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STUDIES ON COMBINATION EFFECTS OF ENVIRONMENTALLY RELEVANT TOXICANTS

Validation of prognostic concepts for assessing the algal toxicity of realistic aquatic pesticide mixtures

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This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I. Junghans, M., Backhaus, T., Faust, M., Scholze, M., Grimme, L.H. (2003) Predictability of combined effects of eight chloroacetanilide herbicides on algal reproduction. Pest Manag. Sci., 59 (10) 1101-1110.

II. Junghans, M., Backhaus, T. Faust, M., Scholze, M., Grimme, L.H. (2003) Toxicity of sulfonylurea herbicides to the green alga *Scenedesmus vacuolatus*. Bull. Environ. Contam., 71 (3) 585-593

III. Junghans, M., Backhaus, T., Faust, M., Meyer, W., Scholze, M., Grimme, L.H. (2004) Predicting the joint algal toxicity of chemical mixtures using a mechanism based two stage prediction (TSP). Manuscript

IV. Junghans, M., Backhaus, T., Faust, M., Scholze, M., Grimme, L.H. (2004) Application and validation of predictive approaches for realistic pesticide mixtures. Manuscript

Denken heißt vergleichen

Walther Rathenau

Note by the author

This thesis is based on four publications of which only two (publications I and II) were published when I submitted the thesis. Hence, the finally published versions of publications III and IV will most probably differ in some aspects from the versions published in this thesis. Thus, the interested reader is kindly asked to look also for the published papers, e.g. in the web of science.

In this context it has also be mentioned that the version of publication III that is included in this thesis has not yet been reviewed by my co-authors. Thus, every inconsistency or error is to be totally blamed on myself.

Abstract

Surface waters are frequently contaminated with several potentially hazardous substances. Hence, aquatic organisms are often exposed to mixtures of toxicants. This thesis aims to analyse whether the joint toxicity of environmentally relevant toxicants can be predicted based on the known toxicity of the single mixture components. Two concepts have been successfully applied so far for the prediction of mixture toxicity: *concentration addition* (*CA*) for mixtures of similarly acting substances and *independent action* (*IA*) for mixtures of dissimilarly acting substances. In environmentally relevant mixtures though, mechanism of action patterns are more complex. Moreover, for many environmentally relevant toxicants the mechanisms of action are unknown. The results of this thesis show, that if (*i*) the mechanisms of action of the mixture components are known and (*ii*) the components can be assigned to discrete mechanism of action groups, the combination of *CA* and *IA* to a two-stage prediction (*TSP*), allows for accurate predictions. The application of the *TSP* on a realistic pesticide mixture led to accurate predictions of the experimentally observed mixture toxicity. However, the application of *CA* and *IA* also led to good mixture toxicity estimations, since the quantitative differences between the predictions according to *CA*, *IA* and *TSP* were rather small. To analyse the quantitative difference between *IA* and *CA* predictions for realistic other exposure scenarios, a mathematical formula was developed, which allows for determining the maximal possible ratio between the *EC50* predicted by *IA* and the *EC50* predicted by *CA*. For this calculation, only the knowledge of the mixture ratio and the *EC50* values of the mixture components are needed. The application of this formula on published agricultural exposure scenarios indicates that small differences between both predictions are rather the rule than an exemption for realistic pesticide mixtures. Two conclusions were drawn: (*i*) the combination of *CA* and *IA* to a *TSP* is a valid approach, which provides accurate predictions of the mixture toxicity in case that the components can be assigned to discrete mechanism of action groups. (*ii*) Even in case that mechanistic knowledge of the mixture components is not provided, the use of *CA* as a precautionary default assumption is a justifiable approach for estimating the mixture toxicity of realistic pesticide mixtures.

Abbreviations and Symbols

BEAM	**B**ridging **E**ffect **A**ssessment of chemical **M**ixtures to ecosystem situations and regulation - research project financed by the commission of the European Communities
CA	concentration addition
CA group	group in which the components share a similar mechanism of action; used in the *TSP* approach
CAS RN	chemical abstracts registry number
c_i	concentration of the individual mixture component *i*
CI	statistically estimated confidence interval
c_{mix}	mixture concentration
CRC	concentration response curve
E	Effect
e^-	electron
$E(c_i)$	effect caused by the concentration of an individual mixture component *i*
$E(c_{mix})$	effect caused by the mixture concentration
ECx_i	effect concentration for a given effect level *x* of the individual mixture component *i*
ECx_{mix}	effect concentration of the mixture for a given effect level *x*
eqn.	Equation
fig.	Figure
HPLC	high performance liquid chromatography
IA	independent action
K*ow*	partition coefficient between octanol and water
LOEC	lowest observed effect concentration
mM	Millimole
MW	molecular weight
NOEC	no observed effect concentration
PEC	predicted environmental concentration
p	Product
p_i	relative proportion of individual mixture component *i* ($p_i = c_i / c_{mix}$)
PNEC	predicted no effect concentration
QSAR	quantitative structure-activity relationship
r	coefficient denoting the correlation of susceptibilities of the test-organisms towards the single mixture components
rp-HPLC	reversed phase high performance liquid chromatography
S	Sum
SSD	species sensitivity distribution
tab.	Table
TSP	two stage prediction
TU	toxic unit
US EPA	environmental protection agency of the United States of America
Y_i	effect of a *CA* group in the *TSP*

Contents

1 Introduction

As monitoring studies reveal, aquatic water bodies are frequently contaminated with a multitude of potentially hazardous substances (e.g. European Commission, 1999; Battaglin and Fairchild, 2002). Accordingly, aquatic organisms are often exposed to contaminant mixtures. However, for the majority of contaminant mixtures eco-toxicity data are available only for single substances.

As a toxicity assessment for every potentially occurring mixture is not feasible, the need arises to estimate its toxicity from toxicity data of the single mixture components. Several studies on the toxicity of multi-component mixtures have revealed that the observed effect of the mixture is usually higher than the highest single effect elicited by the mixture components (e.g. Koenemann, 1980; Altenburger *et al.*, 2000; Silva *et al.*, 2002). Moreover, even mixtures in which each component would cause an effect of only 1% if applied singly led to clear mixture effects (Backhaus *et al.*, 2000b; Faust *et al.*, 2001; Faust *et al.*, 2003). Thus, regarding only the toxicity, which the components would cause if applied singly, may lead to a severe underestimation of the toxicity actually caused by the mixture. However, the above-mentioned studies have also shown that the mixture toxicity can be predicted.

For this purpose, two pharmacological concepts have been adopted in ecotoxicology: *concentration addition* (*CA*) for mixtures of similarly acting substances (Loewe and Muischnek, 1926) and *independent action* (*IA*) for mixtures of dissimilarly acting substance (Bliss, 1939).

The question how wide or how narrow the term similarity respectively dissimilarity of action has to be defined to allow for accurate predictions, has been a matter of discussion (Berenbaum, 1985b; Pöch, 1993). Undebated though, is that mixture toxicity can only be observed, if both substances singly cause an effect for the same studied toxicological endpoint (Loewe and Muischnek, 1926). When defining the term mechanism as a certain crucial biochemical process and/or a xenobiotic-biological interaction, both concepts were shown to provide accurate predictions if the assumptions about the mechanisms of action of the mixture components were met (an overview is given in chapter 2.2).

However, two problems hamper the use of these concepts for the prediction of the toxicity of realistic mixtures: (*i*) the knowledge of the components' mechanisms of action is sometimes lacking and (*ii*) if the knowledge exists, the components' mechanisms of action are in most cases neither strictly similar nor strictly dissimilar and hence do not fulfil the prerequisites of either concept.

In case that the mechanisms of action of the mixture components are known, a way out of this dilemma might be to assign the mixture components to discrete groups according to their mechanism of action. This approach has been introduced by Ankley and Mount (1996), who suggested to subsequently analyse the mixture toxicity of every mechanism of action group by using *CA*. Since it has recently been shown that *IA* accurately predicts the mixture toxicity of multi-component mixtures of dissimilarly acting substances (Backhaus *et al.*, 2000a; Faust *et al.*, 2003), it seems to be reasonable that the overall toxicity may then be predicted by using *IA*, provided that the mechanisms of action between the groups are strictly dissimilar.

In fig. 1.1 this approach is visualized. Due to its stepwise character, it will be called *two-stage prediction* (*TSP*). As can be observed, the *TSP* combines *CA* and *IA* in two stages. In stage 1 of a *TSP,* the mixture components are clustered into groups, in which the mechanisms of action of the components are strictly similar. This grouping is based on *CA*, which assumes that similarly acting substances act like being dilutions of the same substance. Accordingly, the joint effect, which the components of each *CA* group elicit at their common receptor site (R), is then predicted by *CA*. The groups will thus be called *CA* groups for the remainder of the thesis. In stage 2 of a *TSP*, *IA* integrates over all predicted effects ($E_{CA}(R_i)$) of the *CA* groups to predict the integral effect of the mixture. The *TSP* would reduce to *CA* if only one *CA* group existed and to *IA* if every *CA* group consisted of only one mixture component.

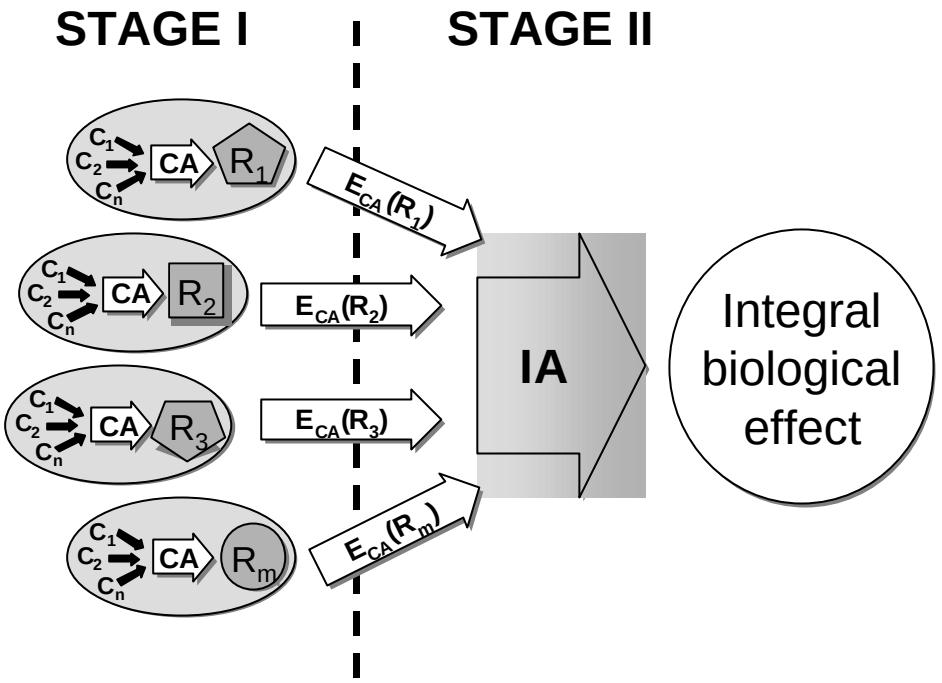

Figure 1.1 The stepwise approach of the *Two Stage Prediction* (*TSP*). *CA* = *concentration addition*; *IA* = *independent action*. C_{1-n} are individual mixture components that exclusively act on one of the distinct receptors (R_{1-m}); $E_{CA}(R_{1-m})$ represents the integral effect the substances C_{1-n} cause through interaction with one of the distinct receptors R_{1-m}, as predicted by *CA*

The validation of the *TSP* approach for the prediction of the mixture toxicity of environmentally relevant toxicants was the first aim of this study. As an example for environmentally relevant toxicants, pesticides were selected. Two reasons led to this selection: (*i*) pesticides are frequent contaminants in surface waters (European Commission, 1999) and (*ii*) knowledge on the mechanism of action is available for most substances. Being a representative of ecologically relevant primary producers, a freshwater alga was selected for all toxicity testing in this study.

The second aim of this study was to validate all three approaches, *CA*, *IA*, and *TSP*, for toxicity predictions for a realistic pesticide mixture. The validity of *CA*, *IA*, and *TSP* for predicting the toxicity of realistic pesticide mixtures was analysed according to two aspects: (*i*) the mechanistic accuracy of the prediction and (*ii*) the value for the use in risk assessment procedures.

The following chapter gives an introduction to concentration-response analysis, mixture toxicity research as well as a brief introduction to the principles of environmental risk assessment procedures. Subsequently the conceptual approach as well as the study design is introduced. Finally, the most important results of this

thesis will be discussed. Scientific publications that are part of this thesis can be found in the appendix and are referred to by their roman numerals.

2 Scientific Background

2.1 Concepts for the prediction of mixture toxicity

Mixture toxicity research dates back to the 19th century but generally Loewe and Muischnek (1926) are referred as the first that introduced a model for the similarly acting substances, and Bliss (1939) as the first for dissimilarly acting substances. Below, the basic models for both concepts are given. In both models no interaction between the mixture components is assumed, *i.e.* it is assumed that neither component does influence the biological action of any other component (Hewlett and Plackett, 1959). Early mixture toxicity research focused on binary mixtures. For the objective of this thesis though, *i.e.* the prediction of mixture toxicity of environmentally relevant mixtures, multi-component mixtures have to be considered. Hence, this thesis will focus on mixture toxicity research with multi-component mixtures. Binary mixture models for both concepts can be found in Bliss (1939).

2.1.1 Concentration addition

As mentioned above, *CA* was introduced to describe the joint toxicity of similarly acting toxicants. It assumes that the mixture components have a common mechanism of action and act at the same molecular target site. The components are assumed to differ only in the relative strength of their toxic effect, like being dilutions of the same substance. Because of the similarity of action, parallelism of the mixture components was regarded as a prerequisite for concentration additive mixture toxicity (Bliss, 1939). However, this assumption was later skipped by Hewlett and Plackett (1959). They argued that even though two chemicals may act in the same way at the same site of action, they might differ in terms of uptake, partitioning and accumulation, binding to unspecific sites, and biotransformation, resulting in concentration-response curves, which may differ in shape and slope.

As the first who extended *CA* for the prediction of multi-component mixtures, Berenbaum (1985a) is usually named. He formulated *CA* for a mixture of *n* substances as:

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1 \qquad [2.1]$$

where ci are the concentrations of the individual components in the mixture and ECx_i is the concentration of the *ith* component that individually would cause the same quantitative effect x as the mixture. The quotients c_i/ECx_i have been termed toxic units (*TUs*) (Sprague, 1970). They represent the concentrations of mixture components as fractions of equi-effective individual concentrations. If *CA* holds, any substance can be substituted by an equally potent concentration of another substance without altering the overall effect.

2.1.2 Independent action

The alternative concept, *IA* is based on the assumption that the mixture components cause a common integral biological effect (e.g. death) through primary interaction with different molecular target sites. *IA* was extended for multi-component mixtures of n substances by Backhaus *et al.* (2000a) and in more detail by Faust *et al.* (2003) to:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \quad \text{with } c_{mix} = c_1 + \ldots\ldots + c_n \qquad [2.2]$$

in which c_i denotes the concentrations of the *i*th mixture component, $E(c_i)$ its corresponding effect and $E(c_{mix})$ the total effect of the mixture.

When introducing the *IA* model for binary mixtures, Bliss (1939) additionally introduced a coefficient to account for possible correlations of susceptibilities of the individual organisms from the population towards the mixture components. This coefficient, usually referred to as *r*, was postulated to may take values between 1 (complete correlation of susceptibilities) and 0 (absence of any correlation). Hewlett and Plackett (1959) later extended this approach to *r*-values ranging from +1 to -1 to account also for complete negative correlation. The limiting cases +1 and -1 have been termed no addition (Koenemann, 1980) and effect summation (Boedeker *et al.*, 1992) respectively. For no addition, the predicted effect of the mixture equals the highest single effect of its components, whereas for effect summation the mixture effect equals the simple algebraic sum of all components' single effects. This extension of the basic *IA* equation (eqn. 2.2) has been formulated originally for binary mixtures. An extension to multi-component mixtures is difficult for two reasons: (*i*) for multi-component mixtures many possible patterns of correlation may exist, and (*ii*) a scientifically sound

assumption for *r* has to be available prior to the predictive toxicity assessment of every individual mixture.

For environmentally relevant mixtures, a scientifically sound assumption for *r* is typically not available *a priori*. Hence, in this thesis, the focus was on the basic form of *IA* (r=0) as given in eqn. [2.2], when assessing its predictive value. When referring to *IA*, it is always meant in the sense of eqn. [2.2].

2.1.3 Quantitative relationship between predictions according *CA* and *IA*

The quantitative relationship between *CA* and *IA* predictions was subject to several studies. Drescher and Boedeker (1995) have shown that *IA* may predict a higher, an equal or a lower toxicity than *CA,* depending on four factors: the steepness of the concentration response curves, the regression model used for their estimation, the effect level under consideration, and the concentration ratio. For multi-component mixtures, Faust (1999) has added the number of mixture components as an influencing factor. In a theoretical study, which was designed to investigate the maximal quantitative difference between *CA* and *IA* predictions, he demonstrated that the ratio between the effect concentration for a given effect level *x* predicted by IA (ECx_{IA}) and the effect concentration predicted by *CA* for the same effect level (ECx_{IA}) will always be equal or lower than the number of mixture components *n*. Furthermore, he showed that this ratio would always be lower than *n* if the mixture components are not mixed according to equal ECx_i values (often referred to as equi-toxic mixture ratio). Thus, in case that *CA* gives higher mixture toxicity estimates than *IA*, the quantitative difference will maximally amount to *n*. For the opposite case, *i.e. IA* predicts a higher toxicity than *CA*, no theoretical limit exists (Faust, 1999).

2.2 Predictive value of CA and IA for multi-component mixtures

Both *CA* as well as *IA* has shown to provide accurate predictions of the mixture toxicity of multi-component mixtures. During the 1980ies and 1990ies, the mixture toxicity of several multi-component mixtures of organic chemical was successfully predicted by using *CA* (Koenemann, 1980; Koenemann, 1981; Hermens *et al.*, 1984; Nirmalakhandan *et al.*, 1997). Later, *CA* was also shown to accurately predict the mixture toxicity of a group of similarly acting pesticides (Faust *et al.*, 2001), of

protonophoric uncouplers (Altenburger *et al.*, 2000), as well as of gyrase-inhibiting pharmaceuticals (Backhaus *et al.*, 2000b).

For multi-component mixtures of dissimilarly acting substances, *IA* was recently shown to provide accurate predictions: for a mixture of 14 substances in a bio-test with *Vibrio fischeri* (Backhaus *et al.*, 2000a), as well as for a mixture of 16 substances in a bio-test with the green alga *Scenedesmus vacuolatus*.

The studies, in which predictions according to both concepts were made, revealed that (*i*) *IA* usually predicts the lower toxicity than *CA* and (*ii*) the predictive quality of either concept is usually lower, if the model assumptions concerning the mechanisms of action were not met.

However, a recent study on the mixture toxicity of phenylureas has shown that *CA* and *IA* may also lead to predictions, which are quantitatively identical (Backhaus *et al.*, 2004): although the mixture components were all assumed to share a similar mechanism of action, the observed toxicity was also in compliance with the *IA* prediction. Hence, the compliance with a prediction is no conclusive argument in itself. In conjunction with non-conformity to *IA* though, the compliance of the observed mixture toxicity with the *CA* prediction may be taken as a strong indicator for a common mechanism of action of the mixture components.

2.3 Principles of environmental risk assessment

In environmental risk assessment, the risk of the occurrence of adverse effects in the environment should be assessed, which are due to the presence of a certain substance. After the initial identification of a hazard, *i.e.* the adverse effects a substance may inherently cause, a risk assessment divides into two parts: the exposure assessment, *i.e.* the derivation of predicted environmental concentration (PEC values), and the effects assessment, *i.e.* the estimation of the relationship between the level of an exposure to a substance, as well as the incidence and severity of the effects (van Leeuwen and Hermens, 1995).

For the effects assessment, the substance under consideration is usually tested in a set of bio-tests. For chemicals that entered the European market after 1981 the effects assessment for the aquatic environment is usually done with short-term toxicity data from algae, daphnids and fish (EU Commission, 2003). The quantitative measures resulting from an effects assessment are *ECx* values, *i.e.*

concentrations that elicit a certain quantitative effect (usually from short term tests), or concentrations which were the highest tested concentration for which no statistically significant effects could be determined in a certain bio-test, the no observed effect concentration (NOEC, usually denoting a long term test). The knowledge of the whole concentration-response function is usually not provided.

Based on these values a concentration is determined below which unacceptable effects on organisms will most likely not occur, the so-called predicted no effect concentration (PNEC) (EU Commission, 2003). Depending on the data availability, the PNECs are established by dividing the toxicity value obtained for the most sensitive species by an arbitrarily chosen factor, which should account for the uncertainty connected to the effects assessment. For every defined source of this uncertainty, usually a factor of ten is applied. These sources are (*i*) the intra- and inter-laboratory variation of toxicity data, (*ii*) differences in species sensitivity, (*iii*) short-term to long-term extrapolation, and (*iv*) laboratory to field extrapolation. The possibility of the occurrence of mixture toxicity in the field is not taken into account.

At the end of a risk assessment a so-called risk quotient is established which gives the ratio between the PEC and the PNEC. The risk quotient serves as a basis for the decision whether a substance presents a risk to organisms in the environment or not (EU Commission, 2003).

3 Conceptual Approach

The study was divided into two parts: (*i*) the experimental validation of the *TSP* and (*ii*) the evaluation of the predictive quality of all three approaches for the toxicity of an agricultural exposure scenario.

For the experimental validation of the *TSP* approach groups of substances were established for which (*i*) a similar mechanism of action within each substance group can be assumed which (*ii*) is different to the mechanism of action of the components of each other mixture. This approach corresponds to the conceptual idea of the *TSP*, which assumes that the mixture components can be assigned to discrete groups in which a similar mechanism of action is shared.

Based on these criteria substances from 5 chemical classes were selected: 18 triazines, 8 chloroacetanilides, and 8 sulfonylurea, all being groups of frequently applied herbicides, as well as 6 quinolones which have found widespread use in aquaculture as antibiotics and 3 quaternary ammonium compounds, being cationic surfactants which may enter the surface water body through household effluents. The last two non-pesticidal substance groups were selected to increase the total number of groups in mixtures composed for the validation of the *TSP*.

To verify the assumed concentration additive mixture toxicity within the groups, single substance as well as mixture toxicity assessments were performed for each group. A subsequent comparison between observed and predicted mixture toxicity according to *CA* and *IA* served as a decision criterion: In conjunction with non-conformity to *IA,* the compliance of the observed mixture toxicity with the *CA* prediction may be taken as a strong indicator for a common mechanism of action of the mixture components. Thus, the tested subgroup mixtures were not only analyzed for compliance with *CA*, but also for their relationship to predictions according to IA. According to this approach, the concentration additive mixture toxicity was analysed for chloroacetanilides (publication I) and sulfonylurea (publication II). Single substances' as well as mixture toxicity data of triazines, quinolones and quaternary ammonium compounds were kindly provided by Dr. Michael Faust, Dr. Thomas Backhaus, and Dipl. Biol. Wiebke Meyer, respectively. Single substances' toxicity data of quinolones and quaternary ammonium compounds is given in appendix 4 of publication III. Data on the single substance' as well as mixture toxicity of 18 triazines has already been published by Faust *et al.*

(2001). Based on these 43 components multi-component mixtures were composed and experimentally tested. Finally, the validity of the *TSP* approach was analysed by comparing the observed toxicity with predictions according to *TSP*, as well as with predictions according to *CA* and *IA* (publication III). Validity criteria were (*i*) accuracy of the *TSP* prediction and (*ii*) higher predictive power than both *CA* and *IA*.

All three approaches, *CA*, *IA*, and *TSP*, were applied for the prediction of the algal mixture toxicity of a modelled agricultural exposure scenario, which reflects the median load of pesticides in field run-off in Central-European agricultural areas after pre-emergency treatments in spring. A list of the scenario components as well as their modelled concentration within the scenario (tab. 1 in publication IV) was kindly provided by Dr. Antonio Finizio and Prof. Dr. Marco Vighi, who did the modelling (Finizio *et al.*, 2004a; Finizio *et al.*, 2004b). The validity of *CA*, *IA*, and *TSP* for predicting the toxicity of realistic pesticide mixtures was analysed according to two aspects: (*i*) the mechanistic accuracy of the prediction and (*ii*) the value for the use in risk assessment procedures.

In all experimental toxicity assessments, the inhibition of reproduction of the freshwater chlorophyte *Scenedesmus vacuolatus* was taken as toxicity parameter.

4 Study Design

4.1. Algal bio-test with Scenedesmus vacuolatus

The selected bio-test had to fulfil four testing requirements to allow for unambiguous results concerning the comparative evaluation of predictive qualities for environmentally relevant toxicant mixtures: (*i*) an *integral toxicity parameter* (*ii*) the testability of substances with *diverse physico-chemical features*, (*iii*) a *high test capacity*, and (*iv*) a rather *low variability* as well as a rather *high reproducibility*. An *integral toxicity parameter* was mandatory because of the mechanistic heterogeneity of mixtures of environmentally relevant toxicants. Furthermore, an *integral toxicity parameter* is needed because *IA* is based on the assumption that the mixture components cause a common integral effect through interaction with different target sites, hence, without allowing for the occurrence of independent action, predictions according to *IA* as well as *TSP* become obsolete. The testability of substances with diverse physico-chemical features is required because of the validation of the predictive approaches for a realistic exposure scenario: in realistic mixtures, the components can be assumed to have diverse physico-chemical properties. For allowing accurate mixture toxicity predictions for mixture effects = 1% a *high test capacity*, a *low variability* as well as a *high reproducibility* of the test results is required.

All experimental single substance as well as mixture toxicity experiments were performed with an algal bio-test using the freshwater chlorophyte *Scenedesmus vacuolatus* (strain 211-15, culture collection of the University of Göttingen, Germany) as test organism (formerly *Chlorella fusca* var. *vacuolata*: Kessler *et al.*, 1997). All four requirements were met for this test, and it was successfully applied in mixture toxicity studies before (Altenburger *et al.*, 1990; Faust *et al.*, 2001). It allows also for the testing of volatile or foaming substances. Toxicity parameter was the inhibition of reproduction in synchronously growing cultures over one generation cycle (24h).

4.1.1 Culture conditions

Stock cultures of *Scenedesmus vac.* were photoautotrophically grown at 28°C, in an inorganic growth medium (tab. 4.1). Under a light/dark regime of 14:10, the cells were synchronised according to procedures described by Altenburger *et al.*

(Altenburger *et al.*, 1990). Illumination occurred with saturating white light (Lumilux Daylight L36 w/11 and Lumilux interna L36 w/41, Osram, Berlin, Germany) with an intensity of 22-33 klux at the surface of the tubes.

Table 4.1 Algal growth medium (modified after Grimme and Boardman, 1972) and medium with tenfold increased phosphate buffer capacity as used in publication IV

	algal growth medium	algal growth medium with 10fold increased buffer capacity
Substance	concentration (mM)	concentration (mM)
KNO_3	8.00	8.00
NaCl	7.00	7.00
$NaH_2PO_4 * H_20$	3.00	9.64
$Na_2HPO_4 * 2\ H_20$	1.00	9.17
$MgSO_4 * 7\ H_20$	1.00	1.00
$CaCl_2 * 2\ H_20$	1.00E-01	1.00E-01
H_3BO_3	8.00E-03	8.00E-03
$MnCl_2 * 4\ H_20$	2.50E-03	2.50E-03
ZnSO4 * 7 H20	6.90E-04	6.90E-04
FeSO4 * 7 H20	2.00E-05	2.00E-05
Na2-EDTA[a]	2.00E-05	2.00E-05
(NH4)2Mo7O24 * 4 H20	1.60E-05	1.60E-05

[a] Titriplex III

4.1.2 Test conditions

The test was performed as described by Faust *et al.* (2001). At the start of toxicity testing, the test culture consisted of a homogeneous population of autospores (young algal cells at the beginning of a growth cycle). Tests were performed in sterilized glass tubes (20x100 mm) with round bottom and a Teflon-lined screwcap. At the beginning of the test (t_0), each tube contained the dissolved test-substance/mixture, an algal suspension (initial cell density $7.5x10^4$ cells/ml), as well as a Teflon-coated magnetic stirring bar. The total volume was 15 ml (without the stirring bar). For CO_2 supply additionally 150µl of an aqueous $NaHCO_3$ solution were added (final concentration: 1.5 mM). Cultures were incubated for 24h under the same conditions as the stock cultures. Sedimentation was avoided by gentle stirring. After the addition of $NaHCO_3$, the initial pH was 6.7, and remained stable during the test: an increase of pH caused by the assimilation of NO_3^- did not exceed 0.2 units.

At the end of the generation cycle (t_{24}) the resulting cell number (CN) was measured (CN_{t24}) and the difference (?CN) to the initial value (CN_{t0}) was calculated: $?CN = CN_{t24} — CN_{t0}$. The inhibition of algal reproduction was calculated by normalising the data from treated cultures ($?CN_{treated}$) to the mean value of untreated controls ($?CN_{control}$):

$$\% \text{ Inhibition of algal reproduction} = (1- ?CN_{treated} / ?CN_{control}) \times 100.$$

Control reproduction yielded average factors of 12, *i.e.* the individual autospores grew up to mother cells and released either 8 or 16 daughter cells at the end of the growth cycle. Cell numbers were determined by means of electronic particle counting (Coulter Electronics, Miami, USA).

