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Algal Toxicity of Binary Combinations of Pesticides

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Pollution of surface waters is rarely a matter of a single toxicant but aquatic organisms are typically exposed to numerous chemicals simultaneously or in sequence. Consequently, hazard assessment in aquatic toxicology cannot be restricted to considerations on individual compounds. Thereby the question arises whether there are rational approaches for a calculation of the toxicities of mixtures from the concentration response relationships of their components.

This paper deals with the predictive value of *concentration addition*, a concept used for the analysis of combination effects (see Boedeker *et al.* 1992 for a review). It is given by the formula:

$$C_1 / ECx_1 + C_2 / ECx_2 = 1 \tag{1}$$

C_1, C_2 - concentrations of the individual agents *1,2* in a mixture which elicits the effect *x*.

ECx_1, ECx_2 - effect concentrations of each agent *1,2* alone which elicit quantitatively the same effect *x* as the mixture (e.g. EC50).

Concentration addition in the most simple case is said to occur when one substance acts like the dilution of another, i.e., the effect of a mixture remains constant when one component is replaced totally or in part by the equieffective amount of another. For the special case of parallel concentration response curves of the individual compounds, concentration addition is equivalent to the model of *simple similiar action* (Bliss 1939). However, concentration addition is commonly thought to be valid in all experimental situations, independent of the shape of the concentration response curves, and has been proposed as a universal reference for the assessment of combination effects (Berenbaum 1985).

The term concentration addition was introduced into aquatic toxicology by Anderson and Weber (1975), however, this concept has been called by many different names in various fields of research and dates back to the beginning of the century (Loewe and Muischnek 1926). Thus, in order to avoid further confusion in terminology *Loewe additivity* has recently been proposed as a consensus term (Greco *et al.* 1992). The *isobologram method* for the assessment of combination effects is a graphical application of this concept (Loewe and Muischnek 1926, Altenburger *et. al.* 1990). Algebraic equivalents used in aquatic toxicology are the *Toxic Unit Summation* (Sprague 1970) and the *Additivity Index* (Marking 1977). Concentration addition is also one of the reference points of Könemann's *Mixture Toxicity Index (MTI)* (Könemann 1981).

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There is consensus that mixtures of substances with a similar mode of action obey to the concept of concentration addition and its incorporation into the definition of water quality objectives has been recommended (Van der Gaag *et al.* 1991, Calamari and Vighi 1992). However, whether concentration addition is a useful concept also for the predictive assessment of substances with different modes of action is still open to dispute.

The majority of published studies on the toxicities of pollutant mixtures to aquatic life deals with the effects of heavy metals and industrial organic chemicals on fish and daphnids (for reviews see EIFAC 1987, Altenburger *et al.* in press). Relatively few studies deal with combination effects of other types of toxicants or on other aquatic species. In a recent paper we reported on a screening of the algal toxicities of 29 binary combinations of 9 different herbicides with various specific modes of action (Faust *et al.* in press). For 85% of these mixtures results were consistent with concentration additivity. Meanwhile we extended our investigations to fungicides (anilazine, prochloraz) and insecticides (lindane, parathion), substances for which a specific mode of action on algae cannot be expected. EC50 values for the inhibition of algal reproduction by 38 binary combinations of these substances with each other and with 8 different herbicides were determined. Results were compared to the toxicities predicted from the concentration addition concept.

MATERIALS AND METHODS

Pesticides used in the mixture toxicity study are listed in Table 1. They were selected from economically important goups with various types of structures and modes of action (see Worthing and Hance 1991). The term "unspecific" is used to label substances where either the primary sites of action which have been identified in the target organisms of pesticide applications do not exist in microalgae or where interference with multiple biochemical processes (anilazine) is expected. Test compounds had analytical standard quality and were purchased from Riedel-de-Häen (Seelze, FRG) or Promochem (Wesel, FRG).

Test species was the unicellular green alga *Chlorella fusca*. Toxicity parameter was the inhibition of reproduction of synchronized cultures during one generation cycle (24 hr). Sensitivity of this test system in terms of EC50 values is comparable to the standard 96-hr OECD algal growth inhibition test (Faust *et al.* 1992) but test duration and biological variances are minimized. Details of the test protocol, test conditions and the statistical analysis of results have been described previously (Altenburger *et al.* 1990).

Experimental data sets comprised a minimum of 11 different test concentrations in the case of single substances and 6 for the mixtures. They were adjusted to allow for a good statistical description of the complete concentration response curves of the individual agents and for a reliable estimation of the EC50 values of the mixtures. Spacing between test concentrations was varied according to slope and shape of the dose response curves. Mixtures were designed to contain the components in the ratio of their individual EC50's.

