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JOINT EFFECTS OF A MIXTURE OF 14 CHEMICALS ON MORTALITY AND INHIBITION OF REPRODUCTION OF *DAPHNIA MAGNA*

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The toxicity to *Daphnia magna* of a mixture of 14 aquatic pollutants with several chemical structures and probable modes of action was determined. The joint effect of this mixture on acute mortality was compared with the effects on inhibition of reproduction. The joint toxicity in the reproduction test was lower in comparison with the mortality test. This study supports the idea that the potential for addition is reduced when more specific sublethal toxicity criteria are studied. Although the joint toxicity at sublethal level is lower than at lethal level, the toxicity of the mixtures remains much higher than that of the individual chemicals and is still near concentration addition.

Key words: mixture; *Daphnia magna*; aquatic toxicity

INTRODUCTION

The aquatic environment in industrial areas often is polluted with a large number of chemicals. In evaluating the joint toxicity of such complex mixtures it is useful to distinguish chemicals with similar from chemicals with dissimilar modes of action.

The joint toxicity of mixtures, composed of similarly acting chemicals, can be predicted theoretically with the concentration addition model (Plackett and Hewlett, 1952; Muska and Weber, 1977; Könemann, 1980). The acute mortality to the guppy (Könemann, 1981) and to *Daphnia magna* (Hermens et al., 1984a) of mixtures of 50 non-reactive, non-ionized organic chemicals, related in action to the volatile anaesthetics, could be predicted successfully with this concentration addition model. Also in mixtures of chlorophenols (Könemann, 1981) and anilines (Hermens et al., 1984b), the joint acute mortality was concentration additive. Only in experiments with inhibition of reproduction of *Daphnia magna* as the type of effect, slight deviations from concentration-addition were found (Hermens et al., 1984a).

In these studies quantitative structure–activity relationships (QSARs) were used

as an indication of similarity of mode of action. The prediction of the joint toxicity of mixtures, composed of many dissimilarly acting chemicals, is much more complex. Only for chemicals with independent actions, the toxicity of mixtures can be predicted (Könemann, 1981). Therefore a more empirical approach was used by Hermens and Leeuwangh (1982) in a study of the joint effects on mortality of the guppy of differently acting pollutants. From this study it appeared that the measure of joint toxicity of mixtures of 8 and 24 chemicals, evaluated with the Mixture Toxicity Index (MTI) as proposed by Könemann (1981), was rather constant.

Information on joint effects on sublethal toxicity level is rather scarce. There is, however, a general feeling that, as concentrations of toxicants in a mixture are reduced to their levels of no effect, or when more specific toxicity criteria are studied, the potential for addition is also reduced (EIFAC, 1980). In the present study we compare the joint effects on mortality and on inhibition of reproduction (as a more specific toxicity parameter) to *Daphnia magna* of a mixture of 14 toxicants with various chemical structures and probably different modes of action.

METHODS

Toxicity experiments

The criteria and conditions in the experiments with *Daphnia magna* are summarized in Table I. A more detailed description of the tests with *Daphnia magna* is given by the Concept NEN reports 6501 and 6502 from the Dutch Standard Organization (1980) and by Canton et al. (1975).

All experiments were carried out in duplicate. Forty-eight-hour LC50 and 16-day EC50 values (see Table I) were first calculated based on the quantities of the chemicals added at the start of the experiments. Also values, corrected for the actual measured concentrations, were calculated. Only the lowest and highest tested concentrations were analysed. For the calculation, first the concentrations found in the duplicate experiments were averaged. Then the mean value of the recoveries at the start of the experiments and after 48 h was calculated and finally the recoveries from the lowest and highest tested concentration were averaged. The calculated concentrations at 50% effect were then corrected for this recovery. These recoveries must not be confused with the recoveries of the analyses.

Mixtures were prepared in about equitoxic concentrations (identical fractions of their LC50 or EC50), based on non-corrected LC50 or EC50 values. Also the concentrations of the components in the mixtures were corrected for their recoveries, in the same way as described above, except that in the mixtures only the highest tested concentrations could be analysed.

The results of the mixture toxicity experiments were evaluated with the MTI and scale as proposed by Könemann (1981) (see Table II). This index is defined as:

$$\mathrm{MTI} = 1 - (\log M / \log M_0) \qquad (1)$$

TABLE I

Criteria and conditions in the toxicological experiments with *Daphnia magna*.

Criteria	Age	Exposure time	Number of organisms per group[a]	Test volume per group[b] (liter)	Food	Temp. (°C)	Test medium	Hardness (mmol/l)	Dosing and ratio of concentrations	Renewing rate
50% Mortality 48-h LC50	< 1 day	48 h	25	1	None	19 ± 1	DSW[b]	ca. 1	static 3.2	-
50% Reproduction 16-day EC50	< 1 day	ca. 16[c] days (3–4 broods)	15[c]	1	Chlorella spec.	19 ± 1	DSW[b]	ca. 1	static 3.2	3 times a week

[a] All tests were carried out in duplicate.