4.1.3 Adjustment of the test conditions for 2.4-D, MCPA, and Clopyralid (publication IV)

In the agricultural exposure study (publication IV), the acids MCPA, 2,4-D, and clopyralid were tested in algal medium with an elevated phosphate buffer capacity (tab. 3.1). This was done to allow for a similar pH value between treated and untreated cultures. The high phosphate concentration led to a small decrease in the reproduction of the untreated controls. Hence, all other substances as well as the mixtures were tested in standard medium. The latter was possible, because the concentrations of MCPA, 2,4-D and clopyralid in the mixture were low, so that the pH during mixture tests was not affected.

4.2 Prediction of mixture toxicity

CA predictions were calculated according to the following equation, which can be derived from eqn. [2.1] by rearrangement

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \qquad [4.1]$$

with p_i being the concentration c of the ith component expressed as relative proportion of the total concentration cmix ($p_i = c_i / c_{mix}$) (Faust *et al.*, 2001).

Predictions according to *IA* were based on eqn. [2.2] and were calculated as described by Backhaus *et al.* (2000a) and in more detail by Faust *et al.* (2003).

TSP predictions were calculated as described in section 2.5 in publication III.[1]

4.3 Tested mixtures

The toxicity of all tested mixtures was analysed according to a fixed ratio design, *i.e.* the molar ratio of the mixture components remained constant, whereas the total mixture concentration was varied. The fixed ratio design was selected, because it allows both, a comparison of the observations with the predictions according to *CA* and *IA* and the statistical estimation of the uncertainty connected to observations and prediction. It has to be noted that statistical uncertainty connected to *TSP* predictions cannot be estimated so far.

4.3.1 Part 1: mixtures to validate the two-stage prediction approach

Mixtures of similarly acting substances

Five groups of substances were established for which it was assumed that the components within each group inhibit the reproduction of *Scenedesmus vac.* with the same mechanism of action: 8 chlorocetanilides, 8 sulfonylureas, 18 *s*-triazines, 6 quinolones, and 3 quaternary ammonium compounds.

The groups have dissimilar mechanisms of action, whereas within these groups the members act strictly similarly. *s-Triazines* inhibit the photosynthetic e^- transport by competitively binding to the same domain of the D1 protein of photosystem II as the e^- acceptor plastoquinone Q_B (Bowyer *et al.*, 1991; Tietjen *et al.*, 1991). For *chloroacetanilides,* the mechanism of action had been recently confined to the inhibition of the synthesis of very long chain fatty acids (Böger *et al.*, 2000). *Sulfonylureas* are reported to inhibit the acetolactate synthase, which is the first common enzyme of branched chain amino acids (Blair and Martin, 1988). *Quinolones* are antibiotics known to inhibit the A-subunit of the DNA gyrase, a protein that introduces negative superhelical twists into the DNA strands of bacteria (Bryan *et al.*, 1989). The recent detection of a gyrase-like protein in plants (Gadelle *et al.*, 2003) may explain the finding that *quinolone* toxicity in algae is higher than the unspecific effect that could be expected to result from hydrophobicity-driven accumulation in cellular membranes alone (Backhaus *et al.*, 2001). *Quaternary ammonium compounds* are cationic surfactants containing a polar headgroup and a hydrophobic tail (in our case C10-C14 alkyl chains). They

[1] TSP predictions given in publication III were calculated by the mathematician Martin Scholze

accumulate at surfaces and interfaces, lowering the surface and interfacial free energies (Versteeg *et al.*, 1997), and are able to solubilize phospholipids in membranes.

For all groups of substances concentration additive mixture toxicity was assumed. In order to confirm this assumption, experimental mixture toxicity studies were performed for every group. All groups were tested in the mixture ratio of the individual components' *EC50* values. For chloroacetanilides, sulfonylureas, and triazines the components were additionally tested in a second mixture ratio: *EC05* (chloroacetanilides and sulfonylureas) respectively *EC01* (triazines).

For chloroacetanilides (publication I), sulfonylureas (publication II), triazines (Faust *et al.*, 2001), and quinolones (Backhaus *et al.*, unpublished results) the concentration additive mixture toxicity was confirmed: *CA* predicted the mixture toxicity in every tested mixture ratio accurately, whereas the predictive power of IA was lower. For quaternary ammonium compounds the results of the mixture toxicity experiments could not be taken as a confirmation for *CA*. Due to a rather high variability of the test results as well as a rather low quantitative difference between *CA* and *IA* predictions, the superior predictive power of *CA* could not be demonstrated (Meyer *et al.*, unpublished results). However, due to their high structural similarity, (the three substances were part of a congeneric series), similarity of action can be assumed.

Mixtures to validate the TSP approach

Two mixtures composed of similarly as well as dissimilarly acting substances were tested. The first mixture (*mixture 1*) was composed only of the groups of substances for which concentration additive mixture toxicity was experimentally demonstrated: *triazines*, *chloroacetanilides*, *sulfonylureas*, and *quinolones*.

This 40-component mixture was tested in two different mixture ratios. One mixture ratio was tailored to reflect the *TSP* approach (fig. 2). According to stage II of the *TSP* (eqn. 6 in publication III) every group has to cause an effect of 15.9%, if all groups are intended to contribute equally to the predicted *EC50* of the mixture: $50\%=0.5=1-[(1-\text{effect})^4]$. Thus, for the remainder of the thesis this mixture ratio will be called "*EC15.9* ratio of *CA* groups". An illustration of this mixture ratio is given in publication III (fig. 2). Within the *CA* groups, the components were present

in the ratio of their *EC50* values. The exact mixture ratio is given in publication III (tab. 1).

In the other ratio of *mixture 1*, the components were present in the ratio of their individual *EC01* values (for *EC01* values please refer to annexes 1-4 in publication III). The *EC01* mixture ratio was selected for two reasons. One reason is that for the 40 selected substances the quantitative difference between *CA* and *IA* predictions is almost maximal in this mixture ratio[2]: the *EC50* predicted by *IA* is by a factor of 4 higher than predicted by *CA* and all three predictions are well discriminable for effect levels ranging from 10 to 100%. The other reason is that it allows the evaluation of mixture effects from low individual concentrations, which would not cause a significant effect if applied singly: *EC01* values for all mixture components were equal to or smaller than the respective NOEC (annexes 1-4).

The second mixture (*mixture 2*) is a mixture composed of 38 components in the ratio of their individual NOECs. This mixture additionally comprised the group of *quaternary ammonium compounds*. Five substances included in *mixture 1* were lacking in *mixture 2*: chlorsulfuron, pretilachlor, and triasulfuron because of undetermined *NOEC* values; norfloxacin and terbutryn because their aqueous stock solutions were not available at the beginning of the mixture experiment. For NOEC values please refer to annexes 1-4. Like the *EC01* ratio in *mixture 1* the NOEC ratio was selected for the evaluation of the mixture toxicity from low concentrations, which would not cause a significant effect, if applied singly.

4.3.2 Part 2: mixture from an agricultural exposure scenario

To validate *CA*, *IA*, and *TSP* for the prediction of the mixture toxicity of a realistic exposure scenario, a mixture was tested which was composed according to a scenario which was modelled to reflect the median load of pesticides in field run-off in central-European agricultural areas after pre-emergency treatments in spring. A list of the scenario components as well as their modelled concentration within the scenario (tab. 1 in publication IV) was kindly provided by Dr. Antonio Finizio and Prof. Dr. Marco Vighi (Finizio *et al.*, 2004a; Finizio *et al.*, 2004b).

[2] simulations for determining the mixture ratio for which the quantitative difference between CA and IA predictions becomes maximal were performed by the mathematician Martin Scholze, which I gratefully acknowledge

Concentration-response analysis[3] was performed as described in the respective publication (publications I-IV and Faust *et al.* 2001). Concentration-response analysis of quinolones (Backhaus *et al*., unpublished data) and quaternary ammonium compounds (Meyer *et al.*, unpublished data) was performed in the same way. Their determined concentration-response functions are given in appendix 4 of publication IV.

[3] Statistical estimations of concentration-response curves were performed by the mathematician Martin Scholze, which I gratefully acknowledge

5 Discussion of Most Important Results

5.1 Mechanistic accuracy of the predictions

5.1.1 *CA* for similarly acting pesticides

In publications I and II the accuracy of *CA* for two groups of environmentally relevant pesticides is shown. The studies were performed on eight chloroacetanilides (publication I) and eight sulfonylureas (publication II). In both studies, *CA* led to accurate predictions, whereas the predictive value of *IA* was lower (fig. 4 A&B in publication I and fig. 1A&B in publication II). These results underline experimental findings on the validity of *CA* for the prediction of the toxicity from mixtures of similarly acting pesticides (Faust *et al.*, 2001; Drost *et al.*, 2003). Furthermore, the results underline that parallelism of the concentration-response curve is no necessary prerequisite for concentration-additive mixture toxicity (c.f. chapter 2.1). As can be observed in fig. 2 in publication I, the concentration-response curves of the eight chloroacetanilides were not parallel. Nevertheless, their mixture toxicity was accurately predictable by *CA*.

5.1.2 Accuracy of the *TSP* approach

As it has recently been shown, the mechanistic accuracy of a prediction may not be judged from compliance with a prediction alone (Backhaus *et al.*, 2004). However, together with the comparatively lower predictive value of *IA* the compliance of the observed toxicity with *CA* may be regarded as a strong indicator for a common mechanism of action.

Based on these criteria, a concentration-additive mixture toxicity was experimentally validated for four of the chemical groups, which were selected for experimentally validating the *TSP* approach (publications I,II, Faust *et al.*, 2001, Backhaus *et al.*, unpublished results). For the quaternary ammonium compounds though, this was not possible due to the rather high observed data variability in combination with comparatively low quantitative differences between *CA* and *IA* predictions (Meyer *et al.*, unpublished results). Therefore, the three quaternary ammonium compounds were not included in the first two mixture experiments in which the predictive value of the *TSP* was analyzed (fig. 3A&B in publication III). Nevertheless, they were included in the NOEC mixture to increase the number of mixture components, as this mixture was additionally designed to give evidence on

the mixture toxicity from low individual effect concentrations. As can be seen in fig. 4 of publication III, the presence of the three quaternary ammonium compounds had no negative influence on the predictive value of the *TSP* approach. This however, may not be regarded as a conclusive experimental validation of concentration additive mixture toxicity of quaternary ammonium compounds.

For both mixtures and mixture ratios tested for its experimental validation, the *TSP* was shown to have a high predictive value (figs. 3 and 4 in publication III). Deviations between predicted and observed toxicity (tab. 4 in publication III) were comparable to deviations observed for *CA* and *IA* in multi-component mixtures for which the respective model assumptions were met (tab. 5 in publication III). The accuracy of the *TSP* approach was underlined by the fact that for every tested mixture and mixture ratio *CA* and *IA* led to less accurate predictions.

It has to be pointed out that in the mixtures tested to validate the *TSP* approach, the mechanisms of action between the *CA* groups were strictly dissimilar. It remains to be clarified whether accurate prediction can also be achieved for mixtures, in which the groups cannot be strictly discriminated, e.g. due to the presence of substances that have more than one mechanism of action.

5.1.3 Accuracy of *TSP*, *CA*, and *IA* for a realistic pesticide exposure scenario

With the mixture tested in publication IV, it was intended to analyze the predictive value of *TSP*, *CA*, and *IA* predictions for a realistic pesticide-exposure scenario.

To allow for a *TSP* prediction, mechanisms of action were assigned to all mixture components according to a procedure outlined in section 2.4 of publication IV. The result of this assignment is listed in tab. 5 (publication IV). However, the accuracy of the *TSP* approach was not discussed in publication IV, because *TSP* and *CA* predictions led to almost identical results. This was due to the finding that the toxicity of this mixture was dominated by a single mechanism of action group: the group of photosystem II inhibitors added 0.80 *TU* to the total sum of *TU* of 0.98 (*TU* = PEC/*EC50* as defined in tab. 5 of publication IV). Accordingly, the dominancy of the PSII inhibitors explains the experimentally shown accuracy of the *CA* prediction for this exposure scenario. The *IA* prediction resulted in a small underestimation of the mixture toxicity, which increased for higher effect levels (fig. 2 in publication IV).

5.2 Value for the use in risk assessment procedures

5.2.1 Relevance of mixture toxicity

The analysis of the mixture toxicity from low, individually not significant effect concentrations, has underlined the relevance of mixture toxicity. As can be observed in publication III (fig. 4) the mixture concentration, at which every mixture component is present in its respective NOEC, inhibited the algal reproduction by over 99%. Even if this mixture concentration was lowered by a factor of 10, statistically significant effects were observed. Also in other mixtures, in which the components were present in their respective NOEC values, a high mixture toxicity was observed. In a study on 11 structurally dissimilar priority pollutants of the aquatic environment an inhibition of algal reproduction by over 60% was observed (Walter *et al.*, 2002). An even higher mixture effect was reported by Backhaus *et al.* (2000b) for a mixture of 10 quinolone antibiotics, which elicited over 99% effect in a biotest with a marine bacterium. As mentioned in chapter 2.3, the effects assessment in risk assessment procedures is based on individual NOEC values. Thus, the high observed mixture effects from individual NOEC values as well as the finding that even concentrations as low as $1/10^{th}$ of individual NOECs may cause a statistically significant effect when present in a mixture, stress the need to account for mixture toxicity in risk assessment procedures.

5.2.2 *CA* as a precautionary default assumption

The *TSP* has proven to be a scientifically accurate tool for the prediction of realistic pesticide mixtures. However, its use within risk assessment procedures may be hampered for three reasons: (*i*) the mechanisms of action of all mixture components have to be known, (*ii*) they have to be assignable to discrete groups according to their mechanism of action, and (*iii*) the concentration response curves have to be known for all components. As neither the knowledge of the mechanism of action, nor the knowledge of the concentration-response curves is usually at hand in risk assessment, the use of the *TSP* as a standard approach for the prediction of mixture toxicity in risk assessment procedures may not seem feasible at the moment. Thus, the question arises whether *CA* or *IA* may be used instead.

Based on a study on the interrelations between *CA* and *IA* predictions for binary mixtures, Boedeker *et al.* (1993) have suggested using *CA* as a reasonable worst-

case estimation even for mixtures of independently acting substances. This conclusion was mainly due to two findings: (*i*) for endpoints used in ecotoxicology *CA* will predict the higher mixture toxicity than *IA* and (*ii*) the quantitative difference between both predictions is small.

The finding that *CA* predicts the higher toxicity was later experimentally confirmed also for multi-component mixtures. In most studies in which predictions according to both concepts were made, *CA* yielded higher toxicity estimates (Altenburger *et al.*, 2000; Backhaus *et al.*, 2000a; Backhaus *et al.*, 2000b; Walter *et al.*, 2002; Lock and Janssen, 2002; Faust *et al.*, 2003; Drost *et al.*, 2003; Altenburger *et al.*, 2003), and Faust *et al.* (2003) have concluded that from a regulatory perspective *CA* may be defendable as a pragmatic approach and a precautionary default assumption. The results of all tested mixtures given in publications I, III, and IV are consistent with that view: if the mechanisms of action of the mixture components were not known, the application of *CA* as a default approach would not have underestimated the mixture toxicity. To our knowledge only in one multi-component mixture study *CA* did not give higher predictions of the mixture toxicity than *IA*: in the study on the mixture toxicity of eight *sulfonylureas* (publication II), for both mixture ratios the application of *IA* resulted in a slightly higher predicted mixture toxicity. Nevertheless, since the mixture toxicity was shown to be concentration additive, even for this mixture the assumption that *CA* gives worst-case estimations of the mixture toxicity was not violated.

The general acceptability of using *CA* as a precautionary default assumption within a regulatory context will mainly depend on two questions: (*i*) whether it can be assumed that the actual mixture toxicity will not be underestimated by *CA* and (*ii*) how much *CA* may overestimate the toxicity of a mixture, in which the mechanisms of action of the mixture components are not strictly similar. Both questions depend on the quantitative relationship between predictions according to the two concepts.

In general, the quantitative relationship between *CA* and *IA* depends on five factors: the number of mixture components, the regression models used for determining their concentration-response curves, the mixture ratio, the steepness of the components' concentration-response curves as well as on the effect level under consideration (c.f. chapter 2.1). The actual relationship between both

concepts depends on all factors and is thus unique for every mixture toxicity assessment. *CA* may give higher, equal, or lower predictions of the mixture toxicity than *IA*. Nevertheless, a few characteristics can be generalised.

For the question whether it can be assumed that the actual mixture toxicity will not be underestimated by *CA* these are: (*i*) there is no theoretical limit for *IA* predicting a higher toxicity than *CA* (Faust, 1999) and (*ii*) higher toxicity estimates by *IA* can only occur for rather flat concentration-response curves of the mixture components (Drescher and Boedeker, 1995; Faust, 1999). Thus, without the knowledge of the concentration-response curves of the mixture components, the question whether a higher toxicity than predicted by *CA* might occur cannot be answered. However, all experimental evidence supports the notion that mixture toxicity higher than predicted by *CA* is rare in ecotoxicological studies on multi-component mixtures. This finding coincides with rather steep concentration-response curves for the integral effect parameters used in ecotoxicological toxicity assessments.

For the question, how much *CA* may overestimate the toxicity of a mixture, in which the mechanisms of action of the mixture components are not strictly similar, it can be generalised that the factor by which *CA* may predict a higher toxicity than *IA* will never exceed a value, which equals the number of mixture components (Faust, 1999). Although the quantitative difference between *CA* and *IA* predictions may thus be high for multi-component mixtures, it never exceeded a factor of 4 in the above named multi-component mixture studies.

Furthermore, in the tested realistic exposure scenario (publication IV), quantitative differences between *CA* and *IA* never exceed a factor of 1.7. To evaluate whether a low quantitative difference between *CA* and *IA* is an exception or rather the rule for realistic pesticide mixtures, in publication IV a mathematical formula was proposed for the calculation of the maximally possible factor, by which *CA* may predict a higher toxicity than *IA* (eqn. 8 in publication IV; for mathematical proof please refer to the annex of this publication).

The application of eqn. [8] (publication IV) on 16 agricultural exposure scenarios (tab. 8 in publication IV), showed that (*i*) the factor never exceeded a value of 3 and (*ii*) was always lower than the number of components within the scenario.

The result of this calculation may not be understood as giving the exact quantitative relationship between *CA* and *IA*. The exact relationship can only be determined in case that the concentration-response curves of all mixture components are known. This knowledge though, is usually not available for risk assessment procedures (c.f. chapter 2.3). In contrast to that the data required for eqn. [8] (publication IV), *i.e. PECs* of all mixture components and *ECx* values, are generally given.

One may thus conclude that the results of this thesis back the approach of using *CA* as a precautionary default assumption for predictive mixture toxicity assessments of realistic pesticide mixtures. On the other hand, all experimental evidence indicates that using *IA* as a default approach would violate the precautionary principle.

However, it has to be clarified in further studies whether using *CA* as a precautionary default assumption is also a valid approach for realistic mixtures in which the substances have rather flat concentration-response curves: as shown in publication II, *IA* may predict a higher mixture toxicity than *CA* for some pesticide mixtures in which flat concentration-response curves of the mixture components are observed.

6 Conclusions and Outlook

The aim of this thesis was to analyse whether the joint toxicity of environmentally relevant toxicants can be predicted based on the known toxicity of the single mixture components. In the light of all results, two conclusions were drawn: (*i*) the combination of *CA* and *IA* to a *TSP* is a valid approach, which provides accurate predictions of the mixture toxicity in case that the components can be assigned to discrete mechanism of action groups. (*ii*) Even in case that mechanistic knowledge of the mixture components is not provided, the use of *CA* as a precautionary default assumption is a justifiable approach for estimating the mixture toxicity of realistic pesticide mixtures.

Concerning the predictability of the mixture toxicity from environmentally relevant toxicants, future research should focus on two questions: (*i*) whether the *TSP* approach also gives accurate predictions if some mixture components have more than one mechanism of action, and (*ii*) whether the precautionary default assumption of *CA* might be refuted for realistic mixtures, in which the components have rather flat concentration-response curves.

7 Zusammenfassung

Wie in Monitoring Studien nachgewiesen wurde, sind Oberflächengewässer häufig mit einer Reihe von potentiell gefährlichen Substanzen verunreinigt. Für aquatische Organismen bedeutet dies die Exposition gegenüber Gemischen von Schadstoffen. Das Ziel dieser Arbeit war es, zu untersuchen, ob die Gemischtoxizität von umweltrelevanten Schadstoffen auf der Grundlage ihrer Einzelstofftoxizität vorhergesagt werden kann. Zur Vorhersage von Gemischtoxizität wurden bislang zwei Konzepte erfolgreich angewandt: *Konzentrationsadditivität* (*CA*) für Gemische ähnlich wirkender Substanzen und Unabhängige Wirkung (*IA*) für Gemische unähnlich wirkender Substanzen. In umweltrelevanten Gemischen kann jedoch nicht von einer strikten Ähnlichkeit beziehungsweise Unähnlichkeit aller Gemischkomponenten ausgegangen werden. Darüber hinaus ist das Wissen über die Wirkmechanismen vieler umweltrelevanter Schadstoffe häufig nicht vorhanden. Die Ergebnisse dieser Arbeit zeigen, dass die Kombination von *CA* und IA zu einer Zweistufenvorhersage (*TSP*) genaue Vorhersagen der Gemischtoxizität ermöglicht, wenn (*i*) die Wirkmechanismen aller Gemischkomponenten bekannt sind und (*ii*) die Gemischkomponenten anhand ihres Wirkmechanismus diskreten Gruppen zugeordnet werden können. Die Anwendung der *TSP* auf ein realistisches Pestizid-Gemisch führte zu einer genauen Vorhersage der beobachteten Gemischtoxizität. Jedoch konnten sowohl mit *CA* als auch mit *IA* ebenfalls gute Vorhersagen erzielt werden. Dies lag daran, dass die quantitativen Unterschiede zwischen den Vorhersagen aller 3 Vorhersagemodelle gering waren. Zur Untersuchung der quantitativen Unterschiede zwischen *CA*- und *IA*-Vorhersagen in anderen realistischen Gemischen, wurde eine mathematische Formel entwickelt, mit der man allein auf der Grundlage des Mischungsverhältnisses und der *EC50*-Werte der einzelnen Gemischkomponenten ermitteln kann, wie groß das Verhältnis zwischen dem nach *IA* vorhergesagten und dem nach *CA* vorhergesagten *EC50* maximal sein kann. Die Anwendung dieser Formel auf veröffentlichte landwirtschaftliche Expositionsszenarien deutet darauf hin, dass für realistische Pestizid-Gemische geringe Unterschiede zwischen *CA*- und *IA*-Vorhersagen eher die Regel als die Ausnahme sind. Aus den Ergebnissen dieser Arbeit wurden 2 Schlussfolgerungen gezogen: (*i*) Die Kombination von *CA* und *IA* zu einer *TSP* ist eine valide Herangehensweise, welche die genaue Vorhersage der Gemischtoxizität erlaubt, vorausgesetzt, dass die Gemischkomponenten diskreten Wirkmechanismusgruppen

zugeordnet werden können. (*ii*) Auch wenn wirkmechanistisches Wissen über die Gemischkomponenten nicht vorliegt, kann die standardmäßige Verwendung von *CA* zur Vorhersage der Gemischtoxiztät von Pestizid-Gemischen ein gerechtfertigter Ansatz sein.

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List of Publications

Continental Shelf Research, 23 1757-1769	**Backhaus,T., Altenburger,R., Arrhenius,A., Blanck,H., Faust,M., Finizio,A., Gramatica,P., Grote,M., Junghans,M., Meyer,W., Pavan,M., Porsbring,T., Scholze,M., Todeschini,R., Vighi,M., Walter,H., Grimme,L.H**, 2003. The BEAM-project: Prediction and assessment of mixture toxicities in the aquatic environment.
Pest Management Science, 59 (10) 1101-1110	**Junghans M., Backhaus T., Faust M., Scholze M., Grimme, L.H.**, 2003. Predictability of Combined Effects of 8 Chloroacetanilide Herbicides on Algal Reproduction.
Bulletin of Environmental Contamination and Toxicology, 71 (3) 585-593	**Junghans M., Backhaus T., Faust M., Scholze M., Grimme, L.H.**, 2003. Toxicity of Sulfonylurea Herbicides to the green alga *Scenedesmus vacuolatus*: Predictability of Combination Effects.
Diploma thesis University of Hamburg	**Junghans, M.**, 1999. Untersuchungen zur Optimierung des Wachstums der Grünalge *Chlorella zofingiensis* und Experimente zur Verbesserung der Wiederergrünung roter Akineten.

Curriculum Vitae

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Predictability of combined effects of eight chloroacetanilide herbicides on algal reproduction

Marion Junghans **37**

Predictability of combined effects of eight chloroacetanilide herbicides on algal reproduction

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Abstract

Chloroacetanilides are pre-emergence herbicides for the control of annual grasses and broad leaf weeds. As a result of their extensive use, residues are often found in surface waters. Observed simultaneous occurrence of different chloroacetanilide herbicides gives reason for concern about potential combination effects on aquatic non-target organisms. This study aimed to clarify whether joint effects of various chloroacetanilide herbicides may be predictable from knowledge of concentration-response relationships of single substances. Whether the chloroacetanilides all share the same mode of action is unclear. Therefore, we investigated the predictive value of two alternative concepts for the prediction of combined effects: *concentration addition*, which assumes a similar mode of action, and *independent action*, which is based on the idea of a dissimilar mode of action of the mixture components. Eight chloroacetanilides (acetochlor, alachlor, butachlor, dimethachlor, metazachlor, metolachlor, pretilachlor, and propachlor) were experimentally tested for their individual as well as for their combined effects in mixtures on the reproduction of the green alga *Scenedesmus vacuolatus*. Individual chloroacetanilides impaired algal reproduction with EC_{50} values ranging from 3 to 232 µg per litre. The differences in EC_{50} values were strongly correlated with lipophilicities of the compounds. Effects of chloroacetanilide mixtures were considerably higher than those of the individual components: a complete inhibition of algal reproduction was observed when every mixture component was present in a concentration that would cause only 5 % effect if applied singly. However, the combined effects proved to be predictable by using the concept of *concentration addition*. The alternative concept, *independent action*, distinctly underestimated the mixture toxicity. These findings (*i*) indicate a similar mechanism of action of

chloroacetanilides in algae and (*ii*) reinforce the view that *concentration addition* is a reasonable assumption for the predictive hazard assessment of groups of similarly acting herbicides.

1 Introduction

1.1 Environmental relevance of chloroacetanilides

Since the 1950s, chloroacetanilide herbicides have been used to control annual grasses and broad leaf weeds, predominantly in corn, soybean, sorghum, cotton, and rice (Tomlin C (ed), 1994; Battaglin and Goolsby, 1999). Battaglin and Goolsby (1999) estimated the use of alachlor, acetochlor and metolachlor in eight states of the Midwestern USA in 1996 at 32 000 metric tons of active ingredients on a planted area of 370.000 km^2. As a result, these herbicides have been frequently detected in local streams and lakes as well as in rivers discharging from that area (Battaglin *et al.*, 2000; Clark and Goolsby, 2000). From monitoring data for freshwaters in member states of the European Communities, alachlor, metazachlor and metolachlor have been identified as contaminants relevant and representative on a European scale (European Commission, 1999). In Japan, pretilachlor and butachlor have been detected in the rivers Shinano (Tanabe *et al.*, 1996) and Kokai (Hatakeyama *et al.*, 1994). Regarding the toxicity to non-target organisms, the US EPA classified alachlor in a "Reregistration Eligibility Decision" as highly toxic to aquatic plants (United States Environmental Protection Agency, 1998). This classification was based upon an EC_{50} of 1.64 µg L^{-1} observed in tests with the freshwater green alga *Selenastrum capricornutum*. A similarly high toxicity was reported for pretilachlor (Kasai and Hatakeyama, 1993) and butachlor (Hatakeyama *et al.*, 1994) in tests with the same species. In comparison with algae and higher plants, aquatic animals have shown to be much less sensitive, with EC_{50} values typically being about 3 orders of magnitude higher (United States Environmental Protection Agency, 1998).

Simultaneous occurrence of different chloroacetanilide herbicides in water bodies is not only likely but has in fact been reported in monitoring studies (Hatakeyama *et al.*, 1994; Tanabe *et al.*, 1996; Boyd, 2000; Clark and Goolsby, 2000). Hence, there is reason for concern that hazard assessment for single substances may underestimate the total risk resulting from exposure to chloroacetanilide mixtures. To evaluate that risk it is neither feasible nor economically sensible to perform

experimental testing on every combination of chloroacetanilide herbicides likely to occur in surface waters. Therefore the question is, whether joint effects of chloroacetanilides on algae and aquatic plants are predictable on the basis of the single substances' effects, by applying a concept for the prediction of combined effects.

1.2 Concepts for the prediction of combined effects

Concepts for the prediction of combined effects are based on alternative assumptions: *concentration addition* (*CA*) assumes a similar mechanism of action of mixture components (Loewe and Muischnek, 1926) whereas *independent action* (*IA)* is based on the hypothesis of dissimilarly acting substances (Bliss, 1939). In several experimental studies with multi-component mixtures both concepts have been successfully applied, *CA* for different types of similarly acting substances (Koenemann, 1980; Koenemann, 1981; Hermens *et al.*, 1984; Nirmalakhandan *et al.*, 1997; Faust *et al.*, 2000; Altenburger *et al.*, 2000; Backhaus *et al.*, 2000b; Faust *et al.*, 2001), and IA in case that all mixture components were known to have strictly different specific mechanisms of action (Faust *et al.*, 2000; Backhaus *et al.*, 2000a; Faust *et al.*, 2003). However, strictly similar or dissimilar action of mixture components may be considered as extreme special cases (Hewlett and Plackett, 1959). Thus, applicability of *CA* or *IA* to many practical problems, concerning the assessment of mixture toxicity, may a priori appear to be doubtful, due to limited knowledge about mechanisms of action of most chemicals, as well as due to complex patterns of action being partly similar and partly dissimilar.