For a given ratio of the components the effect concentration of a binary mixture $ECx_{1,2}$ predicted from the concentration addition concept (Eq. (1)) calculates as

$$1 / ECx_{1,2} = 0.01\, p_1 / ECx_1 + 0.01\, p_2 / ECx_2. \qquad (2)$$

p_1, p_2 - percentages of the substances *1* and 2 in the mixture ($p_1 + p_2 = 100\%$)

To account for experimental variances confidence limits for the predicted $ECx_{1,2}$ were calculated in the same way from the confidence limits of the effect concentrations of the individual substances. This approach is equivalent to the use of a confidence belt within the isobologram method (see Altenburger *et al.* 1990). Experimental results were taken to be compatible with the hypothesis of concentration addition when the 95% confidence intervals of experimental and predicted EC50 values overlapped.

Table 1. Pesticides Used in Mixture Toxicity Studies

Common name (ISO)	CAS RN	Purity	Use	Chemical Group	Mode of Action a) Target Organisms b) Algae
Anilazine	101-05-3	99%	Fungicide	s-Triazines	a) Multi Site Thiol and Amino Groups Inactivator b) unspecific (?)
Prochloraz	67747-09-5	90%	Fungicide	Imidazoles	a) Sterol Biosynthesis Inhibitor b) unspecific (?)
Lindane	58-89-9	99%	Insecticide	Halogenated Hydrocarbons	a) Neurotoxin b) unspecific (?)
Parathion	56-38-2	99%	Insecticide	Organophosphates	a) Neurotoxin b) unspecific (?)
Bentazone	25057-89-0	99%	Herbicide	Benzothiadiazines	a,b) Photosystem II Inhibitor
Chlorotoluron	15545-48-9	≥99%	Herbicide	Phenylureas	a,b) Photosystem II Inhibitor
Methabenzthiazuron	18691-97-9	99%	Herbicide	Benzoylthiazolylureas	a,b) Photosystem II Inhibitor
Simazine	122-34-9	99%	Herbicide	s-Triazines	a,b) Photosystem II Inhibitor
Metazachlor	67129-08-2	98%	Herbicide	Chloroacetanilides	a,b) Lipid Biosynthesis Inhibitor
Tri-allate	2303-17-5	99%	Herbicide	Thiocarbamates	a,b) Lipid Biosynthesis Inhibitor
Glyphosate (isopropylamine salt)	38641-94-0	98%	Herbicide	Aminophosphonates	a,b) Aromatic Amino Acids Biosynthesis Inhibitor
2,4-D	94-75-7	99%	Herbicide	Phenoxyalkanoic Acids	a) Auxin Analogon b) unspecific (?)

ISO - International Organization for Standardization
CAS RN - Chemical Abstracts Services Registry Number

RESULTS AND DISCUSSION

Assessing mixture toxicities by using concentration addition as a reference requires the determination of effect concentrations of each agent alone. Therefore we estimated concentration response functions by probit transformation of data and weighted linear regression analysis. This procedure did not always result in a good fit at high or low effect levels, due to non-symmetrical concentration response curves. However, at intermediate levels data were well fitted and inspection of residuals did not show any systematic departures. EC50 values are given in Table 2, both on a molar and on the more frequently used weight basis. Toxicities of the various toxicants spanned more than four orders of magnitude. Interestingly prochloraz, an inhibitor of the ergosterol biosynthesis in fungi not known for a specific mechanism of action in algae, was the most potent inhibitor of the reproduction of *Chlorella* populations, comparable only to the photosynthesis inhibitor chlorotoluron.

When the observed effect of a given combination of agents on a definite response parameter is compared with the effect expected in the case of concentration additivity, assessments may not be identical for different effect levels and for different ratios of the mixture constituents. However, for the purpose of a screening of a variety of pesticide combinations, we restricted the investigations to the median level of toxicity (EC50) and to mixture ratios given by the ratios of the EC50 values of the individual compounds (equitoxic concentrations). Table 3 displays the combination ratios as well as observed and predicted EC50 values of the 38 test mixtures. 66% of the mixtures showed toxicities as predicted by concentration addition, i.e., they were *Loewe additive* in terms of the proposed consensus terminology (Greco *et al.* in press). The effects of 9 mixtures (24%) were less than concentration additive (*Loewe antagonism*). Only 4 combinations (10%) were more toxic than expected (*Loewe synergism*). However, there were only two cases where the ratio between predicted and observed or observed and predicted EC50 values exceeded a factor of two: the mixture of anilazine and tri-allate was 3.5 times more toxic to the algae than expected while the EC50 value of anilazine+prochloraz accounted for only 48% of the predicted value. All these discrepancies are interesting from a mechanistic point of view, but they are small with respect to inter-laboratory and inter-species differences which have been reported for standard OECD test protocols and numerous algal toxicants.

