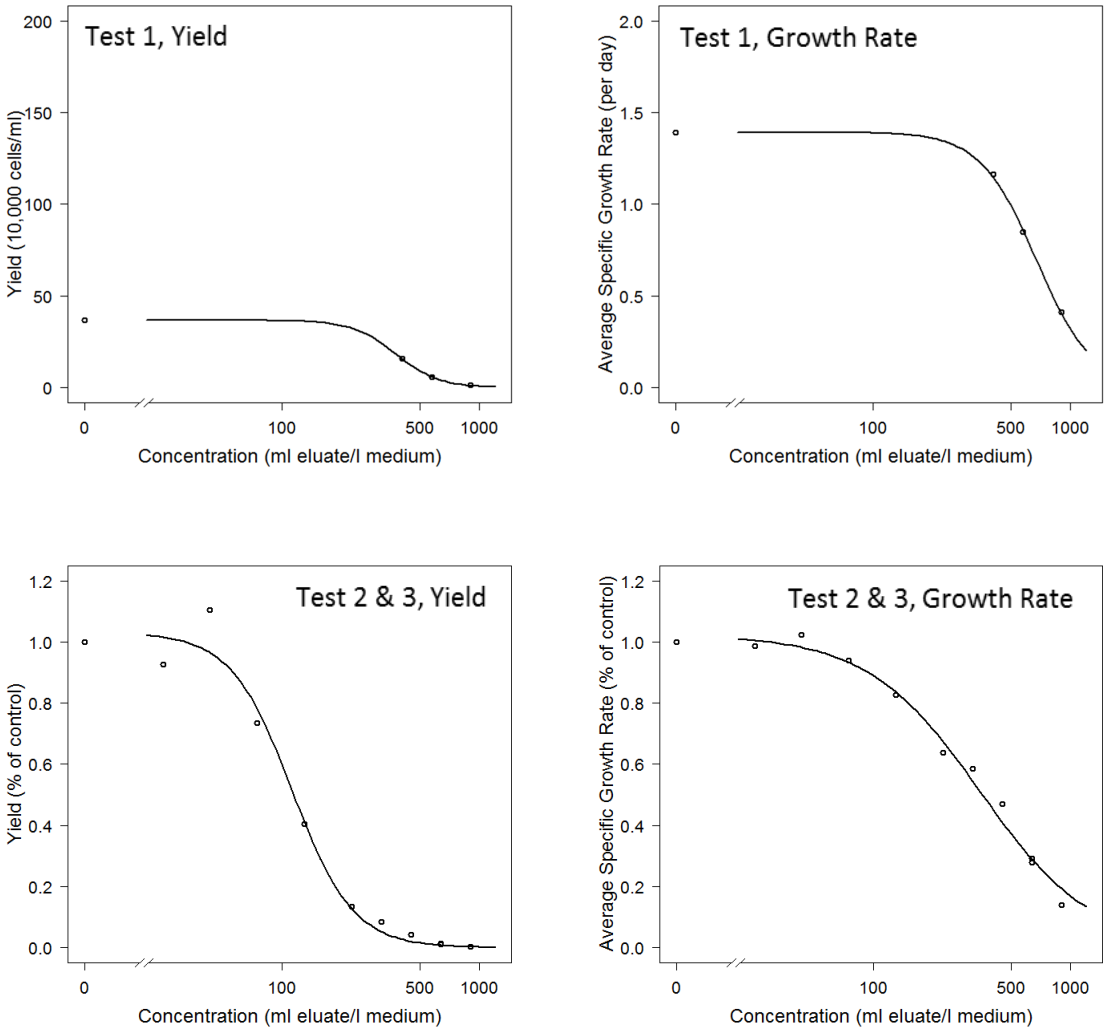
| Eluate | Substance | Concentration in diluted eluate (mg/l) day 0 / day 3 | Re-calculated concentration in eluate (mg/l) | Mean concentration in eluate (mg/l) | Loss within test (%) |
|---|---|---|---|---|---|
| A, painting, test 2 & 3 | propiconazole | 0.18 / n.d.<br>0.18 / n.d.<br>0.68 / n.d. | 0.800<br>0.800<br>0.756 | 0.785 | n.d. |
| | IPBC | 0.0014 / n.d.<br>0.0013 / n.d.<br>0.0052 / n.d. | 0.006<br>0.006<br>0.006 | 0.006 | n.d. |
| | PBC | 0.64<br>0.65<br>0.51 | 2.844<br>2.889<br>0.567 | 2.10 | n.d. |
| | cobalt | 0.041<br>0.037<br>0.150 | 0.182<br>0.164<br>0.167 | 0.171 | n.d. |
| B, painting | propiconazole | 1.02 / n.d.<br>3.3 / 3.0 | 3.56<br>3.67 | 3.614 | 9.1 |
| | fenoxycarb | 0.0024 / n.d.<br>0.0069 / 0.002 | 0.0084<br>0.0077 | 0.008 | 71.0 |
| C, painting | propiconazole | 0.00077<br>0.008<br>0.044 / 0.054 | 4.93<br>6.40<br>4.40 | 5.24 | -22.7 |
| | fenoxycarb | < 0.0000005<br>0.000059<br>0.00044 / 0.00035 | n.d.<br>0.047<br>0.044 | 0.046 | 20.5 |
| | dimethylalkylamine | 0.0018<br>0.016<br>0.113 / 0.095 | 11.52<br>12.80<br>11.30 | 11.87 | 15.9 |
| C, dipping | propiconazole | 0.00096<br>0.009<br>0.066 / 0.061 | 12.29<br>14.40<br>13.20 | 13.30 | 7.6 |
| | fenoxycarb | < 0.0000005<br>0.000058<br>0.00064 / 0.00055 | n.d.<br>0.093<br>0.128 | 0.074 | 14.1 |
| | dimethylalkylamine | 0.0018<br>0.013<br>0.102 | 23.04<br>20.80<br>20.40 | 21.41 | 22.5 |

Given are the concentrations of the substances in at least one concentration level in each of the four eluates as determined by analytical measurements of samples at day 0 (initial concentration) and day 3 (aged test solution), and the measured or from the diluted eluates re-calculated concentrations in undiluted eluates. The percentage loss within the test is the difference of the measured concentration at day 0 and 3 in relation to the initial concentration. n.d.: not determined

The eluate of product A caused concentration-dependent inhibition of algal growth (Figure 9). The concentration-response curve obtained in the first tests with the eluate was not complete,

i.e. the control level of algal growth was not met at any tested concentration. Therefore, the eluate was tested again in two independent tests at different concentration levels of eluate in the test (test 2 & 3). Thereby, a complete concentration-response curve was obtained (Figure 10) that allowed deriving $EC_x$ and NOEC values (Table 21).

Fig. 10: Concentration-response plots of the eluate prepared from product A for the two response variables yield (left) and average specific growth rate (right) in the algal growth inhibition test




Results of test 1 are shown in the top row and those of test 2 & 3 are combined in the graphs in the bottom row. The medium concentration of 225 ml/l was tested in both test 2 and 3. Shown are the mean values at each concentration level after 72 h exposure together with the 3-parameter log-logistic fit. The analysis was based on nominal proportions of the eluates in the test medium

Table 21: Predicted and observed $EC_{50}$ values estimated of the eluate prepared from product A tested for algal growth inhibition and resulting MDR values (test 2 & 3)

| Estimates | Yield | Growth Rate |
|---|---|---|
| Ahead predicted $EC_{50}$ (ml eluate/l test medium) | 1,825.9 | *12,361.9* |
| Observed $EC_{50}$ (ml eluate/l test medium) with [CI] | 112.4 [97.2-127.6] | 346.8 [312.9-380.6] |
| Observed $EC_{50}$ (mg sum substances/l) | 0.089 | 0.274 |
| Revised predicted $EC_{50}$ (mg sum substances/l) | 0.858 | *7.540* |
| MDR based on ahead prediction ($EC_{50}$) | 16.2 | 35.6 |
| MDR ($EC_{50}$) | 9.6 | 27.5 |
| Observed $EC_{20}$ (ml eluate/l test medium) with [CI] | 68.6 [53.7-83.4] | 140.0 [113.2-166.9] |
| Observed $EC_{20}$ (mg sum substances/l) | 0.054 | 0.111 |
| Revised predicted $EC_{20}$ (mg sum substances/l) | 0.212 | 2.220 |
| MDR ($EC_{20}$) | 3.9 | 20.0 |
| Observed $EC_{10}$ (ml eluate/l test medium) with [CI] | 51.4 [36.9-65.8] | 82.4 [60.9-103.9] |
| Observed $EC_{10}$ (mg sum substances/l) | 0.041 | 0.065 |
| Revised predicted $EC_{10}$ (mg sum substances/l) | 0.079 | 0.806 |
| MDR ($EC_{10}$) | 1.95 | 12.4 |
| Observed NOEC (ml eluate/l test medium) | 43.3 | 43.3 |
| Observed NOEC (mg sum substances/l) | 0.034 | 0.034 |
| Revised predicted NOEC (mg sum substances/l) | 0.140 | 0.140 |
| MDR (NOEC) | 4.07 | 4.07 |

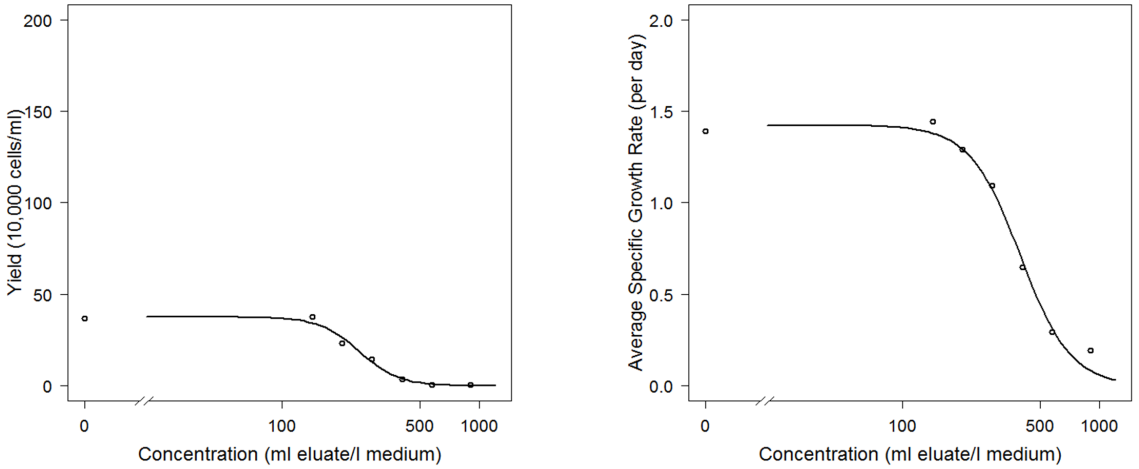
Given are the observed ECx values with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model based on the proportion of the eluate in the test medium in tests 2 & 3. The ahead predicted EC50 values are based on the concentrations of the test substances measured in the eluates ahead of the test (i.e., shortly after preparation), while the revised EC50 values are based on the concentrations measured in the tests. MDR values based on ahead measured concentrations are only reported for EC50 values. All ECx values that were determined by extrapolation beyond tested concentrations are shown in italics. In the case of the growth rate, the EC50 value for propiconazole and thereby predicted values for the mixture are based on extrapolation

With regard to both response variables, an underestimation of the toxicity of the eluate by the CA prediction was indicated. Only the prediction for yield based on the $EC_{10}$ deviated by less than factor 2 from the respective observation. The degree of underestimation of toxicity was higher for the response variable growth rate than for yield. While this is likely due to the fact that the effect estimates for propiconazole with regard to growth rate are extrapolated values, the predictions for yield are all based on reliable effect estimates. The difference in the measured test substance concentrations in the eluate had only a small influence on the agreement between prediction and observation as indicated by the two respective MDR values provided for $EC_{50}$ estimations. This is due to the fact that there was no difference for the more toxic of the two test substances (i.e., IPBC), which demonstrates that a precise concentration

determination is most crucial for the more toxic components in the course of mixture toxicity assessments.

The concentrations of the IPBC metabolite PBC and the formulation additive cobalt were also determined in the test media in tests 2 and 3. The resulting average concentration of PBC in the eluate was 2.1 mg/l, equivalent to 0.236 mg/l at the observed $EC_{50}$ (yield) of the eluate. When assuming a 1:1 stoichiometric transformation of IPBC (281.1 g/mol) to PBC (155.2 g/mol), the initial cumulative concentration of IPBC would have been 0.433 mg/l. Further assuming that PBC is as toxic to algae as IPBC, this concentration would result in a toxic unit of 11.1 for PBC in the eluate, i.e. toxicity more than factor 10 greater than actually observed. This indicates that PBC is less toxic to algae than the parent compound IPBC. The average cobalt concentration of 0.171 mg/l in the eluate equals 0.019 mg/l at the observed $EC_{50}$ (yield) of the eluate, which translates into TU of about 0.21. Hence, in the case of the eluate of product A, both PBC and cobalt may have contributed significantly to the overall toxicity to algae.

Fig. 11: Concentration-response plots of the eluate prepared from product B for the two response variables yield (left) and average specific growth rate (right) in the algal growth inhibition test




Shown are the mean values at each concentration level after 72 h exposure together with the 3-parameter log-logistic fit. The analysis was based on nominal proportions of the eluates in the test medium

The concentrations of the test substances in the eluate of product B as re-calculated from the algal growth inhibition test (Table 20) were about similar to those determined ahead of the test for fenoxycarb, but about twice as high for propiconazole (Table 8). Propiconazole was stable during the test of the eluate of product B, while a considerable amount of fenoxycarb dissipated (Table 20). Clear concentration-response curves were obtained in the test of the eluate of product B (Figure 11) from which $EC_x$ and NOEC values could be estimated with tight confidence intervals and without extrapolation (Table 22). The test results appear therefore reliable enough to be used for MDR calculations, although the test was formally not valid due to slightly too high variations in the growth rate of the controls (Table 9).

Based on measured test concentrations, the MDR values for the response variable yield indicated good agreement between CA prediction and observed toxicity at a 50 % effect level, but overestimation of toxicity at lower effect levels. Based on NOEC values, the toxicity was overestimated by more than factor 5 (Table 22). The MDR based on ahead determined

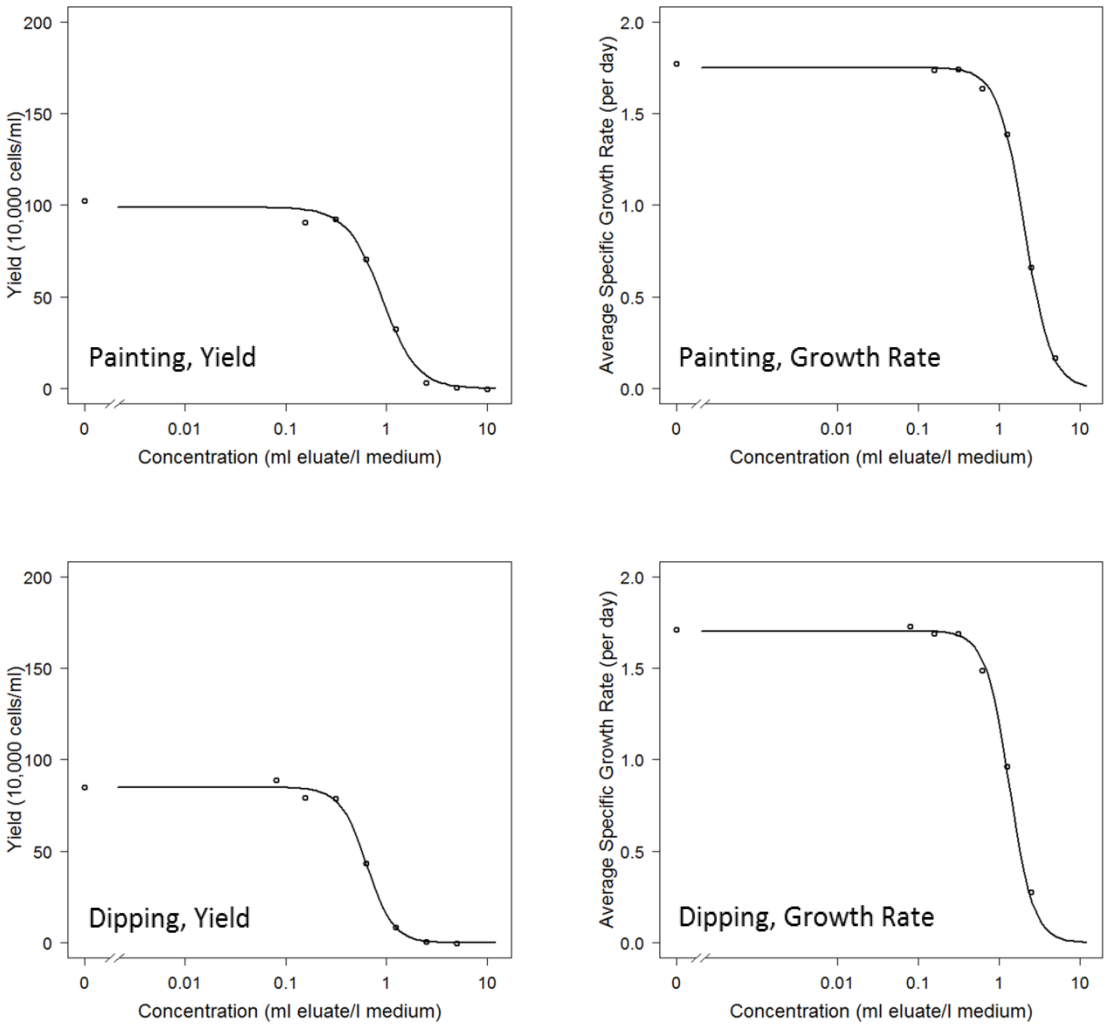
concentrations indicated underestimation of toxicity, but this was solely due to the lower concentration of propiconazole determined ahead of the testing in the eluate compared to the test concentrations. With regard to the response variable growth rate, underestimation of toxicity was indicated at effect levels of 20 % and 50 %. As in the case of the eluate of product A, this may be linked to the fact that the $EC_{50}$ input data for propiconazole was extrapolated and therefore of limited reliability.

Table 22: Predicted and observed $EC_{50}$ values estimated of the eluate prepared from product B tested for algal growth inhibition and resulting MDR values

| Estimates | Yield | Growth Rate |
|---|---|---|
| Ahead predicted $EC_{50}$ (ml eluate/l test medium) | 533.5 | *10,581.3* |
| Observed $EC_{50}$ (ml eluate/l test medium) with [CI] | 246.0 [172.9-3189.0] | 396.3 [351.5-441.0] |
| Observed $EC_{50}$ (mg sum substances/l) | 0.891 | 1.435 |
| Revised predicted $EC_{50}$ (mg sum substances/l) | 1.019 | *20.981* |
| MDR based on ahead prediction ($EC_{50}$) | 2.17 | 26.7 |
| MDR ($EC_{50}$) | 1.14 | 14.6 |
| Observed $EC_{20}$ (ml eluate/l test medium) with [CI] | 178.4 [99.5-257.1] | 263.4 [213.2-313.7] |
| Observed $EC_{20}$ (mg sum substances/l) | 0.646 | 0.954 |
| Revised predicted $EC_{20}$ (mg sum substances/l) | 0.220 | 3.354 |
| MDR ($EC_{20}$) | 0.34 | 3.51 |
| Observed $EC_{10}$ (ml eluate/l test medium) with [CI] | 147.8 [61.7-233.9] | 207.5 [152.8-262.2] |
| Observed $EC_{10}$ (mg sum substances/l) | 0.535 | 0.752 |
| Revised predicted $EC_{10}$ (mg sum substances/l) | 0.080 | 1.010 |
| MDR ($EC_{10}$) | 0.15 | 1.34 |
| Observed NOEC (ml eluate/l test medium) | 202.5 | 202.5 |
| Observed NOEC (mg sum substances/l) | 0.733 | 0.733 |
| Revised predicted NOEC (mg sum substances/l) | < 0.143 | < 0.143 |
| MDR (NOEC) | < 0.20 | < 0.20 |

Given are the observed ECx values with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model based on the proportion of the eluate in the test medium. The ahead predicted EC50 values are based on the concentrations of the test substances measured in the eluates ahead of the test (i.e., shortly after preparation), while the revised EC50 values are based on the concentrations measured in the tests. MDR values based on ahead measured concentrations are only reported for EC50 values. All ECx values that were determined by extrapolation beyond tested concentrations are shown in italics. In the case of the growth rate, the EC50 value for propiconazole and thereby predicted values for the mixture are based on extrapolation

Fig. 12: Concentration-response plots of the two eluates prepared (by painting or dipping) from product C for the two response variables yield (left) and average specific growth rate (right) in the algal growth inhibition tests




Shown are the mean values at each concentration level after 72 h exposure together with the 3-parameter log-logistic fit. The analysis was based on nominal proportions of the eluates in the test medium

In both eluates of product C, the concentration of propiconazole re-calculated from measured test concentrations was about factor 2 higher than those determined shortly after preparation. The concentrations of fenoxycarb and dimethylalkylamine re-calculated from test concentrations, on the other hand, were about similar to previous measurements. Propiconazole was stable during the 3-days exposure period of the tests with eluates from product C, while only about 15 % to 20 % of fenoxycarb and dimethylalkylamine were observed after 3 days (Table 20). For both eluates of product C, clear and complete concentration-response curves were obtained (Figure 12) that allowed a reliable estimation of effect concentrations (Table 23 and 24). All MDR values calculated based on $EC_x$ values indicated for both response variables a compliance between CA prediction and observed toxicity with less than factor 2 deviation. Even for growth rate, based on an extrapolated $EC_{50}$ of propiconazole, the deviation was within this range. Only the MDR based on NOEC values indicated a more than twofold deviation between prediction and observation, with the prediction overestimating the observable toxicity by factor 3.7. However, compliance between prediction and observation

was only found when the dimethylalkylamine was included in the calculations. Without consideration of this formulation additive, the toxicity of the product and the eluates was underestimated by at least factor 10 up to more than factor 1000.