1.2.1 Concentration Addition

The concept of *concentration addition* was first introduced by Loewe and Muischnek (1926) for binary mixtures. Berenbaum (1985) formulated the concept of CA for multi-component mixtures as:

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

where n is the number of mixture components, c_i are the concentrations of the individual agents in the combination and ECx_i the concentration of the ith agent that individually would cause the same quantitative effect x as the combination. The quotients c_i/ECx_i have been termed toxic units (Sprague, 1970). They represent

the concentrations of mixture components as fractions of equi-effective individual concentrations. If the sum of the toxic units equals 1, mixture components act concentration additive, like being dilutions of the same compound. To predict the effect concentrations of mixtures (ECx_{mix}), eqn. [1] can be rearranged (Faust *et al.*, 2001) to give:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \quad [2]$$

with p_i being the concentrations c_i expressed as relative proportions of the total concentration c_{mix} ($p_i = c_i / c_{mix}$).

1.2.2 Independent Action

The concept of *independent action*, sometimes referred to as *response addition*, was introduced by Bliss (1939). *Independent action* assumes that different substances cause a common integral effect (e.g. death) through primary interaction with different molecular target sites. Bliss (1939) formulated *IA* for two substances 1 and 2 as follows:

$$E(c_{mix}) = E(c_1) + E(c_2) - E(c_1) \cdot E(c_2) \quad [3]$$

which may also be written in the equivalent form of

$$E(c_{mix}) = E(c_1) + E(c_2) \cdot (1 - E(c_1)) \quad [4]$$

where $E(c_1)$, $E(c_2)$ are the effects of the substances 1 and 2 if applied singly in concentrations c_1 and c_2. $E(c_{mix})$ is the predicted joint effect caused by the total concentration $c_{mix} = c_1 + c_2$. Effects E are expressed as fractions of a maximum possible effect and thus are scaled from 0 to 1. Equation [3] can be extended for multi-component mixtures to the following equation as described by Faust *et al.*(2003):

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) . \quad [5]$$

For predicting effect concentrations of mixtures, ECx_{mix} eqn. [4] can be rearranged to give:

$$x = 1 - \prod_{i=1}^{n} (1 - F_i(p_i \cdot (ECx_{mix}))) \quad [6]$$

as described in Faust *et al.* (2001), where x is a definite effect level (e.g. 50%) and F_i are the concentration response functions of the individual substances. The value of ECx_{mix} satisfying the equation for a preset effect level x can be calculated by an iterative procedure.

1.2.3 Unpredictable combination effects

Loewe and Muischnek (1926) as well as Bliss (1939) have also discussed mixtures, in which the observed combination effect for a fixed endpoint cannot be predicted by *CA* or *IA*. Plackett and Hewlett (1952) concluded that, irrespective of the similarity of action, compliance with one of the two concepts could only be expected, if there are no interactions between the mixture components. They defined the term "interaction" on a physiological basis by stating, that the mixture components can be ascribed the potential to interact by quantitatively altering the action of another component in the mixture. They postulated that this alteration could take place either by influencing the uptake of this component or by changing the component's physiological action itself. In these mixtures, the deviation of the observed combination effects from the predictions by CA or IA would be strongly dependent on the ratio of the mixture components.

1.3 Mechanism of action

Studies with butachlor (Couderchet *et al.*, 1998), alachlor, metolachlor, and metazachlor showed that chloroacetanilide herbicides affect the metabolism of fatty acids (Möllers and Albrecht, 1994; Couderchet *et al.*, 1998). Couderchet *et al*. (1998) demonstrated that the inhibition of growth of *Scenedesmus acutus* is positively correlated with the intensity of the effect on the fatty acids synthesis in case of alachlor, metolachlor and metazachlor. For butachlor, though, such a correlation was not observed. Thus, it remains unclear whether butachlor acts in a way strictly similar to alachlor, metolachlor and metazachlor. In a recent review, Böger *et al.* (2000) confined the mechanism of chloroacetanilide action to the inhibition of the synthesis of very long chain fatty acids (C20, 22, and 24). Experimental studies gave evidence for a specific binding of selected chloroacetanilides to a fatty acid elongase enzyme system. However, the authors cannot exclude that chloroacetanilides attack other crucial target sites as well. From these findings, the assumption of a strictly dissimilar action seems inappropriate. However, evidence for a perfect compliance with the idea of a

strictly similar interaction with a single molecular binding site may be considered inconclusive as yet.

1.4 Aims and approaches

From the knowledge outlined three possibilities for combined effects of chloroacetanlide mixtures on algae may be envisaged: *(i)* compliance with *CA*, *(ii)* some kind of intermediate effect, approximately predictable by *CA* or *IA*, and, as long as interaction between the mixture components cannot be excluded, also *(iii)* inappropriateness of both concepts. To clarify this point, we studied effects of eight chloroacetanilides combined in two different fixed concentration ratios on the reproduction of the green alga *Scenedesmus vacuolatus*. Experimentally determined concentration-response relationships for mixtures were compared with predictions derived from both concepts (*CA* and *IA*). Predictions were calculated by using concentration-response functions determined for every individual compound.

2 Material and Methods

2.1 Test substances, test solutions and chemical analysis

Test substances included all chloroacetanilides that have been frequently determined in surface waters (cf. section 1.1) plus dimethachlor and propachlor. They were obtained from Riedel de Haën (Seelze, Germany) in analytical purity (tab. 1). Stock solutions were prepared in methanol (chromatography grade, Merck, Darmstadt, Germany) and stored at –30°C. These stock solutions served as standards for the analytical validation of test concentrations. Aqueous stock solutions for toxicity testing were prepared from aliquots of the methanolic solutions after evaporating the organic solvent under a stream of N_2. The crystalline substances were then re-dissolved in bi-distilled water under vigorous stirring at room temperature. Concentrations of aqueous stock solutions were validated by reversed phase high performance liquid chromatography (rp-HPLC) using a LiChrosphere RP 18e column (125 mm length, 4 mm inner diameter, 5 µm pore size, reversed phase, endcapped; Merck, Darmstadt, Germany) and a UV detector at a wavelength of 215 nm. The mobile phases consisted of acetonitrile (Chromasolv, Riedel de Haën, Seelze, Germany) + water (bi-distilled) in various volume ratios (propachlor: 35 + 65; dimethachlor and metazachlor: 40 + 60; acetochlor, alachlor, and metolachlor: 50 + 50; butachlor and pretilachlor: 60 +

40). The flow rate of the mobile phase was always 1 ml min^{-1}. To evaluate the stability of concentrations of substances during the test, the substances were incubated with the algal growth medium under test conditions. For all chloroacetanilides tested recovery rates were greater than 90%. Concentration-response data given in this paper always refer to the analytically validated initial concentrations in the algal cultivation medium.

2.2 Algal toxicity assay

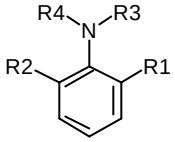
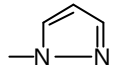
The effect parameter was the inhibition of reproduction of the green alga *Scenedesmus vacuolatus* strain 211-15 (culture collection of the University of Göttingen, Germany) synchronically grown over a period of one generation cycle (24h). The cell number was measured with an electronic particle counter (Coulter Electronics, Bedford UK). The test was conducted as described by Faust *et al.* (2001).

2.3 Concentration response analysis

Experimental concentration-response analyses of mixtures were performed using a fixed ratio design, *i.e.* the total concentration of the mixture was varied, whereas the molar ratio of the constituents remained constant. The fixed ratio design was selected because it allows both a comparison of the observations with the predictions according to both concepts and the statistical estimation of the uncertainty connected to observations and predictions. Two different mixture ratios were tested: the eight chloroacetanilides were present either in the ratio of their individual EC_{50} values or in the ratio of their individual EC_{05} values (tab. 2).

Concentration-response analyses for both single substances and mixtures were performed in essentially the same way. Geometric dilution series were prepared from the aqueous stock solutions. To allow valid statistical estimations of concentration-response functions for the whole effect range, at least 12 different concentrations were tested with three replicates each and compared to 18 untreated controls. The spacing between the test concentrations was adapted to the steepness of the respective concentration-response relationship.

Table 1. Chloroacetanilides used in mixture toxicity studies

Substance	CAS RN [b]	Purity (%)	Structure 				MW [c]	Log Kow [d]
Common Name			R1	R2	R3	R4		
Butachlor	23184-66-9	97.3	C_2H_5	CH_3	$CH_2OC_4H_9$	$COCH_2Cl$	311.9	4.84
Pretilachlor	51218-49-6	98.3	C_2H_5	C_2H_5	$C_2H_4OC_3H_7$	$COCH_2Cl$	311.9	4.29
Acetochlor	34256-82-1	97	C_2H_5	C_2H_5	$CH_2OC_3H_5$	$COCH_2Cl$	269.8	3.37
Alachlor	15972-60-8	99.7	C_2H_5	C_2H_5	CH_2OCH_3	$COCH_2Cl$	269.8	3.37
Propachlor	1918-16-7	99.5	H	H	$CH(CH_3)_2$	$COCH_2Cl$	211.7	2.42
Metazachlor	67129-08-2	98	CH_3	CH_3	CH_2 	$COCH_2Cl$	277.8	2.38
Dimethachlor	50563-36-5	99.8	CH_3	CH_3	$C_2H_4OCH_3$	$COCH_2Cl$	255.7	2.33
Metolachlor [a]	51218-45-2	97	CH_3	C_2H_5	$CH(CH_3)CH_2OCH_3$	$COCH_2Cl$	283.3	3.24

[a] racemate, isomere-ratio unstated, [b] Chemical Abstracts Services Registry Number, [c] Molecular Weight,
[d] Partition coefficient in octanol/wter: calculated with the programm KowWin, Syracuse Research Corporation (http://esc.syrres.com/interkow/kowdemo.htm)

Table 2. Test mixtures.

Components *i*	Relative proportion p_i(%)	
	Mixture 1: *EC05* ratio	Mixture 2: *EC50* ratio
	$p_i = \frac{c_i}{c_{mix}} = \frac{EC05_i}{\sum_{i=1}^{i=8} EC05_i} * 100$	$p_i = \frac{c_i}{c_{mix}} = \frac{EC50_i}{\sum_{i=1}^{i=8} EC50_i} * 100$
Acetochlor	3.30	2.82
Alachlor	7.84	8.77
Butachlor	0.18	0.60
Dimethachlor	22.30	15.20
Metazachlor	7.13	10.49
Metolachlor	43.89	51.17
Pretilachlor	0.70	0.87
Propachlor	14.66	10.08

For p_i rounded values are given, c_i – concentration of individual component *i*, c_{mix} – total concentration of mixture constituents, *EC05*, *EC50* – see tab. 4.

Concentration-response functions were statistically estimated from the experimental data by applying the best-fit procedure introduced by Scholze *et al.* (2001). Three out of ten 2- and 3-parametric models proved best able to describe the chloroacetanilides' concentration-response relationships. They are given in tab. 3. Effect concentrations were calculated using the inverse (F^{-1}) of the best fitting model (tab. 3). Corresponding 95% confidence intervals were estimated by using the bootstrap methodology (Scholze *et al.*, 2001). No observed effect concentrations (NOECs) were determined by applying Dunnett's test (Dunnett, 1964).

2.4 Calculation of mixture toxicity predictions

Predictions of effect concentrations for mixtures by *CA* and *IA* were calculated according to eqns. [2] and [6] respectively, as described previously (Backhaus *et al.*, 2000a; Faust *et al.*, 2001; Faust *et al.*, 2003). They were calculated for numerous effect levels *x*, resulting in graphs of predicted concentration-response curves.

Table 3. Regression models

Name	Function (F)	Inverse Function (F^{-1})
Weibull (W)	$E = 1 - \exp\left(-\exp\left(a + b\,\log_{10}(c)\right)\right)$	$c = 10^{\left((\log_e(-\log_e(1-E)) - \hat{a}) / \hat{b}\right)}$
Generalized Logit (GL)	$E = 1/\left(1 + \exp\left(-a - b\,\log_{10}(c)\right)\right)^{g}$	$c = 10^{\left((-\log_e((1/E)^{(1/\hat{g})} - 1) - \hat{a}) / \hat{b}\right)}$
Box-Cox-Weibull (BCW)	$E = 1 - \exp\left(-\exp\left(a + b\left(\left(c^{g} - 1\right)/g\right)\right)\right)$	$c = \left((\hat{g} / \hat{b}) \bullet (\log_e(-\log_e(1-E)) - \hat{a}) + 1\right)^{(1/\hat{g})}$

E – Effect, expressed as fraction of a maximum possible effect (0 = E = 1); *c* – Concentration; *a, ß, ?* – Model parameters (corresponding statistical estimates marked by ^); exp(x) = e^x

3 Results and Discussion

3.1 Toxicity of single substances

Complete concentration-response curves were determined for all eight chloroacetanilides. As an example for the statistical modelling, fig. 1 shows the experimental concentration-response data for alachlor and the resulting concentration-response curve. In fig. 2 graphs of concentration-response functions for all chloroacetanilide herbicides tested are shown. The corresponding functions are documented in tab. 4. The EC_{50} values of the eight tested chloroacetanilides span over 2 orders of magnitude, ranging from 9.5 to 817.6 nM (3.0 to 232.0 µg L^{-1}) (tab. 4), with butachlor being the most effective and metolachlor being the least effective test substance. Thus, according to current regulatory rules, all tested chloroacetanilide herbicides can be classified as highly toxic to algae. Literature data show that toxicity of chloroacetanilides to green algae is highly species dependent. EC_{50} values for alachlor and pretilachlor vary in ranges from 6µg L^{-1}(Fairchild *et al.*, 1997) to 1430µg L^{-1} (Hawxby *et al.*, 1977) and from 0.94 µg L^{-1} (Hatakeyama *et al.*, 1994) to 3304 µg L^{-1} (Kasai and Hatakeyama, 1993), respectively. A comparison with the data obtained in this study, 37.8 µg L^{-1} and 4.2 µg L^{-1}, shows that *Sc. vacuolatus* belongs to the more sensitive algal species when exposed to chloroacetanilide herbicides.

We compared the differing EC_{50} values of the eight chloroacetanilides observed in this study with a baseline toxicity model developed in the same assay (Walter *et al.*, 2002), *i.e.* the unspecific effect that could be expected to result from hydrophobicity driven accumulation in cellular membranes only.

This assumption of a mere narcotic effect is obviously wrong: the EC_{50} values observed are about three orders of magnitude lower than those predicted by the baseline toxicity model (fig. 3). This excess toxicity over narcosis confirms the view that the chloroacetanilides exert their toxic effect on algae by specific interaction with one or more molecular target sites.

Table 4. Concentration-response-functions and *EC* values for the single chloroacetanilides.

Substance [a] *(in order of EC_{50})*	Concentration-response function				EC_{50} [c]			EC_{05} [c]			NOEC [d]	
	RM [b]	α	β	γ	(nM L^{-1})	[Cl]	(µg L^{-1})	(nM L^{1})	[Cl]	(µg L^{-1})	(nM L^{1})	(µg L^{-1})
Butachlor	GL	18.267	10.570	0.2210	9.5	[8.9; 10.2]	3.0	1.0	[0.6; 1.5]	0.3	1.6	0.5
Pretilachlor	W	8.399	4.716		13.6	[13.6; 14.2]	4.2	3.3	[2.8; 3.9]	1.0	n.d. [e]	n.d. [e]
Acetochlor	GL	27.212	22.079	0.2691	45.1	[43.4; 47.2]	12.2	18.3	[14.9; 22.4]	4.9	18.1	4.9
Alachlor	W	4.009	5.127		140.1	[132.2;147.8]	37.8	43.5	[35.4; 53.5]	11.7	43.1	11.6
Propachlor	W	6.599	8.785		166.1	[147.4;182.0]	35.2	81.4	[66.4; 95.6]	17.2	89.3	18.9
Metazachlor	BCW	6.612	5.780	0.4716	167.6	[162.2;186.7]	46.6	39.6	[27.8; 43.1]	11.0	55.1	15.3
Dimethachlor	W	5.107	8.906		242.9	[222.0;264.1]	62.1	123.9	[91.6;167.7]	31.7	152.6	32.1
Metolachlor	BCW	0.239	3.156	0.4930	817.6	[763.4;868.7]	232.0	243.9	[29.3;377.8]	69.2	422.0	119.8

[a] common name, [b] RM: regression model (see tab. 3), [c] mean effect concentrations with 95% two-sided bootstrap confidence intervals [d] NOEC determined by DUNNETT test, [e] n.d. = not determined

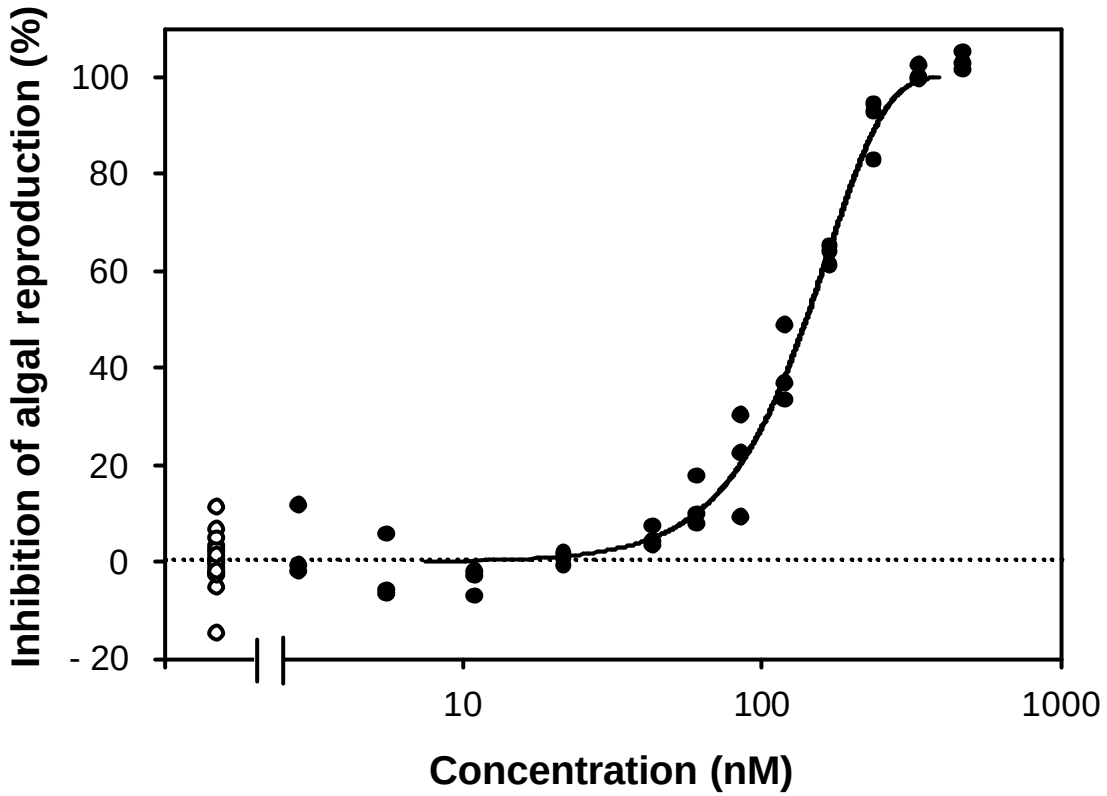


Figure 1. Concentration response relationship for the algal toxicity of alachlor. Experimental data (•), untreated controls (o), statistical fit (- - - -) to the Weibull Model (see tabs. 3 and 4).

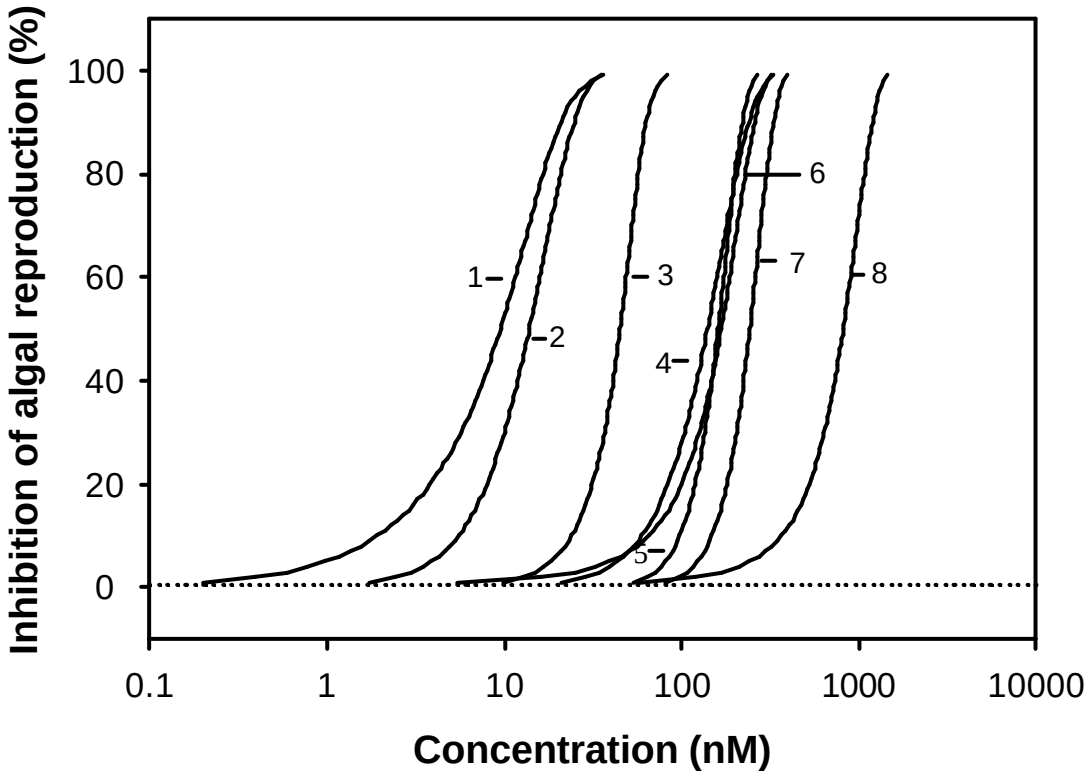


Figure 2. Concentration response curves of the eight chloroacetanilides. The respective functions are given in tab. 4. Individual compounds may be identified using numbers given in tabs. 1 & 4.

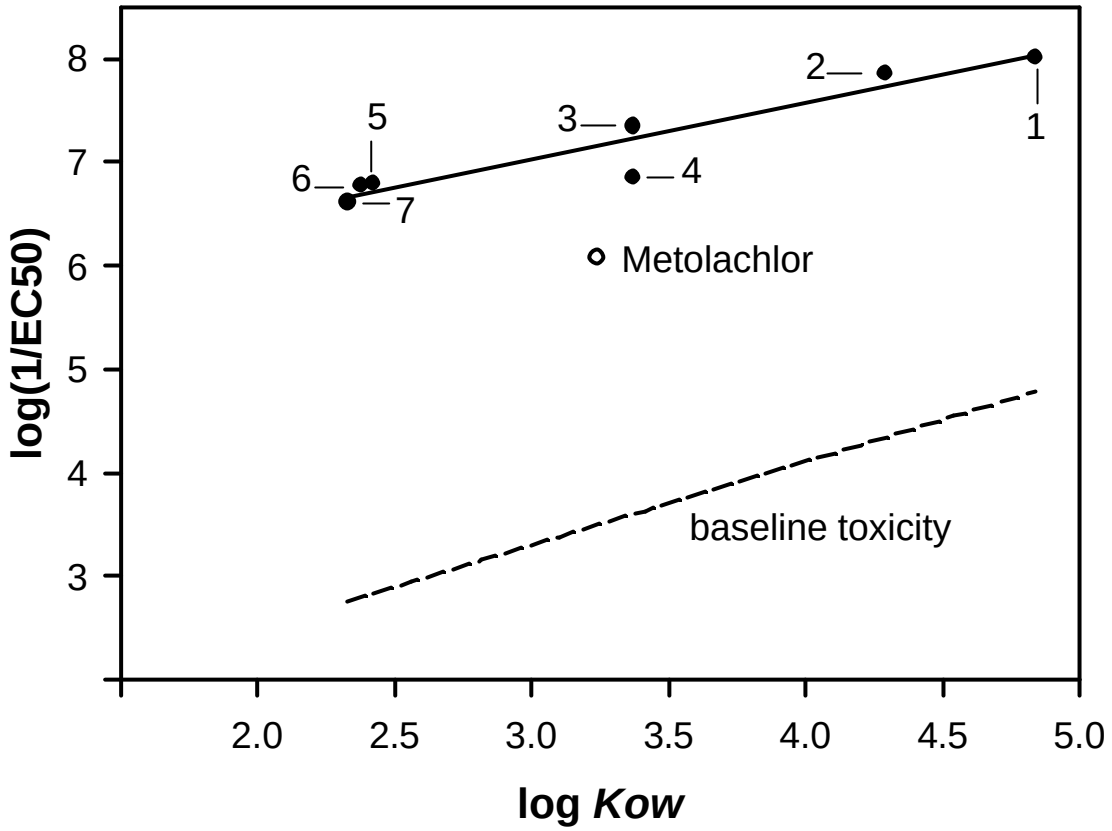


Figure 3. QSAR Analysis of observed toxicity (•) for chloroacetanilides (- - - -)(log 1/*EC50*(mol/L) = 0.54 log*Kow* +5.40) and its relation to the calculated baseline toxicity (- - -) (log 1/*EC50*(mol/L) = 0.81 log*Kow* +0.87). Metolachlor (o) was not included because it was obtained as a mixture of two racemic forms, differing in their toxicity to algae.

Although the absolute EC_{50} values cannot be explained by the compounds' hydrophobicities alone, the relative differences between EC_{50} values are strongly correlated with differences in the octanol/water partition coefficients (*Kow*). Linear regression analysis results in a simple QSAR model (fig. 3):

$$\log 1/EC_{50}(\text{mol L}^{-1}) = 0.54 \log Kow + 5.40 \qquad [7]$$

with the multiple correlation coefficient (R^2) being 0.90, the Fischer statistic *(F)* being 46.6 and a standard error of estimate of 0.19 for $n = 7$ compounds. For an interpretation of this phenomenon, we have to consider that the EC_{50} values refer to the concentrations in the external aqueous algal cultivation medium, not to the concentration at the postulated specific molecular target site. The elongase enzyme system that has been reported to be specifically affected by chloroacetanilide herbicides is located in the membranes of the endoplasmatic

reticulum (ER) (Schmalfuß *et al.*, 2000). Therefore, it seems plausible to assume that the molecular target site is inside the ER membrane, *i.e.* in a lipophilic microenvironment. On this presumption, the linear correlation between lipophilicity and toxicity may mainly reflect the different abilities of chloroacetanilides to pass cellular membranes and to accumulate in the lipid phase of the ER membrane. However, a 1000-fold increase in *Kow* results in less than 50-fold increase in $1/EC_{50}$. In part, this may be due to the fact that octanol is not a completely perfect surrogate for the lipid phase of cellular membranes, but it must also be considered that hydrophobic partitioning into the ER membrane is only a prerequisite for interaction with the postulated target site. Affinity to a binding domain of the target enzyme should be the other crucial factor, and this may be governed by steric and electronic properties of the molecules. Nevertheless, the fact that the differences in chloroacetanilides' EC_{50} values are largely explicable as a function of differing lipophilicity gives a strong additional indication for a similar mechanism of action. The same conclusion has been drawn from analogous results of QSAR analyses for other groups of chemicals (Hermens *et al.*, 1984; Hermens *et al.*, 1985a; Hermens *et al.*, 1985b). It must be emphasized that the QSAR model obtained does also include butachlor. This is remarkable, since an analysis of the relationship between the inhibition of fatty acid metabolism and algal growth by Couderchet *et al.* (1998) indicated a differing mechanism of action for this substance. Our results do not support this view. Metolachlor was left out from our correlation analysis, because the substance was obtained as a mixture of two stereoisomers in an unstated ratio. The R-enantiomer has been reported to be clearly less toxic than the S-enantiomer (Moser *et al.*, 1982; Schmalfuß *et al.*, 2000). The outlier position of metolachlor in fig. 3 is consistent with that finding.

The concentration-response curves of individual chloroacetanilides are not strictly parallel. This is evident from crossings between curves depicted in fig 2. This is important to notice, because many authors take parallelism and non-parallelism as an indicator for similar and dissimilar action, respectively. Bliss (1939) was one of the first who formulated this idea. He considered dosage-mortality curves for populations of organisms, assuming that the slope reflects the variation in individual susceptibility to a chemical. Bliss argued that for components interacting with the same system of receptors, variations in individual susceptibility should be completely correlated (individuals most sensitive to substance A are also most

sensitive to substance B) and hence the curves should be parallel. Based on this assumption he formulated the quantal response model of "similar joint action", which is a special case of *CA*. Bliss' argument seems plausible on a receptor level, but it does not reflect the relation between the external concentration in the environment of an organism and the internal concentration at a specific molecular target site. Although two chemicals may act in the same way at the same site of action, they may differ in terms of uptake, partitioning and accumulation, binding to unspecific sites, and biotransformation. As a result, concentration-response curves may differ in shape and slope. For this reason, Hewlett and Plackett (1959) discarded the assumption of parallel concentration–response curves as a necessary prerequisite for similar action. Experimental examples supporting this view have been reported for different groups of similarly acting chemicals (Backhaus *et al.*, 2000b; Faust *et al.*, 2001). As a consequence, we considered the phenomenon of non-parallel concentration-response curves not to be a conclusive argument for an *a priori* rejection of the hypothesis of *CA* for the joint effects of chloroacetanilide mixtures.

