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The Toxicity of Paired Metal Mixtures to the Nematode *Monhystera disjuncta* (Bastian, 1865)

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ABSTRACT

The toxicity of bipartite Hg, Cu, Zn and Ni metal mixtures was studied to the free-living marine nematode Monohystera disjuncta. *Observed mortality and developmental inhibition responses could not be predicted by independent dissimilar nor simple similar action. The toxic unit concept was also used to evaluate overall mixture toxicity. With regard to mortality all paired metal mixtures act in a less than additive manner. The developmental inhibition response was not so clear-cut: the Zn–Ni mixture acts synergistically; similarly the joint effect of Zn–Cu combinations and Cu–Ni mixtures, containing small amounts of Cu (1 mg litre^{-1}), act synergistically and the response type with regard to the Hg–Cu mixture is not very clear.*

INTRODUCTION

In the field, marine organisms are exposed to complex effluents, e.g. acid iron waste under varying environmental conditions. Therefore, it may seem rather surprising that most toxicological studies deal with dose–response relationships of single toxicants. With some groups of animals, however, such as free-living marine nematodes, our present knowledge even on the mode of action of single toxicants is very poor and at present virtually nothing is known about the toxicity of mixtures. Existing information on single species–single toxicant bioassays with a few nematode species, reveals that these organisms are relatively insensitive, particularly to heavy metals

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and some organics (Bogaert *et al.*, 1984; Vranken *et al.*, 1984; 1985; 1986; Vranken & Heip, 1986), although conflicting results exist (Howell, 1984). Even when the so-called sensitive biological criteria, such as fecundity and developmental inhibition, are used for toxicity ranking, minimum effective concentrations are situated in the lower ppm range.

Predicting environmentally safe limits from such single species–single toxicant assays, however, is highly questionable as the occurrence of interactive effects between the constituents of metal mixtures, both in a positive and negative sense has been reported (e.g. Sprague & Ramsay, 1965; Breittmayer & Guttierez-Galindo, 1981; Negilski *et al.*, 1981 and Howell, 1984). Recently, Hermens (1985) has shown that toxicity caused by complex mixtures, composed of organics with a similar mode of action, can be predicted on the basis of concentration-additivity (Hewlett & Plackett, 1979). This implies that hazard assessment based on the effects of single constituents may be deceptive (Hermens, 1985).

Several models have been proposed to tackle the study of the joint-action of mixtures. One of the most comprehensive approaches is that of Plackett & Hewlett (1952). They distinguished four different types of action among which were two non-interactive (independent dissimilar and simple similar action) and two interactive (complex similar and dependent dissimilar action) models. In the most simple case, the simple (parallel slopes for individual toxicants) similar action model, the response is completely predictable. For example, in an acute-toxicity assay, a mixture composed of two constituents will cause 50% mortality, when the concentrations of the substances are half as strong as their LC_{50}. In other words, the sum of the concentrations of the constituents when expressed as fractions of their LC_{50}, is always equal to 1. In the independent dissimilar action model, the expected response is again predictable. An organism receiving a mixture of two drugs, will only respond if one (or both) of the constituents is present at a concentration higher than the incipient lethal level. Thus in this case organisms will not respond at all at concentrations below the incipient lethal level. Hewlett & Plackett (1979) discussed three different models for independent action. When the tolerances of the organisms to the components of the mixture are positively correlated, the toxicity of the mixture is equal to the most toxic substance. When the tolerances of the organisms are uncorrelated, the proportion responding to a mixture of two drugs can be calculated according to the formula $P = 1 - (1 - P_1)(1 - P_2)$ (Finney, 1971), where P = proportion of organisms expected to respond to the mixture; P_1 and P_2 = percentage of individuals responding to each single toxicant. The expected response in the third model of independent action which assumes negative correlation of tolerances, is $P = P_1 + P_2$ or whichever is least.

However, predicting responses becomes more difficult when the constituents interact with one another. The toxicity of a mixture is then classified as partial-additive, synergistic and antagonistic (Könemann, 1981). Könemann (1981) proposed the use of a mixture toxicity index to evaluate the toxicity of equitoxic mixtures composed of more than two chemicals.