Table 23: Predicted and observed $EC_{50}$ values estimated of the painting eluate prepared from product C tested for algal growth inhibition and resulting MDR values

| Estimates | Yield | Growth Rate |
|---|---|---|
| Ahead predicted $EC_{50}$ (ml eluate/l test medium) | 1.60 (316) | *3.86 (5244)* |
| Observed $EC_{50}$ (ml eluate/l test medium) with [CI] | 0.90 [0.72-1.08] | 2.06 [1.90-2.22] |
| Observed $EC_{50}$ (mg sum substances/l) | 0.0155 (0.005) | 0.0354 (0.011) |
| Revised predicted $EC_{50}$ (mg sum substances/l) | 0.0240 (1.02) | *0.0580 (18.9)* |
| MDR based on ahead prediction ($EC_{50}$) | 1.77 (350) | 1.87 (2543) |
| MDR ($EC_{50}$) | 1.55 (213) | 1.64 (1736) |
| Observed $EC_{20}$ (ml eluate/l test medium) with [CI] | 0.52 [0.34-0.69] | 1.22 [1.06-1.38] |
| Observed $EC_{20}$ (mg sum substances/l) | 0.0089 (0.0027) | 0.0209 (0.0065) |
| Revised predicted $EC_{20}$ (mg sum substances/l) | 0.0091 (0.221) | 0.0221 (3.308) |
| MDR ($EC_{20}$) | 1.02 (80.6) | 1.06 (512) |
| Observed $EC_{10}$ (ml eluate/l test medium) with [CI] | 0.37 [0.21-0.54] | 0.90 [0.74-1.06] |
| Observed $EC_{10}$ (mg sum substances/l) | 0.0064 (0.0020) | 0.0154 (0.0048) |
| Revised predicted $EC_{10}$ (mg sum substances/l) | 0.0063 (0.0805) | 0.0157 (1.0095) |
| MDR ($EC_{10}$) | 0.99 (40.6) | 1.02 (213) |
| Observed NOEC (ml eluate/l test medium) | 0.313 | 0.313 |
| Observed NOEC (mg sum substances/l) | 0.0054 (0.0017) | 0.0054 (0.0017) |
| Revised predicted NOEC (mg sum substances/l) | 0.0014 (0.1435) | 0.0014 (0.1435) |
| MDR (NOEC) | 0.27 (84.4) | 0.27 (84.4) |

Given are the observed ECx values with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model based on the proportion of the eluate in the test medium. The ahead predicted EC50 values are based on the concentrations of the test substances measured in the eluates ahead of the test (i.e., shortly after preparation), while the revised EC50 values are based on the concentrations measured in the tests. MDR values based on ahead measured concentrations are only reported for EC50 values. All ECx values that were determined by extrapolation beyond tested concentrations are shown in italics. In the case of the growth rate, the EC50 value for propiconazole and thereby predicted values for the mixture are based on extrapolation. Values in brackets () were derived without consideration of dimethylalkylamine

Table 24: Predicted and observed $EC_{50}$ values estimated of the dipping eluate prepared from product C tested for algal growth inhibition and resulting MDR values

| Estimates | Yield | Growth Rate |
|---|---|---|
| Ahead predicted $EC_{50}$ (ml eluate/l test medium) | 0.81 (154.0) | *1.97 (2550)* |
| Observed $EC_{50}$ (ml eluate/l test medium) with [CI] | 0.64 [0.56-0.71] | 1.35 [1.23-1.46] |
| Observed $EC_{50}$ (mg sum substances/l) | 0.0222 (0.009) | 0.0468 (0.018) |
| Revised predicted $EC_{50}$ (mg sum substances/l) | 0.0269 (1.018) | *0.0652 (19.87)* |
| MDR based on ahead prediction ($EC_{50}$) | 1.28 (242) | 1.46 (1894) |
| MDR ($EC_{50}$) | 1.21 (120) | 1.39 (1104) |
| Observed $EC_{20}$ (ml eluate/l test medium) with [CI] | 0.42 [0.33-0.51] | 0.84 [0.72-0.96] |
| Observed $EC_{20}$ (mg sum substances/l) | 0.0146 (0.0056) | 0.0292 (0.0112) |
| Revised predicted $EC_{20}$ (mg sum substances/l) | 0.0102 (0.2206) | 0.0248 (3.3301) |
| MDR ($EC_{20}$) | 0.70 (39.3) | 0.85 (297) |
| Observed $EC_{10}$ (ml eluate/l test medium) with [CI] | 0.33 [0.23-0.42] | 0.64 [0.51-0.76] |
| Observed $EC_{10}$ (mg sum substances/l) | 0.0114 (0.0044) | 0.0222 (0.0085) |
| Revised predicted $EC_{10}$ (mg sum substances/l) | 0.0071 (0.0803) | 0.0176 (1.0097) |
| MDR ($EC_{10}$) | 0.62 (18.3) | 0.79 (119) |
| Observed NOEC (ml eluate/l test medium) | 0.313 | 0.313 |
| Observed NOEC (mg sum substances/l) | 0.0109 (0.0021) | 0.0109 (0.0021) |
| Revised predicted NOEC (mg sum substances/l) | 0.0489 (0.1433) | 0.0489 (0.1433) |
| MDR (NOEC) | 0.15 (68.2) | 0.15 (68.2) |

Given are the observed ECx values with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model based on the proportion of the eluate in the test medium. The ahead predicted EC50 values are based on the concentrations of the test substances measured in the eluates ahead of the test (i.e., shortly after preparation), while the revised EC50 values are based on the concentrations measured in the tests. MDR values based on ahead measured concentrations are only reported for EC50 values. All ECx values that were determined by extrapolation beyond tested concentrations are shown in italics. In the case of the growth rate, the EC50 value for propiconazole and thereby predicted values for the mixture are based on extrapolation. Values in brackets () were derived without consideration of dimethylalkylamine

## 5.3 *Daphnia* Acute Immobilisation Tests

All 15 *Daphnia* acute immobilisation tests were valid based on the criteria given in the OECD guideline 202 (Table 25). The results of the 15 tests and those of the accompanying chemical analyses will be reported in the following.

Table 25: Oxygen content and pH determined in the control and the highest test concentration in each of the in total 15 *Daphnia* acute toxicity tests

| Test substance | Oxygen at start (mg/l) | | Oxygen at end (mg/l) | | pH at start | | pH at end | | Mortality in control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Highest test concentration | Control | Highest test concentration | Control | Highest test concentration | Control | Highest test concentration | Blank | Solvent |
| *Validity criterion* | | | *≥ 3* | *≥ 3* | | | | | *≤ 10%* | *≤ 10%* |
| Fenoxycarb | 9.4 | 9.1 | 8.9 | 9.2 | 7.7 | 7.7 | 7.7 | 7.8 | 0.0% | 0.0% |
| Propiconazole | 9.4 | 8.9 | 9.2 | 8.7 | 7.6 | 7.6 | 7.8 | 7.7 | 0.0% | – |
| IPBC | 9.6 | 9.0 | 9.3 | 9.3 | 7.9 | 7.8 | 7.9 | 7.8 | 0.0% | – |
| Dimethylalkylamine | 9.5 | 9.3 | 9.3 | 9.4 | 7.8 | 7.9 | 7.8 | 7.9 | 10.0% | – |
| Product C – Eluate painting | 9.3 | 9.3 | 9.0 | 8.7 | 7.7 | 7.8 | 7.8 | 7.7 | 0.0% | – |
| Product C – Eluate dipping | 9.3 | 9.3 | 8.8 | 9.0 | 7.8 | 7.9 | 7.7 | 7.8 | 0.0% | – |
| Product B – Eluate | 9.6 | 7.8 | 9.7 | 6.1 | 7.7 | 6.8 | 7.8 | 7.1 | 0.0% | – |
| Product A – Eluate | 9.6 | 8.9 | 9.6 | 6.3 | 7.8 | 7.1 | 7.8 | 7.2 | 0.0% | – |
| Product A – Eluate | 8.8 | 8.4 | 9.3 | 5.4 | 8.1 | 7.5 | 7.9 | 7.2 | 0.0% | – |
| Product C | 9.2 | 9.0 | 9.1 | 9.0 | 7.9 | 7.9 | 7.8 | 7.9 | 0.0% | – |
| Product A | 9.1 | 7.7 | 9.3 | 9.2 | 7.8 | 7.8 | 7.8 | 7.9 | 0.0% | – |
| Product B | 8.9 | 7.1 | 8.6 | 9.0 | 7.9 | 7.5 | 7.6 | 7.5 | 0.0% | 0.0% |
| Generic Mix: IPBC / propiconazole 1 | 8.9 | 8.4 | 9.1 | 8.9 | 7.9 | 7.9 | 7.7 | 7.8 | 0.0% | – |
| Generic Mix: IPBC / propiconazole 2 | 8.3 | 8.4 | 9.6 | 8.9 | 7.7 | 7.8 | 7.7 | 7.8 | 0.0% | – |
| Generic Mix: fenoxycarb / propiconazole | 8.6 | 8.0 | 8.7 | 8.2 | 7.7 | 7.8 | 7.7 | 7.7 | 0.0% | 0.0% |

In addition, immobilisation ("mortality") in the control treatments within 48 h is reported. The validity criteria specified in the OECD guideline 202 are not more than 10 % immobilisation in the control and more than 3 mg/l oxygen at the end of the test in all treatments. Shown here are only the control and the highest treatments, which did not differ from the intermediate treatments

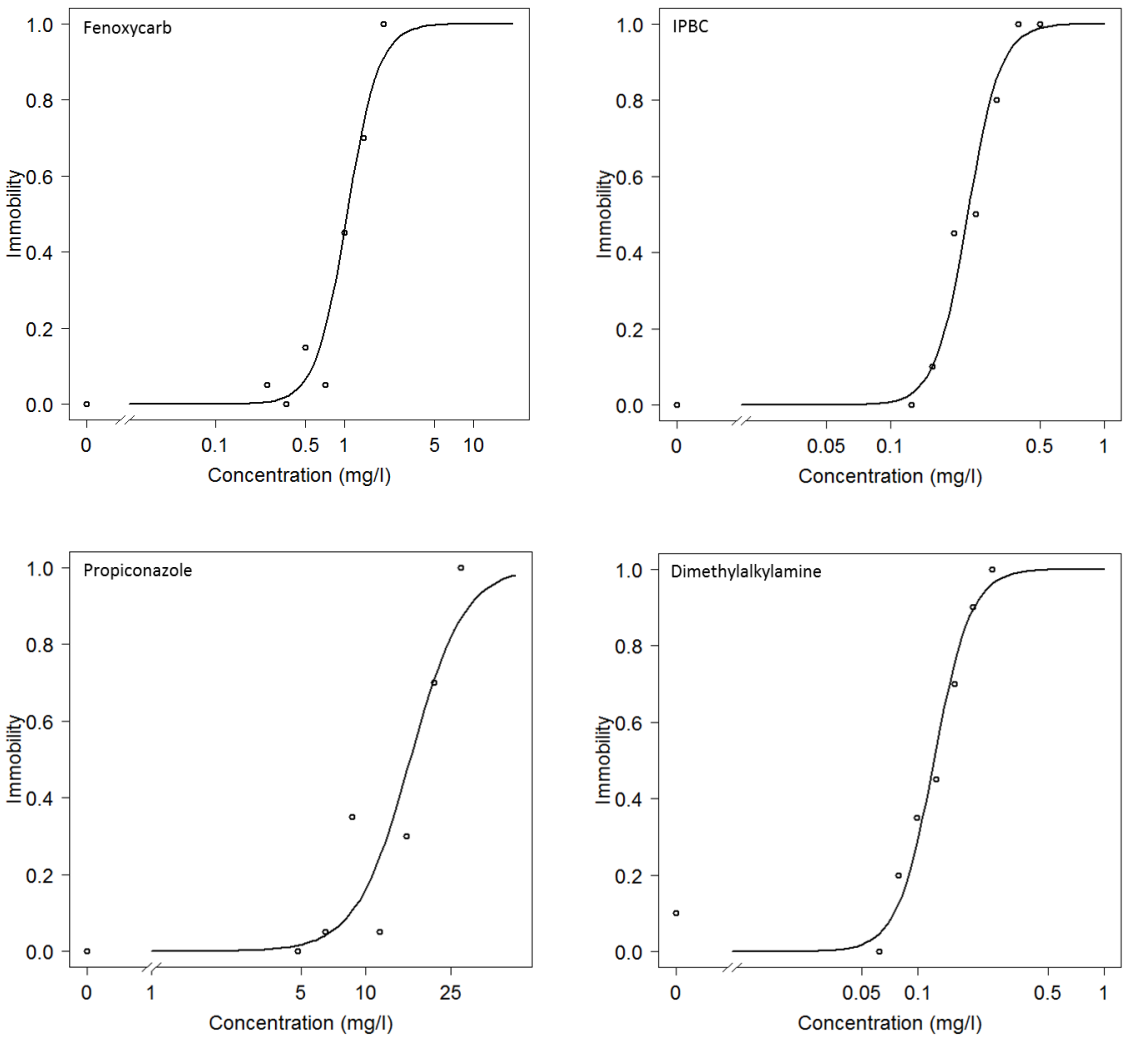
### 5.3.1 Single Substances

The results of the acute immobilisation tests with the four single substances are shown in Figure 13. For all four substances, a clear concentration-response curve was obtained with intermediate responses at several of the seven concentration levels. The estimated $EC_{50}$ values are therefore reliable with tight confidence intervals (Table 26).

The average measured initial concentration of all four single substances was between 80 % and 100 % of the nominal concentration. Hence, the toxicity evaluation can be based on the nominal concentrations according to the respective test guideline. Only in the case of dimethylalkylamine did the loss within the test (2 day exposure) exceed 20 % of the initial concentration. For all three active substances, the $EC_{50}$ value determined in the present study deviated by less than factor 2 from the $EC_{50}$ value used in the regulatory assessment of the EU. The $EC_{50}$ value determined for the formulation additive dimethylalkylamine was below the censored value of <1 mg/l given on the MSDS.

Dimethylalkylamine and IPBC were the most toxic of the four substances in the *Daphnia* acute toxicity test. Fenoxycarb was about ten times less toxic, followed by propiconazole with a difference of about another factor of 10.

Fig. 13: Concentration-response plots for the four tested single substances in the *Daphnia* acute immobilisation test




Shown are the mean proportions of immobile *D. magna* after 48 h at each concentration level together with the 2-parameter log-logistic fit. The analysis was based on nominal concentrations

Table 26: Toxicity and recovery of the single substances in the *Daphnia* acute immobilisation tests

| Substance | Nominal $EC_{50}$ (mg/l) with [CI] | Parameter b (SE) | Recovery (%) | Corrected $EC_{50}$ (mg/l) | Loss within test (%) | Deviation from regulatory $EC_{50}$ |
|---|---|---|---|---|---|---|
| Fenoxycarb | 1.05 [0.90-1.20] | -3.57 (0.59) | 99.0 | 1.05 | 13.6 | 1.75 |
| IPBC | 0.23 [0.21-0.25] | -5.72 (0.90) | 81.0 | 0.19 | -12.2 | 1.18 |
| Propiconazole | 16.18 [13.98-18.39] | -3.43 (0.57) | 87.3 | 14.12 | 16.7 | 1.39 |
| Dimethylalkylamine | 0.12 [0.11-0.13] | -4.44 (0.69) | 80.4 | 0.10 | 33.3 | n.a. |

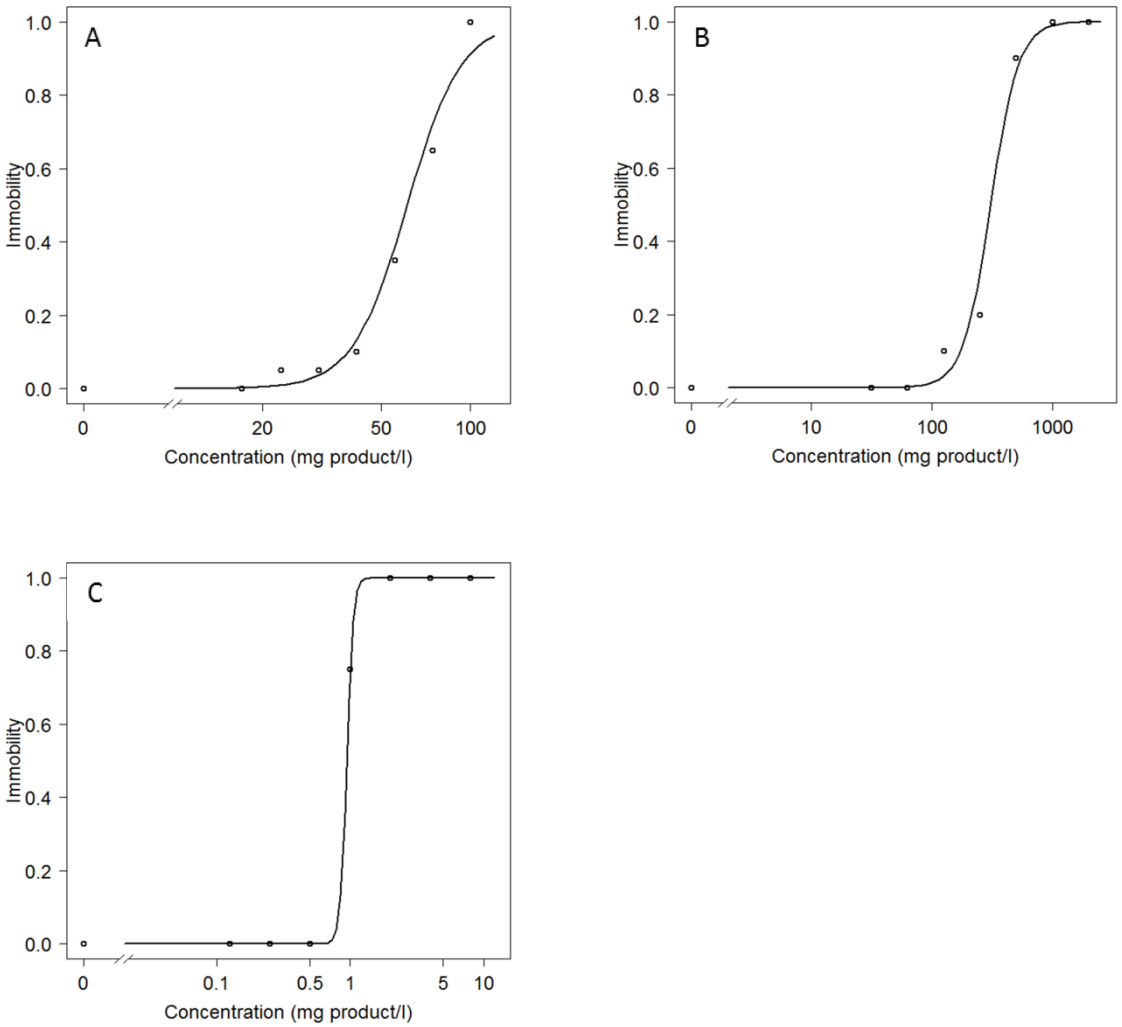
Given are the EC50 values, their 95 % confidence intervals (CI) and the parameter b (standard error, SE) estimated from the fitted 2-parameter log-logistic model. In addition, the mean recovery of each substance is provided (measured initial concentration in percentage of the nominal concentration) and the value for the EC50 after correcting for this recovery. The percentage loss within the test is the difference of the measured concentration at day 0 and 2 in relation to the initial concentration. The last column indicates the fold deviation of the corrected observed EC50 from the EC50 used in the regulatory risk assessment. n.d.: not determined

### 5.3.2 Biocidal Products

Clear concentration-response curves were obtained for all three products in the acute *Daphnia* test (Figure 14). Related to the concentration of the formulated product in the test, the three products differed considerably with product C being the most toxic. This is due to the fact that product C has the highest content of active substances (0.1254 mg sum a.s./mg product) compared to product A (0.0115 mg sum a.s./mg product) and product B (0.00955 mg sum a.s./mg product). Additionally, product C contains the formulation additive dimethylalkylamine, which is more toxic than any of the three active substances.

