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

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Since their commercialization in 1981 sulfonylurea herbicides have been used for pre- and post-emergence 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 n*M* (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. 2003). Because of recommended pre-mixtures (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 (1985) 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 ith compound in the mixture and ECx_i the concentration of the ith compound that individually would provoke the same effect as the mixture.

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

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

where $E(c_{mix})$ describes the predicted effect (scaled from 0-1) of a n compound mixture, c_i is the concentration of the ith 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 the need arises to use one concept as a default approach. Berenbaum (1985) and Boedeker et al. (1993) suggested to use *CA* as a worst case estimation for the combined toxicity of toxicants.

In this study, firstly, the concentration response functions of 8 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.

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. table 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 acetonitrile (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 chemical stability of the substances during the test, the substances were incubated without algae but with the algal growth medium under test conditions. For all tested sulfonylureas, chemical 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, on the basis of 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 Table 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 on the basis of 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)	$E = 1 - \exp(-\exp(\alpha + \beta \log_{10}(c)))$
Generalized Logit (GL)	$E = 1/(1 + \exp(-\alpha - \beta \log_{10}(c)))^{\gamma}$
Box-Cox-Weibull (BCW)	$E = 1 - \exp(-\exp(\alpha + \beta((c^{\gamma} - 1)/\gamma)))$

E – Effect, expressed as fraction of a maximum possible effect ($0 \leq E \leq 1$);
c – Concentration;
α, β, γ – 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 8 sulfonylureas were present either in the ratio of their individual EC_{05} values (Mixture A) or EC_{50} values (Mixture B), respectively (Table 2). For composing the mixture ratios, the relative proportion *p* of each individual 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 equations [1] and [2], respectively. More details can be found in Faust et al. (2003).

RESULTS AND DISCUSSION

For the 8 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 Table 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.* (1.2µ*M*) 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 of 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 Figure 1 together with the results of the mixture toxicity tests. For the sake of simplicity, the fitted concentration-response curves for the mixtures 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 table 3). As indicated by the non-

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

Substance[a] ***(in order of EC_{50})***	**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	GL	BCW	W	W
$\hat{\alpha}$[e]	1.425	1.392	0.093	0.008	-3.333	-1.364	-1.706	-1.968
$\hat{\beta}$[e]	1.376	0.987	1.684	1.863	2.552	0.903	1.929	2.092
$\hat{\gamma}$[e]		-0.027			16.438	-0.018		
EC_{05} µM[f]	0.0006 [0.0003-0.0012]	0.02 [0.01-0.03]	0.02 [0.01-0.02]	0.03 [0.01-0.04]	0.11 [0.09-0.13]	0.17 [0.01-0.56]	0.22 [0.14-0.34]	0.33 [0.13-0.71]
EC_{50} µM[f]	0.05 [0.04-0.06]	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 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

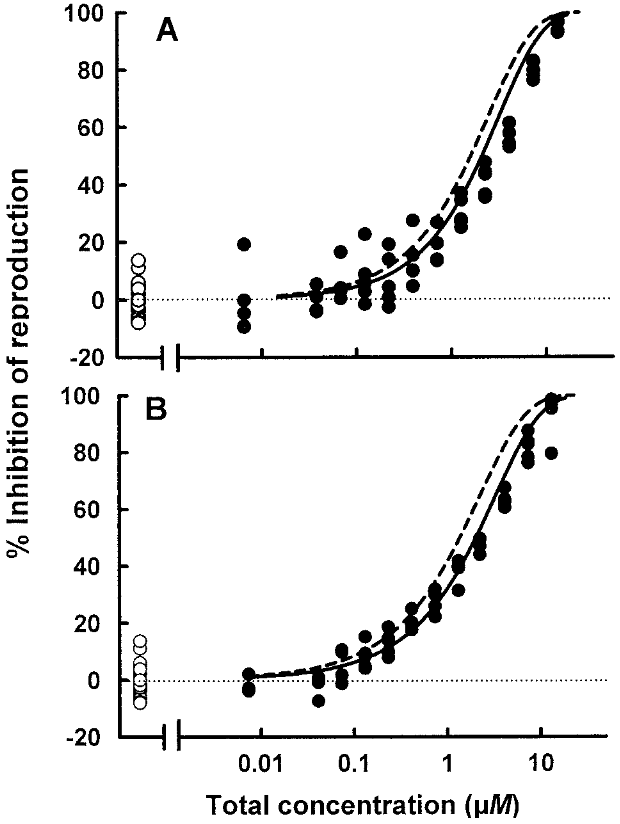

Figure 1. Observed and predicted algal toxicity of sulfonylurea mixtures. (A) Mixture A: components mixed in the ratio of their individual EC_{05} values. (B) Mixture B: components mixed in the ratio of their individual EC_{50} values. (•) experimentally observed toxicity; (o) controls; (———) prediction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

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 experiments 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 8 tested sulfonylureas by *CA* is consistent with other studies on the predictability of multi-component mixtures of similarly acting substances (Faust et al. 2001; Backhaus et al. 2003; Junghans et al. 2003; Escher and Hermens 2002 and references therein).

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

	EC_{50} [μM]					
	by *CA* ^{i}	by *IA* ^{i}	observed ^{f}	RM ^{d}	$\hat{\alpha}$ ^{e}	$\hat{\beta}$ ^{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 table 2

From an empirical point of view it is remarkable that *IA* predicts a higher mixturetoxicity 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. 2002; 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 8 chloroacetanilides (Junghans et al. 2003), 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 1985; Boedeker et al. 1993). The worst-case assumption has not been falsified by this study, since the relatively higher predicted toxicity of the sulfonylurea mixtures 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 8 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 mixture toxicity did not exceed the toxicity predicted by *CA* but (5) was rather accurately predictable by this concept.

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