3.2 Mixture toxicity

Scepticism about the predictability of the joint effects of eight chloroacetanilides on algal reproduction was dispelled by the results of experimental mixture toxicity analyses. Observed concentration response relationships (tab 5) were found to be in fairly good agreement with those predicted by the concept of *CA* (fig. 4.). This applies to both concentration ratios tested. Predictions were most accurate in a median effect range between 30 and 50 %, where observed and predicted mean effect concentrations differed by less than a factor of 1.1. The actual slope of the concentration-response curves was somewhat higher than expected by the assumption of *CA* (fig. 4). As a result, predictions by *CA* tend to give underestimates of low ($< EC_{30}$) and overestimates of high effect concentrations ($> EC_{50}$). However, quantitative differences between predicted and observed effect concentrations did never exceed a factor of 1.5 in the effect range between 10 and 90 %.

Table 5. Algal toxicity of chloroacetanilide mixtures.

Mixtures	Concentration Response Function			EC_{50} [c]			EC_{05} [c]			NOEC [d]	
	RM [b]	$\hat{a}$	$\hat{b}$	(nM L^{-1})	[CI]	(µg L^{-1})	(nM L^{-1})	[CI]	(µg L^{-1})	(nM L^{-1})	(µg L^{-1})
Mixture 1											
EC_{05} ratio (as defined in Table 2)	W	7.480	11.211	199.6	[185.6; 213.0]	52.9	116.9	[87.1; 148.8]	31.0	100	26.5
Mixture 2											
EC_{50} ratio (as defined in Table 2)	W	7.514	10.614	181.0	[172.5; 189.4]	49.2	102.9	[86.2; 119.7]	28.0	117	31.8

signs and abbreviations as given in table 4

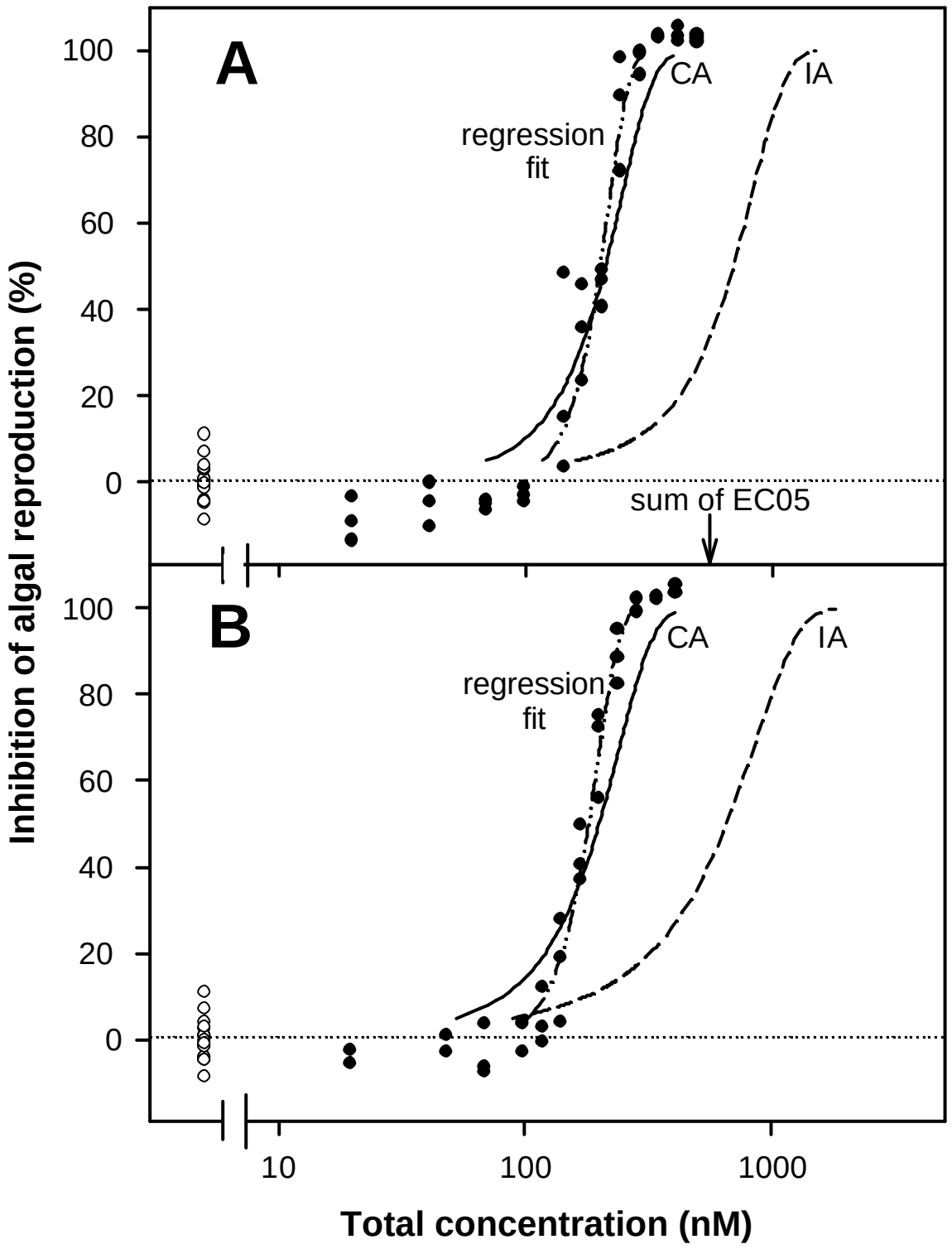


Figure 4. Observed and predicted algal toxicity of chloroacetanilides mixtures. (A) Mixture 1: components mixed in the ratio of their individual *EC05* values. (B) Mixture 2: components mixed in the ratio of their individual *EC50* values. (•) experimentally observed toxicity; (o) controls; (- · · -) statistical fit of experimentally observed toxicity; (- - - -) prediction according to *concentration addition*; (- - -) prediction according to *independent action*.

In contrast to *CA*, the alternative assumption of IA proved to be inappropriate for a predictive effect assessment of chloroacetanilide mixtures. For both mixture ratios tested, predicted *EC50* values underestimated actual ones by a factor of about 3.5 (fig. 4). On the 90 % effect level, observed and predicted effect concentrations differed even by a factor of 4.2 (mixture ratio of *EC05s*) and 4.4 (mixture ratio of *EC50ies*).

However, it may be argued that we only tested a special case of *IA*, based on the implicit assumption of no correlation between the susceptibilities of the individuals of a test population to the different chemicals in a mixture. To account for the possibility of such correlations, Bliss (1939) suggested a more general version of eqn. [4] for independent action of two components by introducing a correlation coefficient *r*:

$$E(c_{mix}) = E(c_1) + E(c_2) \cdot [(1 - E(c_1)) \cdot (1 - r)] \qquad [8]$$

in which *r* may take values between 0 (absence of any correlation) and +1 (complete correlation). For the limiting case of $r = 0$ eqn. [8] reduces to eqn. [4]. For the other limiting case of $r = +1$, which has been termed "no addition" (Koenemann, 1981), the expected joint effect becomes equal to the single effect of the most active constituent alone. Later, Plackett and Hewlett (1948) extended this approach and developed a more general model for independent action, to account also for the possibility of complete negative correlation. Within this extended model *r* may vary in the range between -1 (complete negative correlation) and +1 (complete positive correlation). For the limiting case of $r = -1$, which has been termed "effect summation" (Boedeker *et al.*, 1992), the expected joint effect becomes equal to the simple algebraic sum of the individual effects of mixture constituents.

Prior to the testing of a mixture, information on correlations of susceptibilities is usually unavailable. Even if physiological reasons for assuming such a correlation are given these may not be readily transformable into a quantitative value for the abstract mathematical parameter *r*. Thus, predictions of mixture effects must be based on an assumption for *r*. Furthermore, mathematical treatment and biological reasoning of IA with correlations of susceptibilities has been worked out for binary mixtures only. General application of this approach to mixtures with

more than two components is a difficult and yet unresolved task, due to the resulting multi-dimensional correlation matrix. These are the reasons why we tested the predictive value of the basic version of *IA* with the assumption of no correlations between susceptibilities ($r = 0$).

Nevertheless, just in order to get an impression about the potential influence of such correlations on the prediction quality, we may also test the limiting cases of "effect summation" and "no addition", which are readily calculable. As an example, we may refer to a situation where all eight mixture components are simultaneously present in concentrations that would cause 5% effect if applied alone. These EC_{05} values (tab. 4) add up to a total concentration of about 555 nM, which has been observed to cause a complete inhibition of algal reproduction (fig. 4). In contrast, effects expectable from the assumption of *IA* with correlations of susceptibilities can only range from 5 % (no addition) to 8 x 5 % = 40 % (effect summation). In any case, this would remain a drastic underestimation of the actual joint effect and no substantial improvement of the predictive value of *IA*.

Quantitative differences between predictions of mixture toxicity by *CA* and *IA* depend on the number of mixture components, their concentration ratio (Faust *et al.*, 2001), the effect level considered, as well as on the slopes of the individual concentration-response curves and on the regression models used for their description (Boedeker *et al.*, 1993; Drescher and Boedeker, 1995). Depending on these parameters, both predictions may occasionally be indistinguishable within the ranges of experimental and biological variability (Backhaus *et al.*, 2004). Therefore, compliance of observed joint effects with *CA* alone is no sufficient argument for a similar action of mixture components. This was demonstrated by a review analysing 202 mixtures of similarly as well as of dissimilarly acting pesticides for compliance with *CA* (Deneer, 2000). However, compliance with CA in conjunction with nonconformity to *IA*, as observed in this study, gives a strong indicator for a common mechanism of action. Thus, results of both, QSAR analysis and mixture toxicity analysis, provide consistent evidence for the conclusion that the joint effects of chloroacetanilide herbicides on algae are largely explainable by a common specific mechanism of action and quite accurately predictable by the corresponding concept of *CA*.

To our knowledge, chloroacetanilides are the second group of herbicides for which *CA* could be demonstrated in multi-component mixture experiments. Congruent results have been obtained before with a series of 18 photosynthesis inhibiting *s*-triazines (Faust *et al.*, 2001). We may assume that the principal of *CA* might also be valid for other important groups of pesticides, like sulfonylureas or triazoles, although the experimental proof is yet missing. This expectation is supported by experience gained with binary combinations of pesticides (Deneer, 2000) and with multi-component mixtures of various non-pesticidal toxicants (Koenemann, 1980; Koenemann, 1981; Hermens *et al.*, 1984; Nirmalakhandan *et al.*, 1997; Faust *et al.*, 2000; Altenburger *et al.*, 2000; Backhaus *et al.*, 2000b).

There is an obvious discrepancy between this large body of accumulated scientific evidence and the common regulatory practice to focus hazard and risk assessment of contaminants in the aquatic environment on single substances only, thus largely disregarding the existence of clusters of substances for which *CA* might be anticipated. Although some binary mixtures of similarly acting insecticides have been reported to show an aquatic toxicity that is higher than *CA* **(**Deneer**,** 2000**)**, such cases appear to be exceptions and not the rule. Thus, in the absence of any evidence for specific synergistic interactions, *CA* should be justifiable as a reasonable approach to the predictive assessment of joint effects of similarly acting pesticides.

Conclusions

Risk assessments for individual chloroacetanilide herbicides may underestimate the total risk resulting from simultaneous exposure of aquatic life to different substances from this group. However, reliable estimates of joint effects of different chloroacetanilides can be derived from information about their individual toxicities by applying the concept of *CA*.

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II

Toxicity of sulfonylurea herbicides to the green alga *Scenedesmus vacuolatus*

Toxicity of Sulfonylurea Herbicides to the Green Alga Scenedesmus vacuolatus: Predictability of Combination Effects

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1 Introduction

Since their commercialization in 1981 sulfonylurea herbicides have been used for pre- and postemergence control of broadleaf and sedge weeds as well as of grasses in cereal, rice, maize, and potato crops (Brown and Cotterman, 1994). The first common enzyme of branched chain amino acids, acetolactate synthase, is reported to be the target enzyme in sensitive plants and bacteria (Blair and Martin, 1988). Landstein *et al.* (1990) as well as Nyström and Blanck (1998) demonstrated that interactions with this target site are also responsible for toxic effects of sulfonylureas on algae. Probably because of their low application rates (Brown and Cotterman, 1994) and their rather rapid degradation through hydrolysis (Berger and Wolfe, 1996; Sarmah and Sabadie, 2002) sulfonylurea herbicides are not regarded as relevant contaminants in European surface waters (European Commission, 1999). Nevertheless, they can elicit adverse effects in non-target aquatic organisms at very low concentrations, even below 1 nM (Nyström *et al.*, 1999). The hazard for non-target organisms might even be increased if they are exposed simultaneously to more than one sulfonylurea, as it was shown for mixtures of other herbicides with a common mechanism of action (Faust *et al.*, 2001; Junghans *et al.*, 2003a). Because of recommended premixtures (Blair and Martin, 1988; Brown and Cotterman, 1994) and the broad crop spectrum of sulfonylureas, a multiple exposure is likely in agricultural areas. This study aims to analyse whether the hazard of combinations of sulfonylureas can be predicted using only information on the toxicity of the individual mixture components.

For this purpose two concepts are usually applied: *(i)* concentration addition (*CA*) for mixtures with similarly acting components (Loewe and Muischnek, 1926) and *(ii)* independent action (*IA*) for mixtures of substances with mechanisms of actions

dissimilar from each other (Bliss, 1939). These concepts were originally formulated only for binary mixtures, but have been extended for multi-component mixtures. Berenbaum (1985b) formulated *CA* as:

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

where *n* is the number of mixture components, c_i denotes the concentration of the *i*th compound in the mixture and ECx_i the concentration of the *i*th compound that individually would provoke the same effect as the mixture.

The concept of *IA* can be formulated for multi-component mixtures (Grimme *et al.*, 1998a; Backhaus *et al.*, 2000a) as:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \qquad [2]$$

where $E(c_{mix})$ describes the predicted effect (scaled from 0-1) of a *n* compound mixture, c_i is the concentration of the *i*th compound in the mixture, and $E(c_i)$ describes the effect of that concentration if the compound is applied singly.

The choice between the two concepts depends on the knowledge of the mixture components' mechanisms of action. Since this knowledge is limited for many substances, which can be found in the environment, in ecological risk assessment Boedeker *et al.* (1993) suggested using *CA* as a worst-case estimation for the combined toxicity of toxicants.

In this study, firstly, the concentration response functions of eight sulfonylurea herbicides were determined in an algal bioassay. Based on these functions predictions according to both concepts for two different mixture ratios were calculated and, finally, compared with the experimentally determined mixture toxicity.

2 Materials and Methods

Eight sulfonylurea herbicides were selected: bensulfuron-methyl (BSM), cinosulfuron (CiS), chlorsulfuron (ChS), metsulfuron-methyl (MSM), primisulfuron-methyl (PriSM), prosulfuron (ProS), rimsulfuron (RS), and triasulfuron (TS). It was seen to select at least one representative for each of the main crops: Cereals (ChS, MSM, TS), maize (PriSM, ProS, RS), rice (BSM, MSM), and potatoes (RS) (Brown and Cotterman, 1994). All substances were obtained from Riedel de Haën (Seelze,

Germany) in analytical purity (cf. tab. 2). Stock solutions were prepared in methanol or acetonitrile (both chromatography grade, Merck, Darmstadt, Germany) and stored at –30°C. These stock solutions served as standards for the analytical validation of test concentrations. Aqueous stock solutions for the toxicity testing were prepared from aliquots of the methanolic or acetonitrilic solutions after evaporating the organic solvent under a stream of N_2. The residues were re-dissolved in algal growth medium (Grimme and Boardman, 1972) under vigorous stirring at room temperature. Concentrations of aqueous stock solutions were validated by reversed phase High Performance Liquid Chromatography (rp-HPLC) using a LiChrosphere RP 18e (Merck, Darmstadt, Germany) column (125 mm length 4 mm inner diameter, 5 µm particle size) and an UV detector at a wavelength of 215 nm. The mobile phases consisted of different acetonitril (Chromasolv, Riedel de Haën, Seelze, Germany) / phosphoric acid (0.01 M) ratios (BSM, PriSM, ProS, RS: 50/50; CiS: 40/60; ChS, MSM, and TS: 30/70). The flow rate of the mobile phase was 0.8 ml/minute (1 ml/minute for PriSM, ProS, TS). To evaluate the stability of the substances during the test, the substances were incubated with the algal growth medium under test conditions. For all tested sulfonylureas, stability under test conditions was greater than 87%. Concentration response data given in this paper always refer to the analytically validated initial concentrations. Since the toxicity of sulfonylureas is reported to be pH dependent (Fahl *et al.*, 1995), the pH in the test vessels was kept constant at 6.8 (+/- 0.2).

Effect parameter was the reproduction of a synchronically grown culture of the endospore-building green alga *Scenedesmus vacuolatus* strain 211-15 (culture collection of the University of Göttingen, Germany) over a period of one generation cycle (24h). The cell number was measured with a particle counter (Coulter Electronics, Bedford UK). The test was conducted as described by Faust *et al.* (2001).

Concentration-response analyses for both single substances and mixtures were performed in essentially the same way, as described by Faust *et al.* (2001). Concentration-response functions were estimated from the experimental data by applying the best-fit procedure introduced by Scholze *et al.* (2001). By this approach different nonlinear regression models are used to estimate a concentration-response relationship from a given set of experimental data, *i.e.* for

each tested sulfonylurea ten separate concentration-response relationships were established. Subsequently, based on statistical criteria that function was chosen, which described the experimental data best (the "best fitting model"). It turned out, that three out of ten different regression models proved to describe the sulfonylureas' concentration-response relationships best. They are given in tab. 1. Confidence intervals for the EC_{50} and EC_{05} values were estimated using the bootstrap methodology (Scholze *et al.*, 2001). In case of predicted effect concentrations, the bootstrap samples were generated based on the effect distributions that were estimated within the fitting process for every individual concentration-response function (parametric bootstrap). NOECs (No observed effect concentrations) were determined by applying Dunnett's test (Dunnett, 1964).

Table 1. Regression models.

Name	Function (F)
Weibull (W)	$E = 1 - \mathbf{exp}\left(-\mathbf{exp}\left(a + b\ \mathbf{log}_{10}(c)\right)\right)$
Generalized Logit 2 (GL2)	$E = 1/\left(1 + \mathbf{exp}\left(+a + b\ \mathbf{log}_{10}(c)\right)\right)^{g}$
Box-Cox-Weibull (BCW)	$E = 1 - \mathbf{exp}\left(-\mathbf{exp}\left(a + b\left(\left(c^{g} - 1\right)/g\right)\right)\right)$

E – Effect, expressed as fraction of a maximum possible effect (0 = E = 1); *c* – Concentration; *a, ß, ?* – Model parameters;
$\exp(x) = e^{x}$.

Experimental concentration-response analyses of mixtures were performed using a fixed ratio design, *i.e.* the total concentration is varied, whereas the molar ratio of the constituents remains constant. The concentration ratio was intended to reflect the relative toxicities of individual sulfonylureas. However, in case of non-parallel concentration-response curves this may vary with the effect level *x*. Therefore, we tested two different concentration ratios: the eight sulfonylureas were present either in the ratio of their individual EC_{05} values (Mixture A) or EC_{50} values (Mixture B), respectively (tab. 2). For composing the mixture ratios, the relative proportion *p* of each substance *i* is calculated according to:

$$p_i = \frac{EC}{\sum_{i=1}^{i=8} EC} \qquad [3]$$

for the respective effect level *x*. Predictions of effect concentrations for mixtures by *CA* and *IA* were calculated according to eqns. [1] and [2], respectively. More details can be found in Faust *et al.* (2003).

3 Results and Discussion

For the eight sulfonylurea herbicides complete concentration-response curves were determined. The concentration response functions, the EC_{50}, EC_{05}, and NOEC values of the single substances are given in tab. 2. It can be seen, that sulfonylureas are highly toxic to *Scenedesmus vac.*, with EC_{50} values ranging from 0.05 (bensulfuron-methyl) to 5.8 µM (rimsulfuron).

A comparison of the EC_{50} values obtained in this study with those from Nyström *et al.* (1999), determined for 34 algal species under comparable pH conditions, reveals that in case of chlorsulfuron only one species (*Monoraphidium pusillum*: 0.2 µM) is more sensitive than *Scenedesmus vac.* (1.2µM) and two (*Monoraphidium contortum:* 0.9 µM; *Bumilleriopsis filiformis*: 0.9 µM) can be regarded as being equally sensitive. All other of the 34 algal species were less sensitive to ChS, one species even 3 orders magnitude less sensitive. For metsulfuron-methyl a similar picture can be observed: only 4 algal species were more sensitive (*Amphidinium carterae*: <0.001: *Monoraphidium pusillum*: 0.4 µM; *Prorocentrum minimum*: 0.8 µM; *Staurastrum gracile*: 0.5 µM), 6 equally sensitive, whereas all other 24 species were less sensitive than *Scenedesmus vac.* with an EC_{50} of 3.1 µM. It can be concluded, that *Scenedemus vac.* belongs to the more sensitive algal species for sulfonylurea herbicides. However, it must be mentioned that for metsulfuron-methyl also 4 of the 5 studied cyanobacteria, were more sensitive than *Scenedesmus vac.*, one species even more than 3 orders of magnitude.

The predicted concentration-response curves for *CA* and *IA* are displayed in fig. 1 together with the results of the mixture toxicity tests. For the sake of simplicity, the fitted concentration-response curves for the mixture have been omitted from the figures. Comparing the predicted EC_{50} values according to both concepts, *IA* predicts a mixture toxicity that is 1.3 - 1.5 times higher than the toxicity predicted by the concept of *CA* (see tab. 3).

Table 2. Concentration-response functions, *EC* and NOEC values for the sulfonylurea herbicides.

Substance [a] *(in order of EC50)*	**BSM**	**ProS**	**TS**	**PriSM**	**ChS**	**MSM**	**CiS**	**RS**
CAS RN [b]	83055-99-6	94125-34-5	82097-50-5	86209-51-0	64902-72-3	74223-64-6	94593-91-6	122931-48-0
Purity	99.5 %	98.1 %	99.0 %	99.9 %	99.8 %	99.5 %	99.4 %	99.2 %
MW [c]	410.4	419.4	401.8	468.3	357.8	381.4	413.4	431.4
RM [d]	W	BCW	W	W	GL2	BCW	W	W
$\hat{a}$ [e]	1.425	1.392	0.093	0.008	-3.333	-1.364	-1.706	-1.968
$\hat{b}$ [e]	1.376	0.987	1.684	1.863	2.552	0.903	1.929	2.092
$\hat{g}$ [e]		-0.027			16.438	-0.018		
***EC*$_{05}$ µM** [f]	0.0006 [0.0003;0.0012]	0.015 [0.01;0.03]	0.015 [0.01;0.02]	0.025 [0.01;0.04]	0.111 [0.09;0.13]	0.174 [<0.01;0.56]	0.221 [0.14;0.34]	0.332 [0.13;0.71]
***EC*$_{50}$ µM** [f]	0.05 [0.04;0.068]	0.18 [0.16;0.20]	0.53 [0.48;0.59]	0.63 [0.56;0.71]	1.19 [1.14;1.26]	3.05 [2.38;3.80]	4.95 [4.46;5.46]	5.83 [4.70;7.40]
NOEC µM [g]	0.00024	0.0033	n.d. [h]	0.028	n.d [h].	0.433	0.363	0.508

[a] Abbreviated common name (see Material and Methods), [b] Chemicals Abstracts Services Registry Number, [c] Molecular weight, [d] RM: regression model (see tab. 1), [e] Statistical estimates of model parameters, [f] Mean effect concentrations with 95% two-sided bootstrap confidence intervals, [g] NOEC determined by DUNNETT test, [h] n.d. = not determinable

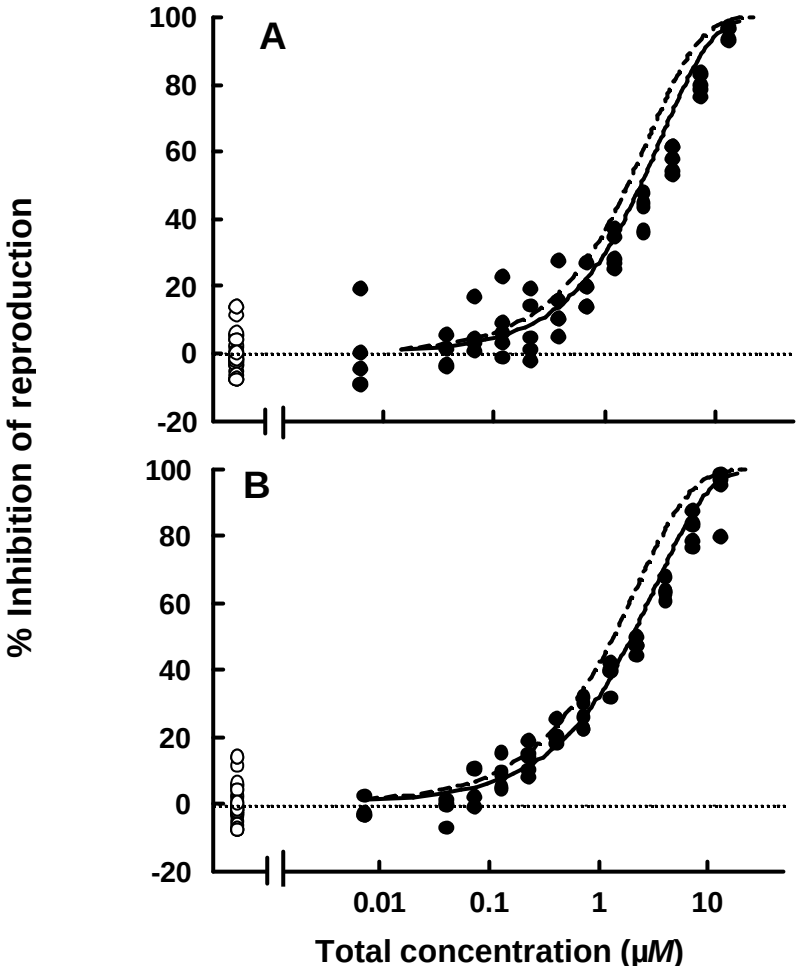

Figure 1. Observed and predicted algal toxicity of sulfonylurea mixtures. (A) Mixture A: components mixed in the ratio of their individual EC_{05} values. (B) Mixture B: components mixed in the ratio of their individual EC_{50} values. (•) experimentally observed toxicity; (o) controls; (- - - -) prediction according to *concentration addition*; (- - -) prediction according to *independent action*.

As indicated by the non-overlapping 95% confidence intervals, the observed EC_{50} values are significantly higher than predicted by both concepts. Nevertheless, the absolute differences between observations and both predictions are quite small. *CA* overestimates the toxicity only by a factor of 1.4 (mixture A), respectively 1.2 (mixture B). On the other hand, *IA* overestimates the toxicity in both mixture ratios by a factor of about 1.8. Thus in both tested mixture ratios *CA* describes the observed mixture toxicity best.

The good predictability of the mixture toxicity of the eight tested sulfonylureas by *CA* is consistent with other studies on the predictability of multi-component mixtures of similarly acting substances (Faust *et al.* 2001; Escher and Hermens 2002 and references therein; Junghans *et al.* 2003; Backhaus *et al.* 2004).

Table 3. Predicted and observed algal toxicity of sulfonylurea mixtures.

	EC_{50} [µM]					
	by *CA* [i]	by *IA* [i]	observed [f]	RM [d]	$\hat{a}$ [e]	$\hat{b}$ [e]
Mixture A	2.09 [1.93-2.19]	1.59 [1.22-1.94]	2.94 [2.67-3.21]	W	-1.382	2.169
Mixture B	2.02 [1.86-2.12]	1.36 [1.04-1.69]	2.37 [2.21-2.54]	W	-0.974	1.618

[i] Predicted effect concentrations with 95% two-sided bootstrap confidence intervals, other signs and abbreviations as given in tab. 2

From an empirical point of view, it is remarkable that *IA* predicts a higher mixture toxicity than *CA*, even though the differences between the predictions are small. In the majority of mixture toxicity studies with multi-component mixtures, in which the observed mixture toxicity was compared with predictions according to both concepts, this relationship was reversed (Backhaus *et al.* 2000; Faust *et al.* 2001; Faust *et al.* 2003; Lock and Janssen 2002; Junghans *et al.* 2003 and references therein). Studies on the quantitative relationship between predictions according to *CA* and *IA* revealed three major factors: the steepness of the individual concentration-response curves (CRCs), the number of mixture components, and the mixture ratio (Boedeker *et al.*, 1993; Drescher and Boedeker, 1995; Faust *et al.*, 2001). In the case of the sulfonylurea mixtures, the rather flat CRCs can be regarded as the decisive factor. Using the ratio between the EC_{05} and the EC_{50} as a measure for the steepness of a CRC, we obtain an average value of 0.051 for all sulfonylureas presented in this paper. In a comparable study on the mixture toxicity of eight chloroacetanilides (Junghans *et al.*, 2003a), the average value was 0.325. This is almost one order of magnitude higher, *i.e.* the slopes of the CRCs of the chloroacetanilides are steeper than that of the sulfonylureas presented in this paper. In contrast to the study with the sulfonylureas, *CA* predicted a higher mixture toxicity than *IA* (by a factor of 3), although the number of mixture components was identical in both studies, and the mixture toxicity was studied in comparable mixture ratios (EC_{05} and EC_{50}). Thus, the different steepness of chloroacetanilide and sulfonylurea concentration-response curves is responsible for the reverse quantitative relationships between *CA* and *IA* predictions.