The concentrations of propiconazole measured in the test with product A at day 0 differed only slightly from the nominal concentrations (Table 27), while those of IPBC indicated less than 1 % recovery. In the test of the WAF of product B, the recovery of propiconazole was about twice as high as the recovery of fenoxycarb. Both were well below 80 % and thereby indicate only partial transfer of the active substances from the solvent-based product to the water phase. The initial measured concentrations in the test with product C exceeded for all three substances 100 % of the nominal concentrations and slightly differed among the three substances. In all three product tests, the loss of active substances over the test duration of two days was negligible with less than 20 % as indicated by the about similar concentrations measured at day 0 and day 2 at the highest concentration level. Only for the amine, a higher loss of about 30 % was observed.

Fig. 14: Concentration-response plots for the three tested wood preservative products (A, B, and C) in the *Daphnia* acute immobilisation test




Shown are the mean proportions of immobile *D. magna* after 48 h at each concentration level together with the 2-parameter log-logistic fit. The analysis was based on nominal concentrations of the product in the test medium. Product B was tested as water accommodated fraction; the nominal concentrations relate to 100 % transfer of the product to the water phase

Table 27: Nominal and measured concentrations of the test substances in the acute *Daphnia* toxicity tests with the three products

| Product | Substance | Nominal test concentration (mg/l) | Measured test concentration (mg/l) day 0 / day 2 | Recovery (%) | Loss within test (%) |
|---|---|---|---|---|---|
| A | propiconazole | 0.048<br>0.116<br>0.280 | 0.051<br>0.100<br>0.210 / 0.200 | 106.2<br>86.2<br>75.0 | n.d.<br>n.d.<br>4.8 |
| | IPBC | 0.149<br>0.360<br>0.870 | < 0.0005<br>0.00072<br>0.0016 / 0.0015 | n.d.<br>0.2<br>0.2 | n.d.<br>n.d.<br>6.3 |
| B | propiconazole | 0.297<br>2.375<br>19.000 | 0.144<br>1.060<br>8.100 / 6.65 | 48.5<br>44.6<br>42.6 | n.d.<br>n.d.<br>17.9 |
| | fenoxycarb | 0.002<br>0.013<br>0.100 | 0.00033<br>0.0028<br>0.0140 / 0.016 | 21.1<br>22.4<br>14.0 | n.d.<br>n.d.<br>-14.3 |
| C | propiconazole | 0.003<br>0.025<br>0.200 | 0.005<br>0.037<br>0.298 / 0.277 | 147.2<br>148.0<br>149.0 | n.d.<br>n.d.<br>7.0 |
| | fenoxycarb | 0.00005<br>0.0004<br>0.0032 | 0.000081<br>0.00065<br>< 0.005 / <0.005 | 162.0<br>162.5<br>n.d. | n.d.<br>n.d.<br>n.d. |
| | dimethylalkylamine | 0.0125<br>0.1000<br>0.8000 | 0.014<br>0.127<br>0.995 / 0.695 | 112.0<br>127.0<br>124.4 | n.d.<br>n.d.<br>30.2 |

Given are the nominal concentrations of the test substances at three of the concentration levels in each of the three product tests, the concentrations in the respective concentration levels as determined by analytical measurements of samples at day 0 (initial concentration) and day 2 (aged test solution), and the resulting recovery as the measured initial concentration in percentage of the nominal concentration. The percentage loss within the test is the difference of the measured concentration at day 0 and 2 in relation to the initial concentration. n.d.: not determined

The comparison of the nominal observed $EC_{50}$ values for the products as estimated from the concentration-response curves (Figure 13) with the predicted values based on the nominal test concentrations resulted in the nominal MDR values (Table 28). In the case of product A, the nominal MDR of 0.43 indicated an overestimation of toxicity by factor 2.3 by the CA prediction. A correction for the measured concentrations of the test substances resulted in a corrected MDR of 61.7, which indicated a considerable underestimation of toxicity. This difference introduced by the correction is due to the very low recovery of IPBC, which is at the same time the more toxic active substance of the two. The nominal MDR below 1 demonstrates that the underestimation of the toxicity of product A suggested by the corrected MDR would be covered by an assessment based on nominal concentrations.

Table 28: Predicted and observed $EC_{50}$ values estimated in the three wood preservative products tested for acute *Daphnia* toxicity

| Estimates | Product A | Product B | Product C |
|---|---|---|---|
| Nominal predicted $EC_{50}$ (mg product/l) | 26.3 | 1387.9 | 0.978 (94.1) |
| Nominal observed $EC_{50}$ (mg product/l) and [CI] | 61.2 [54.4-68.1] | 307.3 [249.4-365.1] | 0.941 [0.37-1.52] |
| Mean recovery (%) | 89.2 (propiconazole) 0.2 (IPBC) | 45.2 (propiconazole) 19.2 (fenoxycarb) | 148.1 (propiconazole) 162.3 (fenoxycarb) 121.1 (amine) |
| Corrected observed $EC_{50}$ (mg sum substances/l) | 0.154 | 1.324 | 0.149 (0.035) |
| Corrected predicted $EC_{50}$ (mg sum substances/l) | 9.495 | 13.738 | 0.128 (11.62) |
| Nominal MDR ($EC_{50}$) | 0.43 | 4.5 | 1.04 (100) |
| Corrected MDR ($EC_{50}$) | 61.7 | 10.4 | 0.86 (328) |

Given are the nominal observed EC50 values for each product with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model. In addition, the mean recovery of each substance is provided (measured initial concentration in percentage of the nominal concentration) and the value for the EC50 after correcting for this recovery. The predicted EC50 values are calculated based on the corrected EC50 values of the two or three (product C) individual substances and nominal or corrected substance concentrations in the test, respectively. Values in brackets () were derived without consideration of dimethylalkylamine

Since very little biologically active IPBC was present in the test medium, other factors such as synergistic interaction or the presence of non-considered toxic substances must have accounted for the expected toxicity of IPBC. Yet, neither PBC nor cobalt has been determined in the *Daphnia* acute toxicity test with product A.

But in order to verify PBC and cobalt concentrations of product A when dissolved in *Daphnia* medium, a high concentration (100 mg product/l *Daphnia* test medium) was subjected to chemical analysis after ultrasonication treatment and stirring overnight. Nominal, measured and re-calculated concentrations of the analytes are shown in Table 29. The concentrations re-calculated for product A from this measurement were in close agreement with those determined when dissolving product A in algae growth medium (Table 16). Cobalt was contained at a relatively low concentration of 15 µg/l, which is about factor 100 below the reported $EC_{50}$ of 1.67 mg/l (Khangarot & Das 2009) for this substance, and it transfers into TU of < 0.00001 at the observed $EC_{50}$ of product A. Hence, cobalt hardly contributed to the overall acute toxicity of product A towards *Daphnia*. The metabolite PBC was detected at higher concentrations than the parent IPBC, indicating relevant transformation. Less than 1 % of the nominal IPBC concentration was determined, which confirmed the previous analytical results in the *Daphnia* test with the product. Based on the re-calculated concentration of 3.4 mg PBC/g product A, the concentration of PBC at the observed $EC_{50}$ of product A was 0.208 mg/l, which is equal to 0.377 mg IPBC/l assuming a 1:1 stoichiometric transformation of IPBC (281.1 g/mol) to PBC (155.2 g/mol). This IPBC concentration transfers to 1.98 TU at the observed $EC_{50}$ of product A. This indicates that PBC considerably contributed to the overall toxicity, but also that it is less toxic than the parent compound because assuming similar toxicity results in a slight overestimation of toxicity (TU of 1.98 alone for PBC). The relative controbution of cobalt and

PBC to the overall toxicity of product A (and its eluate) will be addressed in more detail in the discussion.

Table 29: Nominal, measured and re-calculated concentrations of substances in product A dissolved at 100 mg/l in the *Daphnia* test medium

| Substance | Nominal concentration (mg/l) | Measured concentration (mg/l) | Re-calculated concentration in product A (mg/g) |
|---|---|---|---|
| propiconazole | 0.280 | 0.220 | 2.20 |
| IPBC | 0.870 | 0.001 | 0.01 |
| PBC | unknown | 0.34 | 3.4 |
| cobalt | unknown | 0.015 | 0.15 |

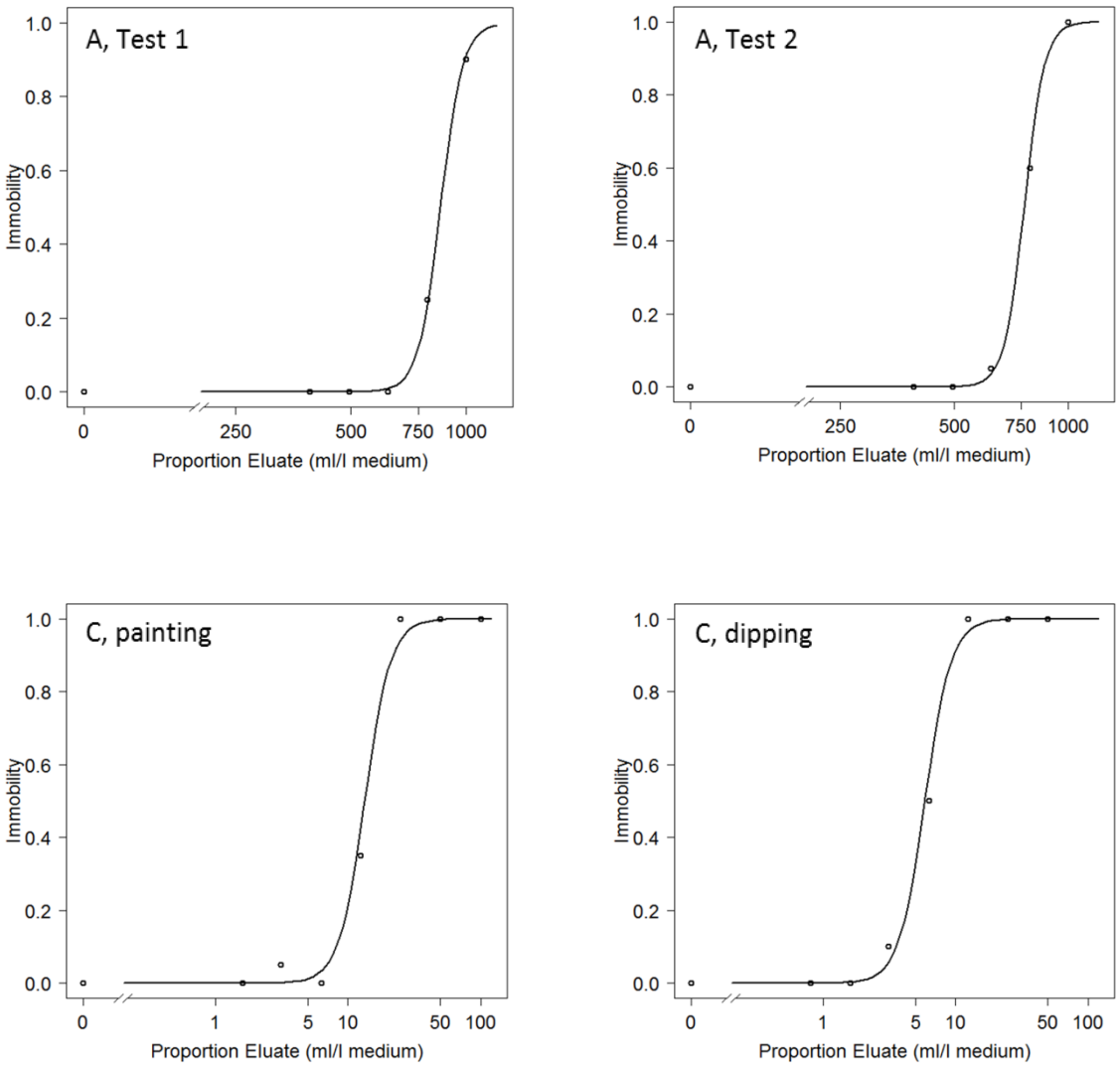
In the case of product B, the nominal MDR indicated an underestimation of product toxicity by the CA prediction. Underestimation of product toxicity based on nominal concentrations provides evidence for synergistic interactions and/or the presence of toxic formulation additives, but no evidence for the relevance of metabolites as it was found in the case of product A. The corrected MDR indicated an even larger degree of underestimation of product toxicity, since for both active substances less than 50 % of the nominal concentration was measured in the test medium. Possible reasons for this finding will be discussed later.

For product C, both the nominal and the corrected MDR indicated a good agreement (less than factor 2 deviation) between observed and predicted toxicity. This was only true if the formulation additive dimethylalkylamine was included in the prediction (as done in Table 28). When excluding the additive, a nominal MDR of 100 and a corrected MDR of 328 indicated a considerable underestimation of toxicity by the CA prediction based only on active substances.

### 5.3.3 Eluates

The eluate of product A was tested in two independent tests (Figure 15). The second test confirmed the unexpected result of the first test: a clear concentration-response curve with about 100 % mortality in the undiluted eluate. Also the measured concentrations of propiconazole and IPBC were about similar in the two tests (Table 30).

Fig. 15: Concentration-response plots for the eluates of the wood preservative products in the *Daphnia* acute immobilisation test




The eluate of product A was tested twice, the eluate of product B did not induce toxicity (data not shown), and from product C two different eluates were tested. Shown are the mean proportions of immobile D. magna after 48 h at each concentration level together with the 2-parameter log-logistic fit. The analysis was based on the volume of the eluate in the test medium

Table 30: The concentrations of the test substances in the four eluates of the three wood preservative products as measured in the *Daphnia* acute toxicity tests

| Eluate | Substance | Concentration in diluted eluate (mg/l) day 0 / day 2 | Re-calculated concentration in eluate (mg/l) | Loss within test (%) |
|---|---|---|---|---|
| A, painting, test 1 | propiconazole | n.d. | 0.80 / 0.72 | 10.0 |
| | IPBC | n.d. | < 0.001 | n.. |
| A, painting, test 2 | propiconazole | n.d. | 1.08 / n.d. | n.d. |
| | IPBC | n.d. | 0.0055 / n.d. | n.d. |
| B, painting | propiconazole | n.d. | 3.5 / 3.0 | 14.3 |
| | fenoxycarb | n.d. | < 0.001 / < 0.001 | n.d. |
| C, painting | propiconazole | 0.0096<br>0.065<br>0.45 / 0.31 | 6.1<br>5.2<br>4.5 / 3.1 | 31.1 |
| | fenoxycarb | < 0.001 | <0.01 | n.d. |
| | dimethylalkylamine | 0.029<br>0.19<br>1.6 / 1.2 | 18.6<br>15.2<br>16.0 / 12.0 | 25.0 |
| C, dipping | propiconazole | 0.0099<br>0.065<br>0.60 / 0.54 | 12.7<br>10.4<br>12.0 | 10.0 |
| | fenoxycarb | < 0.001 | < 0.02 | n.d. |
| | dimethylalkylamine | 0.03<br>0.19<br>1.4 / 1.0 | 38.4<br>30.4<br>28.0 | 28.6 |

Given are the concentrations of the substances in at least one concentration level in each of the four eluates as determined by analytical measurements of samples at day 0 (initial concentration) and day 2 (aged test solution), and the measured or from the diluted eluates re-calculated concentrations in the undiluted eluates. The percentage loss within the test is the difference of the measured concentration at day 0 and 2 in relation to the initial concentration

The propiconazole concentration in the eluate of product A was twice as high in the test compared to the undiluted eluate measured shortly after preparation (Table 8), whereas the concentration of IPBC was similar in all measurements, but around or below the limit of quantification. According to the CA prediction based on the concentrations of the test substances in the eluate measured shortly after preparation, the eluate would have had to be enriched by about factor 17 to produce 50 % mortality in the acute *Daphnia* test (Table 31). Given the observed toxicity, this resulted in MDR values of 20.2 and 22.7, respectively, in the two tests. This considerable underestimation of toxicity was only slightly reduced when

correcting for the actual measured concentrations of the test substances in the eluate test (MDR of 18.8 and 12.4, respectively). Hence, based on CA prediction and measured concentrations of the two active substances, the acute *Daphnia* toxicity of the eluate of product A was underestimated by more than factor 10. Neither cobalt nor PBC were measured in these tests, but their potential contribution to explaining these deviations will be discussed later.