The concept of concentration addition is tied to the idea of "similar acting" agents. However, the understanding of similar action is a controversial issue in the discussion on combined effect analysis: a mechanistic point of view (identical molecular target sites) (Pöch 1993) contrasts with a phenomenological understanding (similar modes of action) (Hermens et al. 1984). All the binary combinations studied here contained a fungicidal or an insecticidal compound with no known specific mechanism of action on algae and were hence classified as "unspecific". In most of the mixtures (84%) the second component was a herbicidal agent proven or assumed to exert a specific effect on green algae on the basis of current knowledge. Thus, although the term "unspecific" is not very precise, at least the majority if not all of the mixtures would hardly qualify as combinations of similar acting agents, whatever the criterion of similarity may be. Therefore, the concept of concentration addition seems to have a good predictive value even for dissimilar acting agents.

The validity of concentration addition for predicting the aquatic toxicities of combinations of similar acting agents has been confirmed by various experimental studies (EIFAC 1987). The results presented here for the algal toxicities of pesticide mixtures indicate that it can also be a valuable tool in case of dissimilar acting substances. This finding is consistent with some other studies on the mixture toxicities of various types of chemicals to fish (e.g., Könemann 1981) and daphnids (e.g., Deneer *et al.* 1988). Thus, we conclude that concentration addition may be a reasonable assumption for the hazard assessment of mixtures of chemicals with unknown modes of action.

Table 2. Algal Toxicity of Individual Pesticides. Parameter: Inhibition of reproduction during one generation cycle (24hr) of synchronized cultures of *Chlorella fusca*

Pesticide	Concentration Response Relationship as Probit Regression Equation P = Probit C = Concentration in µmol/L	EC50 [µmol/L]	(95%-Confidence-Interval)	EC50 [mg/L]
Fungicides				
Prochloraz	P = 8.611 + 1.313 ln C	0.064	(0.055-0.074)	0.024
Anilazine	P = 3.031 + 1.218 ln C	5.04	(3.96-6.41)	1.39
Insecticides				
Lindane	P = 1.652 + 1.252 ln C	14.5	(12.1-17.4)	4.22
Parathion	P = 0.465 + 1.375 ln C	27.0	(17.0-43.1)	7.86
*Herbicides**				
Chlorotoluron	P = 7.088 + 0.950 ln C	0.11	(0.08-0.15)	0.023
Methabenzthiazuron	P = 6.103 + 0.692 ln C	0.20	(0.15-0.27)	0.044
Metazachlor	P = 7.799 + 1.779 ln C	0.21	(0.19-0.23)	0.058
Simazine	P = 6.096 + 1.073 ln C	0.36	(0.32-0.41)	0.073
Tri-allate	P = 0.253 + 2.069 ln C	12.7	(11.6-13.9)	3.87
Bentazone	P = -1.159 + 1.190 ln C	177	(151-207)	42.5
2,4-D	P = -1.900 + 1.151 ln C	402	(371-436)	88.9
Glyphosate (isopropylamine salt)	P = -4.942 + 1.343 ln C	1650	(1470-1840)	377

all values refer to nominal test concentrations
* data from Faust *et al.* in press