Another model which has been used frequently (Sprague & Ramsay, 1965; Negilski *et al.*, 1981; Broderius & Kahl, 1985) to study the effect of mixtures is the toxic-unit concept. This concept has been developed to predict the toxicity of mixtures by adding up the toxic effects of two or more toxicants acting at the same time. The concentration of each single constituent is now expressed in fractions of their incipient levels, which is the highest concentration giving a zero response and not in conventional chemical units (e.g. a concentration of a toxicant equally strong as the incipient level will be given a value of 1 toxic unit). After summing the toxic units assigned to each single constituent, the total toxic unit ascribed to the mixture can then be used to interpret its overall toxicity (Sprague & Ramsay, 1965). This concept consequently assumes that the components contribute in a similar way to the effect caused by the mixture. This implies that the toxic unit concept can be considered as a redefinition of the simple similar action model (Negilski *et al.*, 1981).

In this study we report on the toxicity of Cu/Hg, Zn/Ni, Zn/Cu and Cu/Ni mixtures to the nematode *Monhystera disjuncta*. Two criteria, mortality and developmental inhibition, were studied to evaluate the effect of the paired metal mixtures. We first examined whether the joint effect of the mixtures acted according to the toxic unit concept or according to two popular non-interactive models proposed by Plackett & Hewlett (1952). Between these two non-interactive models, a continuum of non-interactive models exists, which although not considered here are considered elsewhere (Negilski *et al.*, 1981; Broderius & Kahl, 1985). When none of these models matched the experimental figures interactive models were considered.

MATERIAL AND METHODS

Cultivation techniques and test procedures are completely identical to those described in our previous work (Vranken *et al.*, 1984, 1985). Briefly, the nematode, *Monhystera disjuncta*, is cultured in small petri-dishes filled with 0·5% sterile bacto-agar made up in artificial seawater according to Dietrich & Kalle (1957) with a salinity of 30‰. The agar was enriched with a sterol-mixture (Vanfleteren, 1980) and the bacterium *Alteromonas haloplanktis* is administered as food. The test procedure consists in sampling at random 120 J_2-larvae which were distributed equally among four replicates. After a test-period of 96 h the number of dead juveniles and adults were counted. During the test period some worms left the agar bottoms and dried up. Such mortalities, and mortality caused by transferring the larvae from the stocks to the experimental cultures, were not included in the analysis. The experiments were run at 17°C in the dark. In the present assays no mortalities were observed in the controls.

STATISTICAL ANALYSIS

EC_{50} values for developmental inhibition were calculated by minimum logit Chi-square analysis (Hewlett & Plackett, 1979). Confidence limits around the EC_{50} values were corrected for heterogeneity when indicated by goodness of fit analysis (Table 1). The incipient lethal levels (ILL) and the no effect levels with regard to developmental inhibition (NEL) were obtained by inverse prediction from linearized dose–response relationships by transferring the percentages into angles (arc sin $\sqrt{p}$) (Sokal & Rohlf, 1981). ANCOVA and regression analysis through the origin with appropriate ANOVA (Steel & Torrie, 1960) were used to test the toxic unit (TU) concept. For toxicants with parallel dose–response curves expected mortalities according to the simple similar action model were estimated from the dose–response curves of the single metals by expressing the mixture concentrations into an equivalent concentration of one metal: $Z = Z_1 + pZ_2$ where Z_1 and Z_2 are concentrations of two metals and p is the relative potency ratio (Finney, 1971). Expected mortalities to test the independent dissimilar action were again calculated from the individual dose–response relationships. The tolerances of the nematodes to each single metal were considered to be uncorrelated. Both the independent dissimilar action model and the simple similar action model were tested by Chi-square goodness of fit analysis (Finney, 1971). The percentages of juveniles responding when developmental inhibition was used as the criterion were corrected for control response by Abbot's formula (Finney, 1971). The logits of 0% and 100% were calculated as logit ($\frac{1}{2}$m) and logit (1–$\frac{1}{2}$m) respectively (Hewlett & Plackett, 1979).