Table 31: Predicted and observed EC50 values estimated of the four eluates tested for *Daphnia* acute toxicity

| Estimates | A painting | B painting | C painting | C dipping |
|---|---|---|---|---|
| Ahead predicted $EC_{50}$ (ml eluate/l test medium) | 17,348.0 | 7,004.1 | 9.4 (3082) | 4.8 (1500) |
| Observed $EC_{50}$ (ml eluate/l test medium) with [CI] | 856.8 [807.9-905.7] 765.8 [726.6-805.1 | > 1,000.0 | 13.4 [11.1-15.6] | 5.87 [4.87-6.88] |
| Observed $EC_{50}$ (mg sum substances/l) | 0.686 / 0.831 | > 3.5 | 0.293 (0.071) | 0.258 (0.069) |
| Revised predicted $EC_{50}$ (mg sum substances/l) | 12.93 / 10.29 | 14.1 | 0.129 (13.8) | 0.133 (13.8) |
| MDR based on ahead prediction ($EC_{50}$) | 20.2 / 22.7 | < 7.0 | 0.70 (230) | 0.82 (255) |
| MDR ($EC_{50}$) | 18.8 / 12.4 | < 4.0 | 0.44 (195) | 0.52 (201) |

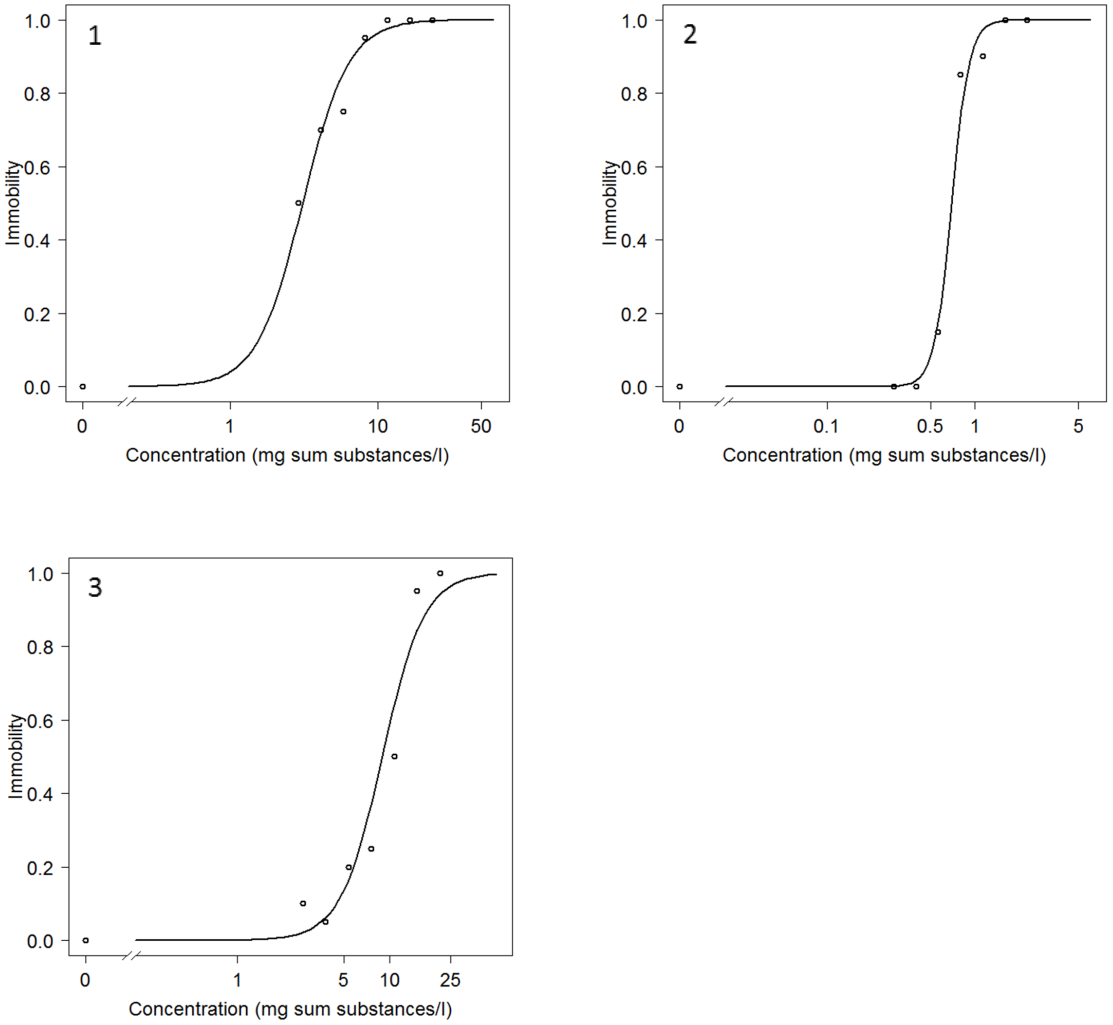
Given are the observed EC50 values with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model of each eluate. The ahead predicted EC50 values are based on the concentrations of the test substances measured in the eluates shortly after preparation, while the corrected EC50 values are based on the concentrations measured in the tests. The eluate of product A was tested in two independent tests. Values in brackets () for eluates of product C refer to calculations not including the formulation additive dimethylalkylamine

The eluate of product B induced no relevant acute toxicity towards *Daphnia*; only one out of 20 animals died in the undiluted eluate (data not shown). Again, propiconazole concentrations were determined to be higher in the tested undiluted eluate than measured ahead of the test, i.e. shortly after preparation (Table 8). In contrast, fenoxycarb was below the limit of quantification in the test and thereby present at a lower concentration in the test compared to the previous measurement. Based on the censored data for the observed toxicity ($EC_{50}$ below 1000 ml eluate/l test medium), a potential underestimation of toxicity was below factor 10 (Table 31). This finding was independent of which measured concentration was used for the prediction.

Both eluates of product C caused concentration-dependent mortality in the *Daphnia* test (Figure 15). Fenoxycarb was below detection limit in both tests, while the concentrations of propiconazole and dimethylalkylamine in the eluate were determined to be higher based on measurements of test media than measured previously (Table 8, Table 30). The deviation between predicted and observed toxicity was below factor 2 for both eluates when based on eluate concentrations measured ahead of the test. The correction of the MDR based on the concentrations measured in the test media resulted in both cases in slightly lower MDR values, but still less than factor 3 overestimation of toxicity by the prediction. Consideration of dimethylalkylamine was crucial for correctly predicting the toxicity of both eluates. Without consideration of the additive, the toxicity was underestimated by more than factor 100.

### 5.3.4 Generic Mixtures

Fig. 16: Concentration-response plots for the eluates of the wood preservative products in the *Daphnia* acute immobilisation test




Shown are the mean proportions of immobile *D. magna* after 48 h at each concentration level together with the 2-parameter log-logistic fit. The analysis was based on the nominal concentration relating to the sum of active substances. Mixtures 1 and 2 represent mixtures of IPBC and propiconazole at different relative proportions (see Table 32), while mixture 3 represents a mixture of fenoxycarb and propiconazole

No deviations between toxicity prediction and observation beyond a threshold of factor 2-3 were observed for product C and the eluates of product C. Therefore, no generic mixtures were tested for the equivalent substance mixtures as no proof of agreement with CA predictability was considered necessary for this combination.

Table 32: Concentrations of the test substances in the *Daphnia* acute toxicity tests of the generic mixtures

| Mixture | Substance (relative nominal proportion) | Nominal test concentration (mg/l) | Measured test concentration (mg/l) day 0 / day 2 | Recovery (%) | Loss within test (%) |
|---|---|---|---|---|---|
| 1 | propiconazole (0.986) | 2.86<br>8.09<br>22.88 | n.d. | n.d. | n.d. |
| | IPBC (0.014) | 0.04<br>0.12<br>0.33 | n.d. | n.d. | n.d. |
| 2 | propiconazole (0.763) | 0.215<br>0.608<br>1.720 | 0.21 / n.d.<br>0.53 / n.d.<br>1.70 / 1.50 | 97.7<br>87.2<br>98.8 | n.d.<br>n.d.<br>11.8 |
| | IPBC (0.237) | 0.067<br>0.189<br>0.535 | 0.075 / n.d.<br>0.20 / n.d.<br>0.55 / 0.48 | 112.2<br>105.7<br>102.8 | n.d.<br>n.d.<br>12.7 |
| | PBC (none) | 0<br>0<br>0 | 0.0011 / n.d.<br>0.002 / n.d.<br>0.011 / 0.035 | n.d. | n.d.<br>n.d.<br>-21.8 |
| 3 | propiconazole (0.931) | 2.50<br>7.06<br>20.0 | 2.2 / n.d.<br>6.7 / n.d.<br>17.0 / 15.0 | 88.1<br>94.9<br>85.1 | n.d.<br>n.d.<br>11.8 |
| | fenoxycarb (0.069) | 0.186<br>0.525<br>1.484 | 0.19 / n.d.<br>0.46 / n.d.<br>0.87 / 0.89 | 102.4<br>87.7<br>58.6 | n.d.<br>n.d.<br>-2.3 |

Given are the concentrations of the substances in at least one concentration level in each of the three generic mixtures as determined by analytical measurements of samples at day 0 (initial concentration) and day 2 (aged test solution). The percentage loss within the test is the difference of the measured concentration at day 0 and 2 in relation to the initial concentration

Since the acute *Daphnia* toxicity of products A and B or that of their eluates appeared to deviate from the CA prediction, generic mixtures of IPBC and propiconazole (mixture 1 and 2) as well as fenoxycarb and propiconazole (mixture 3) were tested. All three mixtures provided clear concentration response curves (Figure 16, Table 32). The recovery of all test substances was between 80 % and 120 % with fenoxycarb at the highest test concentration in mixture 3 being the only exception (Table 32). The PBC concentration increased during the test, which may indicate transformation of IPBC to PBC during the test. However, the related loss of IBPC accounted for only about of the increase.

MDR values determined for mixture 2 and 3 indicated very good agreement between prediction and observation and thereby concentration additive behaviour of binary mixtures of propiconazole and IPBC as well as propiconazole and fenoxycarb (Table 33). The nominal MDR for mixture 1 is slightly above 2. Unfortunately, no analytical measurement could be conducted for this mixture due to accidental loss of samples that may verify if this deviation is simply due to a lower recovery of the test substance in this test.

Overall, the results for the generic mixtures demonstrated that the acute Daphnia toxicity of mixtures of these a.s. can be predicted by the CA within a range of about factor 2 deviation.

Table 33: Predicted and observed EC50 values estimated of the four eluates tested for *Daphnia* acute toxicity

| Estimates | Mixture 1 propiconazole & IPBC | Mixture 2 propiconazole & IPBC | Mixture 3 propiconazole & fenoxycarb |
|---|---|---|---|
| Nominal predicted $EC_{50}$ (mg sum substances/l) | 6.96 | 0.768 | 7.58 |
| Nominal observed $EC_{50}$ (mg sum substances) with [CI] | 3.09 [2.34-3.84] | 0.694 [0.627-0.761] | 8.92 [7.59-12.25] |
| Mean recovery (%) | n.d. | 94.6 (propiconazole) 106.9 (IPBC) | 89.4 (propiconazole) 82.9 (fenoxycarb) |
| Corrected observed $EC_{50}$ (mg sum substances/l) | n.d. | 0.677 | 7.94 |
| Corrected predicted $EC_{50}$ (mg sum substances/l) | n.d. | 0.703 | 7.83 |
| Nominal MDR | 2.25 | 1.11 | 0.85 |
| Corrected MDR | n.d. | 1.04 | 0.99 |

Given are the observed EC50 values with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model of each eluate. The ahead predicted EC50 values are based on the concentrations of the test substances measured in the eluates shortly after preparation, while the corrected EC50 values are based on the concentrations measured in the tests. The eluate of product A was tested in two independent tests. Values in brackets () for eluates of product C refer to calculations not including the formulation additive dimethylalkylamine

## 5.4 *Daphnia* Reproduction Tests

As described in the Material & Methods section, four *Daphnia* reproduction tests were conducted at a low food level and another four at a high food level. The four low-food tests did not meet the validity criterion of the production of at least 60 juveniles per surviving female within 21 days as stated in the OECD guideline 211 (Table 34). The four tests conducted at a high food level met this validity criterion, although in some cases only just. With regard to the validity criterion of at most 10 % mortality of test animals, all eight tests were valid.

Table 34: Cumulative number of offspring per surviving female and mortality of control animals (blank or solvent controls) in the eight *Daphnia* reproduction tests together with the validity criteria stated in OECD guideline 211

| Test substance | Cumulative offspring per surviving female in 21 days | | Mortality in control after 21 days | |
|---|---|---|---|---|
| | Blank | Solvent | Blank | Solvent |
| *Validity criterion* | *≥ 60 %* | *≥ 60 %* | *≤ 20 %* | *≤ 20 %* |
| Fenoxycarb, low food | 47.5 | 37.9 | 0.0 % | 6.7 % |
| Propiconazole, low food | 35.2 | – | 10.0 % | – |
| Dimethylalkylamine, low food | 27.9 | – | 10.0 % | – |
| Product C – Eluate, low food | 48.6 | – | 0.0 % | – |
| Fenoxycarb, high food | 54.9 | 60.0 | 10.0 % | 13.3 % |
| Propiconazole, high food | 109.5 | – | 0.0 % | – |
| Dimethylalkylamine, high food | 142.2 | – | 7.1 % | – |
| Generic mixture, high food | 59.3 | 64.3 | 0.0 % | 6.7 % |

### 5.4.1 Single Substances

The three single substances fenoxycarb, propiconazole and dimethylalkylamine were each tested in two *Daphnia* reproduction tests, once at a low food level and once at a high food level. In each of these six tests, test concentrations were measured twice in freshly prepared and aged test medium (after 2 or 3 days of exposure). The results of the chemical analysis are summarized in Tables 35 and 36. The nominal test concentrations of fenoxycarb were extremely low, ranging from 1 µg/l to 1 ng/l, which was rather challenging for the analytical measurements. The recovery of fenoxycarb based on mean initial concentrations was outside the range of 80-120 % prescribed by the guideline for the Daphnia reproduction test. The very different recoveries between the two tests were mainly due to the large (opposite) deviations from the nominal concentrations at the lower test concentration.

The recovery of propiconazole based on mean initial concentrations was above 80 % in both tests, but generally showed a trend to decreasing recovery with decreasing test concentrations. Propiconazole was stable in the test medium over an exposure period of 2 to 3 days as indicated by the almost negligible loss within the test.

Dimethylalkylamine was mostly measured at lower concentrations than nominally expected. As in the case of propiconazole, there was a trend to decreasing recovery with decreasing test concentrations. Dimethylalkylamine was not always stable in the test medium as indicated by a loss between 4 % and 82 % over 2-3 days. For both propiconazole and dimethylalkylamine, the recovery did not differ much between the two tests.

Table 35: Nominal and measured concentrations of the test substances in the *Daphnia* reproduction tests at a low food level

| Substance | Nominal test concentration (µg/l) | Measured test concentration (µg/l) day 0 | Recovery (%) | Measured test concentration (µg/l) day 2 | Loss within test (%) |
|---|---|---|---|---|---|
| Fenoxycarb | 0.001<br>0.032<br>1.000 | 0.004 / 0.005<br>0.038 / 0.055<br>1.100 / 1.400 | 400.0 / 500.0<br>118.8 / 171.9<br>110.0 / 140.0 | n.d.<br>n.d.<br>0.950 / 0.930 | n.d.<br>n.d.<br>13.6 / 33.6 |
| Propiconazole | 20.0<br>180.0<br>540.0 | 16.0 / 18.2<br>n.d. / 157.0<br>410.0 / n.d. | 80.0 /91.0<br>n.d. / 87.2<br>75.9 / n.d. | n.d. / 18.2<br>n.d. / 157.0<br>410.0 / n.d. | n.d. / 0.0<br>n.d. / 0.0<br>0.0 / n.d. |
| Dimethyl-alkylamine | 12.5<br>35.4<br>70.7<br>100.0 | 3.5 / 7.6<br>12.0 / 22.0<br>n.d. / 40.0<br>38.0 / n.d. | 28.0 / 60.8<br>33.9 / 62.1<br>n.d. / 56.6<br>38.0 / n.d. | n.d.<br>n.d.<br>n.d. / < 1.0<br>6.5 / n.d. | n.d.<br>n.d.<br>n.d. / > 98.2<br>82.9 / n.d. |

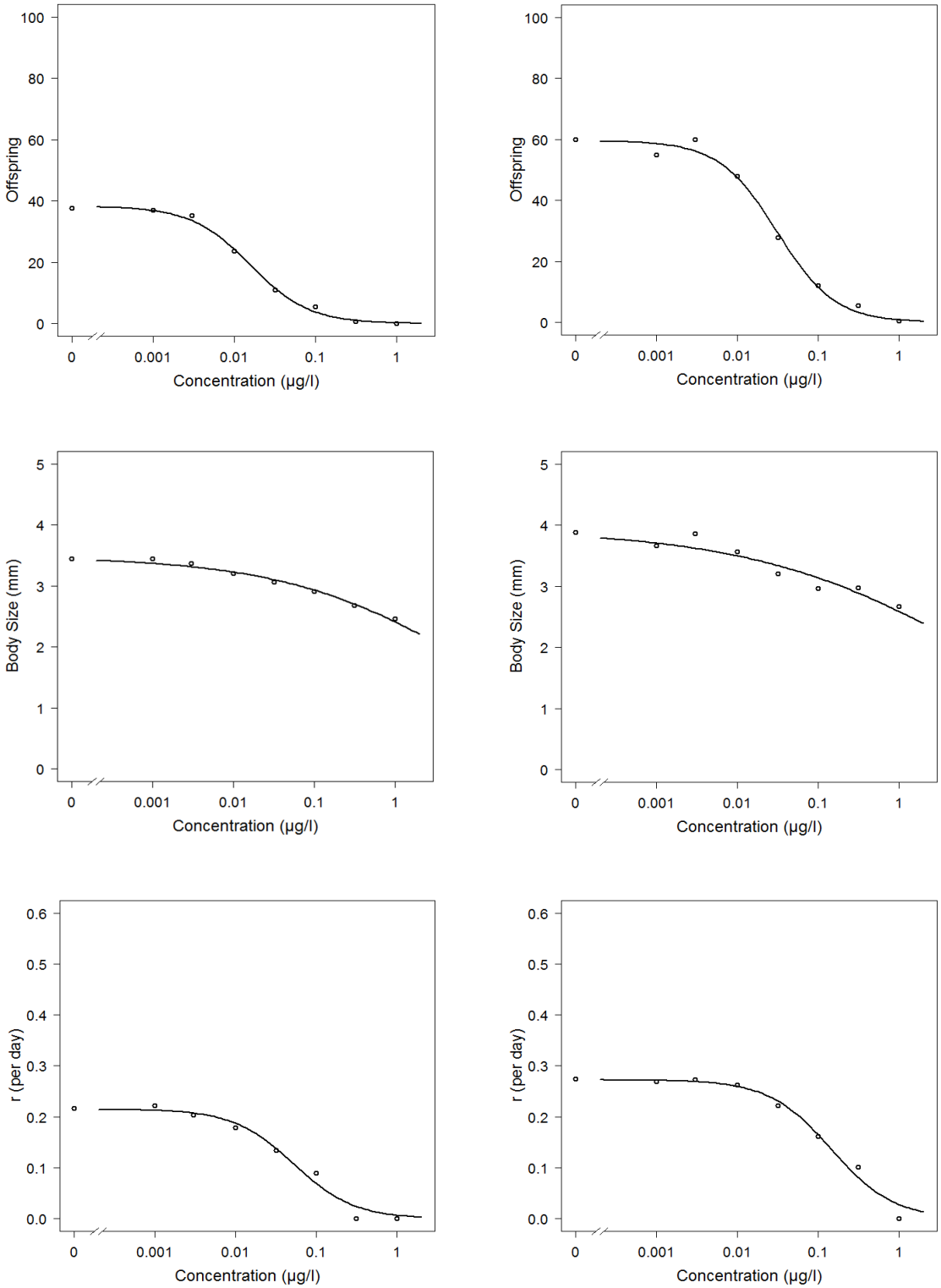
Concentrations were mostly measured twice within the 21 days. The recovery is calculated as the measured initial concentration in percentage of the nominal concentration. The percentage loss within the test is the difference of the measured concentration at day 0 and 3 in relation to the initial concentration. n.d.: not determined

Table 36: Nominal and measured concentrations of the test substances in the *Daphnia* reproduction tests at a high food level

| Substance | Nominal test concentration (µg/l) | Measured test concentration (µg/l) day 0 | Recovery (%) | Measured test concentration (µg/l) day 2 | Loss within test (%) |
|---|---|---|---|---|---|
| Fenoxycarb | 0.001<br>0.032<br>1.000 | < 0.001 / < 0.0005<br>0.002 / 0.001<br>0.94 / 0.79 | n.d.<br>6.3 / 3.1<br>94.0 / 79.0 | n.d.<br>n.d.<br>0.38 / 0.49 | n.d.<br>n.d.<br>59.6 / 40.0 |
| Propiconazole | 13.0<br>48.3<br>180.0 | 9.6 / 8.4<br>44.0 / 45.0<br>180.0 / 160.0 | 73.8 / 64.6<br>91.1 / 93.2<br>100.0 / 88.9 | n.d.<br>n.d.<br>170.0 / 170.0 | n.d.<br>n.d.<br>5.5 / -6.2 |
| Dimethyl-alkylamine | 6.2<br>17.7<br>50.0 | 3.2 / 1.4<br>17.0 / 7.6<br>50.0 / 33.0 | 51.6 / 22.6<br>96.0 / 42.9<br>100.0 / 66.0 | n.d.<br>n.d.<br>48.0 / < 10.0 | n.d.<br>n.d.<br>4.0 / > 69 |

Concentrations were mostly measured twice within the 21 days. The recovery is calculated as the measured initial concentration in percentage of the nominal concentration. The percentage loss within the test is the difference of the measured concentration at day 0 and 3 in relation to the initial concentration. n.d.: not determined

Fig. 17: Concentration-response plots for fenoxycarb tested in the *Daphnia* reproduction test




Shown are the mean number of offspring per surviving female within 21 days (top), the body size of surviving females after 21 days (middle), and the intrinsic rate of population increase, r, (bottom) at each concentration level together with their 3-parameter log-logistic fits. The left column relates to the test at low food level and the right column to the test at high food level. The analysis was based on nominal concentrations

Despite the highly varying recovery of fenoxycarb among tests and concentration levels, clear and mostly complete concentration-response curves were obtained in both tests with fenoxycarb for the three response variable reproduction, body size and population growth rate (Figure 17). For all three response variables, $EC_x$ values could be determined along with NOEC values. The NOEC for fenoxycarb did not depend on the food level or used *Daphnia* clone but was identical in both tests (Tables 37 and 38), while the $EC_x$ values were about factor 2 higher in the test at high food level compared to the low-food test. The determined NOEC for fenoxycarb differed only by factor 2 from the NOEC used in the regulatory assessment. No relevant mortality occurred in the first test with fenoxycarb and only little mortality (30 %) at the highest test concentration in the second test. This demonstrates that the chosen test concentrations allowed detecting sub-lethal effects, which occurred across a wide concentration range spanning three orders of magnitude. The highly specific, endocrine mode of action of fenoxycarb in *Daphnia magna* is confirmed by the extremely high acute-to-chronic ratio of 328,125 ($EC_{50}$ divided by NOEC, according to Ahlers et al. 2006). The number of offspring per surviving female was the most sensitive endpoint, followed by the population growth rate r and the body size of test animals.