[b] Dutch standard water (Canton and Slooff, 1982).

[c] Deviating from Concept NEN 6502 (1980).

TABLE II

Mixture toxicity scale after Könemann (1981).

	Classification for toxicity of mixtures (possible types of joint action)
MTI < 0	Antagonism
MTI = 0	No addition (independent action, $r = +1$)[a]
0 < MTI < 1	Partial addition
MTI = 1	Concentration addition (simple similar action)
MTI > 1	Supra addition (potentiation of the toxic action(s) of one or more of the compounds in the mixture)

[a] Positive correlation between susceptibilities of the individual organisms to the single toxicants.

in which $M = \Sigma f_i$ at 50% mortality in the mixture; $f = c/\text{LC50}$ (concentration as fraction of the LC50); and $M_0 = M/f_{max}$; f_{max} is the highest f value in a mixture.

Chemical analyses

The analytical methods, used to determine the actual concentrations of the compounds in the toxicity experiments, are given in Table III. If necessary the water samples were extracted with an appropriate organic solvent and eventually concentrated. The concentration of potassium dichromate was not determined because in earlier experiments 100% of this compound was always found.

RESULTS

Tables IV and V summarize the results of the toxicity experiments with *Daphnia magna* of the single compounds, respectively of a mixture of the compounds. Con-

TABLE III

Analytical methods.

	Method	Special conditions (coating of the column, detector)
di-isopropylamine	GC[a]	Chromosorb 103, 80–100 mesh, FID
2,6-dimethylquinoline	GC	Tenax-GC, 160–180 mesh, FID
2,4-dichloroaniline and 3-methylaniline	GC	See Wegman and de Korte (1981)
dinitro-*o*-cresol	HPLC[b]	See Wegman and Wammes (1983)
sodium bromide	GC	See Wegman et al. (1981)
dimethoate	GC	3% OV-101, FPD (P mode)
pentachlorophenol	GC	See Wegman and Hofstee (1979)
1,3-dinitrobenzene and 1,3-dichlorobenzene	GC	5% OV-210, 5% OV-17, ^{63}Ni ECD
lindane	GC	See Wegman and Greve (1978)
phenol	GC	20% SP-2100, 0.1% Carbowax, FID
malathion	GC	3% OV-101, FPD (P mode)

[a] Gas chromatography.

[b] High-performance liquid chromatography.

TABLE IV

Results of the experiments with *Daphnia magna* of the single compounds.

Compound	LC50[a] (mg/l)	LC50[b] (mg/l)	Rec.[c] (%)	EC50[a] (mg/l)	EC50[b] (mg/l)	Rec.[c] (%)
di-isopropylamine	457	448	98	61	60	99
2,6-dimethylquinoline	40	38	95	2.4	2.4	100
potassium dichromate	1.1	1.1	-[d]	0.27	0.27	-[d]
2,4-dichloroaniline	0.85	0.71	84	0.090	0.080	89
dinitro-*o*-cresol	3.4	3.3	97	2.2	2.1	95
sodium bromide	13500	13500	100	29	29	100
dimethoate	6.4	6.4	100	0.31	0.31	100
pentachlorophenol	0.28	0.30	107	0.15	0.13	91
3-methylaniline	0.75	0.73	97	0.038	0.043	114
1,3-dinitrobenzene	46	43	93	3.2	3.2	99
lindane	1.5	1.0	68	0.50	0.34	68
phenol	23	23	100	10	10	101
malathion	0.052	0.033	63	0.00056	0.00036	64
1,3-dichlorobenzene	5.6	1.7	30	1.8	1.4	76

[a] Concentration at 50% mortality (LC50) or 50% inhibition of reproduction (EC50); not corrected for recoveries.

[b] LC50 and EC50 values; corrected for measured recoveries (see Methods).

[c] Recoveries of the added quantities during the LC50 and EC50 experiments (see Methods).

[d] Potassium dichromate was not analysed. For the calculation of corrected values for the LC50 and EC50 the recovery was taken as 100%.

centrations at 50% mortality (LC50) and at 50% inhibition of reproduction (EC50) were calculated based on the added quantities as well as on experimental measured concentrations (see Methods).

For some chemicals in the mixture, as for instance 2,4-dichloroaniline, pentachlorophenol and 1,3-dinitrobenzene, the recoveries of the added quantities were rather low.

With the sum of the *f*-values *(M)* from Table V, MTI values were calculated. The advantage of using MTI instead of *M* is that the MTI gives constant values for the same types of joint actions (Könemann, 1981). The MTI is normalized for the number of chemicals in a mixture and their relative concentrations, which makes MTI values better comparable with each other than *M* values.

The MTI of the experiments with mortality (LC50) and inhibition of reproduction (EC50) of *Daphnia magna* of the mixtures of 14 chemicals are given in Table VI. As in Tables IV and V also MTI values were calculated based on the added quantities as well as on measured concentrations.