RESULTS

Developmental inhibition

EC_{50}s based on development inhibition for each single metal constituent of the mixtures tested are shown in Table 1. Using this criterion, Cu and Hg

TABLE 1

Monhystera disjuncta. Minimum Logit Chi-square Analysis of the Logit of the Percentage Juveniles (l) Against the Logarithm of the Concentration (C):

Metal	t	$f(\pm SE)$	EC_{50} (95% CI)	χ^2 (df)
Hg	−2·93	8·14 (±1·309)	2·3 (1·70–3·09)	12·6** (3)
Cu	−2·17	6·38 (±4·566)[a]	2·2 (0·025–193·160)	6·7** (1)
Zn	−9·34	12·40 (±1·668)	5·7 (5·26–6·09)	1·6 NS (1)
Ni	−7·20	5·51 (±0·507)	20·3 (18·25–22·49)	7·1 NS (3)

[a] Slope in NS ($t_s = 1·573 < 12·706$).

** ($0·001 < P < 0·01$).

$l = t + f \log_{10} C$; $t =$ Intercept; $f =$ slope; $m = EC_{50}$ in mg litre^{-1}; SE = standard error; CI = confidence interval; χ^2 = Chi-square for goodness of fit; NS = not significant.

appeared to be equitoxic. Zn is intermediate toxic and Ni is relatively non-toxic.

Copper mercury mixtures

Mortality. Observed mortalities of *Monhystera disjuncta* exposed to Cu/Hg mixtures were significantly different from expected mortalities based on both independent dissimilar action and simple similar action ($P < 0·001$). The potency ratio (PR) used to express the Hg content of the mixture into an equivalent Cu concentration is estimated as PR = 0·431 (Table 2). The simple similar action model overestimated mortality in all cases (Table 3). At 1 combination (2·5 ppm Hg/1 ppm Cu) the observed mortality was higher when compared with the expected death rate according to the independent dissimilar action (Table 4). The incipient lethal levels (ILL) of Cu and Hg were determined as 0·7 and 1·1 mg litre^{-1}, respectively (Table 5). Percentage mortality (transformed into logits) caused by each single metal and by the mixture is plotted against the logarithms of the toxic units in Fig. 1a. The response curves of each single metal are almost identical (Fig. 1a); hence, a single line can be plotted through the data-points (Fig. 1a). The toxic units of the mixture varied between 2·3 and 5·8. They overestimated in all cases the overall mixture toxicity significantly (Fig. 1a, Table 6). The mixture with a strength of 3·7 TU containing, respectively, 2·5 mg litre^{-1} Hg and mg litre^{-1} Cu is more toxic than the mixture with a strength of 4·5 TU composed out of 1 mg litre^{-1} of the less toxic Hg and 2·5 mg litre^{-1} of the relatively more toxic Cu. As a result the curve depicting mortality against the TU of the mixture shows an indented pattern (Fig. 1a). From Fig. 1a it is obvious that the mixture's ILL is considerably higher than the ILL's of each single metal.

TABLE 2

Monhystera disjuncta. Combined Slopes (b) with Standard Error and Potency Ratios (PR) with 95% Confidence Limits for Cu/Hg, Zn/Ni, Zn/Cu and Cu/Ni 96 h Dose–Mortality Curves, χ^2 (Heterogeneity) Tests the Goodness of Fit, the Second χ^2 and/or the Variance Ratio Test, Examines Whether the Dose–Response Curves are Parallel; NP = Not Parallel

	Cu/Hg	*Zu/Ni*	*Zn/Cu*	*Cu/Ni*
b (±SE)	8·35 (0·842)[a]	7·17 (0·553)	NP	NP
PR (±95% CI)	0·431(0·388–0·497) (0·349–0·533)[a]	0·234 (0·209–0·263)		
χ^2 (heterogeneity)	15·75 (df = 6; P = 0·015)	7·44 (df = 5; P = 0·190)	11·83 (df = 6; P = 0·066)	8·17 (df = 5; NS)
χ^2 or F_s (test for parallelism)	6·24 (df = 1, 6; P = 0·047)	0·39 (df = 1; P = 0·531)	16·60 (df = 1; P < 0·001)	7·07 (df = 1; P = 0·008)

[a] Corrected for heterogeneity.