Table 3. Algal Toxicity of Binary Combinations of Pesticides

Pesticide Combination (Mixture Ratio p_1:p_2)*	EC50 [µmol/L] (95% Confidence Interval) predicted**		observed		Assessment***
Fungicide + Herbicide					
Anilazine + Bentazone (2.42 : 97.58)	97.0	(79.7-117.8)	167	(123-227)	-
Anilazine + Chlorotoluron (97.8 : 2.2)	2.55	(1.96-3.32)	2.67	(2.47-2.88)	additive
Anilazine + 2,4-D (1.03 : 98.97)	222	(190-258)	379	(273-527)	-
Anilazine + Glyphosate (0.26 : 99.74)	891	(750-1054)	542	(378-776)	additive
Anilazine + Metazachlor (96.26 : 3.74)	2.69	(2.28-3.17)	2.19	(2.04-2.34)	additive
Anilazine + Methabenzthiazuron (96.54 : 3.46)	2.76	(2.13-3.58)	2.74	(2.39-3.15)	additive
Anilazine + Simazine (93.52 : 6.48)	2.73	(2.26-3.30)	2.91	(2.67-3.18)	additive
Anilazine + Tri-allate (27.8 : 72.2)	8.91	(7.54-10.47)	2.53	(2.30-2.78)	+
Prochloraz + Bentazone (0.0305 : 99.9695)	96.0	(82.5-111.7)	87.5	(68.8-111.4)	additive
Prochloraz + Chlorotoluron (35.3 : 64.7)	0.0876	(0.0710-0.1085)	0.0769	(0.0658-0.0898)	additive
Prochloraz + 2,4-D (0.0128 : 99.9872)	223	(200-249)	615	(473-800)	-
Prochloraz + Glyphosate (0.0032 : 99.9968)	902	(795-1024)	831	(763-905)	additive
Prochloraz + Metazachlor (24.1 : 75.9)	0.135	(0.120-0.152)	0.090	(0.073-0.110)	+
Prochloraz + Methabenzthiazuron (25.6 : 74.4)	0.130	(0.105-0.165)	0.124	(0.089-0.173)	additive
Prochloraz + Simazine (15.1 : 84.9)	0.212	(0.184-0.244)	0.267	(0.246-0.289)	-
Prochloraz + Tri-allate (0.472 : 99.528)	6.56	(5.83-7.37)	7.33	(6.25-8.60)	additive
Insecticide + Herbicide					
Lindane + Bentazone (6.46 : 93.54)	103	(86.7-121.5)	189	(126-282)	-
Lindane + Chlorotoluron (99.2 : 0.8)	7.12	(5.61-9.01)	5.94	(5.26-6.70)	additive
Lindane + 2,4-D (2.82 : 97.18)	229	(202-260)	216	(176-264)	additive

Table 3 continued

Lindane + Glyphosate (0.72 : 99.28)	909	(787-1049)	981	(920-1046)	additive
Lindane + Metazachlor (98.63 : 1.37)	7.46	(6.51-8.53)	12.6	(9.61-16.60)	-
Lindane + Methabenzthiazuron (98.73 : 1.27)	7.65	(6.07-9.63)	7.67	(6.53-9.02)	additive
Lindane + Simazine (97.6 : 2.4)	7.46	(6.36-8.75)	6.69	(5.94-7.53)	additive
Lindane + Tri-allate (51.8 : 48.2)	13.6	(11.8-15.5)	14.8	(11.6-18.9)	additive
Parathion + Bentazone (22.2 : 77.8)	79.3	(54.9-112.2)	65.9	(59.9-72.6)	additive
Parathion + Chlorotoluron (99.805 : 0.195)	18.4	(12.2-27.5)	15.3	(12.5-18.7)	additive
Parathion + 2,4-D (10.7 : 89.3)	162	(115-221)	307	(279-338)	-
Parathion + Glyphosate (2.91 : 97.09)	600	(421-831)	799	(539-1184)	additive
Parathion + Metazachlor (99.665 : 0.335)	18.9	(13.1-26.4)	14.4	(11.0-18.9)	additive
Parathion + Methabenzthiazuron (99.69 : 0.31)	19.2	(12.7-28.9)	11.9	(9.6-14.7)	additive
Parathion + Simazine (99.4 : 0.6)	18.7	(12.9-26.6)	14.6	(12.1-17.6)	additive
Parathion + Tri-allate (81.6 : 18.4)	22.4	(15.6-31.0)	13.3	(12.0-14.8)	+
Fungicide + Fungicide / Fungicide + Insecticide / Insecticide + Insecticide					
Anilazine + Prochloraz (98.78 : 1.22)	2.59	(2.13-3.13)	5.39	(4.47-6.51)	-
Anilazine + Lindane (26.38 : 73.62)	9.69	(7.84-12.0)	8.88	(8.01-9.84)	additive
Anilazine + Parathion (8.0 : 92.0)	20.0	(13.4-29.6)	36.5	(32.7-40.6)	-
Prochloraz + Lindane (0.439 : 99.561)	7.28	(6.17-8.58)	8.10	(6.94-9.45)	additive
Prochloraz + Parathion (0.107 : 99.893)	18.6	(12.8-26.6)	19.7	(13.8-28.1)	additive
Lindane + Parathion (19.5 : 80.5)	23.1	(15.7-33.5)	12.9	(11.1-15.0)	+

* Mixture ratio $p_1 : p_2 \cong$ EC50 Substance 1 / EC50 Substance 2 [mol/mol] ; (p_1, p_2 - percentages of components *1* and *2)*

** predicted by the concentration addition model

*** additive - experimental data are consistent with the hypothesis of concentration addition (overlapping confidence intervals)

+ , - - EC50 of the combination is significantly higher (+) or lower (-) than expected from the concentration addition model

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