Also from a regulatory point of view the higher toxicity predicted by *IA* is remarkable, as it has been suggested to use *CA* as a reasonable worst case

approach for the predictive hazard assessment of chemical mixtures (Berenbaum, 1985b; Boedeker *et al.*, 1993). The worst-case assumption has not been falsified by this study, since the relatively higher predicted toxicity of the eight sulfonylureas according to *IA* was with a factor of 1.5 quantitatively small. Moreover, the observed toxicity did not exceed the toxicity predicted by *CA*. But it seems that the worst-case assumption might be challenged with mixtures dominated by dissimilarly acting substances with very flat concentration-response relationships.

The following conclusions can be drawn from the presented algal toxicity study with eight sulfonylurea herbicides: (1) the concentration-response relationships of the tested sulfonylureas are rather flat compared to those obtained for other herbicides with the same test organism, resulting (2) in a rather unexpected higher predicted toxicity by the concept of *IA*, which (3) challenges but not falsifies the worst-case assumption for *CA* since (4) the observed toxicity did not exceed the toxicity predicted by *CA* but (5) was rather accurately predictable by this concept.

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III

Predicting the joint algal toxicity of chemical mixtures using a mechanism based two stage prediction (*TSP*)

Predicting the joint algal toxicity of chemical mixtures using a mechanism based Two Stage Prediction (*TSP*)

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In preparation

Abstract

Two concepts have shown to provide accurate predictions for mixture toxicity, *concentration addition* (*CA*) for similarly acting substances and *independent action* (*IA*) for dissimilarly acting substances. However, in environmentally relevant chemical mixtures similarly as well as dissimilarly acting substances have an impact on organisms at risk at the same time. As a step towards greater knowledge concerning the predictability of the toxicity of mixtures containing similarly as well as dissimilarly acting substances we studied 40-component mixtures, in which the components can be clustered into four groups: *chloroacetanilides*, *sulfonylurea*, *quinolones* and *triazines*. The groups have dissimilar mechanisms of action, whereas within these groups the components act strictly similarly. Additionally another mixture containing as a fifth group 3 congeneric *quaternary ammonium compounds* was tested in the same way. The mixtures were studied for their effects on the reproduction of the green alga *Scenedesmus vacuolatus*. Predictions were made according to three approaches: *CA*, *IA*, and, according to a new approach, the *two stage prediction (TSP)* where the mixture toxicity of each group with a similar mechanism of action is predicted by *CA* and the overall toxicity is then predicted by assuming *IA* between the groups. For all mixtures and mixture ratios, the *TSP* approach led to accurate predictions. Moreover, predictions according to *CA* and *IA* were distinctly less good: *CA* overestimated the mixture toxicity whereas *IA* underestimated it. This may be seen as the first experimental proof of the *TSP* approach. The analysis of the mixture toxicity from low individual effect concentrations underlines the importance of mixture toxicity assessments.

1 Introduction

In ecotoxicology, the relevance of mixture toxicity has been recognised (Eggen *et al.*, 2004). It has been shown that a hazard assessment only for single substances may lead to clear underestimations of the toxicity (Backhaus *et al.*, 2000b; Faust *et al.*, 2001; Walter *et al.*, 2002; Faust *et al.*, 2003): a significant mixture toxicity may result even from concentrations at which the corresponding effects of the single mixture components are below their statistical significance level. Thus, the assessment of the mixture toxicity seems to be mandatory. However, the direct testing of every possibly occurring mixture is not feasible. If both the composition and the toxicity of the components are known, the prediction of the mixture toxicity has shown to be a promising alternative to direct testing.

For predictive mixture toxicity assessments, generally two concepts have found widespread use: *concentration addition* (*CA*) and *independent action* (*IA*).

CA was introduced by Loewe and Muischnek (1926) to describe the joint toxicity of similarly acting toxicants. It assumes that the mixture components have a common mechanism of action and act at the same molecular target site. The components are assumed to differ only in the relative strength of their toxic effect, like being dilutions of the same substance. Berenbaum (1985) formulated *CA* for multi-component mixtures of *n* substances as:

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

where c_i are the concentrations of the individual mixture components and ECx_i is the concentration of the ith component that individually would cause the same quantitative effect x as the mixture. The quotients c_i/ECx_i have been termed toxic units (TUs) (Sprague, 1970). They represent the concentrations of mixture components as fractions of equi-effective individual concentrations. If CA holds, any substance can be substituted by an equally potent concentration of another substance without altering the overall effect.

IA can be regarded as the alternative concept, because it is based on the assumption that the mixture components have dissimilar mechanisms of action, *i.e.* they cause a common integral biological effect (e.g. death) through primary interaction with different molecular target sites. First introduced by Bliss (1939)

for binary mixtures, IA was extended for multi-component mixtures of n substances by Backhaus *et al*. (2000a) and in more detail by Faust *et al*. (2003) to:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \quad \text{with } c_{mix} = c_1 + \ldots\ldots + c_n \qquad [2]$$

in which c_i denotes the concentrations of the *i*th mixture component, $E(c_i)$ its corresponding effect and $E(c_{mix})$ the total effect of the mixture.

In several experimental studies with multi-component mixtures *CA* and *IA* have been successfully applied for the predictive assessment of the mixture toxicity of reference mixtures composed of similarly acting substances (Altenburger *et al.*, 2000; Rajapakse *et al.*, 2002; Escher and Hermens, 2002 and references therein; Drost *et al.*, 2003, Junghans *et al.* 2003a) and dissimilarly acting substances (Backhaus *et al.*, 2000a; Faust *et al.*, 2003), respectively.

However, these studies also reveal that for both concepts the similarity respectively dissimilarity of the mechanisms of action of the mixture components seems to be a crucial factor for the prediction quality of the concepts. Both concepts gave good predictions, if the pharmacological assumptions were met, but *CA* tends to overestimate the mixture toxicity of dissimilarly acting substances and *IA* tends to underestimate the toxicity of similarly acting substances.

For accurate predictions of the toxicity of environmentally relevant mixtures, this poses a problem: strict similarity respectively dissimilarity of the mixture components' mechanisms of action cannot be generally expected. This has also been recognized by Ankley and Mount (1996). To overcome this problem they suggested assigning the mixture components to discrete mechanism of action groups prior to mixture toxicity assessments according to *CA*. We would like to adopt this approach and extend it to a *two-stage prediction* (*TSP*) for the integral joint toxic effect of mixtures, in which the components can be assigned according to their mechanisms of action to discrete groups. As visualized in fig. 1 the *TSP* combines *CA* and *IA* in a stepwise approach. In stage 1 of a *TSP,* the mixture components are clustered into groups, in which the mechanisms of action of the components are strictly similar. This grouping is based on *CA*, which assumes that similarly acting substances act like being dilutions of the same compound. Accordingly, the joint effect, which the components of each *CA* group elicit at their common receptor site (R), is then predicted by *CA*. The groups will thus be

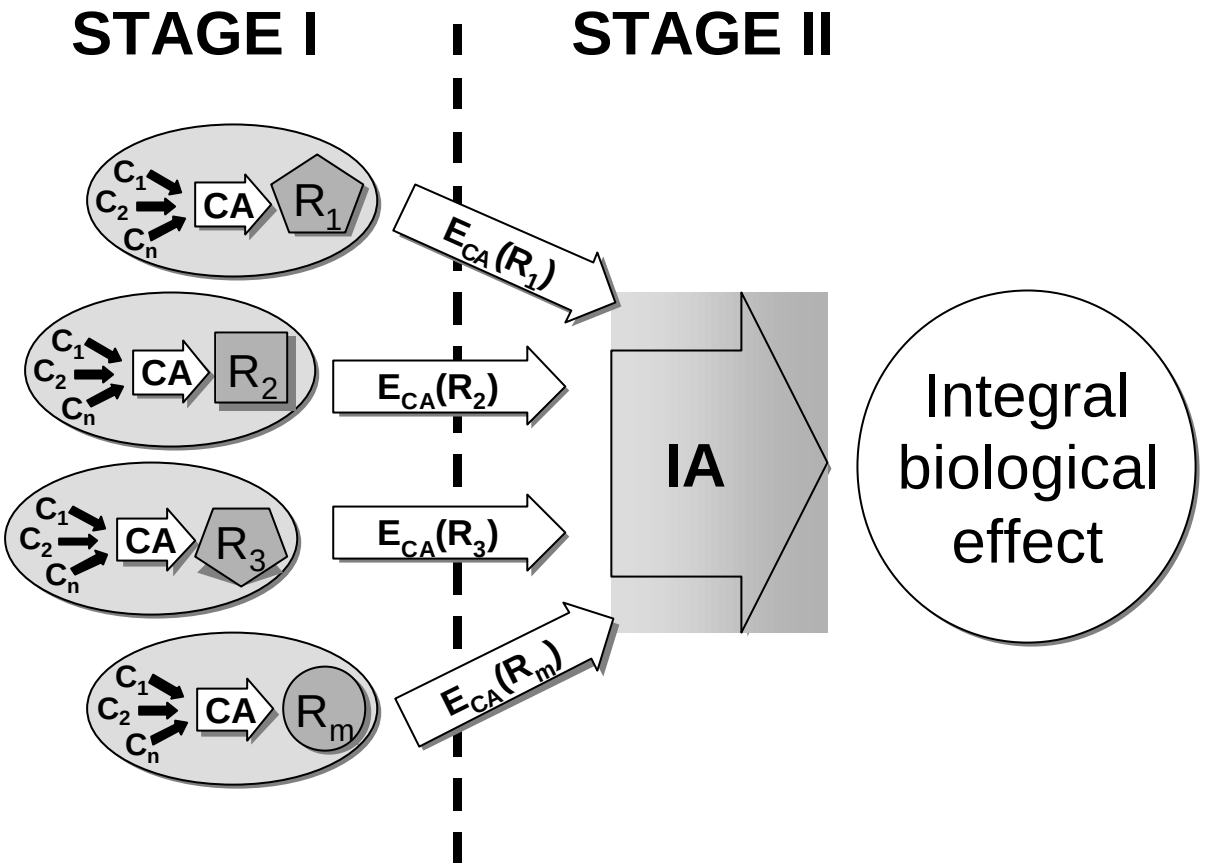

Figure 1. The stepwise approach of the *Two Stage Prediction* (*TSP*). *CA* = *concentration addition*; *IA* = *independent action*. C_{1-n} are individual mixture components that exclusively act on one of the distinct receptors (R_{1-m}); $E_{CA}(R_{1-m})$ represents the integral effect the substances C_{1-n} cause through interaction with one of the distinct receptors R_{1-m}, as predicted by *CA*

called *CA* groups for the remainder of the article. In stage 2 of a *TSP*, *IA* integrates over all predicted effects ($E_{CA}(R_i)$) of the *CA* groups to predict the integral effect of the mixture. The *TSP* would reduce to *CA* if only one *CA* group exist and to *IA* if every *CA* group consists of only one mixture component. A similar approach was suggested by Traas *et al.* (2002) for predicting species sensitivity distributions (SSDs) of mixtures. Since the validity of both *CA* and *IA* has been shown for mixtures in which the model assumptions concerning the mechanisms of action of the mixture components were met, the combination of *CA* and *IA* to a *TSP* seems to be a reasonable approach. This study aims to analyze the validity of this approach. For this purpose, mixtures have to be composed according to three criteria: (*i*) based on their mechanism of action, the mixture components have to be assignable to discrete groups in which a single mechanism of action is shared, (*ii*) the mechanism of action between the groups have to be strictly dissimilar, and (*iii*) the

predictions according to *CA*, *IA*, and *TSP* have to be quantitatively discriminable. This last criterion is important to allow for unambiguous results, since it has recently been shown that compliance with a prediction is no conclusive argument for the mechanistic validity of a prediction (Backhaus *et al.*, 2004). Based on these criteria, 43 substances from five environmentally relevant chemical classes were selected for testing. For all of them single substance as well as mixture toxicity assessments have been performed in previous studies within the same algal biotest to confirm their concentration additive mixture toxicity. The toxicological endpoint was the inhibition of reproduction of the freshwater chlorophyte Scenedesmus vac. Based on the known concentration-response relationships of these substances predictions according to *TSP*, *CA*, and *IA* were made, and two mixtures were selected for experimental mixture toxicity analysis. The predictive quality of all three approaches was evaluated by comparing the observed toxicity with the predictions. Additionally, mixture effects from low individual effect concentrations were analysed.

2 Material and Methods

2.1 Algal bio-test

Test organism for the single substance and mixture experiments was the chlorococcal algae *Scenedesmus vac.* (strain 211-15, culture collection of the University of Göttingen, Germany). As toxicological endpoint, the inhibition of reproduction of synchronously growing cells was recorded after an exposure period of one-generation cycle (24h). Cell numbers were determined by electronic cell counting (Coulter Electronics, Miami, USA). The original test protocol was described by Altenburger *et al.* (1990). It was used with the modifications by Faust *et al.* (2001).

2.2. Test substances: mechanisms of action and environmental relevance

The components of four mixtures of similarly acting toxicants, for which the predictability by *CA* has previously been demonstrated within the same bio-test, were selected as test substances: 18 *s-triazines* (Faust *et al.*, 2001), eight *chlorocetanilides* (Junghans *et al.*, 2003a), eight *sulfonylureas* (Junghans *et al.*, 2003b), and six *quinolones* (Backhaus *et al.*, unpublished results). For all four mixtures, the predictive power of *IA* was distinctly lower. As a fifth group 3

quaternary ammonium compounds were selected for testing. Due to their high structural similarity, (the three substances were part of a congeneric series), similarity of action can be assumed. However, because of a rather high variability of the test results as well as a rather low quantitative difference between *CA* and *IA* predictions, the superior predictive power of *CA* could not be demonstrated (Meyer *et al.*, unpublished results).

The groups have dissimilar mechanisms of action, whereas within these groups the members act strictly similarly. *s-Triazines* inhibit the photosynthetic e^- transport by competitively binding to the same domain of the D1 protein of photosystem II as the e^- acceptor plastoquinone Q_B (Bowyer *et al.*, 1991; Tietjen *et al.*, 1991). For *chloroacetanilides,* the mechanism of action had been recently confined to the inhibition of the synthesis of very long chain fatty acids (Böger *et al.*, 2000). *Sulfonylureas* are reported to inhibit the acetolactate synthase, which is the first common enzyme of branched chain amino acids (Blair and Martin, 1988). *Quinolones* are antibiotics known to inhibit the A-subunit of the DNA gyrase, a protein that introduces negative superhelical twists into the DNA strands of bacteria (Bryan *et al.*, 1989). The recent detection of a gyrase-like protein in plants (Gadelle *et al.*, 2003) may explain the finding that *quinolone* toxicity in algae is higher than the unspecific effect that could be expected to result from hydrophobicity-driven accumulation in cellular membranes alone (Backhaus *et al.*, 2001). *Quaternary ammonium compounds* are cationic surfactants containing a polar headgroup and a hydrophobic tail (in our case C10-C14 alkyl chains). They accumulate at surfaces and interfaces, lowering the surface and interfacial free energies (Versteeg *et al.*, 1997), and are able to solubilize phospholipids in membranes.

All five chemical classes are of environmental relevance for surface waters. *s-Triazines*, *chloroacetanilides* and *sulfonylureas* are commonly used herbicides. A compilation of monitoring data for freshwaters in member states of the European Communities (European Commission, 1999) identified eight *s-triazines* (atrazine, cyanazine, prometryn, propazine, sebuthylazine, terbuthylazine, and terbutryn) and three *chloroacetanilides* (alachlor, metazachlor and metolachlor) as being relevant and representative on a European scale. *Quinolones* have a widespread use as antibiotics in aquaculture and in human as well as in veterinary medicine

(Backhaus *et al.*, 2000b and references therein). Because of their application as fabric softener or disinfectant, *quaternary ammonium compounds* may enter surface water bodies through household effluents (Garcia *et al.*, 2001).

2.3 Test solutions and chemical analysis

All substances were obtained from Sigma (Seelze, Germany) in analytical purity (cf. annexes 1-4). Stock solutions were prepared in methanol; acetonitrile, or acetone (all chromatography grade, Merck, Darmstadt, Germany) and stored at –30°C. These stock solutions served as standards for the analytical validation of test concentrations.

Aqueous stock solutions of *triazines*, *chloroacetanilides* and *sulfonylureas* were prepared in algal growth medium (Grimme and Boardman, 1972) according to procedures described in previous publications (Faust *et al.*, 2001; Junghans *et al.*, 2003a; Junghans *et al.*, 2003b), which also state the conditions for the chemical analysis as well as the stability under test conditions.

For *quinolones* and *quaternary ammonium compounds* aqueous stock solutions were prepared from aliquots of acetonic stock solutions by evaporating the organic solvent under a stream of N_2 and re-dissolving the residue in algal growth medium (Grimme and Boardman, 1972) under vigorous stirring at room temperature.

Concentrations of aqueous stock solutions of *quinolones* were validated by reversed phase High Performance Liquid Chromatography (rp-HPLC) using a LiChrosphere RP 18e (Merck, Darmstadt, Germany) column (125 mm length 4 mm inner diameter, 5 µm particle size) and an UV detector at different wavelengths (enoxacin: 270 nm; flumequine: 240 nm; lomefloxacin: 288 nm; nalidixic acid: 256 nm; norfloxacin: 280 nm; ofloxacin: 294 nm). The mobile phases consisted of different acetonitrile[4] / (0.1 M phosphoric acid[5] + 0.01 M ammonium acetate[6]) ratios: 20/80 (lomefloxacin), 25/85 (enoxacin, norfloxacin, ofloxacin), 30/70 (nalidixic acid), 40/60 (flumequine). To evaluate the stability of the substances during the test the substances were incubated with the algal growth medium under test conditions. For all tested *quinolones,* stability under test conditions was higher than 90%.

[4] chromatography grade, Merck, Darmstadt, Germany
[5] Janssen Chimica, Geel, Belgium
[6] Riedel de Haën, Seelze, Germany

For *quaternary ammonium compounds,* a chemical analysis by HPLC was not feasible. Test concentrations were therefore analysed experimentally, by validating the *EC20*, *EC50*, and *EC80* of the respective concentration-response curve. Stability under testing conditions was analysed by bio-testing a single aqueous stock solution twice: prior and after incubation under the testing conditions (without algae). As both experiments resulted in a similar toxicity (Meyer *et al.*, unpublished results), stability under testing conditions was assumed.

Concentrations given in this paper always refer to the analytically or experimentally validated initial concentrations.

Since the toxicity of *sulfonylureas* is reported to be pH dependent (Fahl *et al.*, 1995), the pH in the test vessels was kept constant at 6.8 (+/- 0.2).

2.4 Tested mixtures

Two mixtures composed of similarly as well as dissimilarly acting substances were tested. The first mixture (*mixture 1*) was composed only of the groups of substances for which concentration additive mixture toxicity has been experimentally demonstrated: *triazines*, *chloroacetanilides*, *sulfonylureas*, and *quinolones*. This 40-component mixture was tested in two different mixture ratios. One ratio was tailored to reflect the *TSP* (fig. 2). This mixture ratio will be called "*EC15.9* ratio of *CA* groups" for the remainder of the article, because all *CA* groups are present in their predicted *EC15.9* at the *EC50* predicted by *TSP* thus contributing with equal effects to the predicted *EC50* (fig. 2). Within the *CA* groups, the components were present in the ratio of their *EC50* values. The exact mixture ratio is given in tab. 1. In the other ratio of *mixture 1*, the components were present in the ratio of their individual *EC01* (for *EC01* values please refer to annexes 1-4). The *EC01* mixture ratio was selected for two reasons. One reason is that for the 40 selected substances the quantitative difference between *CA* and *IA* predictions is almost maximal for this mixture ratio: the *EC50* predicted by *IA* is by a factor of 4 higher than predicted by *CA* and all three predictions are well discriminable for effect levels ranging from 10 to 100%. The other reason is that it allows the evaluation of mixture effects from low individual concentrations, which

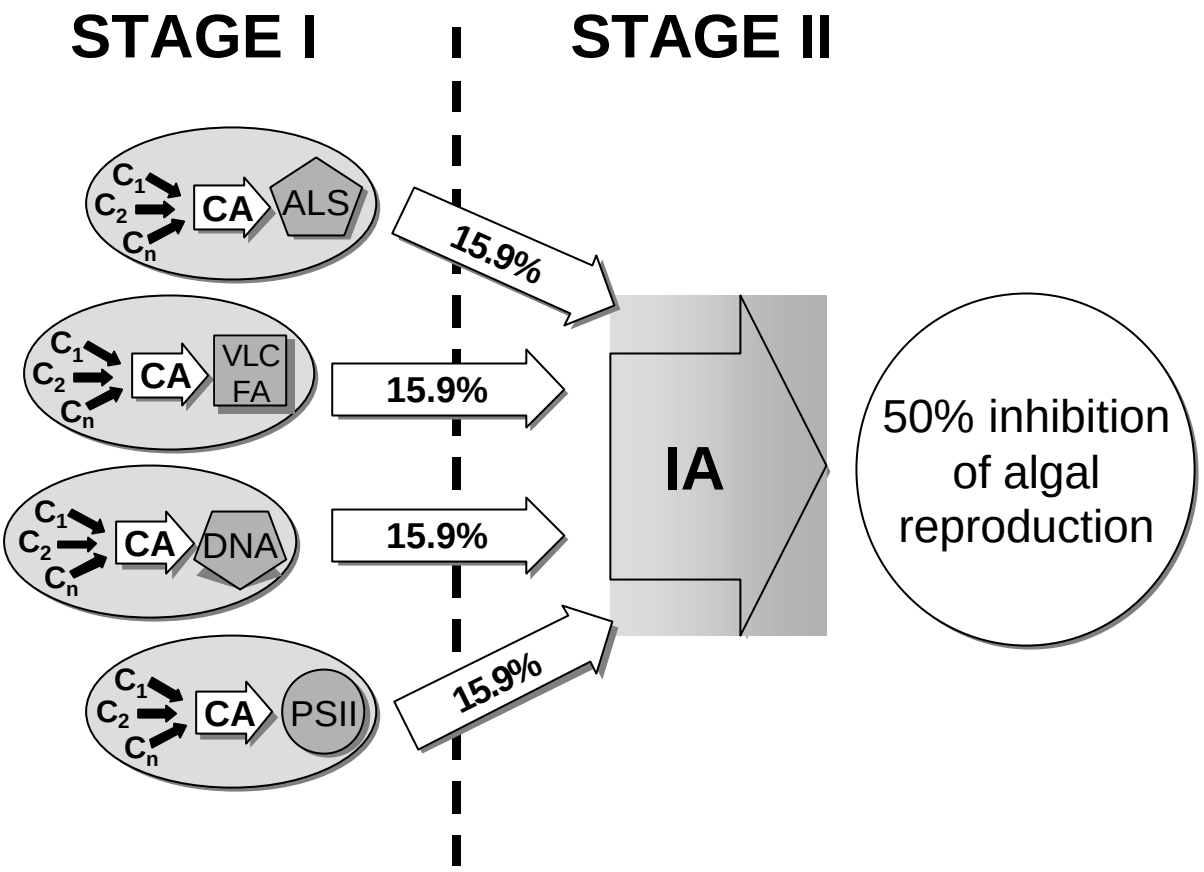

Figure 2. Mixture ratio of *EC15.9* of *CA* groups. *CA* = *concentration addition*; *IA* = *independent action*. $C_{1\text{-}n}$ are individual mixture components that exclusively act on one of the distinct receptors: ALS = inhibition of acetolactate synthase, the first common enzyme of branched chain amino acids; VLCFA = inhibition of very long chain fatty acid synthesis; DNA = Inhibition of the A-subunit of the DNA gyrase (GyrA) (topoisomerase II), a protein which introduces negative superhelical twists into the DNA strands of bacteria and chloroplasts; PSII = Inhibition of photosynthetic e^- transport by competitively binding to the same domain of the D1 protein of the photosystem II as the e^- acceptor plastoquinone Q_B. Equal effect contribution of the four groups to the *EC50* predicted by *IA* can be calculated by: $50\%=0.5=1-[(1-\text{effect})^4]$; the effect has been computed by iterative procedure and resulted in 0.159=15.9%. Within the *CA* groups, the components are present in the ratio of their individual *EC50* values.

Table 1
***EC15.9* ratio of *CA* groups**

Component [a]	fraction [b]	component	fraction	component	fraction	component	fraction
acetochlor	6.616E-05	**desmetryn**	1.611E-05	**metsulfuron-methyl**	1.538E-03	**prosulfuron-methyl**	8.840E-05
alachlor	2.055E-04	**dimethachlor**	3.561E-04	**nalidixic acid**	1.477E-01	**rimsulfuron**	2.936E-03
ametryn	6.876E-06	**dimethametryn**	2.262E-05	**norfloxacin**	2.268E-01	**sebuthylazin**	5.828E-05
atraton	1.660E-04	**dipropetryn**	3.517E-05	**ofloxacin**	3.868E-01	**secbumeton**	7.372E-05
atrazin	7.889E-05	**enoxacin**	7.787E-02	**pretilachlor**	1.989E-05	**simazin**	1.238E-04
bensulfuron-methyl	2.513E-05	**flumequin**	2.208E-02	**primisulfuron-methyl**	3.173E-04	**simetryn**	2.099E-05
butachlor	1.397E-05	**lomefloxacin**	1.269E-01	**prometon**	2.400E-04	**terbumeton**	4.541E-05
chlorsulfuron	5.969E-04	**metazachlor**	2.456E-04	**prometryn**	2.255E-05	**terbuthylazin**	3.040E-05
cinosulfuron	2.492E-03	**methoprotryn**	3.032E-05	**propachlor**	2.362E-04	**terbutryn**	1.420E-05
cyanazin	4.967E-05	**metolachlor**	1.198E-03	**propazin**	1.359E-04	**triasulfuron**	2.688E-04

[a] common name [b] relative proportion of the respective component in the mixture; all fractions sum up to 1

would not cause a significant effect if applied singly: *EC01* values for all mixture components were equal to or smaller than the respective NOEC (annexes 1-4). The second mixture (*mixture 2*) is a mixture composed of 38 components in the ratio of their individual no observed effect concentrations (*NOECs*). This mixture additionally comprised the group of *quaternary ammonium compounds*. Five substances included in *mixture 1* were lacking in *mixture 2*: chlorsulfuron, pretilachlor, and triasulfuron because of undetermined *NOEC* values; norfloxacin and terbutryn because their aqueous stock solutions were not available at the beginning of the mixture experiment. For NOEC values please refer to annexes 1-4. Like the *EC01* ratio in *mixture 1* the NOEC ratio was selected for the evaluation of the mixture toxicity from low concentrations, which would not cause a significant effect if applied singly.

For both mixtures and all mixture ratios experimental concentration-response analyses were performed using a fixed ratio design, *i.e.* the total concentration of the mixture is varied, whereas the molar ratio of the constituents remains constant. The fixed ratio design was selected for two reasons: (*i*) it allows for a comparison of the observations with the predictions according to *CA*, *IA*, and *TSP* and (*ii*) it allows the statistical estimation of the uncertainty connected to observations and predictions according to *CA* and *IA*. For *TSP* predictions statistical uncertainty cannot be estimated so far.

2.5 Prediction of mixture toxicity

The concentration-response curves of the single mixture components have been determined in previous studies (Faust *et al.*, 2001; Junghans *et al.*, 2003a; Junghans *et al.* 2003b; Backhaus *et al.*, unpublished results; Meyer *et al.* unpublished results). In these studies, the concentration-response analysis was designed to allow for statistical estimates of *ECx* values with $x = 1\%$. To exclude shifts in the concentration-response-curves, the respective concentration-response curve of every mixture component was confirmed experimentally by testing the *EC20*, *EC50*, and *EC80* of prior to the mixture toxicity assessments.

Based on statistical estimates of the mixture components' concentration-response relationships (parameters of regression models are given in annexes 1-4; the model equations in tab. 2), predictions according to *CA*, *IA*, and *TSP* were calculated.