TABLE 3

Monhystera disjuncta. Concentrations of Bipartite Mixtures of Hg, Cu, Ni and Zn in mg litre^{-1}; Expected Mortalities Based on Simple Similar Action and Observed Mortalities

Mixture concentrations (mg litre^{-1})		*%Mortality expected by simple similar action*	*%Observed mortality*
Hg +	Cu		
1	1	12·38	0
2·5	1	35·31	11·32
1	2·5	65·51	0
2·5	2·5	79·64	2·56
		χ^2: 694·2 ($P < 0{\cdot}001$)	
Ni +	Zn		
5	1	0·05	0
5	5	1·32	0
2·5	1	1·83	0
5	10	7·84	0
2·5	10	20·22	5·10
		χ^2: 26·3 ($P < 0{\cdot}001$)	

Developmental inhibition. For this criterion only the independent dissimilar action model was tested as there exists significant heterogeneity ($P < 0{\cdot}001$) around the common Cu/Hg slope (Table 7). Expected numbers of juveniles lowering their development rate, as based on independent dissimilar action, were significantly different from the observed numbers ($P < 0{\cdot}001$; Table 8). Except for the 2·5 mg litre^{-1} Hg + 1 mg litre^{-1} Cu mixture the model overestimated developmental inhibition. The threshold NEL for developmental inhibition were estimated as 0·65 and 0·7 mg litre^{-1} for, respectively, Hg and Cu. The toxic units of the mixture are higher than 1. However, as the developmental inhibition (percentage individuals remaining juvenile) versus toxic unit plots of both single metals cannot be fitted by a single line (Table 9), the toxicity of the mixture is a function of the proportion of each constituent. Nevertheless, on statistical grounds no arguments exist to reject the toxic unit concept for mixtures possessing a strength of 1 TU (Table 10). As for mortality, the mixture composed proportionally out of more Hg, causes a greater effect on development.

Zinc–nickel mixtures

Mortality. The common slope and PR are given in Table 2. Observed and expected mortalities for both non-interactive models tested are significantly different (Tables 3 and 4). The independent dissimilar action model overestimated slightly the observed mortalities ($0{\cdot}05 > P > 0{\cdot}01$). Differences

TABLE 4

Monhystera disjuncta. Concentrations of Bipartite Mixtures of Hg, Cu, Ni and Zn in mg litre^{-1}; Expected Mortalities for Each Single Metal; Expected Mortalities According to Independent Dissimilar Action; Observed Mortalities and Toxic Units of the Mixture Concentrations

Mixture concentrations (mg litre^{-1})		*%Mortality expected for each single metal*		*%Mortality expected by independent dissimilar action*	*%Observed mortality*	*Toxic units*
Hg + Cu		Hg	Cu			
1	1	0·50	1·13	1·62	0	2·34
2·5	1	7·65	1·13	8·70	11·32	3·70
1	2·5	0·50	55·42	55·64	0	4·48
2·5	2·5	7·65	55·42	58·83	2·56	5·84
				$\chi^2 = 302{\cdot}5$ ($P < 0{\cdot}001$)		
Ni + Zn		Ni	Zn			
5	1	0·004	0·1	0·01	0	0·32
5	10	0·004	6·12	6·13	0	1·82
25	1	0·81	0·1	0·81	0	0·93
25	10	0·81	6·12	6·88	5·10	2·43
5	5	0·004	0·79	0·80	0	0·99
				$\chi^2 = 9{\cdot}0$ ($0{\cdot}05 > P > 0{\cdot}01$)		
Zn + Cu		Zn	Cu			
1	1	0·01	1·13	1·14	0	1·6
5	1	0·79	1·13	1·92	0	2·3
10	1	6·12	1·13	7·19	3·74	3·1
1	2·5	0·01	55·42	55·43	4·85	3·7
10	2·5	6·12	55·42	58·15	25·89	5·2
				$\chi^2 = 160{\cdot}0$ ($P < 0{\cdot}001$)		
Cu + Ni		Cu	Ni			
1	5	1·13	0·004	1·14	0	1·58
1	25	1·13	0·81	1·93	0	2·20
2·5	5	55·42	0·004	55·42	0	3·72
2·5	25	55·42	0·81	55·78	27·08	4·34
				$\chi^2 = 178{\cdot}4$ ($P < 0{\cdot}001$)		

TABLE 5

Monohystera disjuncta. Incipient Lethal Levels (ILL) in mg litre^{-1} with 95% CI and No Effect Levels (NEL) as Measured by Developmental Inhibition of Different Metals as Obtained from Mortality/Dose and Developmental Inhibition/Dose Response Curves