DISCUSSION

The non-corrected and the corrected (for recovery) MTI values within one experi-

TABLE V

Results of the experiments with *Daphnia magna* of a mixture of the 14 compounds from Table IV.

Compound	f (LC50)[a]	f (LC50)[b]	f (EC50)[a]	f (EC50)[b]	Rec.[c] (%)
di-isopropylamine	0.087	0.067	0.494[d]	0.379[d]	76
2,6-dimethylquinoline	0.087	0.078	0.146	0.124	85
potassium dichromate	0.092	0.092	0.162	0.162	-[e]
2,4-dichloroaniline	0.050	0.023	0.129	0.057	39
dinitro-*o*-cresol	0.138	0.108	0.210	0.168	76
sodium bromide	0.087	0.084	0.143	0.137	96
dimethoate	0.087	0.101	0.044	0.051	116
pentachlorophenol	0.087	0.016	0.261	0.057	20
3-methylaniline	0.082	0.085	0.122	0.107	-[f]
1,3-dinitrobenzene	0.087	0.037	0.164	0.066	40
lindane	0.087	0.095	0.231	0.251	74
phenol	0.057	0.035	0.164	0.099	61
malathion	0.087	0.087	0.164	0.161	63
1,3-dichlorobenzene	0.087	0.154	0.164	0.114	53
sum of f values *(M)*	1.2	1.1	2.6	1.9	

[a] Concentrations of the compounds in the mixture experiment at 50% response, expressed as fractions of their LC50 or EC50 *(f)*; not corrected for the recoveries. f = Concentration calculated from the added quantities/not corrected LC50 or EC50 values.

[b] As in [a]; corrected for the recoveries. f = Concentrations corrected for the recoveries/corrected LC50 or EC50 values.

[c] Recoveries of the added quantities during the EC50 experiments (see Methods). The same values are used for the LC50 experiment.

[d] The relatively high concentration of di-isopropylamine was caused by a mistake in the preparation of the solutions.

[e] Potassium dichromate was not analysed. For the calculation of corrected f-values the recovery was taken as 100%.

[f] The analysis of 3-methylaniline in the mixture was not performed because the added concentration was below the limit of detection. For the calculation of corrected f-values the recovery was taken as 100%.

ment differ only slightly (Table VI). That in both cases (LC50 and EC50) the corrected values are higher is related with the lower recoveries of the added quantities in the mixture experiments. The acute mortality of the mixture is near concentration addition because the MTI is very near to 1.0. This relatively high joint toxicity was also found in acute mortality tests with guppies in mixtures of 8 and 24 chemicals by Hermens and Leeuwangh (1982) which led to the conclusion that the lethal effects of the mixture must result from a complex system of joint actions, and that independent joint actions and potentiation are probably rare phenomena in toxicity studies of mixtures with mortality as overall criterion of effect. The relatively high joint toxicity to *Daphnia magna* in the LC50 experiment can be explained in the same way. The MTI value of the experiment with mortality as the criterion of effect does not deviate from concentration addition (MTI $\sim$ 1.0). The MTI of the reproduction experiment deviates from concentration addition (MTI $<$ 1.0).

TABLE VI

Results of the experiments with *Daphnia magna* of a mixture of 14 compounds, expressed in MTI values.

	LC50 (mortality)		EC50 (reproduction)	
	not corrected[a]	corrected[b]	not corrected[a]	corrected[b]
Number of compounds	14	14	14	14
f_{max}[c]	0.138	0.154	0.49	0.38
M[c]	1.2	1.1	2.6	1.9
M_0[c]	8.7	7.1	5.3	5.0
MTI[c]	0.92	0.95	0.43	0.60
st. dev. in MTI[d]	0.11	0.12	0.18	0.16

[a] Not corrected for recoveries.

[b] Corrected for recoveries.

[c] See equation 1.

[d] SD in MTI calculated with an estimated error in log LC50 and log EC50 of 0.10 and according to the procedure described by Könemann (1981).

Although the estimated errors in MTI are rather high this deviation from concentration addition is quite obvious. Also with other chemicals a reduced joint toxicity was found in experiments with inhibition of reproduction as a parameter in comparison with mortality experiments (Hermens et al., 1984a). Therefore, it cannot be excluded that the potential for addition is reduced when more specific sublethal effects are studied, as suggested in the EIFAC report (EIFAC, 1980).

Such a reduced joint toxicity can be explained in the following way. Inhibition of reproduction can be considered to be based on a number of more specific modes of action of compounds in comparison with mortality. It is of interest to note in this respect that for compounds such as malathion and sodium bromide the effect on reproduction is at very much lower levels than for acute mortality. It is likely that in studies with specific effects, joint toxicity may result either from simple similar action or from independent action (see Table II). Independent actions generally result in a lower joint toxicity of mixtures. Because the chemicals in the mixture we have tested have various chemical structures and probably various modes of action, independent action will be more likely.

Although the joint toxicity at sublethal level is lower than at lethal level, the toxicity of the mixture remains much higher than of the individual chemicals and is still rather near concentration addition.

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