Table 2 Regression models (RM)

Name	Function (F)	Inverse Function (F^{-1})
Logit	$E = 1 / (1+\exp(-a - b \log_{10}(c)))$	$c' = 10^{\left((-\log_e((1/1-E)-\hat{a})/\hat{b}\right)}$
Weibull (W)	$E = 1-\exp(-\exp(a + b \log_{10}(c)))$	$c' = 10^{\left((\log_e(-\log_e(1-E))-\hat{a})/\hat{b}\right)}$
Generalized Logit (GL)	$E = 1 / (1+\exp(-a - b \log_{10}(c)))^g$	$c' = 10^{\left((-\log_e((1/E)^{(1/\hat{g})}-1)-\hat{a})/\hat{b}\right)}$
Generalized Logit 2 (GL2)	$E = 1 / (1+\mathbf{exp}(a + b \log_{10}(c)))^g$	$c' = 10^{\left(\left(\log_e\left((1/1-E)^{(1/\hat{g})}-1\right)-\hat{a}\right)/\hat{b}\right)}$
Box-Cox-Weibull (BCW)	$E = 1-\exp(-\exp(a + b((c^g-1)/g)))$	$c' = \left((\hat{g}/\hat{b}) \bullet (\log_e(-\log_e(1-E)) - \hat{a}) + 1\right)^{(1/\hat{g})}$
Box-Cox-Probit (BCP)	$E = 1/\sqrt{2p} \int_{-\infty}^{a+b((c^g-1)/g)} \exp(-u^2/2)du$	$c' = \left((\hat{g}/\hat{b}) \bullet (\text{Probit}\ (E) - \hat{a}) + 1\right)^{(1/\hat{g})}$

E – Effect, expressed as fraction of a maximum possible effect (0 = E = 1);
c' – Concentration;
a, b, g – Model parameters (corresponding statistical estimates marked by ^);
Probit – Inverse standard normal (Gaussian) distribution function;
$\exp(x) = e^x$

CA predictions were calculated according to the following equation, which can be derived from eqn. [1] by rearrangement

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \qquad [3]$$

with p_i being the concentration c of the ith component expressed as relative proportion of the total concentration c_{mix} ($p_i = c_i/c_{mix}$) (Faust *et al.*, 2001).

Predictions according to *IA* were based on eqn. [2] and were calculated as described previously (Backhaus *et al.*, 2000a; Faust *et al.*, 2003).

For the prediction of the mixture effect for a given mixture concentration c_{mix} according to *TSP* the n components of the mixture can be arranged in k subgroups so that the components in each subgroup share a similar mechanism of action, but not components **between** subgroups. If the total mixture concentration of the ith subgroup is z_i ($c_{mix}=z_1+...+z_k$) and $c_{i,j}$ is the individual concentration of the jth component within z_i, then it is possible to predict the combined effect Y_i for z_i according to *CA* on the basis of the components belonging to the ith subgroup as

$$\sum_{j=1}^{k_i} \frac{c_{i,j}}{EC_{Y_i}^{j}} = 1 \text{ with } z_i = c_{i,1} + \ldots + c_{i,k}. \quad [4]$$

The necessary information about the effect concentration $EC_{Y_i}^{j}$ of the *j*th individual component of the *i*th subgroup can be derived from the inverse of the concentration-response function $F_{j(i)}$, *i.e.*

$$\sum_{j=1}^{k_i} \frac{c_j}{F_{j(i)}^{-1}(Y_i)} = 1 \text{ with } z_i = c_{i,1} + \ldots + c_{i,k}. \quad [5]$$

The value of Y_i satisfying eqn. [5] has to be computed by iterative methods. In the second stage of the *TSP*, the mixture effects Y_i of all *k* subgroups are used to predict the overall mixture effect by applying *IA*, *i.e.*

$$E(c_{mixture}) = 1 - \prod_{i=1}^{k} (1 - Y_i). \quad [6]$$

Predictions according to *CA*, *IA* and *TSP* were generated for numerous effect levels *x*, resulting in graphs of predicted concentration-response curves.

2.6 Concentration response analysis

Aqueous stock solutions of the mixtures were prepared from aqueous stock solutions of the mixture components according to the mixture ratios defined in section 2.4. From these stock solutions, geometric dilution series were prepared. To allow valid statistical estimations of concentration-response functions for the whole effect range (1%-99%) 12 different concentrations were tested with at least three independent replicates each and compared to six untreated controls. Concentration-response functions were estimated from the experimental data by applying the best-fit procedure introduced by Scholze *et al.* (2001). Five out of ten 2- and 3-parametric models (tab. 2) proved to describe the concentration-response relationships of the mixtures (tab. 3) and their components (annexes 1-4) best. Effect concentrations were calculated using the inverse (F^{-1}) of the best fitting model (tab. 2). Corresponding 95% confidence intervals were estimated by using the bootstrap methodology (Scholze *et al.*, 2001). No observed effect concentrations (*NOECs*) were determined by applying Dunnett's test (Dunnett, 1964).

3 Results

The results of the experimental mixture toxicity assessments as well as predictions according to *CA*, *TSP* and *IA* are shown in figs. 3 and 4. In fig. 3A (mixture ratio of *EC15.9* of *CA* groups) as well as in fig. 4 (NOEC mixture ratio) the results from two independent experiments are depicted. All experimental mixture toxicity analyses led to the determination of complete concentration-response curves. The corresponding concentration-response functions, as well as statistical estimates of the *EC01*, *EC50* and *NOEC* values are given in tab. 3.

For both mixtures and all mixture ratios, the *TSP* predicted the observed toxicity almost accurately. On the level of the *EC50*, differences between predicted and observed values were small, and never exceeded a factor of 1.2 (tab. 4). For effects higher than 80% the difference increased in both ratios of *mixture 1*, the mixture ratio of *EC15.9* of *CA* groups (fig. 3A) and the mixture ratio of *EC01* of the mixture components (fig. 3B). On the level of the *EC90* the *TSP* overestimates the mixture toxicity up to a factor of 1.7. For *mixture 2*, in which the components were mixed in the ratio of their individual *NOEC* values (fig. 4), the differences between the observed and predicted *EC90* values remained below a factor of 1.2.

Below the 20% effect level, though, in every mixture and mixture ratio the observed toxicity was increasingly lower than predicted. However, differences between predicted and observed *EC10* values never exceeded a factor of 2 (*EC01* mixture ratio in tab. 4).

CA and *IA* led to less good predictions of the mixture toxicity: *CA* overestimated the mixture toxicity, whereas *IA* underestimated it. On the level of the *EC50*, maximal quantitative differences between observed and predicted mixture toxicity occurred for both concepts in the *EC01* ratio of *mixture 1*: *CA* overestimated the observed toxicity by a factor of 2 and *IA* underestimated it by a factor of 1.9.

For effect levels below 20%, predictions according to all three approaches increasingly overestimated the mixture toxicity. However, judged from overlapping confidence intervals of *EC10* values predicted according to *IA* and observed *EC10* values (tab. 4), this overestimation is significant only for the mixture ratio *EC15.9* of *CA* groups of *mixture 1*.

Table 3
Toxicity of the tested mixtures to *Scenedesmus vacuolatus*

Mixtures	Concentration Response Function				*EC01* [c]		*EC50* [c]		NOEC [d]
	RM [a]	$\hat{a}$ [b]	$\hat{b}$ [b]	$\hat{g}$ [b]	(µM L^{-1})	95% CL	(µM L^{-1})	95% CL	(µM L^{-1})
				Mixture 1					
EC15.9 ratio of *CA* groups (as defined in table 1)	GL	-4.461	3.468	2.4975	6.37	3.51-10.18	41.20	37.81-44.34	7.15
EC01 ratio (for *EC01* values refer to Annexes 1-4)	BCP	-39.58	27.046	-0.6357	26.55	22.2-30.91	66.02	62.18- 70.23	29.40
				Mixture 2					
NOEC ratio (for NOEC values refer to Annexes 1-4)	W	-6.931	4.009		3.81	1.65- 8.69	43.40	37.02-51.44	14.58

[a] RM: regression model (see table 2), [b] Statistical estimates of model parameters,
[c] Mean effect concentrations, for 95% confidence intervals see Scholze et al.2001, [d] NOEC determined by DUNNETT test

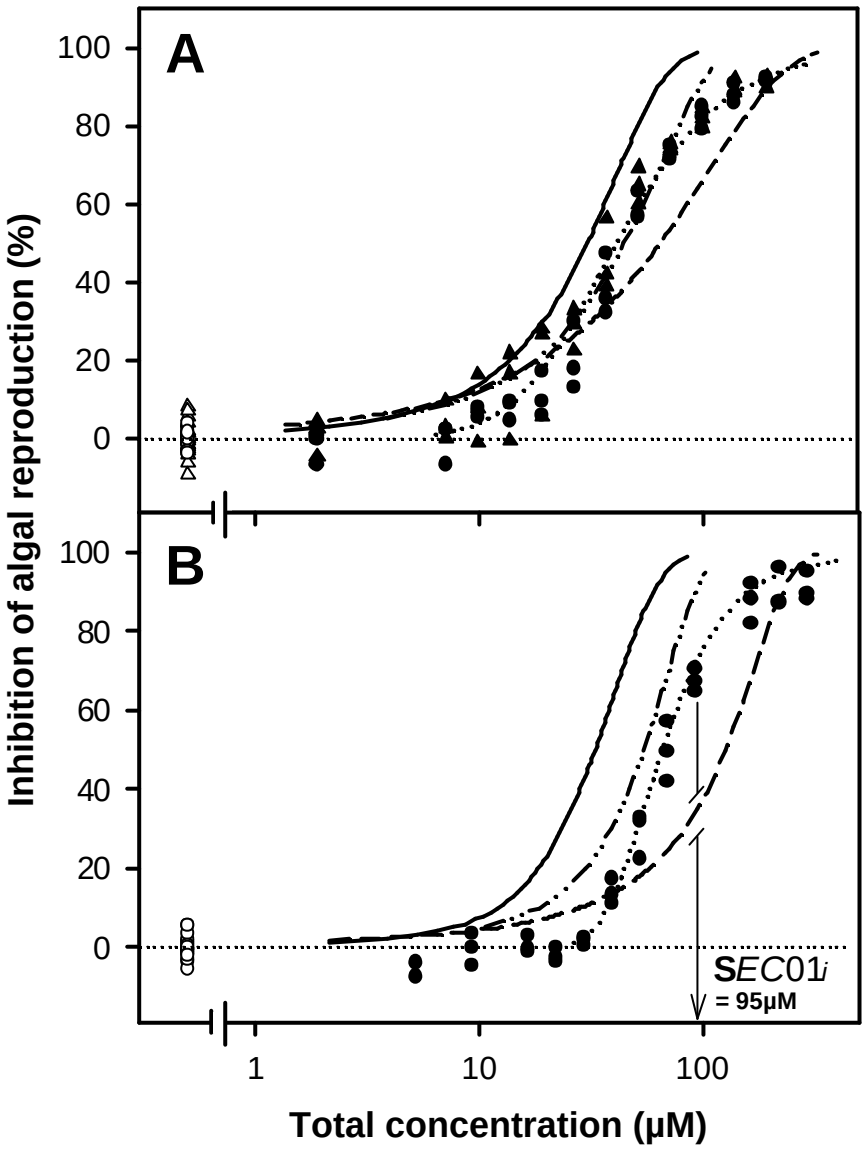

Figure 3. Observed and predicted algal toxicity of *mixture 1*: A ratio *EC15.9* of *CA* groups (independent experiments A and B), B ratio of the *EC01* of the single substances (the arrow highlights the mixture concentrations in which each component is present in its *EC01* value*).* (- · · -) *two stage prediction*; (- - - -) prediction according to *concentration addition*; (- - -) prediction according to *independent action*; (· · ·) statistical fit of experimentally observed toxicity; (•) experimentally observed toxicity; (o) untreated controls; (?) experimentally observed toxicity in second experiment; (?) untreated controls in second experiment (applies to fig. 3A only).

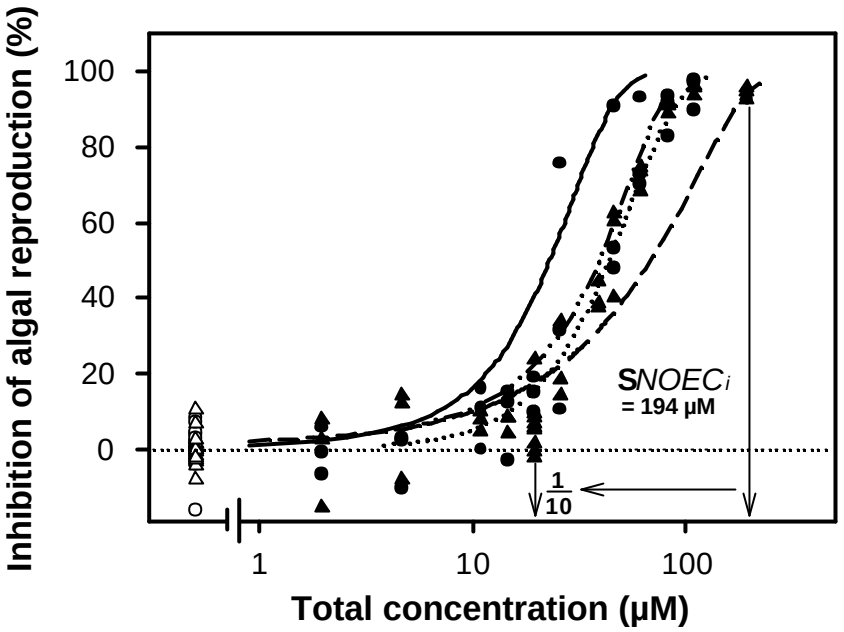

Figure 4. Observed and predicted algal toxicity of *mixture 2*: ratio of the NOECs of the single substances (independent experiments A and B). Arrows highlight the mixture concentrations in which each component is present in its NOECs or in 1/10th of its NOECs. (- · · -) *two stage prediction*; (- - - -) prediction according to *concentration addition*; (- - -) prediction according to *independent action*; (· · · ·) statistical fit of experimentally observed toxicity; (•) experimentally observed toxicity; (o) untreated controls; (*?*) experimentally observed toxicity in second experiment; (?) untreated controls in second experiment.

Two mixture ratios were designed to additionally give evidence on the mixture toxicity from low effect concentrations: the *EC01* ratio of *mixture 1* (fig. 3B) as well as *mixture 2*, in which the components were mixed in the fixed ratio of their individual NOEC values (fig. 4). The arrows indicate the design point, *i.e.* the mixture concentration, at which every mixture component was present at its *EC01* or NOEC value respectively. *EC01* values were always equal to or lower than the respective NOEC. Hence, mixture concentrations below 95µM (*EC01* mixture ratio) respectively 194 µM (NOEC mixture ratio) represent individual concentrations of the mixture components which would not cause a significant effect if applied singly. However, the algal reproduction was inhibited by over 60% at the design point of the *EC01* mixture ratio and by over 99% at the design point of the *NOEC* mixture ratio.

Table 4 Statistical uncertainty of predicted and observed effect concentrations for mixtures

	Effect mixture concentration in µM							
Effect	Observed		Predicted by *TSP*		Predicted by *CA*		Predicted by *IA*	
level	Mean	95% CL [a]	Mean	95% CL [a]	Mean	95% CL [a]	Mean	95% CL [a]
				Mixture 1				
EC15.9 ratio of CA groups (as defined in tab. 1)								
10%	14.68	12.25-17.66	8.70	n.d.	7.38	6.36-7.90	7.27	1.35-9.73
50%	41.20	37.81-44.34	44.95	n.d.	30.64	29.92-31.18	64.32	52.70-75.75
90%	155.93	146.79-166.16	96.70	n.d.	63.70	61.90-65.85	196.09	184.61-214.77
EC01 ratio of compounds (for EC01 values refer to Annexes 1-4)								
10%	37.52	34.17-41.81	18.52	n.d.	11.60	10.86-11.99	28.06	7.49-34.14
50%	66.02	62.18-70.23	54.68	n.d.	31.94	31.30-32.59	126.88	110.36-135.36
90%	160.83	147.85-173.49	92.24	n.d.	58.32	56.05-60.26	228.60	217.63-241.03
				Mixture 2				
NOEC ratio of compounds (for NOEC values refer to Annexes 1-4)								
10%	14.71	9.59-22.55	9.33	n.d.	6.86	5.18-7.28	10.00	1.90-13.63
50%	43.40	37.02-51.44	39.22	n.d.	22.98	22.36-23.38	68.36	52.62-79.97
90%	86.50	69.21-109.10	74.58	n.d.	44.20	42.88-45.67	169.60	157.30-184.58

[a] 95% confidence intervals, see Scholze *et al.* 2001

In the latter mixture ratio, even for a mixture concentration which was by a factor of 10 lower than the design point (19.4 µM), a significant effect was observed. This concentration is the lowest observed effect concentration (*LOEC*), as it the lowest tested mixture concentration that is higher than the NOEC (tab. 3).

4 Discussion

For two mixtures of environmentally relevant substances, the high predictive power of the *TSP* approach was shown: for mixture effects ranging from 20% to 80%, the *TSP* predicted the observed toxicity accurately. Deviations between observed *EC50* values and values predicted according to *TSP*, ranged from factors of 0.9 to 1.2 (tab. 4). These deviations were comparable to deviations between predicted and observed effects reported in the literature for multi-component mixtures, in which the model assumptions concerning the mechanism of action of the mixture components were met (tab. 5): on the level of the *EC50* the deviation between *CA* predictions and observed toxicity of similarly acting substances ranged from a factor of 1.04 to a factor of 1.4, and for dissimilarly acting substances the

deviation between *IA* predictions and observed toxicity was 1.1 and lower. For this compilation, only studies were selected, in which predictions according to *CA* as well as *IA* were made and observed and predicted values were given. The availability of predictions according to both concepts was regarded as a necessary prerequisite for the evaluation of the predictive quality, since it has recently been shown that a compliance with a respective prediction may also result from a quantitative similarity of *CA* and *IA* predictions (Backhaus *et al.*, 2004). For all mixtures reported in tab. 5 as well as for every mixture and mixture ratio tested in this study, the model for which the assumptions concerning the mechanisms of action of the components were met was shown to predict the mixture toxicity best.

The tendency for overestimation of mixture effects below 20% may be due to the high number of mixture components and the resulting uncertainties connected with the estimation of rather low effects. As the overestimation was highest in the *EC01* ratio of *mixture 1*, we may take the *EC10* predicted according to *TSP* for this mixture ratio (tab. 4) as an example: if the individual concentrations c_i of the mixture components at the mixture concentration c_{mix} ($c_{mix} = \sum_{i=1}^{n} c_i$) that is predicted to cause 10% effect were inserted into the respective concentration-response curves of the mixture components, estimated individual effects ranging from 0.7% to 8E-5% would result. The experimental single substance concentration-response analysis however, was only designed to allow for statistical estimates of *ECx* values with *x* down to 1%. It may be argued that for the *TSP* not the individual effects of the mixture components are used to predict the mixture toxicity, but effects predicted by *CA* for the *CA* groups. The *CA* prediction though, is based on the estimation of effect concentrations for a given effect level *x* (c.f. eqn. 3). In other words, if according to *CA* an effect of *x*% is predicted, the uncertainty connected with the *CA* prediction depends on the uncertainty of the estimated *ECx* value of every mixture component. In our example the effects predicted according to *CA* for each *CA* group ranged from 5% (18 *triazines*) to 1% (6 *quinolones*) and were hence based on estimations of individual *EC05* (18 *triazines*) to *EC01* (6 *quinolones*) values.

Table 5 Mixtures composed of strictly similarly, respectively dissimilarly acting substances: comparison with predictions

Mixture components	Test organism	Mechanism of action	Mixture ratio [a]	$EC50_{obs}/EC50_{CA}$ [b]	$EC50_{obs}/EC50_{IA}$ [b]	reference
similar mechanism of action						
16 phenol type compounds	*Vibrio fischeri*	Uncoupling of oxidative phosphorylation	*EC50*	0.9	0.5	(Altenburger *et al.*, 2000)
			EC01	0.9	0.3	
10 quinolones	*Vibrio fischeri*	DNA Gyrase	*EC50*	1.2	0.4	(Backhaus *et al.*, 2000b)
			EC01	1.2	0.4	
			NOEC	1.2	0.4	
18 triazines	*Scenedesmus vacuolatus*	PSII [d]	*EC50*	=1.04	0.5	(Faust *et al.*, 2001)
			EC01	=1.04	0.4	
4 triazines	*Lemna minor*	PSII	*EC50*	0.95	0.6	(Drost *et al.*, 2003)
8 chloroa-cetanilides	*Scenedesmus vacuolatus*	VLCFA [e]	*EC50*	=1.1	0.3	(Junghans *et al.*, 2003a)
			EC05	=1.1	0.3	
8 sulfonylureas	*Scenedesmus vacuolatus*	ALS [f]	*EC50*	1.2	1.7	(Junghans *et al.*, 2003b)
			EC05	1.4	1.9	
dissimilar mechanism of action						
8 dissimilar	*Vibrio fischeri*	dissimilar	*EC50*	1.4	0.9	(Backhaus *et al.*, 2000a)
			EC01	2.4	0.97	
16 dissimilar	*Scenedesmus vacuolatus*	dissimilar	*EC50*	1.9	0.9	(Faust *et al.*, 2003)
			EC01	2.6	1.04	

[a] substances were mixed in the ratio of their individual *ECx*/NOEC values [b] values =1 indicate underestimation, values =1 overestimation of the observed toxicity; values for CA correspond to M values introduced by Andersen and Weber (1975) [c] toxicity of single substances was describable by a single quantitative structure-activity relationship [d] inhibition of D1 protein in photosystem II [e] inhibition of very long chain fatty acid formation [f] inhibition of acetolactate synthase

As mentioned above, the concentration-response analysis for the single substances was designed to allow for the estimation of effect concentrations down to *EC01*. Hence, in a strict sense, the *EC10* value predicted by *TSP* seems to be reliable. It has to be taken into account though, that due to a frequently observed heteroscedasticity the uncertainty of *ECx* estimations increases with decreasing effects levels *x* (Scholze *et al.*, 2001). Thus the uncertainty of the *TSP* becomes higher the lower the considered effect level of the *CA* groups becomes. Concurrently, estimations of the uncertainty become more important for the

interpretation of observed deviations from *TSP* predictions. Alas, the estimation of the uncertainty connected to *TSP* predictions is yet an unresolved task.

Despite these general considerations, it has to be stressed that the *TSP* gave accurate predictions for almost the complete concentration-response range. The high predictive quality of *TSP* for the tested mixtures together with the overestimation of the observed toxicity by *CA* and its underestimation by *IA* may be seen as a first experimental proof for the validity of the *TSP* approach.

This study has shown that severe joint toxicity may result from mixture concentrations at which neither mixture component is present in concentrations that would elicit a statistically significant effect if applied singly. The fact that a mixture concentration, which equals the sum of NOECs of the individual mixture components, was shown to inhibit the algal reproduction by over 99% underlines that NOECs may not be misunderstood as concentrations at which no effects occur. They are only a measure of statistical significance, which has already been pointed out by several authors (e.g. Moore and Caux, 1997; van der Hoeven *et al.*, 1997). This becomes especially evident when the joint toxicity of multi-component mixtures is assessed. Irrespective of the similarity of the mixture components' mechanisms of action, joint effects were shown to result from individual NOECs, which were similarly severe as the effects observed in this study. A mixture composed of 11 structurally dissimilar priority pollutants of the aquatic environment caused an inhibition of algal reproduction by over 60%. An even higher joint effect was reported by Backhaus *et al.* (2000b) who observed over 99% effect in a biotest with a marine bacterium after exposure to a mixture of 10 *quinolone* antibiotics. Also for individual concentrations lower than the NOEC severe joint effects were observed. In the same study for a mixture concentration at which every component was present in its respective *EC01* value, Backhaus *et al.* (2000b) still measured a joint effect of over 50%. For 16 dissimilarly acting substances, also present at sum of their individual *EC01* values, Faust *et al.* (2003) detected a joint effect, which was with 16% still statistically significant. These findings are consistent with the results obtained in this study, which have shown that even from individual concentrations as low as $1/10^{th}$ of the NOEC significant mixture effects cannot be excluded. This further underlines the relevance of mixture toxicity and stresses the need for a hazard assessment of mixtures. Using the *TSP* the observed

severe mixture toxicity from low individual effect concentrations was accurately predicted.

Conclusions

The results of this study demonstrate the validity of the combination of *CA* and *IA* to a *TSP* for the prediction of the joint toxicity of mixtures in which the components can be assigned according to their mechanisms of action to discrete groups, and dissimilarity of action between the groups is provided. The observed severe joint toxicity from low individual effect concentrations underlines the importance of regarding mixture toxicity when assessing the hazard of mixtures of environmentally relevant toxicants.

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Annex 1. Chloroacetanilides used in mixture toxicity studies

Substance [a]	CAS RN [b]	Purity	MW [c]	RM [d]	$\hat{a}$ [e]	$\hat{b}$ [e]	$\hat{g}$ [e]	EC_{01} nM [f]	EC_{50} nM [f]	*NOEC* nM [g]
Acetochlor	34256-82-1	97.0%	269.8	GL	27.212	22.079	0.2691	9.83	45.1	18.1
Alachlor	15972-60-8	99.7%	269.8	W	4.009	5.127		20.93	140.1	43.1
Butachlor	23184-66-9	97.3%	311.9	GL	18.267	10.57	0.221	0.2	9.5	1.6
Dimethachlor	50563-36-5	99.8%	255.7	W	5.107	8.906		81.29	242.9	152.6
Metazachlor	67129-08-2	98.0%	277.8	BCW	6.612	5.78	0.4716	5.39	167.6	55.1
Metolachlor [h]	51218-45-2	97.0%	283.3	BCW	0.239	3.156	0.493	57.23	817.6	422
Pretilachlor	51218-49-6	98.3%	311.9	W	8.399	4.716		1.75	13.6	n.d [i]
Propachlor	1918-16-7	99.5%	211.7	W	6.599	8.785		53.11	166.1	89.3

[a] common name, [b] Chemical Abstracts Services Registry Number, [c] Molecular Weight, [d] RM: regression model (see table 1), [e] Statistical estimates of model parameters, [f] Mean effect concentrations for confidence intervals see Junghans et al. 2003a, [g] NOEC determined by DUNNETT test, [h] racemate, isomere-ratio unstated, [i] n.d. = not determinable; data taken from Junghans et al. 2003a

Annex 2. Concentration-response functions, *ECx* and *NOEC* values for the sulfonylurea herbicides

Substance [a]	CAS RN [b]	Purity	MW [c]	RM [d]	$\hat{a}$ [e]	$\hat{b}$ [e]	$\hat{g}$ [e]	EC_{01} nM [f]	EC_{50} nM [f]	*NOEC* nM [g]
bensulfuron-methyl	83055-99-6	99.5 %	410.4	W	1.425	1.376		0.00004	0.050	0.00024
cinosulfuron	94593-91-6	99.4 %	413.4	W	-1.706	1.929		0.025	1.185	0.36300
chlorsulfuron	64902-72-3	99.8 %	357.8	GL2 [i]	-3.333	2.552	16.438	0.032	4.948	n.d. [h]
metsulfuron-methyl	74223-64-6	99.5 %	381.4	BCW	-1.364	0.903	-0.018	0.031	3.052	0.43347
primisulfuron-methyl	86209-51-0	99.9 %	468.3	W	0.008	1.863		0.0034	0.630	0.02800
prosulfuron	94125-34-5	98.1 %	419.4	BCW	1.392	0.987	-0.027	0.0036	0.175	0.00334
rimsulfuron	122931-48-0	99.2 %	431.4	W	-1.968	2.092		0.055	5.825	0.50800
triasulfuron	82097-50-5	99.0 %	401.8	W	0.093	1.684		0.0016	0.533	n.d. [h]

[a] common name, [b] Chemicals Abstracts Services Registry Number, [c] Molecular weight, [d] RM: regression model (see table 1), [e] Statistical estimates of model parameters, [f] Mean effect concentrations with 95% two-sided bootstrap confidence intervals, [g] NOEC determined by DUNNETT test, [h] n.d. = not determinable; data taken from Junghans et al. 2003b, [i] corrected, in Junghans et al. 2003b wrongly GL is named

Annex 3. *s*-Triazines used in mixture toxicity analyses

Substance[a]	CAS RN [b]	Purity	MW [c]	RM [d]	$\hat{a}$ [e]	$\hat{b}$ [e]	$\hat{g}$ [e]	*EC01* nM [f]	*EC50* nM [f]	*NOEC* nM [g]
ametryn	834-12-8	98 %	227.33	GL	13.68	8.63	0.3402	0.7	15.6	2.1
atraton	1610-17-9	95 %	211.27	GL	1.30	11.06	0.2037	6.9	378	57
atrazine	1912-24-9	99 %	215.69	GL	6.76	17.39	0.1118	1.7	180	36
cyanazine	21725-46-2	99 %	240.69	BCP	8.64	7.40	0.6482	6.9	113	21
desmetryn	1014-69-3	98 %	213.31	W	5.36	3.99		3.2	36.8	7.1
dimethametryn	22936-75-0	97 %	255.39	W	5.72	4.73		6.6	51.5	18
dipropetryn	4147-51-7	99 %	255.39	BCW	5.64	3.03	0.2005	3.6	80.1	18
methoprotryne	841-06-5	99 %	271.39	GL	31.19	32.44	0.1074	5.2	69.1	18
prometon	1610-18-0	99 %	225.29	W	0.43	3.04		22	547	154
prometryn	7287-19-6	99 %	241.36	GL	256.97	251.11	0.0105	1.7	51.8	3.4
propazine	139-40-2	99 %	229.71	GL	1.95	14.44	0.1281	2.4	310	28
sebuthylazine	7286-69-3	95.9 %	229.71	W	2.76	3.56		8.6	133	33
secbumeton	26259-45-0	99 %	225.29	GL	5.85	14.15	0.1354	1.5	168	12
simazine	122-34-9	99 %	201.66	W	0.83	2.18		3.2	282	19
simetryn	1014-70-6	99 %	213.30	W	3.63	3.03		1.9	47.8	4.2
terbumeton	33693-04-8	99 %	225.29	W	2.62	3.03		4.1	103	11
terbuthylazine	5915-41-3	99 %	229.71	W	4.16	3.91		5.7	69.3	9.7
terbutryn	886-50-0	99 %	241.36	GL	26.28	20.00	0.1948	3.2	32.3	6.6