Table 37: Toxicity of fenoxycarb in the *Daphnia* reproduction test at low food level

| Estimates | Reproduction | Size | r |
|---|---|---|---|
| Parameter b (SE) | 1.19 (0.227) | 0.38 (0.037) | 1.16 (0.235) |
| Parameter d (SE) | 38.2 (1.74) | 3.48 (0.040) | 0.21 (0.011) |
| Nominal $EC_{50}$ (ng/l) and [CI] | 15.9 [9.7-22.1] | n.d. | 52.9 (20.8-84.9) |
| Nominal $EC_{20}$ (ng/l) and [CI] | 5.0 [1.7-8.2] | 220.6 [122.5-318.8] | 15.9 [0-32.6] |
| Nominal $EC_{10}$ (ng/l) and [CI] | 2.5 [0.3-4.7] | 25.7 [5.8-45.6] | 7.9 [0-18.8] |
| Nominal NOEC (ng/l) | 3.2 | 3.2 | n.d. |
| Mean initial recovery (%) | 240.1 | | |
| Corrected $EC_{50}$ (ng/l) | 38.2 | n.d. | 126.9 |
| Deviation from regulatory NOEC | 2.0 | n.d. | n.d. |

Results are shown for the three response variables reproduction (number of offspring per surviving female within 21 days), size (body size after 21 days), and r (intrinsic rate of population increase). Given are the parameters b and d (standard error, SE) from the fitted log-logistic models, the ECx values with their 95 % confidence intervals (CI) at three effects levels and the no observed effect concentrations (NOEC). In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the EC50 corrected for this recovery. n.d.: not determined

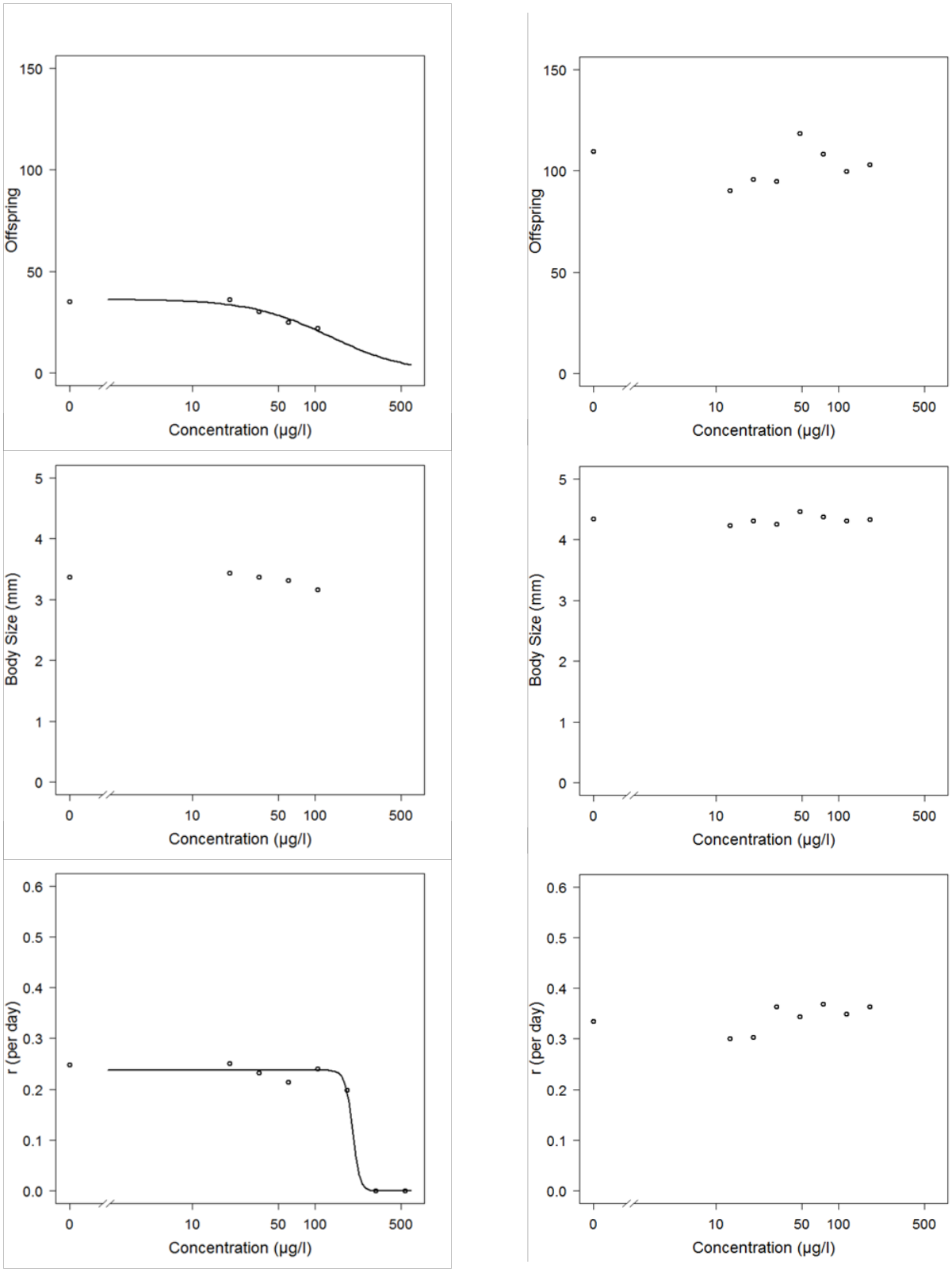
Table 38: Toxicity of fenoxycarb in the *Daphnia* reproduction test at high food level

| Estimates | Reproduction | Size | r |
|---|---|---|---|
| Parameter b (SE) | 1.21 (0.206) | 0.31 (0.041) | 1.13 (0.184) |
| Parameter d (SE) | 59.5 (2.17) | 3.92 (0.070) | 0.28 (0.009) |
| Nominal $EC_{50}$ (ng/l) and [CI] | 31.1 [21.3-40.8] | n.d. | 146.1 [77.7-214.5] |
| Nominal $EC_{20}$ (ng/l) and [CI] | 9.9 [4.5-15.2] | 100.2 [22.7-177.7] | 42.7 [8.5-76.8] |
| Nominal $EC_{10}$ (ng/l) and [CI] | 5.1 [1.3-8.8] | 7.6 [0-17.5] | 20.8 [0-42.99] |
| Nominal NOEC (ng/l) | 3.2 | 3.2 | n.d. |
| Mean initial recovery (%) | 45.6 | | |
| Corrected $EC_{50}$ (ng/l) | 14.2 | n.d. | 66.6 |
| Deviation from regulatory NOEC | 2.0 | n.d. | n.d. |

Results are shown for the three response variables reproduction (number of offspring per surviving female within 21 days), size (body size after 21 days), and r (intrinsic rate of population increase). Given are the parameters b and d (standard error, SE) from the fitted log-logistic models, the ECx values with their 95 % confidence intervals (CI) at three effects levels and the no observed effect concentrations (NOEC). In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the EC50 corrected for this recovery. n.d.: not determined

While the measured concentrations of propiconazole were in good agreement with the nominal test concentrations, the effects of propiconazole are not so straight forward (Figure 18). All test animals died at the three highest test concentrations (180 to 500 µg/l) in the first test until day 21. Some animals reproduced before they died, resulting in a population growth rate above zero. Because of the high mortality, a slightly lower concentration range was tested in the second test with 180 µg/l as the highest concentration. Mortality was negligible in the second test, even at this highest test concentration. In both tests, almost no effects were observed at the sub-lethal test concentrations. This indicates that the concentration-response curve for sub-lethal effects of propiconazole on Daphnia magna is extremely steep, spanning a range of less than one order of magnitude. Consequently, most $EC_x$ values could only be extrapolated beyond tested concentrations or estimated as censored values (Table 39 and 40), e.g. as > 180 µg/l in the second test. An exception was the effect on reproduction in the first test for which $EC_{20}$ and $EC_{10}$ could be estimated, but with rather wide confidence intervals. Likewise, no definitive NOEC could be determined in the second test. The NOEC for reproduction in the first test was about factor 10 lower than the NOEC used in regulatory settings. Based on these NOECs, moderate acute-to-chronic ratios of <90 up to 468 were determined. The cumulative number of offspring per surviving female was the most sensitive response variable, followed by population growth rate r. Here, effects were mainly due to the high mortality, which is integrated into r.

Fig. 18: Concentration-response plots for propiconazole tested in the *Daphnia* reproduction test




Shown are the mean number of offspring per surviving female within 21 days (top), the body size of surviving females after 21 days (middle), and the intrinsic rate of population increase, r, (bottom) at each concentration level together with their 3-parameter log-logistic fits. The left column relates to the test at low food level and the right column to the test at high food level. The analysis was based on nominal concentrations

Table 39: Toxicity of propiconazole in the *Daphnia* reproduction test at low food level

| Estimates | Reproduction | Size | r |
|---|---|---|---|
| Parameter b (SE) | 1.35 (0.48) | 2.15 (0.79) | 15.0 (49.5) |
| Parameter d (SE) | 36.26 (2.34) | 3.40 (0.02) | 0.24 (0.006) |
| Nominal $EC_{50}$ (µg/l) and [CI] | *> 104.3* | n.d. | *200.9* [17.2-384.7] |
| Nominal $EC_{20}$ (µg/l) and [CI] | 46.7 [15.7-77.8] | *> 104.3* | *183.2* [155.8-210.7] |
| Nominal $EC_{10}$ (µg/l) and [CI] | 25.6 [0-51.7] | *> 104.3* | *173.6* [115.4-231.7] |
| Nominal NOEC (µg/l) | 34.6 | 60.0 | n.d. |
| Mean initial recovery (%) | 81.3 | | |
| Corrected $EC_{50}$ (µg/l) | *106.4* | n.d. | *163.4* |
| Deviation from regulatory NOEC | 0.11 | n.d. | n.d. |

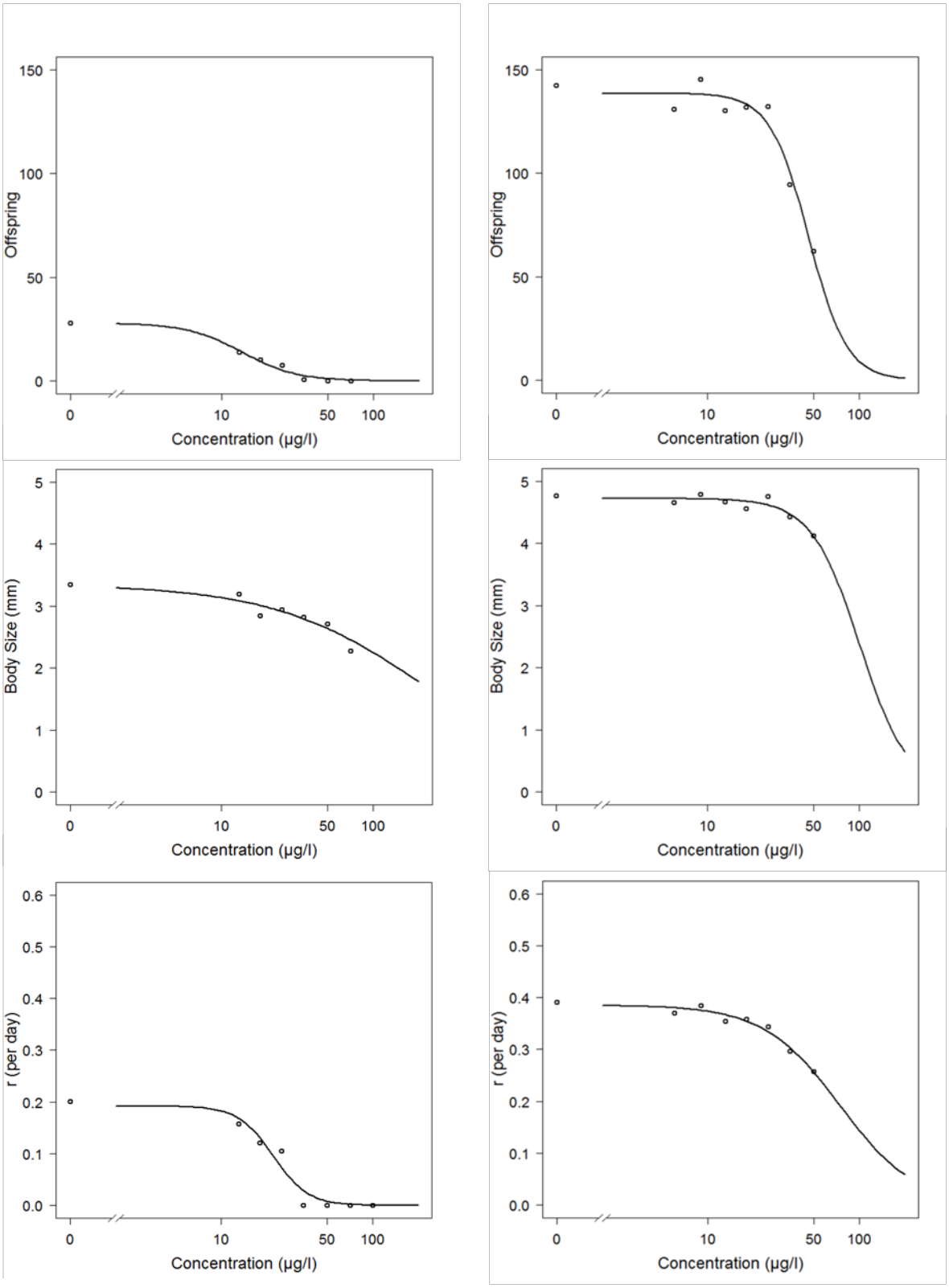
Results are shown for the three response variables reproduction (number of offspring per surviving female within 21 days), size (body size after 21 days), and r (intrinsic rate of population increase). Given are the parameters b and d (standard error, SE) from the fitted log-logistic models, the ECx values with their 95 % confidence intervals (CI) at three effects levels and the no observed effect concentrations (NOEC). In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the EC50 corrected for this recovery. Values in italics are extrapolated beyond tested concentrations. n.d.: not determined

Table 40: Toxicity of propiconazole in the *Daphnia* reproduction test at high food level

| Estimates | Reproduction | Size | r |
|---|---|---|---|
| Parameter b (SE) | n.d. | n.d. | n.d. |
| Parameter d (SE) | n.d. | n.d. | n.d. |
| Nominal $EC_{50}$ (µg/l) and [CI] | > 180.0 | > 180.0 | > 180.0 |
| Nominal $EC_{20}$ (µg/l) and [CI] | > 180.0 | > 180.0 | > 180.0 |
| Nominal $EC_{10}$ (µg/l) and [CI] | > 180.0 | > 180.0 | > 180.0 |
| Nominal NOEC (µg/l) | > 180.0 | > 180.0 | > 180.0 |
| Mean initial recovery (%) | 85.3 | | |
| Corrected $EC_{50}$ (µg/l) | > 153.5 | n.d. | > 153.5 |
| Deviation from regulatory NOEC | > 0.5 | n.d. | n.d. |

Results are shown for the three response variables reproduction (number of offspring per surviving female within 21 days), size (body size after 21 days), and r (intrinsic rate of population increase). Given are the parameters b and d (standard error, SE) from the fitted log-logistic models, the ECx values with their 95 % confidence intervals (CI) at three effects levels and the no observed effect concentrations (NOEC). In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the EC50 corrected for this recovery. n.d.: not determined

Fig. 19: Concentration-response plots for dimethylalkylamine tested in the *Daphnia* reproduction test




Shown are the mean number of offspring per surviving female within 21 days (top), the body size of surviving females after 21 days (middle), and the intrinsic rate of population increase, r, (bottom) at each concentration level together with their 3-parameter log-logistic fits. The left column relates to the test at low food level and the right column to the test at high food level. The analysis was based on nominal concentrations

Table 41: Toxicity of dimethylalkylamine in the *Daphnia* reproduction test at low food level

| Estimates | Reproduction | Size | r |
|---|---|---|---|
| Parameter b (SE) | 2.40 (0.58) | 0.86 (0.19) | 3.76 (1.27) |
| Parameter d (SE) | 27.79 (1.58) | 3.34 (0.06) | 0.19 (0.02) |
| Nominal $EC_{50}$ (µg/l) and [CI] | 13.7 [10.8-16.5] | n.d. | 22.0 [14.5-29.5] |
| Nominal $EC_{20}$ (µg/l) and [CI] | *7.7* [4.3-11.0] | 46.3 [32.2-60.5] | 15.2 [6.2-24.3] |
| Nominal $EC_{10}$ (µg/l) and [CI] | *5.5* [2.2-8.7] | 18.0 [8.5-27.5] | *12.3* [2.9-21.7] |
| Nominal NOEC (µg/l) | < 12.5 | < 12.5 | n.d. |
| Mean initial recovery (%) | 46.6 | | |
| Corrected $EC_{50}$ (µg/l) | 6.4 | n.d. | 10.2 |
| Deviation from regulatory NOEC | n.d. | n.d. | n.d. |

Results are shown for the three response variables reproduction (number of offspring per surviving female within 21 days), size (body size after 21 days), and r (intrinsic rate of population increase). Given are the parameters b and d (standard error, SE) from the fitted log-logistic models, the ECx values with their 95 % confidence intervals (CI) at three effects levels and the no observed effect concentrations (NOEC). In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the EC50 corrected for this recovery. Values in italics are extrapolated beyond tested concentrations. n.d.: not determined

Table 42: Toxicity of dimethylalkylamine in the *Daphnia* reproduction test at high food level

| Estimates | Reproduction | Size | r |
|---|---|---|---|
| Parameter b (SE) | 3.42 (0.544) | 2.71 (0.768) | 1.73 (0.351) |
| Parameter d (SE) | 138.5 (2.74) | 4.72 (0.034) | 0.385 (0.008) |
| Nominal $EC_{50}$ (µg/l) and [CI] | 46.4 [42.6-50.2] | n.d. | 74.4 [52.7-96.1] |
| Nominal $EC_{20}$ (µg/l) and [CI] | 31.0 [26.9-35.0] | 60.8 [51.5-70.1] | 33.4 [25.3-41.5] |
| Nominal $EC_{10}$ (µg/l) and [CI] | 24.4 [19.6-29.2] | 45.1 [40.3-49.9] | 20.9 [11.5-30.4] |
| Nominal NOEC (µg/l) | 25.0 | 25.0 | n.d. |
| Mean initial recovery (%) | 63.2 | | |
| Corrected $EC_{50}$ (µg/l) | 29.3 | n.d. | 47.0 |
| Deviation from regulatory NOEC | n.d. | n.d. | n.d. |

Results are shown for the three response variables reproduction (number of offspring per surviving female within 21 days), size (body size after 21 days), and r (intrinsic rate of population increase). Given are the parameters b and d (standard error, SE) from the fitted log-logistic models, the ECx values with their 95 % confidence intervals (CI) at three effects levels and the no observed effect concentrations (NOEC). In addition, the mean recovery of the test substance is provided (in percentage of the nominal concentration) and the values for the EC50 corrected for this recovery. Values in italics are extrapolated beyond tested concentrations. n.d.: not determined

Clear concentration-dependent responses were observed for dimethylalkylamine in the *Daphnia* reproduction tests (Figure 19). However, the curves, spanning about one order of

magnitude, were not complete in both tests. There was either no test concentration without effect (first test) or the highest test concentration induced an effect well beyond 100 % (second test). Consequently, the NOEC and $EC_x$ values differed somewhat among the tests (Tables 41 and 42). High mortality (100 % and 80 %, respectively) was observed in the two highest test concentrations in the first test. The second test did not include these concentration levels. Effects on reproduction and population growth rate were in about similar concentration levels in the two tests, while body size turned out as a less sensitive response variable. The acute-to-chronic ratio was between 4.8 and 9.6 for dimethylalkylamine.