Metal	*Mortality* *ILL in mg litre*$^{-1}$	*Developmental inhibition* *NEL in mg litre*$^{-1}$
Hg	1·1 (95% CI: 0–3·26)	0·65 (95% CI: 0·022–2·042)
Cu	0·7 (95% CI: 0·106–1·745)	0·7 (95% CI: 0·103–1·638)
Zn	6·0 (95% CI: 3·4–9·2)	1·6 (95% CI: 0·294–3·569)
Ni	32·6 (95% CI: 10·9–47·9)	4·0 (95% CI: 0·625–10·64)

between observed and theoretical frequencies based on simple similar action are more pronounced which is expected because the response for similar action is always higher than the independent dissimilar action when the logit slopes ≥ 1·43 for the individual toxicants (Table 2) (Christensen *et al.*, 1985). The ILL of Zn and Ni are 6·0 and 32·6 mg litre^{-1}, respectively. The mortality TU curves for each single metal match and their plots can be fitted by a single curve (Fig. 1b). The mixture TU's ranged between 0·3 and 2·4. In two

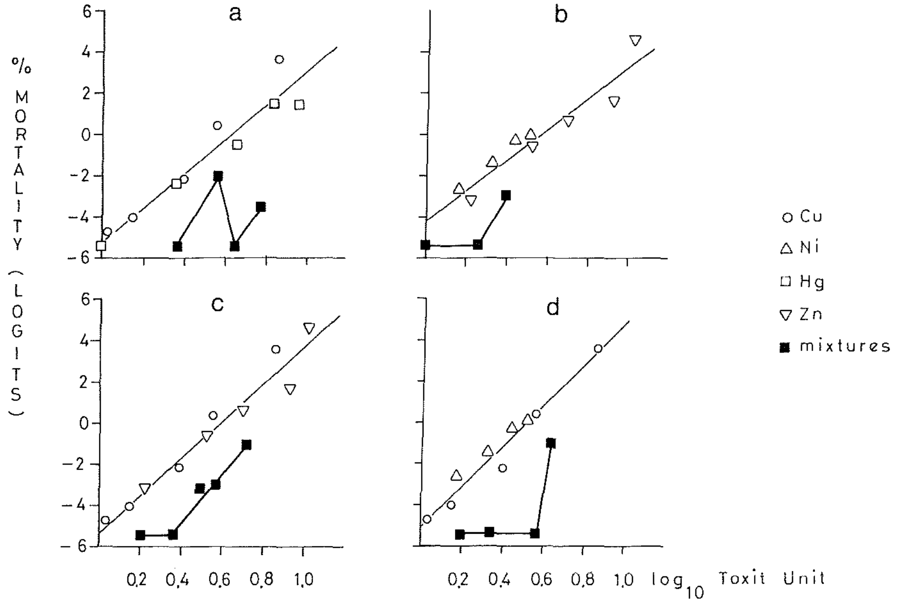

Fig. 1. *Monhystera disjuncta*: percentage mortality of juveniles exposed to Cu, Hg, Zn, Ni and paired mixtures of these metals.

TABLE 6

Monhystera disjuncta. Expected Mortalities Based on the Toxic Unit Concept Compared with Observed Mortalities

Mixture	*Toxic units*	*Expected mortality (%) and 95% CI*	*Observed mortality (%)*
Hg/Cu	2·3	10·7 (5·59–19·57)	0·0
	3·7	37·9 (23·24–55·08)	11·3
	4·5	54·6 (35·94–71·98)	0·0
	5·8	75·5 (55·76–88·22)	2·6
Zn/Ni	0·3	0·04 (0·01–0·29)	0·0
	0·9	1·15 (0·36–3·59)	0·0
	1·0	1·41 (0·47–4·19)	0·0
	1·8	9·7 (4·91–18·09)	0·0
	2·4	21·7 (13·32–33·28)	5·1
Zn/Cu	1·6	3·2 (1·5–6·55)	0·0
	2·3	12·1 (7·03–20·09)	0·0
	3·1	30·9 (20·82–43·29)	3·7
	3·7	47·4 (41·72–53·09)	4·9
	5·2	77·5 (65·52–86·22)	25·9
Cu/Ni	1·6	4·2 (2·38–7·22)	0·0
	2·2	15·7 (10·51–22·78)	0·0
	3·7	65·1 (51·21–76·86)	0·0
	4·3	78·6 (65·43–87·72)	27·1

combinations 5 mg litre^{-1} Ni + 5 mg litre^{-1} Zn and 25 mg litre^{-1} Ni 1 mg litre^{-1} Zn with a TU very close to 1, no mortality was observed. Nevertheless, the TU model overestimated the overall mixture toxicity (Table 6).