[a] common name, [b] Chemical Abstracts Services Registry Number, [c] Molecular Weight, [d] RM: regression model (see table 1), [e] Statistical estimates of model parameters, given for concentrations *c* expressed in µmol/L (rounded values), [f] Mean effect concentrations for confidence intervals see Faust et al. 2001, [g] NOEC determined by DUNNETT test, data taken from Faust et al. 2001

Annex 4. Quinolones and quaternary ammonium compounds used in mixture toxicity studies

Substance [a]	CAS RN [b]	Purity	MW [c]	RM [d]	$\hat{a}$ [e]	$\hat{b}$ [e]	$\hat{g}$ [e]	*EC01* µM [f]	[CL]	*EC50* µM [f]	[CL]	*NOEC* µM [g]
Quinolones												
Enoxacin	74011-58-8	n.g.[i]	320.33	L	-11.73	6.84		11.065	[8.218; 13.582]	52.018	[49.036; 54.980]	27.18
Flumequine	42835-25-6	n.g.	261.25	BCW	-6.48	3.24	-0,28	1.885	[0.889; 2.993]	14.748	[13.824; 15.702]	2.76
Lomefloxacin	98079-51-7	n.g.	351.36	W	-6.08	2.96		3.151	[1.166; 7.250]	84.801	[72.304; 97.635]	12.66
Nalidixic acid	389-08-2	>99.5 %	232.24	W	-6.89	3.27		5.012	[3.587; 6.860]	98.678	[94.319; 103.629]	14.31
Norfloxacin	70458-96-7	n.g.	319.34	BCW	-7.41	1.77	0.096	5.603	[0.00005; 19.846]	151.522	[117.869; 194.913]	20.25
Ofloxacin	82419-36-1	n.g.	361.38	GL	-5.05	3.45	18.5	67.860	[51.542; 101.533]	258.357	[222.537; 287.250]	134.86
quaternary ammonium compounds												
BD 10[7]	965-32-2	>97 %	311.93	GL	5.018	10.08	0.2685	0.006	[0.00007; 0.026]	0.180	[0.156; 0.215]	0.05
BD 12[8]	139-07-1	>99 %	339.99	W	2.614	3.074		0.005	[0.003; 0.020]	0.107	[0.078; 0.162]	0.02
BD 14[9]	139-08-2	>99 %	368.05	W	1.596	2.513		0.003	[0.001; 0.008]	0.165	[0.141; 0.191]	0.04

[a] common name, [b] Chemical Abstracts Services Registry Number, [c] Molecular Weight, [d] RM: regression model (see table 1), [e] Statistical estimates of model parameters, [f] Mean effect concentrations, [g] NOEC determined by DUNNETT test, [h] n.d. = not determinable, [i] n.g. = not given by the manufacturer (Sigma, Germany), [j] only mixture component in Quaternary ammonium compound EC50 mixture, [k] Partition Coefficient in Octanol/Water: calculated with the programm KowWin, Syracuse Research Corporation (http://esc.syrres.com/interkow/kowdemo.htm); unpublished data by Backhaus et al. (quinolones) and Meyer et al. (quaternary ammonium compounds)

[7] benzyl-dimethyl-**decyl**-ammonium-chloride
[8] benzyl-dimethyl-**dodecyl**-ammonium-chloride
[9] benzyl-dimethyl-**tetradecyl**-ammonium-chloride

IV

Application and validation of predictive approaches for realistic pesticide mixtures

Application and validation of predictive approaches for realistic pesticide mixtures

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In preparation

Abstract

In freshwater systems located in agriculturally used areas organisms are exposed to a multitude of toxicologically and structurally different substances. For regulatory purposes it is of major importance whether the combined hazard of these substances can be predictively assessed from the single substance toxicity. For reference mixtures it has been shown so far, that the mixture toxicity can be predicted by using the concept of *concentration Addition* (*CA*) for mixtures of similarly acting and the concept of *independent Action* (*IA*) for mixtures of dissimilarly acting substances. This study aims to analyse whether these concepts may also be used to predictively assess the toxicity of environmentally realistic mixtures. For this purpose a realistic mixture, reflecting pesticide run-off to surface waters in agriculturally used areas, was studied for its effects on the reproduction of the freshwater green alga *Scenedesmus vacuolatus.* The toxicity of the tested mixtures showed a good predictability by the concept of *CA*. This is in accordance with the finding that a group of photosystem II inhibitors dominates the mixture toxicity. Further more, a tool to determine the maximal factor, by which *IA* may predict a lower toxicity than *CA* is presented. Its application on 16 realistic agricultural exposure scenarios reveals that the use of *CA* as a precautionary default assumption for a predictive hazard assessment of mixtures from agricultural exposure scenarios is justifiable.

1. Introduction

In agriculturally used areas, pesticides frequently contaminate surface waters through run-off processes. At peak application times water bodies discharging from

agricultural areas have shown to transport a cocktail of diverse pesticides (Battaglin and Goolsby, 1999; Clark *et al.*, 1999; Thomas *et al.*, 2001; Battaglin and Fairchild, 2002; Hunt *et al.*, 2003).

Since pesticides are designed to exert a toxic effect on target-organisms, it is likely that pesticide contaminations in water bodies are hazardous to non-target, aquatic organisms as well.

Previous studies demonstrated that the exposure towards mixtures of pesticides leads to a toxic effect, which is higher than that of each single pesticide alone (Carder and Hoagland, 1998; Faust *et al.*, 2001; Backhaus *et al.*, 2004). To predict the potential mixture toxicity of pesticides detected in water bodies, several authors have applied the toxic unit (*TU*) approach (Steen *et al.*, 1999; Thomas *et al.*, 2001; Battaglin and Fairchild, 2002; George *et al.*, 2003; Hunt *et al.*, 2003; Anderson *et al.*, 2003).

According to this approach, which was proposed by Sprague (1970), the toxicological strength of an individual substance in a given mixture may be expressed by scaling the concentration (*c*) of the substance for its relative toxicity, given e.g. as a certain effect concentration (*ECx*):

$$TU = \frac{c}{ECx} \qquad [1].$$

Sprague postulated that the toxicological strength of the mixture (TU_{mix}) may then be calculated by summing up the *TUs* of the individual mixture components *i* (*i* = *1,2,…,n*)

$$TU_{mix} = \sum_{i=1}^{n} TU_i \qquad [2]$$

By inserting eqn. [1] into eqn. [2] we will get

$$\frac{c_{mix}}{ECx_{mix}} = \sum_{i=1}^{n} \frac{c_i}{ECx_i} \qquad [3]$$

where *n* is the number of mixture components, c_i is the concentration of the individual component *i* (*i* = 1,*2,…,n*) in the mixture, and ECx_i is the concentration of the *i*th component that alone would induce the same quantitative effect *x* as the mixture.

The TU summation has its origin in the concept of *CA* (Loewe and Muischnek, 1926). The basic idea of this concept is, that all components share an identical mechanism of action in the exposed organism. Accordingly, any component of the mixture can be replaced by another chemical without changing the overall mixture toxicity, as long as the corresponding *TUs* are identical.

If *CA* accurately describes the observed mixture toxicity, the *TUs* sum up to unity at the mixture concentration, which elicits *x*% effect, *i.e.* when $c_{mix}=ECx_{mix}$:

$$\sum_{i=1}^{n}\frac{c_i}{ECx_i}=1 \qquad [4]$$

CA has been successfully applied to predict the combined effects of multi-component mixtures of different types of similarly acting pesticides (Faust *et al.*, 2001; Drost *et al.*, 2003; Junghans *et al.*, 2003a; Junghans *et al.*, 2003b; Backhaus *et al.*, 2004). In addition, *CA* has shown to give precise predictions of the mixture toxicity also for multi-component mixtures of non-pesticidal similarly acting substances: for mixtures of organic narcotics (Koenemann, 1980; Koenemann, 1981; Hermens *et al.*, 1984; Nirmalakhandan *et al.*, 1997), protonophoric uncouplers (Altenburger *et al.*, 2000), gyrase-inhibiting pharmaceuticals (Backhaus *et al.*, 2000b), and xenoestrogens (Silva *et al.*, 2002).

Recent studies on multi-component mixtures have shown that *CA* may overestimate the actual mixture toxicity of dissimilarly acting substances (up to a factor of 2.6), whereas *IA*, a concept based on the assumption of a dissimilar mechanism of action of all mixture components, was shown to predict the observed mixture toxicity accurately (Backhaus *et al.*, 2000a; Faust *et al.*, 2003).

IA was first proposed by Bliss (1939). It assumes that the mixture components cause a common effect through primary interaction with different target sites. In contrast to *CA*, where *TUs* are added, *IA* calculates with effects. For multi-component mixtures, the concept can be formulated as (Grimme *et al.*, 1998; Backhaus *et al.*, 2000a):

$$E(c_{mix})=1-\prod_{i=1}^{n}(1-E(c_i)) \qquad [5]$$

where $E(c_i)$ are the effects of the individual mixture components *i* (*i* = *1,2,…,n*) if applied singly in the concentrations c_i. Some authors have extended this basic

version of *IA* by including a so-called coefficient of correlation (generally denoted by *r*), in order to account for possible correlations between the susceptibilities of the individuals within a population (Bliss, 1939; Plackett and Hewlett, 1948). *r* is thought to cover a range from -1 (complete negative correlation) over *r*=0 (no correlation, eqn. 5) to *r*=1 (complete correlation). This extension of the basic *IA* equation (eqn. 5) has been formulated originally for binary mixtures and quantal endpoints only. An extension to multi-component mixtures is difficult, because many possible patterns of correlation may exist. Furthermore, a scientifically sound assumption for *r* has to be available prior to the predictive toxicity assessment of every individual mixture. However, a scientifically sound reasoning is *a priori* typically not available. Hence, for the remainder of this article we will focus on the basic form of *IA* as given in eqn. [5]

Although precise predictions were obtained by *IA* for mixtures of dissimilarly acting substances, for several multi-component mixtures of similarly acting substances this concept was shown to underestimate the toxicity (Altenburger *et al.*, 2000; Backhaus *et al.*, 2000b; Faust *et al.*, 2001; Silva *et al.*, 2002; Junghans *et al.*, 2003a).

Hence, for both concepts, the similarity, respectively dissimilarity of the mechanisms of action of the mixture components seems to be the governing factor for the prediction quality of the concepts. Both concepts gave good predictions if the pharmacological assumptions were met, but *CA* tends to overestimate the mixture toxicity of dissimilarly acting substances, and *IA* tends to underestimate the toxicity of similarly acting substances. This may pose a problem for the predictive toxicity assessment of environmentally relevant mixtures, as they cannot be expected to be composed of substances for which the mechanisms of action are either strictly similar or strictly dissimilar.

The comparative evaluation of the predictive potential of *CA* and *IA* has so far been largely restricted to specifically designed mixtures, in which the components were either strictly similarly or strictly dissimilarly acting - selected on a precise knowledge of their pharmacology (e.g. PSII inhibiting herbicides) or their chemical classification (e.g. congeneric groups). However, our knowledge on the predictive value of the concepts with respect to environmentally realistic mixtures is limited. Hence, this study aims to analyse the predictive value of both concepts for an

example of a typical environmentally realistic mixture. It is based on a modelled exposure scenario that reflects the median load of pesticides in field run-off in central-European agricultural areas after pre-emergency treatments in spring (tab. 1), and was modelled by Finizio *et al.* (2004a; Finizio *et al.*, 2004b).

Table 1. Substances modelled within the agricultural exposure scenario.

Substance[a]	CAS RN[b]	MW[c]	use	PEC (µM)[d]	Log *Kow*[e]
2,4-D	94-75-7	221.04	herbicide	2.28E-02	2.62
aclonifen	74070-46-5	264.67	herbicide	7.56E-04	3.88
alachlor	15972-60-8	269.77	herbicide	1.58E-02	3.37
atrazine	1912-24-9	215.69	herbicide	4.87E-02	2.82
bromoxynil	1689-84-5	276.91	herbicide	1.30E-02	3.39
carbofuran	1563-66-2	221.26	insecticide	1.08E-02	2.30
carbosulfan	55285-14-8	380.54	insecticide	2.45E-02	5.57
chloridazon	1698-60-8	221.65	herbicide	4.59E-02	0.76
clopyralid	1702-17-6	192.00	herbicide	3.66E-02	1.63
cycloxydim	101205-02-1	325.47	herbicide	2.27E-03	3.88
ethofumesate	26225-79-6	286.35	herbicide	6.22E-03	2.89
ioxynil	1689-83-4	370.92	herbicide	1.98E-03	3.94
isofenphos	25311-71-1	345.39	insecticide	1.16E-03	4.65
isoproturon	34123-59-6	206.29	herbicide	1.45E-03	2.84
isoxaflutole	141112-29-0	359.32	herbicide	3.09E-03	2.25
lenacil	2164-08-1	234.30	herbicide	4.48E-03	3.09
linuron	330-55-2	249.10	herbicide	5.20E-03	2.91
MCPA	94-74-6	200.62	herbicide	1.58E-02	2.52
metamitron	41394-05-2	202.22	herbicide	1.19E-01	1.44
metolachlor	51218-45-2	283.80	herbicide	2.60E-02	3.24
pendimethalin	40487-42-1	281.31	herbicide	5.23E-05	4.82
terbuthylazine	5915-41-3	229.71	herbicide	2.12E-02	3.27
thifensulfuron-methyl	79277-27-3	387.38	herbicide	2.14E-04	1.27
triasulfuron	82097-50-5	401.82	herbicide	7.71E-04	2.44
tribenuron-methyl	101200-48-0	395.39	herbicide	1.47E-03	2.55

[a] Common name [b] Chemical Abstracts Services Registry number [c] Relative molecular mass [d] modelled concentration in the scenario (predicted environmental concentration, PEC) in run-off water (Finizio *et al.*, 2004a&b) [e] Partition Coefficient in Octanol/Water: calculated with the programme KowWin, Syracuse Research Corporation (http://esc.syrres.com/interkow/kowdemo.htm)

As the majority of the 25 scenario components are herbicides, the freshwater chlorophyte *Scenedesmus vacuolatus* was selected as test-organism. To allow for a comparison of the observed mixture toxicity with the predictions according to *CA* and *IA*, as a first step single substance concentration-response relationships were

analysed. In a second step, the mechanism of action of each mixture component was determined. Subsequently, predictions according to *CA* and *IA* were made and the predictive quality of each concept was analysed, by a comparison of the predictions with the experimentally observed mixture toxicity.

2. Material and Methods

2.1. Bio-test

As the exposure scenario was dominated by herbicides, the toxicities of single substances as well as mixtures were determined in a bio-test with the chlorophyte *Scenedesmus vacuolatus* (strain 211-15, culture collection of the University of Göttingen, Germany). After an exposure period of one generation cycle (24h) the inhibition of reproduction in synchronous cultures of *Scenedesmus vac.* was determined by means of electronic cell counting (Coulter Electronics, Miami USA). The original test protocol was described by Altenburger *et al.* (1990). It was used with the modifications by Faust *et al.* (2001), as this includes the use of gas tight test-vessels with a low headspace and therefore allowed the testing of volatile substances.

2.2. Test-substances, test-solutions and chemical analysis

The test substances and their modelled concentration in the scenario (predicted environmental concentration, PEC) for field run-off are given in tab. 1. All substances were obtained by Riedel de Haën (Seelze, Germany) in the highest available purity (carbofuran 92%, all other substances >97%). Stock solutions were prepared in methanol or acetonitrile (chromatography grade, Merck, Darmstadt, Germany) and stored at -30ºC. These organic solutions also served as standards for the analytical validation of test-concentrations (tab. 2). For toxicity testing and the analysis of chemical stability under test conditions, aqueous stock solutions were prepared from aliquots of the organic solvent stock solutions. For this purpose, the organic solvent was evaporated from the aliquot and the pure substance was then re-dissolved in algal growth medium (Grimme and Boardman, 1972).

Table 2. Analytical conditions for HPLC analyses of test concentrations and substance stability

Substance	Stationary phase	Mobile phase		Absorbance detection (nm)
Common name		Eluent A	% Eluent B[c]	
2,4-D	L[a]	0.01M H_3PO_4	50	225
aclonifen	L	0.01M NaH_2PO_4	50	238
alachlor	L	H_20	50	215
atrazine[a]	S[b]	0.001M $NH_4C_2H_3O_2$	60	220
bromoxynil	L	H_20	80	254
carbofuran	L	H_20	60	272
chloridazon	S	H_20	70	230
cycloxydim	L	0.01M H_3PO_4	40	280
ethofumesate	L	H_20	40	275
ioxynil	L	0.01M H_3PO_4	50	235
isofenphos	L	H_20	40	220
isoproturon	L	0.01M H_3PO_4	60	278
isoxaflutole	L	H_20	40	220
lenacil	L	H_20	70	245
linuron	L	0.01M H_3PO_4	50	250
MCPA	L	0.01M H_3PO_4	50	225
metamitron	L	0.001M $NH_4C_2H_3O_2$	80	225
metolachlor	L	H_20	50	215
pendimethalin	L	H_20	25	240
terbuthylazine	S	0.001M $NH_4C_2H_3O_2$	60	220
thifensulfuron-methyl	L	0.01M H_3PO_4	60	225
triasulfuron	L	0.01M H_3PO_4	70	225
tribenuron-methyl	L	0.001M $NH_4C_2H_3O_2$	80	236

[a] L: Lichrosphere RP 18e 125-4 (5µm) [b] S: Supersphere RP 18e 125-3.1 (4µm) [c] acetonitrile (chromasolv) all Merck, Darmstadt, Germany

In order to keep the pH stable, the acids MCPA, 2,4-D, and clopyralid were tested in algal medium with an elevated phosphate buffer capacity (the NaH_2PO_4 concentration was increased from 3.00 to 9.64 mM, the Na_2HPO_4 concentration from 1.00 to 9.17 mM). This high phosphate concentration led to a small decrease in the reproduction of the untreated controls. Hence, all other substances as well as the mixtures were tested in standard medium. The latter was possible, because the concentrations of MCPA, 2,4-D and clopyralid in the mixture were low, so that the pH during mixture tests was not affected.

HPLC based stability tests for the substances under testing conditions (without algae) led to recovery rates greater than 75% (metamitron), 87% (tribenuron-methyl), and 90% for all other substances.

2.3. Concentration-response analysis of single mixture components

Based on the results from previously conducted range-finding tests, geometric dilution series were prepared from the aqueous stock solutions for each single mixture component. The inhibition of reproduction of at least 12 different test concentrations was measured in three replicates; each replicate was compared to the reproduction measured in six untreated controls.

From the experimental concentration-response data, concentration-response functions were estimated using the best fitting model out of a set of 10 different 2- and 3-parametric regression models (Scholze *et al.*, 2001). The five models used to describe the concentration-response functions are given in tab. 3. Effect concentrations EC_x were calculated using the inverse (F^{-1}) function of the respective model. No observed effect concentrations (NOECs) were determined according to Dunnett's test (Dunnett, 1964).

Table 3. Regression models

Name	Function (F)	Inverse Function (F^{-1})
Logit (L)	$E = 1 / (1+\exp(-a - b \log_{10}(c)))$	$c' = 10^{\left((\log_e(E/(1-E)) - \hat{a})/\hat{b}\right)}$
Probit (P)	$E = \frac{1}{2p} \int_{-\infty}^{a+b\log_{10}(c)} \exp(-u^2/2)du$ $= \Phi(a + b \log_{10}(c))$	$c' = 10^{((\Phi^{-1}(E) - \hat{a})/\hat{b}))}$
Weibull (W)	$E = 1 - \exp(-\exp(a + b \log_{10}(c)))$	$c' = 10^{\left((\log_e(-\log_e(1-E)) - \hat{a})/\hat{b}\right)}$
Generalized Logit (GL)	$E = 1 / (1+\exp(-a - b \log_{10}(c)))^g$	$c' = 10^{\left((-\log_e((1/E)^{(1/\hat{g})} - 1) - \hat{a})/\hat{b}\right)}$
Box-Cox-Weibull (BCW)	$E = 1 - \exp(-\exp(a + b((c^g - 1)/g)))$	$c' = \left((\hat{g}/\hat{b}) \bullet (\log_e(-\log_e(1-E)) - \hat{a}) + 1\right)^{(1/\hat{g})}$

E – Effect, expressed as fraction of a maximum possible effect (0 = E = 1);
c' – Concentration; F – cumulative normal (Gauss) distribution;
a, b, g – Model parameters (corresponding statistical estimates marked by ^);
exp(x) = e^x

2.4. Assignment of a mechanism of action

All substances were classified according to their mechanism of action that is reported in the literature - if it is likely to apply to green algae. In case that no such mechanism was found, the experimental *EC50* was compared to a baseline QSAR for *Scenedesmus vac.* published by Walter *et al.* (2002)

$$log\ 1/EC50(\text{mol/L}) = 0.81\ logKow + 0.87 \qquad [6].$$

If both *EC50* values did not differ by more than a factor of 10 the substance was classified as showing non-polar narcosis. Otherwise, it was classified as having an unknown mechanism of action.

2.5. Mixture toxicity determination and calculation of predictions to derive effect concentrations of mixtures

The scenario components were tested in the fixed ratio of their PECs. The concentration-response analysis was performed as described for the single mixture components, but the mixture concentration which equals the sum of the PECs was diluted separately. Based on the concentration-response relationships of the single components, predictions for the toxicity of the exposure scenario were generated. *CA* predictions were calculated according to the following equation, which can be derived from eqn. [3] by rearrangement

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \qquad [7]$$

with p_i being the concentration c of the ith component expressed as relative proportion of the total concentration c_{mix} ($p_i = c_i / c_{mix}$).

Predictions according to *IA* were based on eqn. [6] and were calculated as described previously (Backhaus *et al.*, 2000a; Faust *et al.*, 2003). Predictions according to both concepts were generated for numerous effect levels x, resulting in graphs of predicted concentration-response curves.

2.6. Predictions with incomplete or missing concentration-response functions of single components

Due to experimental restraints, e.g. solubility limits, not for all scenario components the individual toxicity could be assessed experimentally for the whole effect range. In case that a relationship between the applied concentration and the

toxic response was determined, the concentration-response function was extrapolated for the upper effect range. Of the substances for which the concentration-response relationships were extrapolated, the maximally recorded effect was at least 45% (Isoxaflutole). Substances for which no concentration-response relationships could be determined (carbosulfan and clopyralid) were left unaccounted for in the predictions: the predictions were based only on the toxic substances and subsequently corrected for the elevated total mixture concentration by multiplying the resulting effect concentrations by the factor of 1.17. The validity of this approach for the present mixture will be discussed in section 4. To analyse the influence on the prediction quality, two mixtures were tested: a mixture composed only of the toxic substances and a mixture composed of all substances.

3. Results

3.1. Toxicity of single substances

For 23 of the 25 tested substances concentration-response functions were determined. They are given in tab. 4 together with the corresponding individual *EC50* and NOEC values. On the level of the *EC50,* the toxicity of the substances spans over five orders of magnitude. The toxicity of pendimethalin varied between independent experiments: the *EC50* values of six independent experiments ranged from 0.003 µM to 0.025 µM. To increase the accuracy of the predictions, the pendimethalin concentration-response curve was always recorded in parallel to the mixture experiment and an identical pendimethalin stock solution was used for both experiments. For carbosulfan and clopyralid, a concentration-response relationship could not be established. Carbosulfan showed at the limit of solubility only an effect of 20%, whereas clopyralid, tested up to a concentration, which considerably lowered the pH even of the modified test medium, did not show effects, which were higher than 10%. In the concentration range, in which they were present in the mixtures, both substances maximally showed effects of approximately 5%, which is within the range of the control variability.

Table 4. Toxicity of single pesticides to *Scenedesmus vacuolatus.*

Substance$_i$ [a]		Concentration-response function			EC50$_i$ [c]		NOEC [d]
	RM [b]	$\hat{a}$	$\hat{b}$	$\hat{g}$	(µM)	[Cl] (µM)	(µM)
2,4-D	3-GL	-37.540	11.106	0.1392	856.0	[739.3;991.0]	51.38
Aclonifen	3-BW	2.402	0.408	-0.3400	0.0297	[0.0274;0.0320]	0.0053
Alachlor	2-W	4.060	5.193		0.1404	[0.1328;0.1481]	0.043
Atrazin	3-GL	6.765	17.391	0.1118	0.1797	[0.1678;0.1932]	0.0364
Bromoxynil	2-L	-19.600	9.267		130.3	[117.9;142.9]	75.73
Carbofuran	2-W	-4.564	1.978		132.3	[111.9;160.2]	16.04
Carbosulfan				no effects			
Chloridazon	2-W	-2.375	2.777		5.2866	[4.9613;5.5983]	0.0998
Clopyralid				no effects			
Cycloxydim	2-W	-5.232	1.990		278.5	[222.4;325.9]	14.61
Ethofumesate	2-W	-2.126	1.108		38.7	[24.5;66.9]	0.00472
Ioxynil	2-W	-3.785	2.229		34.18	[30.29;38.58]	0.7105
Isofenphos	3-GL	-3.373	2.186	0.4219	7.7556	[5.7560;10.754]	0.2424
Isoproturon	3-BW	1.246	1.073	-0.0235	0.2284	[0.2146;0.2440]	0.0233
isoxaflutole [e]	2-W	-5.313	2.529		90.3524	[39.4243;7795.0	11.633
Lenacil	3-GL	14.991	14.338	0.1845	0.0494	[0.0468;0.0528]	0.0072
Linuron	2-W	1.769	2.020		0.0877	[0.0797;0.0959]	0.00134
MCPA	2-P	-4.501	1.551		798.34	[575.58;1252.0]	103.6
Metamitron	2-W	-0.995	1.912		2.1317	[1.9261;2.3295]	0.0929
Metolachlor	3-BW	0.239	3.156	0.4930	0.8176	[0.7634;0.8687]	0.422
Pendimethalin [e]	2-W	5.752	2.957		0.0085	[0.0075;0.0097]	0.0024
terbuthylazin	2-W	4.165	3.908		0.0693	[0.0658;0.0725]	0.0097
thifensulfuron-methyl	2-L	-2.093	1.837		13.7756	[11.3232;16.807]	0.0222
triasulfuron	2-W	0.093	1.684		0.5333	[0.4777;0.5860]	n.d. [d]
tribenuron-methyl	2-W	0.670	1.735		0.2527	[0.2317;0.2762]	0.00104

[a] Common name [b] RM: Regression model (see table 4) [c] Mean effect concentrations with 95% two-sided bootstrap confidence intervals
[d] NOEC determined by Dunnett test [d] n.d. not determined [e] please refer to paragraph *3.1.*

Table 5. Mechanism of Action in *Scenedesmus vacuolatus* (see Material and Methods), and Toxic Units

Substance [a] by order of *TU* [b]	Mechanism of action	*TU* [b]	Substance [a] by order of *TU* [b]	Mechanism of action	*TU* [b]
terbuthylazine	PSII [c]	3.07E-01	etofumesate	VLCFA [d]	1.61E-04
atrazine	PSII [c]	2.71E-01	isofenphos	narcotic [h]	1.49E-04
alachlor	VLCFA [d]	1.12E-01	bromoxynil	PSII [c]	9.98E-05
lenacil	PSII [c]	9.06E-02	carbofuran	unknown [i]	8.12E-05
linuron	PSII [c]	5.93E-02	ioxynil	PSII [c]	5.79E-05
metamitron	PSII [c]	5.59E-02	isoxaflutol	plastoquinone [j]	3.42E-05
metolachlor	VLCFA [d]	3.18E-02	2,4-D	narcotic [h]	2.66E-05
aclonifen	Porphyrin [e]	2.55E-02	MCPA	narcotic [h]	1.98E-05
chloridazon	PSII [c]	8.68E-03	thifensulfuron-methyl	ALS [g]	1.55E-05
isoproturon	PSII [c]	6.37E-03	cycloxydim	narcotic [h]	8.16E-06
pendimethalin	Microtubule [f]	6.13E-03	clopyralid	no effects	
tribenuron-methyl	ALS [g]	5.81E-03	carbosulfan	no effects	
triasulfuron	ALS [g]	1.44E-03		?	9.82E-01

[a] Common name [b] Toxic unit (PEC/*EC50*) [c] inhibition of the D1 protein in the photosystem II (Devine *et al.*, 1993; Tomlin C (ed), 1994; Faust *et al.*, 2001) [d] inhibition of very long chain fatty acid formation (Devine *et al.*, 1993; Böger *et al.*, 2000) [e] inhibition of the porphyrin synthesis (Tomlin C (ed), 1994) [f] inhibition of the microtubule formation (Devine *et al.*, 1993) [g] inhibition of the acetolactate synthase (Blair and Martin, 1988; Landstein *et al.*, 1990; Nyström and Blanck, 1998) [h] narcotic effect that could be expected to result from hydrophobicity-driven accumulation in cellular membranes only (c.f. chapter 2.5) [i] unknown but higher toxicity than expected for a narcotic effect (c.f. chapter 2.5) [j] depletion of plastoquinone (Viviani *et al.*, 1998; Pallet *et al.*, 1998)

According to the decision procedure outlined in section 2.4 the scenario components can be arranged into eight groups of similar action (tab. 5). Hence, the mixture from the agricultural exposure scenario does fulfil neither the prerequisites of *CA* nor those of *IA*: the components neither share all the same mechanism of action nor has every mixture component a unique one.