Summarizing, clear concentration-dependent effects could be observed for the three test substances in the *Daphnia* reproduction tests. The effects did differ between the two tests for each substance. The individual impact of either food limitation or genetic differences (*Daphnia magna* clone) cannot be separated from each other, because the two tests for each substance differed in both factors. However, the degree of the difference was overall not large, but rather similar to the degree of deviation from the available regulatory data.

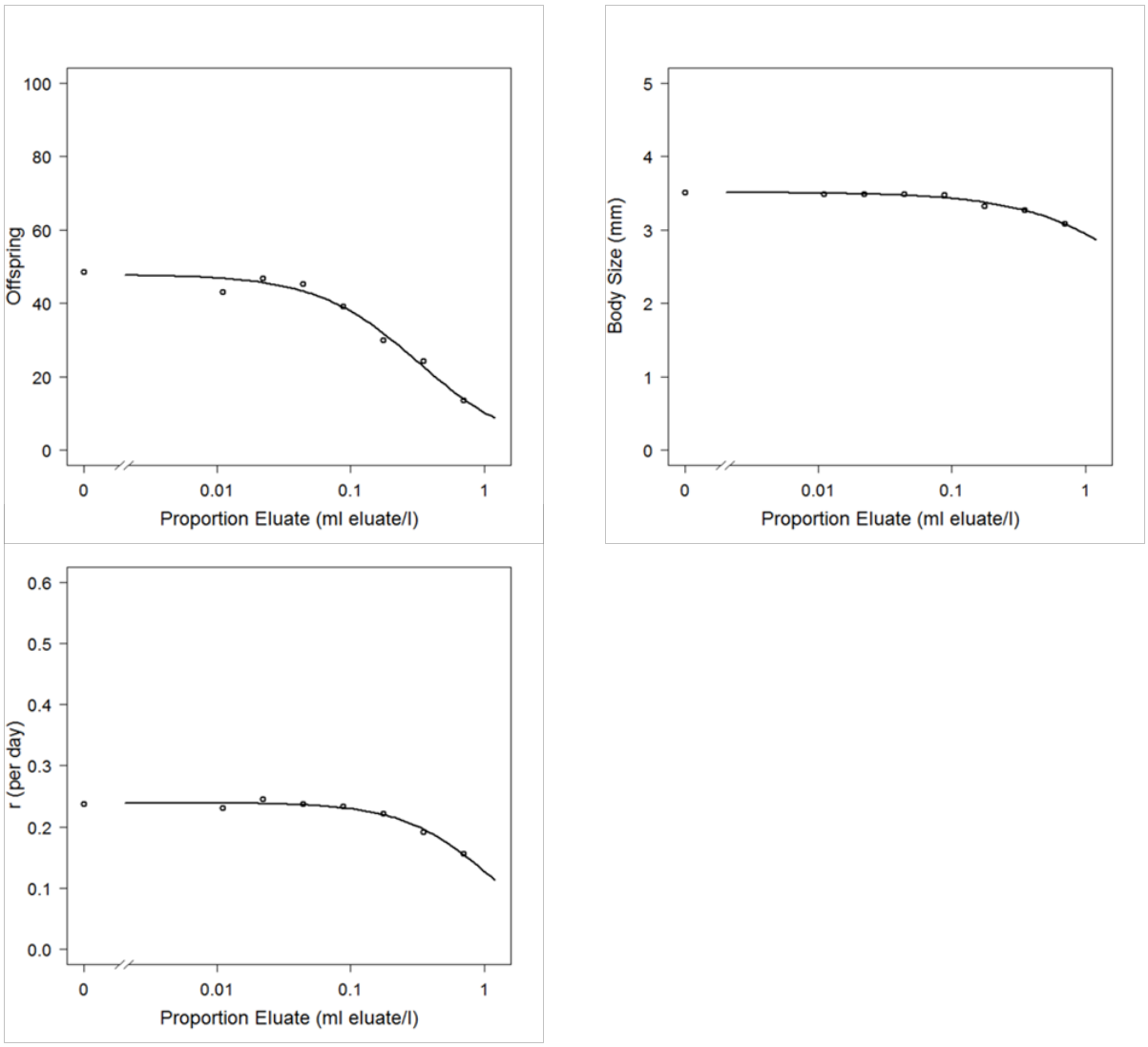
The chronic toxicity of fenoxycarb to *D. magna* was more than three orders of magnitudes higher than that of either propiconazole or dimethylalkylamine, which were about in the same range of toxicity.

### 5.4.2 Eluate

The test with the painting eluate of product C was conducted at a low food level. Consequently, the effect concentrations estimated in the low food single-substance tests were used for CA predictions. Increasing eluate proportions in the test medium caused increasing effects, which were strongest with regard to offspring production (Figure 20).

The concentrations of fenoxycarb, propiconazole, and dimethylalkylamine were measured twice in two independent preparations of test media. Results are summarized in Table 43. The determined concentrations differed hardly among the two batches. They were also in good agreement (only 1.3-fold greater) with the concentrations determined in the undiluted eluates ahead of the testing (Table 8). The loss of propiconazole during the exposure of 2 days was negligible, the loss of fenoxycarb highly variable (likely related to the low concentration in relation to the detection limit), and the loss of dimethylalkylamine considerable with more than 80 % of this substance being lost in the aged solutions.

Fig. 20: Concentration-response plots for the painting eluate of product C tested in the *Daphnia* reproduction test at low food level




Shown are the mean number of offspring per surviving female within 21 days (top left), the body size of surviving females after 21 days (top right), and the intrinsic rate of population increase, r, (bottom) at each concentration level together with their 3-parameter log-logistic fits. The analysis was based on the proportions of the eluate in the test medium

Table 43: The concentrations of the test substances in the painting eluate of product C as measured in the *Daphnia* reproduction test

| Substance | Proportion of eluate in test medium (ml/l) | Concentration in diluted eluate (µg/l) day 0 | Concentration in diluted eluate (µg/l) day 2 | Mean concentration in undiluted eluate (mg/l) | Loss within test (%) |
|---|---|---|---|---|---|
| Fenoxycarb | 0.011<br>0.088<br>0.701 | 0.002 / < 0.001<br>0.005 / n.d.<br>0.042 / 0.042 | n.d.<br>n.d.<br>0.044 / 0.026 | 0.058 / 0.060 | -4.8 / 38.1 |
| Propiconazole | 0.011<br>0.088<br>0.701 | 0.056 / 0.059<br>0.450 / n.d.<br>3.5 / 3.8 | n.d.<br>n.d.<br>3.6 / 3.6 | 5.08 / 5.40 | -2.9 / 5.3 |
| Dimethyl-alkylamine | 0.011<br>0.088<br>0.701 | < 0.2 / < 0.1<br>1.2 / n.d.<br>< 1.0 / 9.6 | n.d.<br>n.d.<br>1.0 / < 1.0 | 13.4 / 13.7 | 89.1 / > 89.6 |

Given are the concentrations of the substances in at least one concentration level as determined by analytical measurements of samples at day 0 (initial concentration) and day 2 (aged test solution) for two sampling dates. From these measured concentrations the concentrations in the undiluted eluates are re-calculated. The percentage loss within the test is the difference of the measured concentration at day 0 and 2 in relation to the initial concentration

Based on the nominal proportions of the eluate in the test medium, ECx and NOEC values were determined together with resulting MDR values (Table 44). Only the EC50 for population growth rate had to be extrapolated beyond measured test concentrations. The nominal values were re-calculated in terms of the sum of substances, based on mean initial concentrations measured in the test. The MDR values resulting from the comparison with predicted toxicity were for all response variables and endpoints within a range of factor 2 around 1, i.e., indicated good agreement between observed and CA-predicted toxicity in the Daphnia reproduction test. Censored MDR values (< 0.58) were observed based on NOECs, which indicated that the CA prediction using NOEC would rather overestimate but not underestimate the toxicity of the eluate.

Table 44: Predicted and observed $EC_{50}$ values estimated for the painting eluate of product C in the *Daphnia* reproduction test at a low food level together with resulting model deviation ratios (MDR)

| Estimates | Reproduction | Size | r |
|---|---|---|---|
| Ahead predicted $EC_{50}$ (ml eluate/l test medium) | 0.315 | n.d. | 0.667 |
| Observed $EC_{50}$ (ml eluate/l test medium) with [CI] | 0.324 [0.240-0.408] | n.d. | *1.111* [0.785-1.437] |
| Observed $EC_{50}$ (mg sum substances/l) | 6.10 | n.d. | *20.93* |
| Revised predicted $EC_{50}$ (mg sum substances/l) | 5.05 | n.d. | *10.27* |
| MDR based on ahead prediction ($EC_{50}$) | 0.97 | n.d. | 0.60 |
| MDR ($EC_{50}$) | 0.83 | n.d. | 0.49 |
| Observed $EC_{20}$ (ml eluate/l test medium) with [CI] | 0.096 [0.048-0.144] | 1.350 [0.911-1.789] | 0.387 [0.289-0-485] |
| Observed $EC_{20}$ (mg sum substances/l) | 1.81 | 25.44 | 7.29 |
| Revised predicted $EC_{20}$ (mg sum substances/l) | 1.37 | 31.81 | 4.04 |
| MDR ($EC_{20}$) | 0.76 | 1.25 | 0.55 |
| Observed $EC_{10}$ (ml eluate/l test medium) with [CI] | 0.047 [0.014-0.080] | 0.545 [0.420-0.671] | 0.209 [0.117-0.301] |
| Observed $EC_{10}$ (mg sum substances/l) | 0.88 | 10.27 | 3.94 |
| Revised predicted $EC_{10}$ (mg sum substances/l) | 0.71 | 6.06 | 2.18 |
| MDR ($EC_{10}$) | 0.80 | 0.59 | 0.55 |
| Observed NOEC (ml eluate/l test medium) | 0.088 | 0.088 | n.d. |
| Observed NOEC (mg sum substances/l) | 1.65 | 1.65 | n.d. |
| Revised predicted NOEC (mg sum substances/l) | < 0.92 | < 0.95 | n.d. |
| MDR (NOEC) | < 0.58 | < 0.58 | n.d. |

Shown are the results for the three response variables reproduction (number of offspring per surviving female within 21 days), size (body size after 21 days), and r (intrinsic rate of population increase). Given are the observed ECx values with their 95 % confidence intervals [CI] estimated from the fitted 2-parameter log-logistic model based on the proportion of the eluate in the test medium. The ahead predicted EC50 values are based on the concentrations of the test substances measured in the eluate ahead of the test (i.e., shortly after preparation), while the revised EC50 values are based on the concentrations measured in the tests. MDR values based on ahead measured concentrations are only reported for EC50 values. All ECx values that were determined by extrapolation beyond tested concentrations are shown in italics.

### 5.4.3 Generic Mixture

The recovery of the test substances in the test with a generic mixture of propiconazole, fenoxycarb and dimethylalkylamine was highly variable (Table 45). At the highest test concentration measured and nominal concentrations showed relatively good agreement, but not at low concentration levels. This will be largely due to the very low test concentrations and the related challenge to the analytical determination. Issues with test media preparation may additionally play a role, e.g. incomplete mixing of the test substances into the test media (all

dissolved together in an acetone dilution series). Because of the observed high variation in recovery, the nominal $EC_{50}$ estimates were not corrected for measured initial concentrations.

Table 45: The concentrations of the test substances in the *Daphnia* reproduction toxicity tests of the generic mixture

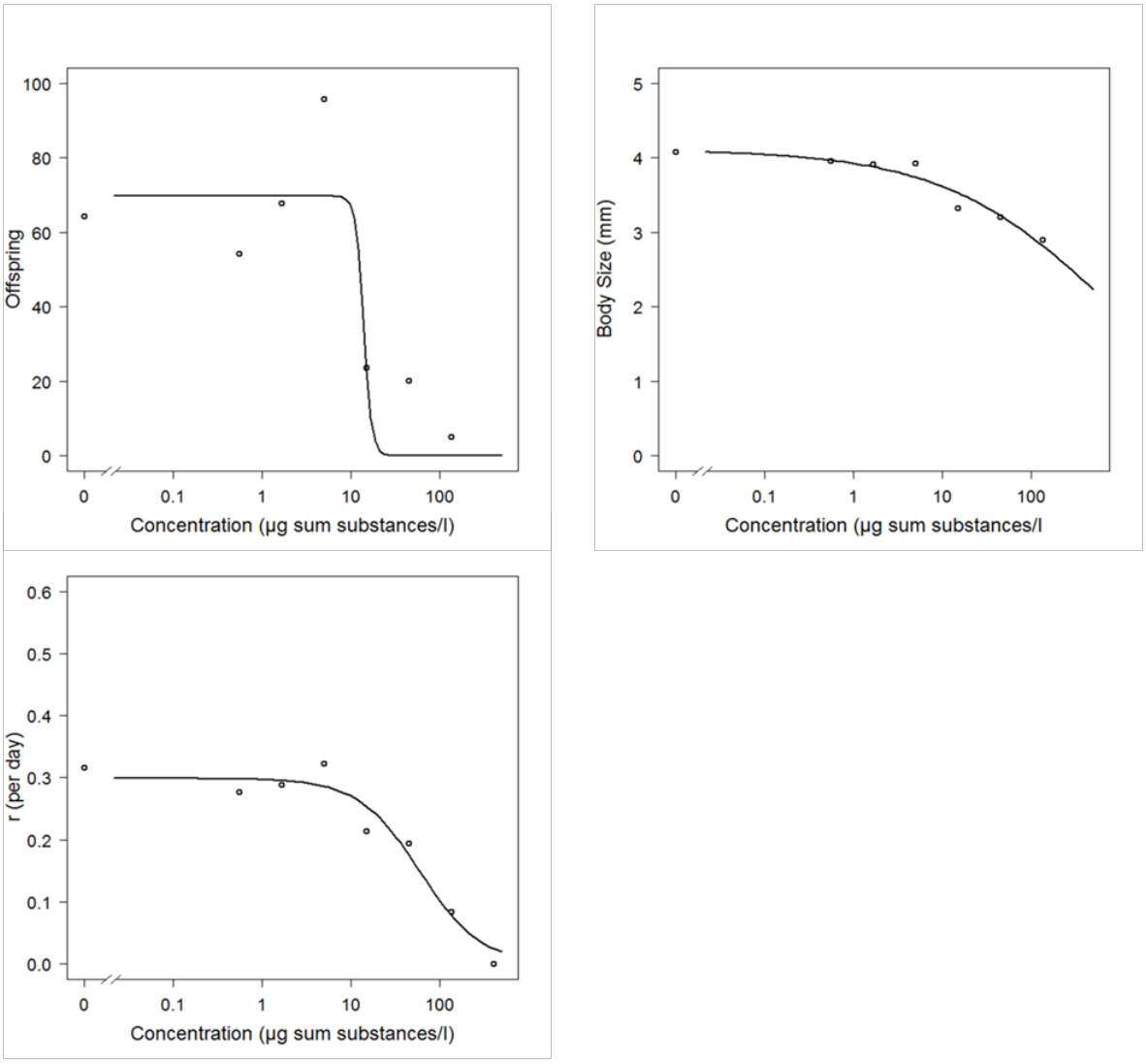
| Substance (relative nominal proportion) | Nominal concentration (µg/l) | Measured concentration (µg/l) day 0 | Measured concentration (µg/l) day 2 | Recovery (%) | Loss within test (%) |
|---|---|---|---|---|---|
| Propiconazole (0.7771) | 0.433<br>11.69<br>315.5 | 7.2 / 1.0<br>0.45 / 7.3<br>285.0 / 285.0 | n.d.<br>n.d.<br>290.0 / 250.0 | 1663.6 / 225.3<br>3.9 / 62.5<br>90.3 / 90.3 | n.d.<br>n.d.<br>-1.7 / 12.3 |
| Fenoxycarb (0.0004) | 0.00025<br>0.0068<br>0.183 | 0.0014 / 0.14<br>< 0.0005 / 11.1<br>0.31 / 0.32 | n.d.<br>n.d.<br>0.37 / 0.39 | 537.8 / 55770<br>< 7.4 / 163770<br>169.4 / 172.1 | n.d.<br>n.d.<br>-19.4 / -21.9 |
| Dimethyl-alkylamine (0.2225) | 0.124<br>3.34<br>90.3 | 2.8 / < 1.0<br>< 1.0 / 2.0<br>84.0 / 79.0 | n.d.<br>n.d.<br>24.5 / 2.7 | 2260.5 / < 806<br>< 29.9 / 59.8<br>93.0 / 87.5 | n.d.<br>n.d.<br>70.8 / 96.6 |

Given are the concentrations of the substances in three concentration levels (as mean of two analytical measurements per samples) for two batches of test media in consecutive weeks prepared from the same dilution series in acetone. Given are concentrations at day 0 (initial concentration) and at day 2 (aged test solution). The percentage loss within the test is the difference of the measured concentration at day 0 and 2 in relation to the initial concentration

Despite the variation in measured concentrations, clear concentration-dependent effects of the generic mixture were observed in the Daphnia reproduction test (Figure 21). There was one concentration level that appeared to be an outlier with regard to the number of offspring and, less strong, with regard to body size. Yet, this outlier was not removed from the concentration-response modelling.

The effects on reproduction and body size were strong enough to allow estimating $EC_{50}$ values without extrapolation (Table 46). Since the comparisons with predicted mixture effects was based on the single-substance effects from the high food tests (which obtained censored effect values), the MDR values calculated for the generic mixture test were censored as well. They all clearly indicated that the toxicity of the mixture was at most underestimated by factor 3.0 (MDR for $EC_{50}$ of reproduction). At lower effect levels, the censored MDR values rather indicated overestimation of toxicity than underestimation. Hence, the testing of the generic mixture demonstrated (based on nominal concentrations), that no synergistic interaction is to be expected from the joint chronic toxicity of these substances towards *Daphnia magna*.

Fig. 21: Concentration-response plots for the generic mixture of fenoxycarb, propiconazole and dimethylalkylamine tested in the *Daphnia* reproduction test at high food level




Shown are the mean number of offspring per surviving female within 21 days (top left), the body size of surviving females after 21 days (top right), and the intrinsic rate of population increase, r, (bottom) at each concentration level together with their 3-parameter log-logistic fits. The analysis was based on nominal concentrations

Table 46: Predicted and observed $EC_x$ and NOEC values estimated of the generic mixture tested in the *Daphnia* reproduction test at high food level together with resulting model deviation ratios (MDR)

| Estimates | Reproduction | Size | r |
|---|---|---|---|
| Nominal observed $EC_{50}$ (µg sum substances/l) with [CI] | 14.0 [9.1-18.9] | n.d. | 60.0 [15.3-104.6] |
| Nominal predicted $EC_{50}$ (µg sum substances/l) | > 42.4 | n.d. | > 96.2 |
| Nominal MDR ($EC_{50}$) | < 3.02 | n.d. | < 1.60 |
| Nominal observed $EC_{20}$ (µg sum substances/l) with [CI] | 12.1 [0-24.9] | 37.4 [20.5-54.2] | 19.9 [0-45.6] |
| Nominal predicted $EC_{20}$ (µg sum substances/l) | > 17.5 | > 80.2 | > 46.4 |
| Nominal MDR ($EC_{20}$) | < 1.45 | < 2.15 | < 2.33 |
| Nominal observed $EC_{10}$ (µg sum substances/l) with [CI] | 11.1 [0-27.4] | 6.6 [1.4-11.7] | 10.4 [0-28.4] |
| Nominal predicted $EC_{10}$ (µg sum substances/l) | > 9.8 | > 14.6 | > 27.3 |
| Nominal MDR ($EC_{10}$) | < 0.88 | < 2.23 | < 2.61 |
| Nominal observed NOEC (µg sum substances/l) | 5.01 | 5.01 | n.d. |
| Nominal predicted NOEC (µg sum substances/l) | > 6.5 | > 6.5 | n.d. |
| Nominal MDR (NOEC) | < 1.29 | < 1.29 | n.d. |

Given are the observed EC50 values with their 95 % confidence intervals [CI] estimated from the fitted 3-parameter log-logistic for each of the three response variables based on nominal concentrations. n.d.: not determined

# 6 Discussion

The present study clearly demonstrated that the toxicity of mixtures of biocides (products and eluates) can in principle be correctly and reliably predicted by the concept of concentration addition. The quantitative measure for assessing compliance between predicted and observed toxicity, the MDR, rarely deviated by more than factor two from 1 (with 1 indicating perfect compliance). There were some cases of larger deviations that will be discussed in the following. Ahead of the mixture toxicity issue, some relevant background aspects will be discussed. These are namely analytical aspects and the individual toxicity of the mixture components together with the reliability of their toxicity estimates.