Developmental inhibition. The slopes of the curves depicting the numbers not maturing against the concentration of each single metal constituent of this mixture were not parallel. The independent dissimilar action model significantly underestimated the response ($P < 0{\cdot}001$) (Table 8). The threshold NELs for developmental inhibition were determined at 1·6 and 4·0 mg litre^{-1} for Zn and Ni, respectively. It seems that the toxicity of the mixture is more than additive (Fig. 2b). This apparent synergistic effect is not pronounced enough to be statistically significant (ANCOVA; $P = 0{\cdot}087$).

Zinc–copper mixtures

Mortality. Observed mortalities over the entire range tested were significantly less ($P < 0{\cdot}001$) than the expected death rate based on

TABLE 7

Monhystera disjuncta. Combined Slopes (b) with Standard Error and Potency Ratios (PR) with 95% Confidence Limits for Cu/Hg, Zn/Ni, Cu/Zn and Cu/Ni Dose–Developmental Inhibition Curves; χ^2 and F_s as in Table; NP = Not Parallel

	Cu/Hg	*Zn/Ni*	*Cu/Zn*	*Cu/Ni*
b (±SE)	7·88 (1·30)	NP	9·20 (2·33)	5·59 (0·90)
PR (±95% CI)	0·872 (0·549–1·771)		0·330 (0·166–0·657)	0·115 (0·071–0·240)
χ^2 (heterogeneity)	19·31 (df = 4, $P < 0·001$)	8·73 (df = 4; NS)	8·34 (df = 2; $P = 0·015$)	13·79 (df = 4; $P = 0·008$)
χ^2 or F_s (test for parallelism)	0·223 ($f = 1, 4$; NS)	15·63 (df = 1, $P < 0·001$)	1·66 (df = 1,2; NS)	< 1; NS

TABLE 8

Monhystera disjuncta. Concentrations of Bipartite Mixtures of Hg, Cu, Ni, and Zn in mg litre⁻1; Observed % Juveniles for each Single Metal; Expected % Juveniles According to Independent Dissimilar Action; Observed % Juveniles, Corrected for Control Response with the Formulae (*P*–*C*)/(1–*C*) (Hewlett & Plackett, 1979), and Toxic Units

Mixture concentrations (*mg litre*$^{-1}$)		*% Juveniles responding for each single metal*		*% Juveniles expected by independent dissimilar action*	*% Juveniles observed*	*Toxic units*
Hg + Cu		Hg	Cu			
1	1	3·18	10·96	13·79	7·47	3·0
2·5	1	71·30	10·96	74·45	99·49	5·3
1	2·5	3·18	99·31	99·33	87·04	5·1
2·5	2·5	71·30	99·31	99·80	99·08	7·4
				χ^2: 77·8 ($P < 0{\cdot}001$)		
Ni + Zn		Ni	Zn			
5	1	4·42	0	4·42	4·62	1·9
5	10	4·42	97·24	97·36	99·36	7·5
25	1	57·35	0	57·35	99·40	6·9
25	10	57·35	97·24	98·82	99·33	12·5
5	5	4·42	33·28	36·23	96·81	4·4
				χ^2: 190·5 ($P < 0{\cdot}001$)		
Zn + Cu		Zn	Cu			
1	1	0	10·96	10·96	30·43	2·1
5	1	33·28	10·96	40·59	99·53	4·6
10	1	97·24	10·96	97·54	99·48	7·7
1	2·5	0	99·31	99·31	99·46	4·2
10	2·5	97·24	99·31	99·98	99·50	9·8
				χ^2: 67·0 ($P < 0{\cdot}001$)		
Cu + Ni		Cu	Ni			
1	5	10·96	4·42	14·90	53·54	2·7
1	25	10·96	57·35	62·02	99·41	7·7
2·5	5	99·31	4·42	99·34	99·47	4·8
2·5	25	99·31	57·35	99·71	99·37	9·8
				χ^2: 23·8 ($P < 0{\cdot}001$)		

Slopes of Cu and Ni are not significantly different from zero, therefore experimental percentages have been used to determine expected frequencies.