3.2. Toxicity from the agricultural exposure scenario and predictability of mixture toxicities

Two mixtures were tested: a mixture composed of the 23 toxic components, for which concentration-response functions were determined, and a mixture composed of all 25 scenario components. For both mixtures, complete concentration-response functions were determined. The regression model, the model parameters, the *EC50* and the NOEC value of each mixture are given in tab. 6.

Table 6. Toxicity of pesticide mixtures to *Scenedesmus vacuolatus.*

mixture	Concentration-response function				*EC50*[c]		NOEC[d]
	RM[b]	$\hat{a}$	$\hat{b}$	$\hat{g}$	(µM)	[CL]	(µM)
mixture of 23 substances	BCW	1.090	1.896	0.3659	0.40584	[0.3732;0.44138]	0.022578
mixture of 25 substances	W	0.675	3.521		0.50622	[0.46298;0.5628]	0.093126

Signs and abbreviations as given in tab. 5

The experimental data variability for both mixtures is comparable to that observed for the single substances. The 23-component mixture showed a slightly lower *EC50* than the 25-component mixture (0.41 µM and 0.51 µM respectively). However, after the correction for the concentration of the two non-toxic substances (by the factor of 1.17), the *EC50* of the 25 component mixture is with 0.43 µM almost identical to the *EC50* of the 23 component mixture and they show overlapping confidence limits. Accordingly, the effects caused by each sum of the PECs are similar: 46% and 42%, respectively. It can be concluded that carbosulfan and clopyralid did not influence the algal toxicity of the mixture. For the remainder of the results section we will thus focus on the 23-component mixture. The results for the 25-component mixture are essentially the same.

In fig. 1 the individual effects are depicted, which each component's modelled concentration in the scenario (PEC) would elicit if applied singly. These effects were estimated by inserting the PEC (tab. 1) into the respective concentration-response function (tab. 4). Only 11 substances show an effect of at least 1% and only four of them show significant effects, *i.e.* being present in a concentration above their NOEC.

With 17%, atrazine elicits the highest single PEC-effect. This is distinctly lower than the observed mixture toxicity of 46%. When comparing the observed toxicity (46%) with the predictions, it becomes clear that it is only slightly lower than predicted according *CA* (49%) and higher than predicted according to *IA* (39%).

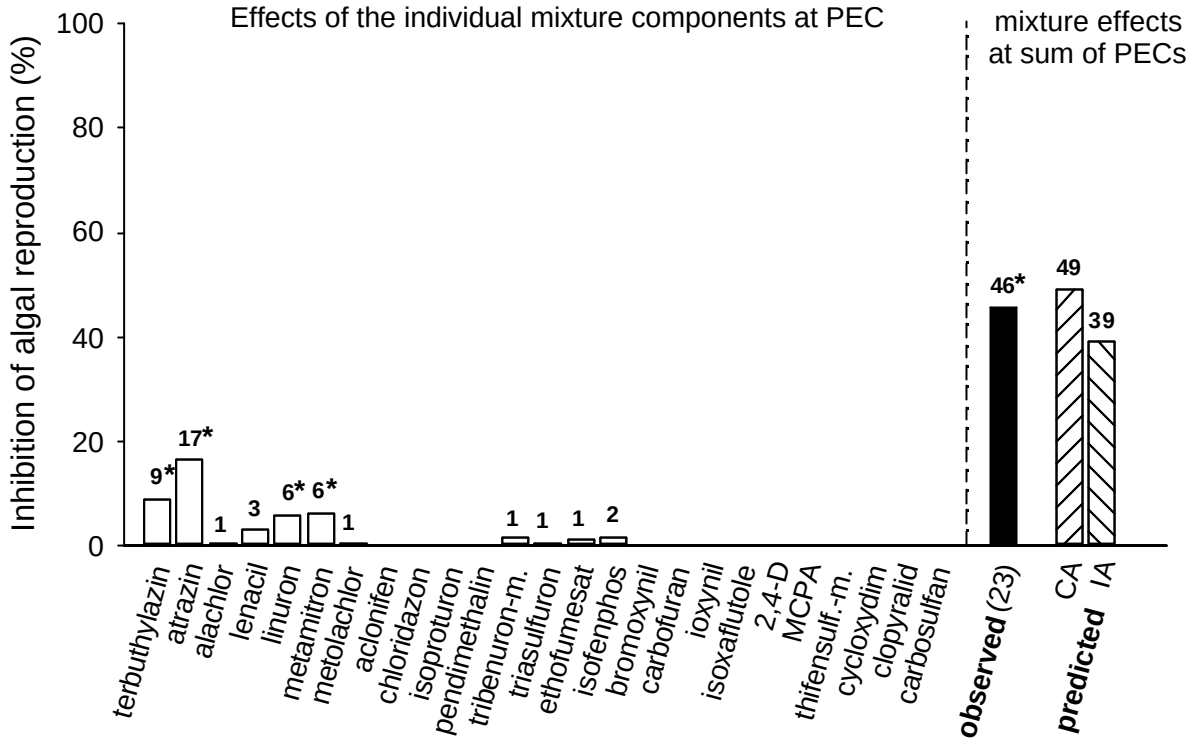

Figure 1. Comparison of effects by the single mixture components at their PEC with the observed effect at the sum of PECs in the mixture composed of 23 components, as well as with the effects predicted according to *CA* and *IA*; in case of missing values on the bars the effects are lower than 1; the single mixture components are arranged according to decreasing toxic units from left to right. Effects marked with ***** are significantly different from controls (PEC > NOEC c.f. tabs. 1 & 5/7).

For a direct graphical assessment of the whole concentration-response range, we depicted the experimental data points, their statistical fit as well as the predictions according to *CA* and *IA* (fig. 2). When comparing the experimental concentration-response curve with the predictions it becomes clear that *CA* shows good predictive quality over the complete range of effects. *IA* on the other hand underestimates the mixture toxicity slightly, although at the level of the *EC50*, the confidence intervals of the *EC50* predicted according to *IA* still overlaps with the confidence interval of the experimental *EC50* (tab. 7). At the higher effect levels, the underestimation by *IA* becomes slightly more prominent.

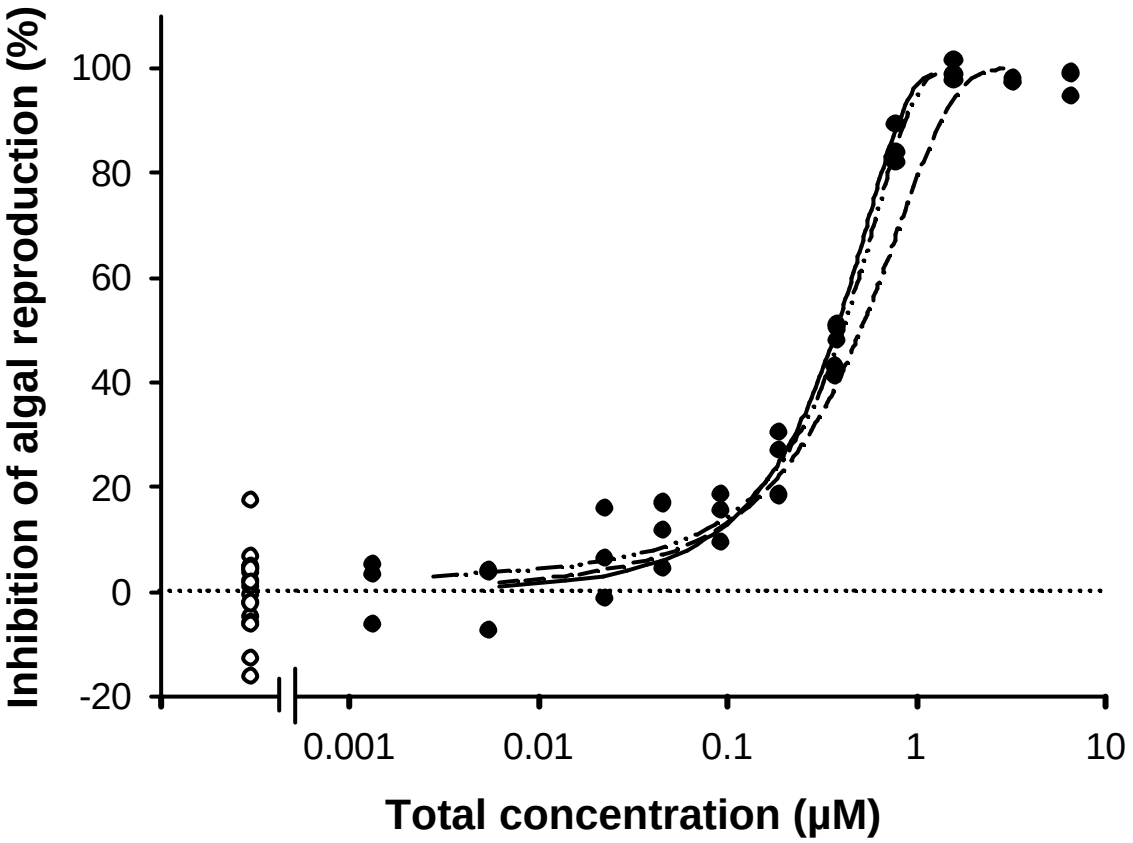

Figure 2. Observed and predicted mixture toxicity of 23 "effective" substances: (•) experimentally observed toxicity; (o) untreated controls; (- · · -) fit of experimentally observed data (- - - -); prediction according to *concentration addition*; (- - -) prediction according to *independent action*. For the sake of relevance and graphical clarity, the lowest tested concentration is not shown: this concentration is distinctly below the NOEC.

Table 7. EC_{50} values of the mixtures – confidence limits have been calculated by a bootstrap method (Scholze *et al.*, 2001)

	EC_{50} (µM)	upper confidence limit (µM)	lower confidence limit (µM)	EC_{50} predicted by CA (µM)	[CL]	EC_{50} predicted by IA (µM)	[CL]
mixture of 23 substances	0.4058	0.3732	0.4414	0.3750	0.3645;0.3847	0.50	0.4231;0.5487
mixture of 25 substances	0.5062	0.4630	0.5628	0.4359	0.4265;0.4501	0.58	0.4950;0.6420

4. Discussion

The exposure scenario that is given in tab. 1 is the result of modelling a general average European agricultural situation during spring using standard FOCUS surface water scenarios (FOCUS, 2001) and assuming an average distribution of major crops (Vighi, 2004). The exact procedure for calculating the composition of the resulting pesticide mixtures is described by Finizio *et al.* (2004a). The listed PEC values indicate the concentrations in run-off water that enter a particular stream. Hence, the resulting mixture (SPECs of the individual components) represents the overall load of pesticides that is emitted from the total agricultural area to the aquatic system.

The tested mixture caused a severe inhibition of algal reproduction by almost 50% at the SPECs of the individual components. The resulting algal toxicity in the receiving water can be assumed to be lower, depending on the actual dilution after entry into a given stream. Using the concentration-response fit for the mixture that is given in tab. 6, the actual toxicity to *Scenedesmus vac*. might be easily calculated for any mixture concentration. Nevertheless, substance specific degradation and sorption processes might change the mixture ratio. Hence, a refinement of the PECs for the individual components by incorporating environmental fate estimations (considering e.g. the actual temperature, UV-intensity etc.) might be indicated.

The fact that the individual concentration-response relationships were not determinable for two components (carbosulfan and clopyralid) has to be considered during the application of both concepts. According to *IA* only those mixture components contribute to the mixture toxicity, that are present in the mixture in a concentration, which would provoke an effect if applied singly ($E(c_i) > 0$, see eqn. 5). Thus, the question arises, whether carbosulfan and clopyralid elicit effects in the concentration-ranges in which they were present in the mixture. As mentioned above, neither of these substances showed effects higher than 5% when applied single in the concentration range, in which they were present in the mixtures. As this can be regarded to fall within the range of the control variability, no influence on the mixture toxicity according to *IA* was expected.

According to *CA,* the overall mixture toxicity can only be predicted if for all substances effect concentrations are available (eqn. 3). If this is not the case in a

given mixture study, a case-by-case analysis on how to circumvent this problem is needed. Two principal situations might be distinguished here. If the data are simply missing, QSAR-based toxicity estimations might provide a mean to surrogate missing experimental toxicity data. For the agricultural mixture that is analysed in this paper, though, the toxicity of carbosulfan and clopyralid was actually assessed but the data did not allow the estimation of a concentration-response curve, as no effects > 20% could be determined (see above). Hence, *ECx* values are principally not available for carbosulfan and clopyralid. Consequently, this makes a *CA* prediction for the 25-substance mixture unfeasible in a strict sense.

Here, the only option is to simply ignore any possible toxicity contribution of both chemicals to the *CA* prediction. The following procedure allows assessing the maximum possible error of the calculation of the *CA*-prediction under these circumstances: For both chemicals, no effects greater than 20% were observed in the tested concentration range. Thus, we can safely postulate for both components, that their *ECx* (x=20-99) values have to be above or at least equal to the highest tested concentrations. Hence, the maximum possible *TU* of each chemical for an effect level > 20% can be estimated as the ratio between the corresponding PEC and the maximum tested concentration. The PECs of carbosulfan and clopyralid are 2.45E-02 µM and 3.66E-02 µM respectively, the maximum tested concentrations were 91.5 and 4574.3 µM. Consequently, the *TUs* at any effect level are always equal to or smaller than 2.7E-4 and 8.0E-6. A comparison with the sum of *TUs* of the 23 components, for which a complete concentration response curve was determined (e.g. 0.98 at the 50% effect level) then allows to quantify the maximum error of ignoring the contributions of clopyralid and carbosulfan. It can be safely concluded, that this maximum possible error is negligible for the actual mixture.

The fact that neither according to *IA* nor according to *CA* an apparent influence on the mixture toxicity is expected is in accordance with the experimental finding that the presence of carbosulfan and clopyralid did not alter the toxicity of the mixture.

It has been shown that *CA* almost accurately predicts the mixture toxicity, although the mixture components do not fulfil the prerequisite of strict similarity of action. Nevertheless, the predictive quality of *CA* is comparable to that observed for multi-

component mixtures composed exclusively of similarly acting substances (Altenburger *et al.*, 2000; Backhaus *et al.*, 2000b; Drost *et al.*, 2003; Junghans *et al.*, 2003a; Junghans *et al.*, 2003b). The reason for this accordance can be found in the fact that the mechanisms of action present in the mixture are not distributed evenly in terms of *TUs*. If the *TUs* are summed up for every mechanism of action, it becomes evident that the group of PSII inhibitors contributes 0.80 to the total sum of *TUs* of 0.98. Assuming that the only toxic components in the mixture were PSII inhibitors, the *EC50* predicted by *CA* would be raised for the 23-component mixture by a factor of only 1.2 to 0.43µM. It can be concluded that the dominancy of PSII inhibitors, makes almost concentration-additive mixture toxicity seem reasonable.

The finding for the present mixtures that *IA* predicts a lower toxicity than *CA* is in accordance with the majority of multi-component mixtures (Altenburger *et al.*, 2000; Backhaus *et al.*, 2000a; Backhaus *et al.*, 2000b; Walter *et al.*, 2002; Silva *et al.*, 2002; Faust *et al.*, 2003; Drost *et al.*, 2003; Altenburger *et al.*, 2003; Junghans *et al.*, 2003a).

Hence, the application of *IA* for the prediction of any mixture that is not composed of only strictly dissimilarly acting substances runs the risk of an underestimation of the actual mixture toxicity. An application of *IA* for mixtures with components of unknown or even partly similar mechanisms of action therefore conflicts with the precautionary principle.

In contrast, in none of the multi-component studies known to us *CA* underestimated the actual mixture toxicity, even if applied to mixtures in which the components' mechanisms of action were not similar. Some authors have thus suggested that *CA* may be used as a precautionary default assumption in the context of predictive hazard assessment of mixtures (Boedeker *et al.*, 1993; Faust *et al.*, 2003).

The acceptability of this approach might depend on the quantitative error that can possibly occur when using *CA* for mixtures in which the components are not strictly similarly acting. Under the assumption of no interaction between the mixture components, this error may equal the quantitative difference between *CA* and *IA* predictions. The factors influencing this quantitative difference are reported to be the mixture ratio, the steepness of the individual concentration-response curves, the number of mixture components as well as the effect level under consideration (Boedeker *et al.*, 1993; Drescher and Boedeker, 1995; Faust *et al.*, 2001; Backhaus

et al., 2004). As shown by Faust (1999) the maximal factor by which *CA* may predict a higher mixture toxicity than *IA* equals the number of mixture components. For a 23 component mixture of strictly dissimilarly acting substances the quantitative error made when using *CA* to predict the toxicity can thus maximally equal a factor of 23.

However, this maximum possible ratio between the *EC50* values predicted by *CA* and *IA* may be further refined on the basis of the data, which are already used as an input for the calculation of a *CA* prediction: the mixture ratio and the *ECx* values of all mixture components. The maximal factor by which *CA* may predict a higher *ECx* value than *IA* can be calculated by:

$$\frac{\mathrm{EC}_{\mathrm{X}}^{\mathrm{IA}}}{\mathrm{EC}_{\mathrm{X}}^{\mathrm{CA}}} \leq \frac{\sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{TU}_{\mathrm{i}}}{\max\limits_{\mathrm{i}\in(1,\ldots,\mathrm{n})} \{\mathrm{TU}_{\mathrm{i}}\}} \qquad [8]$$

where again TU_i is the concentration of the ith substance scaled for its *EC50* and n is the number of mixture components. ECx_{IA} and ECx_{CA} are predictions for the mixture concentration that would provoke *x*% effect according to *IA* and *CA* respectively. The mathematical proof for eqn. [8] is given in the appendix.

For the specific case of a mixture in which the components all have identical *TUs*, *i.e.* $TU_1 = TU_2 = TU_n$ the following relation holds:

$$\frac{\mathrm{EC}_{\mathrm{X}}^{\mathrm{IA}}}{\mathrm{EC}_{\mathrm{X}}^{\mathrm{CA}}} \leq \frac{\sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{TU}_{\mathrm{i}}}{\max\limits_{\mathrm{i}\in(1,\ldots,\mathrm{n})} \{\mathrm{TU}_{\mathrm{i}}\}} = n \qquad [9]$$

as previously shown by Faust (1999). If any mixture component has a *TU* different from the other *TUs*, the maximal factor will always be smaller than *n*.

As a thought experiment, we might assume that only the individual *EC50s* were at hand for the mixture that is given in tab. 1. On the basis of eqn. [9] we still can conclude that the maximal factor by which *CA* predicts a lower *EC50* than *IA* is only 3. So, even assuming a complete independence of the mixture components (*i.e.* a predictability of the mixture effects by *IA*), the error by using the wrong concept (*CA*) might be considered small from a regulators point of view. The actually observed ratio of 1.3 is even somewhat smaller than this theoretical maximum, due

to the specific characteristics of the tested mixture, *i.e.* the steepness of the individual concentration-response curves.

To clarify whether this small maximal difference in *CA* and *IA* predictions is an exception or rather the rule, a corresponding calculation was exemplarily made for other agricultural exposure scenarios, also on the level of the *EC50* value. Only exposure scenarios were selected, for which for all mixture components' *EC50* values were reported in the same publication. The results are listed in tab. 8: 16 different taxon – exposure scenario combinations were evaluated. For every scenario the maximal possible ratio between the *EC50* predicted by *IA* and the *EC50* predicted by *CA* was lower than *n* and never exceeded a factor of 3. Thus in case that a predictive hazard assessment is to be made for the given exposure situations on the basis of *EC50* values alone, *CA* seems to be a reasonable approach for the prediction of mixture toxicity in all analysed scenarios. Based on these results the sole use of *CA* can be justified. However, it has to be pointed out here, that all these scenarios concern pesticide mixtures. Whether mixtures of other environmental chemicals show the same characteristics, remains to be investigated.

From the fact that in neither scenario the maximal possible value of *n* resulted, it can be deduced that the mixture components did not have equal *TUs*, *i.e.* under the assumption of *CA* some substances have a stronger influence on the mixture toxicity than others do.

This is shown in fig. 3 in which a ranked *TU* summation is given for four exposure scenarios: the scenario tested in this study (fig. 3A), as well as for three scenarios taken from tab. 8 (figs. 3B-D). Additionally, we have also plotted the theoretical case that all components have identical *TUs*. It can be seen, that the actual conditions in the analysed scenarios clearly deviate from this situation. Only a few mixture components dominate the mixture. Hence, if the mixture components have similar mechanisms of action, this ranked *TU* summation may assist the determination of the substances contributing most to the mixture toxicity. However, it has to be pointed out here that due to differences in the slopes of the individual concentration response curves of the mixture components the relative ranking of *TUs* might change in dependence of the effect level.

Table 8. Evaluation of agricultural exposure scenarios for the maximal value by which *CA* may predict a higher toxicity than *IA*

Number of mixture components	taxon	$\frac{\sum_{i=1}^{n} TU_i}{\max_{i\in(1,\ldots,n)}\{TU_i\}}$ [a]	citation
9	crustacea	1.20	George *et al.* 2003
8	algae	1.07	Steen *et al.* 1999
8	crustacea	2.01	Steen *et al.* 1999
8	fish	2.48	Steen *et al.* 1999
6	crustacea	1.06	Steen *et al.* 1999
6	fish	1.23	Steen *et al.* 1999
5	crustacea	1.01	Steen *et al.* 1999
5	crustacea	1.54	Steen *et al.* 1999
5	fish	1.42	Steen *et al.* 1999
5	fish	1.98	Steen *et al.* 1999
4	algae	1.66	Steen *et al.* 1999
4	algae	1.76	Steen *et al.* 1999
4	crustacea	1.05	Steen *et al.* 1999
4	crustacea	1.44	Thomas *et al.* 2001
4	crustacea	2.00	Thomas *et al.* 2001
4	fish	1.75	Steen *et al.* 1999
3	crustacea	1.13	Steen *et al.* 1999
3	fish	1.83	Steen *et al.* 1999

[a] calculated according to eqn. [8]: the ratio between EC_x^{IA} and EC_x^{CA} can not exceed the given value

The extendibility of this approach to mixtures, in which the mechanisms of action of the components are unknown or known to be not strictly similar, is subject to several factors, e.g. the steepness of the components' concentration-response curves and TU vs. mechanism of action patterns. Thus, a sensitivity analysis should be performed to give evidence on the general applicability of a ranked *TU* summation for the determination of the substances contributing most to the mixture toxicity.

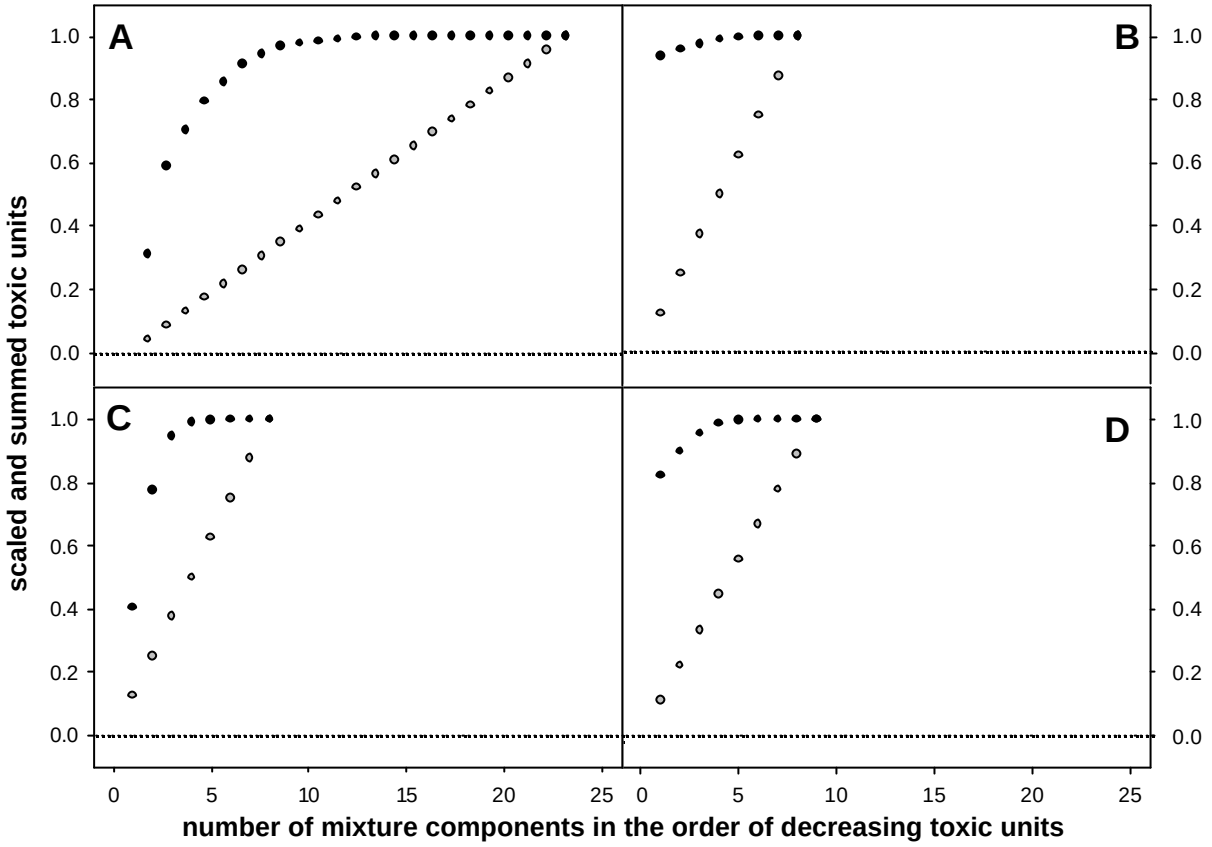

Figure 3. Ranked toxic unit summation for 4 exposure scenarios: scenario tested in this study (A), 6 herbicides and 2 insecticides detected in River Ebro (Steen *et al.* 1999) – *EC50* for algae (B), 8 herbicides detected in the River Scheldt (Steen *et al.* 1999) – *EC50* for fish (C), worst case scenario for 9 detected athmospherically transported pesticides (6 Herbicides, 2 Insecticides and PCP) (George *et al.* 2003) – *EC50* for crustacea (D). Each total sum of toxic units was scaled for 1 for the sake of comparatibility. (•) original mixture ratio (o) theoretical equitoxic mixture ratio.

5. Conclusions

In view of all the results of this study, it can be concluded that the use of *CA* as a precautionary default assumption for the hazard assessment of mixtures from agricultural exposure scenarios is justified.

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Appendix

If for an *n*-component mixture the relative mixture ratio **p**={p_1,…,p_n} between the compounds is exactly known and also the concentrations for all mixture compounds given which produce the same effect *X* (EC_X^i, i=1,…,n), then the maximal possible relative deviation between effect concentrations calculated by concentration addition (EC_X^{CA}) and independent action (EC_X^{IA}) can be estimated by

$$\frac{EC_X^{IA}}{EC_X^{CA}} \le \left(\max_{i\in(1,\ldots,n)} \left\{ p_i / EC_X^i \right\} \right)^{-1} * \sum_{i=1}^{n} \frac{p_i}{EC_X^i}. \qquad [1]$$

The proof is based mainly on the quantitative relationship between the combined effect calculated by independent action and the maximal individual effect: as the effects are based on a fractional scale, joint effects of independent action can be estimated by the inequality

$$E(c_{IA}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \ge 1 - \min_{i\in(1,\ldots,n)} \{1 - E(c_i)\} = \max_{i\in(1,\ldots,n)} \{E(c_i)\}, \qquad [2]$$

where $E(c_{IA})$ is the combined effect produced by the total mixture concentration c_{IA} according to IA, and $E(c_i)$ the effect of the i^{th} individual mixture component if applied singly in the concentration c_i. The maximal individual effect as an estimate for the description of the overall mixture effect has been termed "no addition" (Koeneman, 1981) and is regarded by some authors as a special case of Independent action (see more in Plackett/Hewlett, 1948, etc.). As for the i^{th} individual mixture concentration $c_i = p_i * c_{IA}$,

$$E(c_{IA}) \ge \max_{i\in(1,\ldots,n)} \{E(c_i)\} = \max_{i\in(1,\ldots,n)} \{E(p_i * c_{IA})\}. \qquad [3]$$

This inequality can easily be rephrased for effect concentrations to

$$EC_X^{IA} \le \min_{i\in(1,\ldots,n)} \{EC_X^i / p_i\} = \min_{i\in(1,\ldots,n)} \left\{ \frac{1}{p_i / EC_X^i} \right\} = \frac{1}{\max_{i\in(1,\ldots,n)} \{p_i / EC_X^i\}}. \qquad [4]$$

Thus, the ratio between the effect concentrations calculated by IA and CA (as defined in eqn. 7 in section 2.5) becomes to

$$\frac{EC_X^{IA}}{EC_X^{CA}} \le \frac{1}{EC_X^{CA} * \max_{i\in(1,\ldots,n)} \{p_i / EC_X^i\}} = \frac{\sum_{i=1}^{n} \frac{p_i}{EC_X^i}}{\max_{i\in(1,\ldots,n)} \{p_i / EC_X^i\}}. \qquad [5]$$

In terms of toxic units (see introduction for more details), eqn. [5] can be simplified to

$$\frac{EC_X^{IA}}{EC_X^{CA}} \le \frac{\sum_{i=1}^{n} TU_i}{\max_{i\in(1,\ldots,n)} \{TU_i\}}. \qquad [6]$$

It should be noted that no knowledge is necessary about the underlying concentration effect models of the individual compounds.