## 6.1 Analytical verification of test substance concentrations

The verification of the test substance concentration in the here conducted aquatic ecotoxicological tests is required according to the relevant OECD guidelines. While not all guideline requirements regarding analytical measurements were fulfilled in the present study (i.e., limited measurement of aged test solutions and no measurement of all concentration levels in case of low recovery), the conducted measurements mostly allowed correcting for actual measured initial concentrations at the median concentration level and assessing the stability of the test substance over the test duration.

The fungicide propiconazole as technical material could be dissolved directly in the aqueous media of both test systems (algae and *Daphnia*). Between 81.3 and 87.3 % of the nominal concentration was measured in the various single substance tests as average initial concentration. Propiconazole was generally stable over the duration of 2 to 3 days, which is in agreement with a previous study (Coors et al. 2011). Hence, single-substance toxicity estimates for propiconazole can be related to the measured as well as to the nominal concentrations according to the OECD guidelines for the here conducted tests.

The formulation additive dimethylalkylamine could also be dissolved directly in the aqueous media despite its reported low water solubility. In the single-substance tests, the measured concentrations reached mostly above 80 % of the nominal concentrations. An exception was the *Daphnia* reproduction test at low food level and two cases where the lowest test concentration was out of the range of 80-120 % recovery. The loss of dimethylalkylamine over the exposure periods was somewhat higher than for propiconazole, particularly in the *Daphnia* reproduction test. This may indicate that the test substance adsorbs to test vessels and/or the algae which are fed in the *Daphnia* reproduction test. Adsorption to glass test vessels would also explain the less than 100 % recovery for initial concentrations. Adsorption to solid material is likely because of the low water solubility and high lipophilicity of dimethylalkylamine (calculated log Kow value of 5.47). Due to the method of chemical analysis, substances adsorbed to or taken up by algae are not included in the analysis.

Fenoxycarb was added to all test media using acetone as solvent. The initial measured concentration was generally within 80-120 % of the nominal concentrations. Exceptions were the lower concentration levels in the *Daphnia* reproduction tests, where extremely high or low recoveries were found. Since the lowest test concentration level was 1 ng/l (close to the detection limit of the analytical method, with reasonable effort), these extreme deviations from nominal concentrations were most likely due to analytical uncertainties. They should not be over-interpreted since analytical uncertainties are not considered separately alongside the

allowed 20 % deviation from nominal concentrations. Hence, in the case of high analytical uncertainty due to very low test concentrations, the 20 % deviation criterion may be misleading. Observed concentration-response curves in these tests with low concentrations did not indicate any deviations from the nominal dilution series (with the exception of one unexplained outlier). Loss of fenoxycarb during the exposure period was relatively large in the *Daphnia* reproduction test with up to 60 % and very variable (7-71 %) in the algae growth inhibition tests, but lower in the *Daphnia* acute toxicity tests. Loss of fenoxycarb particularly in tests that involved algae either as food or as test organism may be caused by uptake into or adsorption to algae cells. Such adsorption and subsequent uptake of fenoxycarb to the algae used as cladoceran food was discussed before as a reason for decreased toxicity at food limiting conditions (Rose et al. 2002). In the present study, this dependency of fenoxycarb toxicity on food availability was not confirmed, but rather the opposite trend was found, i.e. lower toxicity at higher food level. Again, the low concentrations of fenoxycarb in the *Daphnia* reproduction tests hampered the exact determination of fenoxycarb concentrations and loss over time, which are therefore to be seen with caution.

The fungicide IPBC is reported in the final assessment report of the EU (SC 2008) to be “stable to direct and indirect photolysis in the aquatic environment” together with hydrolytic stability. In the present study, IPBC as technical material could be dissolved in the aqueous test media reaching as mean initial concentration between 70 and 81 % of the nominal concentration. This indicates that only little dissipation of IPBC occurred during the preparation of the test media (stirring in the dark overnight). The loss of IPBC in the acute *Daphnia* test was negligible, but considerable in the algal growth inhibition test (about 79 %). An earlier study found a loss of about 40 % over 2 days of exposure in the fish embryo test (Coors et al. 2011). Hence, IPBC appears to be not as stable in aquatic biotests as expected from the statement in the assessment report (SC 2008).

Less than 1 % of the nominal concentration of IPBC was determined in the biotests with product A, which contained IPBC together with propiconazole. Similarly, very little IPBC was detected in the eluate of product A (see below). A replication of the analyses with product A being dissolved in test media at a high concentration confirmed the very low concentration of IPBC in the product. Since IPBC did not dissipate to a large amount during the preparation of test media when using the technical material, the (almost complete) absence of IPBC in the analyses of the product proves that it must already have dissipated in the product. This must further have happened during the storage of the product since previous tests with the same batch of product detected IPBC in accordance with nominal concentrations (Coors et al. 2011). There was no expiring date stated for product A on its label and no specific, unabated storage conditions; therefore, the instability of IPBC during storage was not foreseen. Prop-2-ynyl-N-butylcarbamate (PBC) has been reported as the sole and terminal bacterial transformation product of IPBC (Cook et al. 2002). Indeed, PBC was found at considerable amounts in test media to which product A or eluate of product A was added. A PBC concentration of 0.33 mg/mg product was consistently determined in two independent analyses using the two different test media for dissolving the product. This equals 70.5 % of the expected PBC concentration when assuming a complete and 1:1 stoichiometric transformation of IPBC to PBC (Cook et al. 2002). With regard to the mixture toxicity predictions it is therefore important to keep in mind that PBC instead of IPBC was present in product A.

In the two biotests with a water-accommodated fraction of product B about 45 % of propiconazole and 28 or 19 % of fenoxycarb, respectively, were determined with regard to their concentrations assuming 100 % transfer into the test media. These recoveries are in general in agreement with a previous study that found 37-42 % and 24-40 % recovery for propiconazole and fenoxycarb, respectively (Coors et al. 2011, 2012). However, the difference for the recovery of fenoxycarb in the two tests as well as to the previous study remains unexplained. With regard to mixture toxicity predictions it is important to keep in mind that predictions based on nominal concentrations are expected to overestimate the toxicity of product B, because the actual test concentrations were considerably lower than foreseen in the mixture toxicity prediction. This holds of course only if other reasons for enhanced toxicity such as synergistic interaction or relevance of non-considered mixture components can be excluded.

The three test substances fenoxycarb, propiconazole and dimethylalkylamine were measured at different recovery rates in the two biotests that were conducted with product C. While the more than 100 % recovery rates in the algal growth inhibition test and, particularly, in the *Daphnia* immobilisation test may relate to a slightly higher than planned weight-in, the relation of the recovery rates differed also among the three substances. This applied particularly to fenoxycarb (once with higher and once with lower recovery than propiconazole), which may point at some analytical problems with fenoxycarb determination. Slightly higher-than-nominal concentrations of fenoxycarb in product C were also observed in previous fish embryo tests with product C (Coors et al. 2011, 2012). In general, such deviations may result from deviations of substance concentrations in the product itself from the labelled concentrations (which is normal and legal within certain ranges). Therefore, the here observed deviations of more than 20 % between measured and nominal concentrations are not considered to render the respective tests unreliable, which is supported by the unambiguous concentration-response curves.

Summarizing, the analytical verification of the test substance concentrations provided mostly reliable and unequivocal data that can be used to correct mixture toxicity predictions. In doing so, the present study advances many other studies on mixture toxicity published in the literature that base mixture toxicity assessments on nominal concentrations only. There are currently no mixture toxicity models available that allow taking account of varying exposure concentrations over time. Therefore, the mixture toxicity predictions corrected for measured concentrations were restricted in the present study to measured initial concentrations. As long as the dissipation of substances during the tests does not depend on whether this substance is present alone or in a mixture with others, this restriction to initial measured concentrations does not make any difference for the comparisons between predicted and observed mixture toxicity.

## 6.2 Leaching rates of test substances from treated wood

The leaching rates determined in the present study differed for the same substance (i.e., propiconazole) among the four prepared eluates. This clearly demonstrates that the leaching rate of a substance in a wood preservative product depends on the formulation of the product as well as on the method of eluate preparation and, hence, the product application method (painting or dipping). Leaching from wood treated with solvent-based preservative products seems to be lower compared to water-based formulations, at least under the here applied

conditions of eluate preparation. This may indicate that solvents rather improve the penetration of the active substances into the wood instead of enhance leaching, which could be explained by the evaporation of the solvents. In addition, water-based products may contain additives that enhance penetration but do not evaporated similarly to solvents and therefore enhance also leaching. The leaching rates of the various substances in the same product partly differ from each other, demonstrating that substance properties also determine the leaching behaviour. However, similar leaching rates for fenoxycarb and propiconazole in the respective two products and an only slightly lower leaching rate of the amine indicate that the difference between products is larger than the difference in leaching behaviour between substances within the same product (at least in the here investigated small sample size of products and a.s.). The low leaching rate determined for IPBC is due to the considerable dissipation of the substance already in the product, as discussed before. This is supported by a previous study where the leaching rate of IBPC was considerably higher with 14 % (Schoknecht 2010). Hence, with regard to an environmental risk assessment it cannot be assumed that IPBC leaches from treated wood at a rate of less than 1 % as indicated by the present study. The determination of 2.1 mg PBC/l eluate of product A is equal to 3.8 mg IPBC/l eluate assuming 1:1 stoichiometric transformation. This IPBC concentration in the eluate transfers to a leaching rate of 10 %, which is only slightly lower than in a previous study (Schoknecht 2010). The comparisons for the other substances showed an about twofold lower leaching rate in the present study than in the previous study with the same products (Schoknecht 2010). While the leaching tests were conducted according to the same protocol in the same laboratory, other parameters differed between the two studies: the analytical determination was conducted by different laboratories, the eluates were stored differently until analysis, the pine sapwood was stored 1.5 years longer for the present study than for the previous one, and the salt composition and pH of the used elution medium differed (for example: pH 8.3 in this study and pH 6.6-7.0 in the previous study). Several of these factors, particularly wood storage time and pH are known to influence the leaching of wood preservatives from treated wood (Lupsea et al. 2012).

Cobalt, contained as cobalt(II) salts in product A, was not directly measured in the eluate or product, but re-calculated from measured concentrations in test media. Based on these calculations, a leaching rate of 28.3 % was derived for cobalt, which considerably exceeds the leaching rate of the two active substances in this product and may be related to the ionic nature of cobalt(II) salts.

The concentrations of the test substances in the eluates appeared to differ between the two determinations, i.e., when analysed in not pH-adjusted subsamples shortly after preparation or when re-calculated from test concentrations based on analysis of pH-adjusted test media. The concentration of propiconazole in the eluates was 1.4 to 2.0-fold higher when re-calculated from test media. Fenoxycarb concentrations, on the other hand, tended to be lower (range from 0.6 to 1.1 compared to concentrations measured ahead of testing). For dimethylalkylamine the concentrations differed by about factor 1.3 to 1.4. These differences are overall rather small and are within the range of variations of analytical methods. The pH adjustment of the solutions did apparently not play a role as verified by dissolving product A in algae test medium and analysing a pH-adjusted and not-adjusted subsample. The determined concentrations were almost identical in the pH-adjusted and not-adjusted subsamples. Therefore, it can be concluded that pH adjustment of the samples for the analytical measurement had little influence on the determined concentration of the test substances.

## 6.3 Toxicity of single substances

$EC_x$ values as toxicity estimates for the single substances could be determined in the present study with tight confidence intervals and are therefore seen as precise and reliable. The reliability is further supported by the finding that the here estimated $EC_x$ values do not deviate by more than factor 2 from respective values in the regulatory risk assessment reports. Therefore, the originally foreseen repetition of the single substance tests was not deemed necessary in the end. The deviations with regard to available regulatory NOEC values were somewhat larger, with a maximum of almost factor 10 in the case of propiconazole. However, NOEC values depend very much on the chosen test concentrations and can therefore hardly be compared among tests that used different concentration ranges.

The $EC_{50}$ of propiconazole with regard to algal growth rate was the only single substance acute toxicity estimate that could not be determined with sufficient precision and reliability due to an incomplete concentration response curve. Repetitions of the test (not reported here) could not solve this issue, which is likely caused by the rather flat concentration response curve and limited water solubility of propiconazole. The extrapolated $EC_{50}$ for growth rate, however, did not deviate by more than factor 3 from the updated regulatory $EC_{50}$ for algal growth rate.

Estimates for chronic toxicity towards *Daphnia* had occasionally to be extrapolated beyond measured concentrations for propiconazole and dimethylalkylamine in the present study. Even by testing more than the required five concentration levels in the reproduction tests, this problem could not be completely avoided. It is basically due to the, in this case steep, concentration response curves with regard to chronic effects. Namely in the case of propiconazole, this indicates that the mode of action is related to acutely toxic effects rather than to long-term effects on reproduction.

The four test substances can be ranked (based on mass concentrations) according their increasing toxicity to algae as dimethylalkylamine > IPBC > fenoxycarb > propiconazole. The toxicity rank order was identical with regard to acute *Daphnia* toxicity. For chronic *Daphnia* toxicity, however, the rank order of toxicity changed to fenoxycarb > dimethylalkylamine > propiconazole (IPBC was not tested). Because the molar masses of the four substances do not differ much, the rank orders of toxicity do not change when toxicity is compared on the more meaningful molar basis instead of the mass basis (data not shown here). The rank order of toxicity did also not differ between the two response variables regarding algae or the three response variables regarding chronic *Daphnia* toxicity. The change in the rank order from acute to chronic endpoints in *Daphnia* is linked to the well-known highly specific (i.e., endocrine) mode of action of fenoxycarb.

The determined NOEC values were overall in the range of the $EC_{10}$ and $EC_{20}$ values. Mostly, the NOEC for reproduction was between the $EC_{10}$ and $EC_{20}$ in the *Daphnia* reproduction test, but this relationship was not consistent enough to allow assuming e.g. that the NOEC generally equals 15 % effect. Yet, such a strong relationship would be required to scientifically defend usage of the NOEC instead of an $EC_x$ estimate in the CA prediction (Kortenkamp et al. 2009). In addition, it has to be kept in mind that more than the usual five concentrations levels and a rather small spacing factor were used in the present study in all tests. Typical regulatory data will therefore even less reliable fit into a presumed constant relationship between NOEC and $EC_x$ because they usually employ fewer test concentrations. The results of the present study,

however, do not suggest that a pragmatic usage of NOEC instead of $EC_x$ values in regulatory risk assessment schemes is not meaningful at all.

## 6.4 Predictability of mixture toxicity for assessing products and eluates

When it comes to assessing the predictability of the mixture toxicity of biocidal products and their eluates, there are two principle approaches that will be discussed in the following. In a retrospective approach, comparisons of predicted and experimentally observed mixture toxicity serves to detect non-additive interactions and to identify so far not recognized ingredients that significantly contribute in a given mixture to the overall toxicity. In a prospective approach, the potential of all known ingredients to significantly contribute to the overall toxicity is assessed without actually testing the mixture. Such an approach can serve in a regulatory risk assessment context to better target potential testing of a mixture and to derive thresholds for the need to include ingredients in mixture toxicity predictions. The present project was set up to apply the first approach, but the results will also be applied to inform threshold derivation with respect to the second approach.

### 6.4.1 Identification of relevant mixture components

Mixtures of technical material of active substances (so-called generic mixtures) were only tested for *Daphnia* acute toxicity in the present study. The results clearly demonstrated that propiconazole & IPBC as well as propiconazole & fenoxycarb interact in a concentration additive manner. Only in the case of the propiconazole & IPBC mixture, the MDR was slightly above 2 (2.25). However, this MDR was based on nominal concentrations and IPBC was often recovered in test media at a rate below 100 %; lower-than-nominal actual test concentration can completely explain this slight deviation. The here observed concentration additive behaviour of these active substance mixtures supports previous findings of CA predictability of their mixture toxicity in the fish embryo test (Coors et al. 2001, 2012). This finding is further in agreement with a large body of literature (reviewed in Deneer 2000, Belden et al. 2007) that reports concentration additive mixture toxicity for the large majority of mixtures of technical pesticides.

#### Products with propiconazole and fenoxycarb

The toxicity of propiconazole & fenoxycarb towards algae can also be predicted with good precision by the concept of CA as demonstrated in the present study by product B and the eluate of product B: the MDR values for the $EC_{50}$ of yield ($E_bC_{50}$) were between 1 and 2. MDR values for the response variable growth rate were considerably larger, but this was most likely due to the extrapolated estimate for propiconazole. Using the revised regulatory $E_rC_{50}$ estimate of 0.46 mg/l (personal communication D. Frein, UBA, 13 July 2012), the MDR values for growth rate were below 1. Hence, the toxicity of product B and eluates thereof can be explained solely on the basis of the concentration additive toxicity of the contained active substances; there is no indication that formulation additives have to be considered.

In contrast to its toxicity to algae, the acute toxicity of product B to *Daphnia* was underestimated by the CA prediction by about factor 10 after correcting for measured concentrations. Even before correcting, the nominal MDR was above 2, while a value clearly below 1 was expected for this WAF test as discussed above. Fish embryo toxicity tests conducted

with product B obtained similarly corrected MDR values above 2, indicating unexplained underestimation of product toxicity (Coors et al. 2011, 2012). Because the testing of the generic mixture of propiconazole & fenoxycarb for *Daphnia* (as well as for fish embryo) acute toxicity proved concentration additive behaviour, the underestimation of toxicity for the product provides clear evidence for the influence of formulation additives. This may either be ingredients that are toxic to *Daphnia* (and fish) or ingredients that interact with the toxicity of the active substances. A possible explanation would be that the solvents, which are present at a high concentration in product B, enhanced the uptake of the active substances into *Daphnia* (and fish). Such a situation would represent a synergistic interaction on a toxicokinetic level. For the eluate of product B, the MDR value was determined as "< 4", which indicates little or even no underestimation of toxicity by the CA prediction. Together with the results for the product, this leaves two possible explanations: (i) the presence of a *Daphnia* (and fish)-toxic formulation additive in product B and (ii) the presence of a formulation additive in product B that interacts with the active substances in a synergistic way. In both cases, the formulation additive is either not leaching from the treated wood in relevant amounts or not taken up into the wood in the first place. The organic solvent naphtha contained in product B in high amounts may exhibit effects such as enhanced uptake. Naphtha was separated from the actual tested water phase in the production step of the water accommodated fraction, but may have remained at small amounts in the test medium. Interestingly, ultrasonication was used in the preparation of the WAF only for the *Daphnia* but not for the algae test. Hence, if ultrasonication resulted in a better dispersion of the organic solvent in the water and subsequently less efficient separation, an influence of the organic solvent may also explain the difference in the MDR values between the algae and the *Daphnia* test. However, toxicokinetic interactions are expected to be more relevant in *Daphnia* or fish embryos compared to unicellular organisms such as green algae, which provides an additional explanation for the difference in MDR between these taxonomic groups. Summarizing, there is evidence for the relevance of formulation additives in product B with regard to *Daphnia* and fish toxicity, but not with regard to algal toxicity. In a meta-analysis of the mixture toxicity of plant protection products (Coors & Frische 2011), it was shown that the mixture toxicity predictability of formulated pesticides correlated between fish and daphnids, but not between algae and *Daphnia* or algae and fish. Hence, the biocidal product B is another example underlining that correct CA-predictability of the toxicity of formulated pesticides on one trophic level does not indicate correct CA-predictability of toxicity of the same product for another trophic level, with the exception of the correlation between fish and *Daphnia*.