TABLE 9

Monhystera disjuncta. Linear Least Squares Unweighted Regression Analysis of the Logit of the Percentage Juveniles Responding (1) Against the Logarithm of the Toxic Units (TU) of single Metals and Mixtures $1 = a + b \log_{10} \mathrm{TU}$; a = Intercept; b = Slope: r^2 = Coefficient of Determination; F_s = Variance Ratio Testing the Significance of Regression; P = Probability

Metals	*a(SE)*	*b (95% CI)*	r^2	F_s	P
Hg	−4·76 (0·68)	8·47 (2·50)	0·97	116·3	0·002
Cu	−5·44 (3·45)	16·66 (108·70)	0·79	3·8	0·302
Zn	−5·35 (1·93)	10·20 (29·5)	0·95	19·3	0·143
Ni	−4·53 (1·40)	7·41 (5·69)	0·85	17·2	0·025
Cu/Hg	−11·03 (4·87)	19·27 (30·56)	0·80	7·85	0·107
Zn/Ni	−6·40 (1·26)	13·76 (8·03)	0·97	57·9	0·017
Zn/Cu	−6·57 (0·77)	18·48 (17·59)	0·99	178·3	0·048
Cu/Ni	−8·41	19·98			

independently dissimilar action. The dose–mortality curves of Zn and Cu are not parallel. The TU concept significantly overestimated toxicity (Table 6; Fig. 1c) both at a mixture strength equal to 1 TU ($0{\cdot}01 < P < 0{\cdot}05$) and greater than 1 TU (ANCOVA; $P < 0{\cdot}001$). Thus Zn and Cu when together, act in a less than additive manner. The lower toxicity of the mixture when compared with the single metals is clearly shown in Fig. 1c.

TABLE 10

Monhystera disjuncta. F-test Examining Whether the Regression of the Numbers Responding, Corrected for Control Response and Transformed to Arc sin $\sqrt{p}$, Against the Logarithm of the Toxic Units of Metal Mixtures, Passes through the Origin. When the Intercept of this Relationship is Significantly Different from Zero, as Indicated by a High F_s-value, Metal Mixture Concentrations Corresponding with a Toxic Unit = 1 Induce Mortalities Significantly Different From the Expected Zero Response

	Metal mixture			
Criterion	Cu/Hg	Zn/Ni	Zn/Cu	Cu/Ni
Mortality	CR	ND	$F_s = 22{\cdot}3$ (df = 1, 2; $0{\cdot}01 < P < 0{\cdot}05$)	ND
Development	$F_s = 2{\cdot}76$ (df = 1, 2; NS)	$F_s = 0{\cdot}63$ (df = 1, 2; NS)	$F_s = 4{\cdot}09$ (df = 1, 1; NS)	ND

ND: not determinable from the present data set.
CR: curvilinear relationship.
df = degrees of freedom.