The toxicity of product C and eluates thereof towards algae and *Daphnia* could only be reliably and correctly predicted by CA if the formulation additive dimethylalkylamine was included in the prediction. This holds for both of the investigated trophic levels, different response variables and acute as well as chronic endpoints. The good compliance of prediction and observation regarding this three component mixture supports again the non-synergistic behaviour of propiconazole & fenoxycarb and demonstrates that no other ingredients relevant for an aquatic environmental risk assessment are contained in product C. Overall, this finding is in good agreement with the results of the fish embryo test carried out with product C (Coors et al. 2011, 2012).

### Product with propiconazole and IPBC

IPBC is known to be degraded to PBC (prop-2-ynyl-N-butylcarbamate) by biotic and abiotic mechanisms with PBC being the terminal and only metabolite in biotic degradation tests with bacteria (Cook et al. 2002). A complete loss of fungicidal activity by this transformation was observed (Cook eta al. 2002). IPBC was almost non-detectable in tests with product A and its eluate. Chemical analysis demonstrated that IPBC was presumably completely transformed to PBC already in the product. Yet, MDR values based on nominal concentrations indicated only slight overestimation of product toxicity (by up to factor 3). This was observed for the toxicity towards algae as well as to *Daphnia*, and provides evidence for the presence of an ingredient that contributes to the overall toxicity (in the absence of IPBC), but that is not considered in the prediction. Using measured concentrations of IPBC (or instead the detection limit) in the prediction resulted in considerable underestimation of toxicity of the product as well as of the eluate, again for algal growth inhibition as well as for *Daphnia* acute toxicity. This supports the finding that an ingredient not considered in the mixture prediction was contributing to the overall toxicity. In principle, this can either be PBC or a formulation additive contained in product A (or a combination of both). Given the finding of nominal MDR values slightly below 1, either PBC or the hypothetical formulation additive or the two together would exhibit then toxicity almost identical to that of IPBC alone if it were present at its nominal concentration. However, it cannot be decided which ingredient contributed significantly to the mixture toxicity without knowledge about their individual toxicity. Testing of PBC was beyond the scope of the present study and is hampered anyway by limited availability of the substance. According to the labelling of product A, it contained cobalt(II) salts as formulation additives. Analysis of some samples for cobalt could indeed detect this metal and derive a concentration for total cobalt in the product and the eluate. In a toxic unit approach, the contribution of this formulation additive and thereby its relevance for the overall toxicity of the product and eluate can be evaluated based on toxicity estimates submitted to ECHA (see next chapter).

The example of product A illustrates the importance of analytical measurements for mixture toxicity predictions. Without measurement, the absence of the active substance IPBC would have gone unnoticed. As a result, the evidence for the presence of a relevant mixture component in the product as expressed in the MDR values based on measured concentrations would have gone unnoticed too. Such a relevant mixture component may be a formulation additive, as convincingly demonstrated by the case of product C, or a transformation product as indicated in the case of product A. Either way, the verification of the presence of active substances in the mixture toxicity test delivers clues about the presence of relevant mixture components by unravelling unexplained toxicity. Quantification and testing of transformation products and formulation additives would be the last step to fully decipher the reason of mixture toxicity underestimation and thereby exclude synergistic interactions as explanation.

### Differences between the various endpoints of acute and chronic toxicity

There was a clear tendency to increasing overestimation of mixture toxicity with regard to algal growth inhibition when predictions were based on increasingly lower effect levels ($EC_{20}$ or $EC_{10}$). This trend was true for both response variables and observed for all tested mixtures. The most extreme increase was found for product B where the MDR based on the $EC_{50}$ indicated good compliance between prediction and observation, but more than 10-fold overestimation of product toxicity based on $EC_{10}$ estimates. The same pattern was observed for the MDR values

derived in the two *Daphnia* reproduction tests with mixtures. Here, the trend was less strong with nominal MDR values indicating less than 3-fold overestimation of mixture toxicity. Hence, the correctness of the CA-prediction varies with the effect level. On a mathematical basis, this is related to non-parallel concentration-response curves, which are the pre-requisite for dose- or concentration-independent CA predictions (Finney 1978, Villeneuve et al. 2000). On a biological level, one possible explanation for overestimation of mixture toxicity at sublethal (low effect) concentrations may be that compensation reactions in the organisms add up for the individual, differently acting mixture components. For reproduction of *Daphnia magna*, less-than-additive mixture toxicity has been observed previously (Hermens et al. 1984).

Although the here determined NOEC values did not relate to a constant effect level in the employed test systems (algal growth inhibition and *Daphnia* reproduction test), the mixture toxicity prediction based on nominal NOEC value did not indicate much larger deviations between prediction and observation than the respective MDR based on $EC_{10}$ or $EC_{20}$ estimates. They tended to overestimate toxicity, but the degree was with about factor 10 at most as large as predictions based on $EC_{10}$ values (i.e., the case of product B). Hence, the here obtained results support the use of NOEC instead of $EC_{10}$ or $EC_{20}$ values for mixture toxicity predictions. Yet, they also call for care when doing so because mixture toxicity based on (no) effect estimates at sublethal (low effect) concentrations apparently tend to overestimate mixture toxicity.

### 6.4.2 Which substances should be included in the mixture toxicity prediction?

For a prospective approach, toxic units (TU) can be calculated for each substance in a mixture that may be relevant and for which toxicity estimates are available. To illustrate this approach, it was conducted here for the products and eluates with regard to the three most commonly used endpoints $E_bC_{50}$ for algal growth inhibition (yield), $EC_{50}$ for *Daphnia* acute toxicity and NOEC for *Daphnia* reproduction (Table 47). The TU calculation is based on nominal toxicity estimates for the mixture components and their nominal concentrations in the products (or eluates, as measured ahead of testing). Hence, the TU calculation is here independent of any biological testing of products or eluates (i.e., as if it were done ahead of experimental biotesting) to illustrate identification of relevant mixture components and target the testing. The relative TU presented in Table 47 indicates how much each mixture component contributes to the overall expected toxicity based on CA. All data used in these calculations were presented before in the Material & Methods section and the Results section and will not be repeated here. Hence, Table 47 is basically a repetition of already presented results and pre-information just in a different way for illustration purposes.

The sum of TU (absolute values) demonstrates that the wood preservative products are, on a volume basis, by several orders of magnitude more toxic than their eluates. The sum of TU further demonstrates that chronic *Daphnia* toxicity is the most sensitive endpoint for all products and eluates, and that it is always followed by algal growth inhibition as the second most sensitive endpoint.

Table 47: Relative Toxic Units (individual TU in % of the sum of TU) for the components of the tested products and eluates together with the sum of TU

| | Algal growth inhibition | Acute *Daphnia* toxicity | Chronic *Daphnia* toxicity |
|---|---|---|---|
| Product A (eluate) | | | |
| propiconazole | 11.9 (14.9) | 4.2 (9.0) | 81.3 (82.2) |
| IPBC | 85.6 (4.1) | 94.4 (8.0) | 18.1 (0.7) |
| cobalt | 2.5 (81.3) | 1.5 (83.1) | 0.6 (17.0) |
| sum of TU | 59,482 (2.3) | 12,902 (0.3) | 309,430 (14.8) |
| Product B (eluate) | | | |
| propiconazole | 99.2 (99.3) | 92.5 (93.5) | 1.7 (2.0) |
| fenoxycarb | 0.8 (0.7) | 7.5 (6.5) | 98.3 (98.0) |
| sum of TU | 7,787.7 (1.6) | 634.8 (0.1) | 15,899,566 (2,742) |
| Product C | | | |
| propiconazole | 0.2 | 0.2 (0.3) | 3.4 |
| fenoxycarb | < 0.01 | < 0.01 | 58.9 |
| dimethylalkylamine | 99.8 | 99.8 | 37.7 |
| sum of TU | 8,716,029 | 834,916 | 21,222,543 |
| Eluate of product C painting (dipping) | | | |
| propiconazole | 0.3 (0.4) | 0.3 (0.3) | 0.6 (0.6) |
| fenoxycarb | <0.01 (<0.01) | < 0.1 (< 0,1) | 95.1 (95.3) |
| dimethylalkylamine | 99.7 (99.6) | 99.7 (99.7) | 4.4 (4.1) |
| sum of TU | 907.5 (1,780) | 87.0 (170.6) | 19,067 (39,357) |

Shown are the TU estimates for algal growth inhibition (EbC50), acute toxicity to *D. magna* (EC50), and chronic toxicity to *D. magna* (NOEC of reproduction). Calculations are based on nominal concentrations (assuming the density of water for the products) and concentrations measured ahead of testing for the eluates together with nominal toxicity estimates, all as reported in the present study. Literature data were used for cobalt

The relative TU illustrate that in most mixtures one component dominates the overall toxicity by contributing more than 80 % of the toxicity. With regard to algal and acute *Daphnia* toxicity, this is propiconazole in product B and its eluate, but IPBC in product A and dimethylalkylamine in product C and its eluates. Cobalt contributes more than 80 % of the toxicity in the eluate of product A, but less than 5 % in the product. This difference is related to the relatively high leaching rate of cobalt. However, it must be noted that PBC is not included in these calculations as it would not be known as ingredient in such a stage of a prospective assessment. With regard to chronic *Daphnia* toxicity, fenoxycarb dominates in most cases the toxicity by contributing more than 95 %. A noticeable share of more than 10 % is only presented by dimethylalkylamine in product C (but not in the eluates), IPBC in product A and cobalt in the eluate of product A.

While it must be a regulatory decision how large the share of a mixture component to the overall toxicity must be to trigger its consideration in the risk assessment (e.g. 1, 5, or 10 %), the theoretical relative toxic unit distribution in the here investigated mixtures provides some evidence that the aquatic toxicity of wood preservative products and their eluates may typically be dominated by just one component. However, this one dominating component may differ in a given case among trophic levels and acute or chronic endpoints, and it may differ considerably between a product and its eluate as the environmentally relevant mixture.

# 7 Conclusions and outlook

The present study confirmed the predictability of the mixture toxicity of wood preservative products and their eluates by the concept of concentration addition for other organisms (i.e., *Daphnia* and green algae) than previously tested (i.e., fish embryos, Coors et al. 2011).

MDR values considerably larger than 2 indicate synergistic interaction or the need to include so-far neglected substances in the prediction. Such neglected substances may be formulation additives of either organic (dimethylalkylamine) or inorganic (cobalt) nature as demonstrated here. Organic solvents as formulation additive may in theory also influence the overall toxicity of a product by enhancing the uptake of active substances. However, in the present study there was some evidence, but no definitive proof for the occurrence of such a synergistic effect of solvents. In the context of an environmental risk assessment, it is important to consider that formulation additives, particularly ionic compounds such as cobalt, may only be relevant for the assessment after leaching from treated wood, while they may not be identified as relevant in a risk assessment solely of the product. This was illustrated in the present study for cobalt using a TU approach. The absence of IPBC and the presence of PBC demonstrated that next to formulation additives also transformation products may influence the mixture toxicity prediction. The toxicity of a product, however, will not be underestimated if the assessment is based on the nominal concentrations of the (typically more toxic) parent compound. If the mixture assessment, however, is to be based on measured concentrations it needs to be assured that toxic transformation products of active substances (and eventually relevant formulation additives) are also taken into account. Based on the present results the comparison of CA-predicted and observed product toxicity appears as a sufficient and reliable tool for the identification of formulation additives and transformation products that are relevant for the overall toxicity. Because of these product components (i.e. formulation additives and transformation products), testing of generic mixtures of technical material of active substances alone can only exclude synergistic interactions between these substances.

If all relevant formulation additives are known, theoretical CA-predictions without actual product testing appear reliable enough for usage in a risk assessment context (assuming the absence of synergistic interaction). Based on the here investigated range of products, it seems to be sufficient to consider known formulation additives, i.e., those that are labelled because of their (eco)toxicological relevance. Information about confidential formulation additives was not necessary for any of the here investigated products to explain the observed toxicity. However, the case of the underestimated *Daphnia* and fish embryo toxicity of product B may be an exception to this finding, unless the hypothesised role of organic solvents can be verified.

With regard to an environmental risk assessment, it can be concluded from the present results (in agreement with previous results) that findings of compliance with CA predictions cannot be extrapolated between trophic levels, particularly not between unicellular autotrophic and multicellular heterotrophic organisms. Hence, theoretical mixture toxicity assessment should rather be carried out within these taxonomic groups only.

It is further important to note that the substances dominating the toxicity may differ between acute and chronic endpoints as well, which is due to substance-specific acute-to-chronic ratios. This aspect was clearly illustrated by the case of fenoxycarb acting as endocrine disruptor at extremely low concentrations in the chronic *Daphnia* test, but exhibiting little acute toxicity. If

such specifically acting substances are present, the mixture toxicity of acute and chronic endpoints may therefore rather be conducted separately in the context of a risk assessment. For the assessment of chronic mixture toxicity, it should be kept in mind that CA-predictions tend to overestimate the toxicity of mixtures at low effect levels by up to about factor 10 based on the present study.

If sufficient single-substance toxicity estimates are available, the toxic unit approach exemplified here for the products and eluates may help to better identify cases where actual toxicity testing of products or eluates is needed. Even censored data (i.e., toxicity below a threshold concentration of e.g. 100 mg/l) are helpful in this context. The key drivers of toxicity (i.e., substances dominating the mixture toxicity) for the different endpoints and assessed mixtures (products or eluates) can be identified by this way. Consequently, substances may be left out from further testing or laborious fate assessments if it is established in such a toxic unit approach beforehand that they are not expected to contribute in a significant way to the overall predicted toxicity.

# 8 References

Ahlers J., Riedhammer C., Vogliano M., Ebert R.-U., Kühne R., Schüürmann G. (2006) Acute to chronic ratios in aquatic toxicity – variation across trophic levels and relationship with chemical structure. Environmental Toxicology and Chemistry 25, 2937-2945.

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 of the Federal Environment Agency (UBA). FKZ 3709 65 404.

Anonymous (2008) Draft evaluation report on fenoxycarb for use in wood preservatives (product type 8). Prepared by Syngenta Ltd.

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

Cook SR, Sullivan J, Dickinson DJ (2002) The bacterial biotransformation of IPBC. The International Research Group on Wood Preservation. 33rd Annual Meeting Cardiff, Wales.

Coors A., Dobrick J., Küster E. (2011) Development of ecotoxicological tests with biocidal products and eluates: investigating the suitability of the fish embryo test with the zebrafish (*Danio rerio*, *Dar*T) [Entwicklung von ökotoxikologischen Test mit Biozid-Produkten und Eluaten: Prüfung des Anwendbarkeit des Embryotests mit dem Zebrabärbling (Danio rerio, DarT)]. Final Project Report on behalf of the Federal Environment Agency (UBA), Germany. FKZ 360 04 029.

Coors A., Dobrick J., Möder M., Kehrer A. (2012) Mixture toxicity of wood preservative products in the fish embryo toxicity test. Environmental Toxicology & Chemistry 31(6), 1239-1248.

Coors A., Frische T. (2011) Predicting the aquatic toxicity of commercial pesticide mixtures. Environmental Sciences Europe 23:22. doi:10.1186/2190-4715-23-22.

Cousyn C, De Meester L, Colbourne JK, Brendonck L, Verschuren D, Volckaert F (2001) Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. PNAS 98, 6256-6260.

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

EC (1998) Directive 98/8/EC of the European Parliament and the Council of 16 February 1998 concerning the placing of biocidal products on the market. (OJ L 123, 24.4.1998).

ECB (2008) Technical notes for guidance in support of Annex IV of directive 98/8/EC of the European Parliament and the Council concerning the placing of biocidal products on the market. Common principles and practical procedures for the authorisation and registration of products. European Chemicals Bureau.

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

EU (2012) Regulation 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.

Finney D.J. (1978) Statistical Method in Biological Assays. 3rd edition. Charles Griffin & Company Ltd, London and High Wycombe.

FRAC (2008) Mode of action of fungicides - FRAC classification on mode of action 2007. Fungicide Resistance Action Committee. www.frac.info.

Hermens J., Canton H., Janssen P., De Jong R. (1984) Quantitative structure-activity relationships and toxicity studies of mixtures of chemicals with anaesthetic potency: acute lethal and sublethal toxicity to *Daphnia magna*. Aquatic Toxicology 5, 143-154.

IRAC (2008) Insecticide mode of action classification. Insecticide Resistance Action Committee. www.irac-online.org.

ISO (2004) Water quality – Freshwater algal growth inhibition test with unicellular green algae. ISO Guideline 8692. International Standard Organization, Paris.

Khangarot BS, Das S (2009) Acute toxicity of metals and reference toxicants to a freshwater ostracod, *Cypris subglobosa* Sowerby, 1840 and correlation to EC50 values of other test models. Journal of Hazardous Materials 172, 641-649.

Kortenkamp A., Backhaus T., Faust M. (2009) State of the art report on mixture toxicity. Report prepared under contract No. 070307/2007/485103/ETU/D.1 of the European Commission. December 2009.

Kuhl A, Lorenzen H (1964) Handling and culturing of *Chlorella*. In: Prescott DH (ed) Methods in Cells Physiology, Academic Press, New York, London.

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

Lonza (2009) Material Safety Data Sheet Barlene 12/14 containing 100% w/w C12-C16-alkyl dimethylamine. Canada Colors and Chemicals Limited, Brampton, ON.

Lotka AJ (1913) A natural population norm. Journal of the Washington Academy of Sciences, 3, 241-248.

Lupsea MO, Mathies H, Schoknecht U, Tiruta-Barna L, Schiopu N (2012) Leaching from new generation treated wood: a chemical approach. In: Brebbia CA, Aravossis K, Brooks CN, Chon By T-S (eds.) Environmental Impact. Ecology and the Environment 162.

OECD (2004) OECD Guidelines for the testing of chemicals, OECD Guideline 202, *Daphnia sp.*, Acute Immobilisation Test.

OECD (2007) OECD Guidelines for the testing of chemicals, OECD Guideline 313, Estimation of Emissions from Preservative - Treated Wood to the Environment: Laboratory Method for Wooden Commodities that are not Covered and are in Contact with Fresh Water or Seawater.

OECD (2008) OECD Guidelines for the testing of chemicals, OECD Guideline 211, *Daphnia magna* Reproduction Test.

OECD (2011) OECD Guidelines for the testing of chemicals, OECD Guideline 201, Freshwater Alga and Cyanobacteria, growth inhibition test.

R Development Core Team (2010) R: A Language and Environment for Statistical Computing. Version 2.11.1. R Foundation for Statistical Computing, Vienna, Austria.

Ritz C., Streibig J.C. (2005) Bioassay Analysis using R. J. Statist. Software 12. Version 1.9-6. Obtained from author in June 2010.

Rose RM, Warne MStJ, Lim RP (2002) Food concentration affects the life history response of *Ceriodaphnia cf. dubia* to chemicals with different mechanisms of action. Ecotoxicol Environ Safe 51, 106-114.

SC (2007) Final assessment report on propiconazole (PT 8) in view of its inclusion in Annex I to Directive 98/8/EC. Standing Committee on Biocidal Products. 29 November 2007.

SC (2008) Final assessment report on IPBC (PT 8) in view of its inclusion in Annex I to Directive 98/8/EC. Standing Committee on Biocidal Products.

Schoknecht (2010) Leaching tests with wood preservatives on the basis of OECD 313 and quantitative determination of propiconazole, IPBC and fenoxycarb according to BAM-test procedures. Test Report BAM Federal Institute for Materials Research and Testing Division IV.1 on behalf of Federal Environment Agency Germany, FKZ 363 01 275. Berlin, 07 July 2010.

Singer M.M., Aurand D., Bragin G.E., Clark J.R., Coelho G.M., Sowby M.L., Tjeerdema R.S. (2000) Standardization of the preparation and quantification of water-accommodated fractions of petroleum for toxicity testing. Mar. Pollut. Bull. 40, 1007-1016.

Tomlin CDS, ed. (2008) The e-Pesticide Manual. 14th Edition. British Crop Protection Council.

Villeneuve D.L., Blankenship A.L., Giesy J.P. (2000) Derivation and application of relative potency estimates based on in vitro bioassay results. Environmental Toxicology and Chemistry 19, 2835-2843.