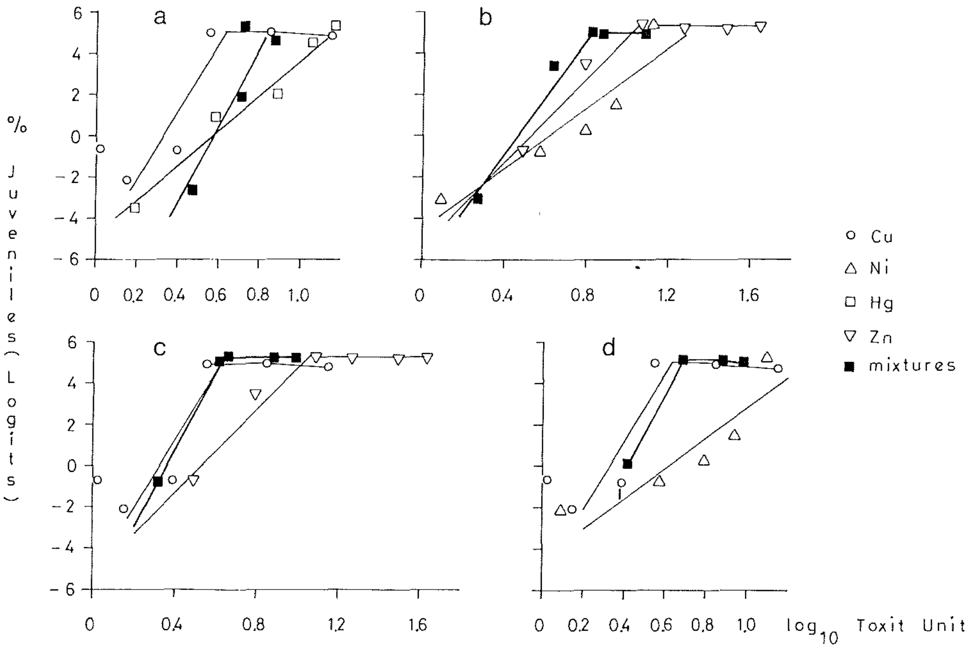

Fig. 2. *Monhystera disjuncta*: percentage worms remaining juvenile after exposure to Cu, Hg, Zn, Ni and paired mixtures of these metals.

Developmental inhibition. The number of worms not maturing were not predictable by independent dissimilar action ($P < 0{\cdot}001$). At two mixture concentrations—1 mg litre^{-1} Zn + 1 mg litre^{-1} Cu and 5 mg litre^{-1} Zn + 1 mg litre^{-1} Cu—the observed numbers remaining juveniles were twice as high as expected. Simple similar action might result in biased predictions as there exists significant heterogeneity around the combined Zn/Cu curves (Table 2). Especially at higher Cu concentrations (Cu < 1·75 mg litre^{-1}) predictions based on the logit-response curve are unreliable. For mixtures containing 1 mg litre^{-1} Cu more or less reliable expected frequencies can be determined by using the simple similar action model. The expected percentage worms remaining juvenile are for the three combinations tested, containing 1 mg litre^{-1} Cu (namely, 1 mg litre^{-1} Zn + 1 mg litre^{-1} Cu, 5 mg litre^{-1} Zn + 1 mg litre^{-1} Cu and 10 mg litre^{-1} Zn + 1 mg litre^{-1} Cu) less than those observed (20% expected versus 30% observed; 63% expected versus 99·5% observed and 87% expected versus 99·5% observed). The response curve of the mixture lies very close to that of Cu, when the metal amount is expressed in TU's. This, together with the fact that simple similar action underestimates developmental inhibition, reveals that the overall mixture toxicity is more than expected from independent dissimilar joint action (Table 8).

Copper–nickel mixtures

Mortality. The independent dissimilar action model overestimated in all mixture combinations tested the observed mortalities ($P < 0{\cdot}001$). In two mixtures containing copper concentrations higher than the 96 h LC_{50}, observed mortality was significantly less ($P < 0{\cdot}01$) when compared with expected mortalities caused by copper alone. The dose–mortality curves of Cu and Ni are not parallel (Table 2). The TU mortality curves of both single metals were fitted conveniently by one single curve (Fig. 1c). The TU overestimated in all combinations the overall toxicity of the mixture (Table 6). Again, the TU mortality curve lies completely under the curve depicting the response of each single metal (Fig. 1c). Therefore, Cu and Ni when together act in a less than additive manner.

Developmental inhibition. Observed percentages not reaching adulthood were significantly different from those predicted by independent dissimilar action ($P < 0{\cdot}001$). At 1 mg litre^{-1} Cu + 5 mg litre^{-1} Ni and 1 mg litre^{-1} Cu + 25 mg litre^{-1} Ni the observed % juveniles was considerably higher than expected. At these combinations the response type of the mixture appears to be more than additive as expected frequencies according to simple similar action were smaller than those observed (29% expected versus 54% observed and 77% expected versus 99% observed). Toxic units ranged between 2·7 and 9·8. As the TU developmental inhibition curve of both single metals cannot be fitted by one single line, the interpretation of the mixture toxicity based on the toxic unit concept is rather difficult.

DISCUSSION

Knowledge of the mode of toxic action of heavy metals to nematodes is very poor. Therefore we do not know which of the non-interactive models proposed by Plackett & Hewlett (1952), simple similar action or independent dissimilar action, is the more relevant. According to several authors (Barnes & Stanburry, 1948; Corner & Sparrow, 1956 and Negilski *et al.*, 1981) toxicants are acting on a similar site when the dose mortality curves of the single metals are parallel. Recently Christensen *et al.* (1985) have shown that parallel dose–response curves are not required for concentration addition (similar systems affected). Nevertheless, according to the present results one might expect, when mortality is considered as a criterion, that Cu and Hg on the one hand and Zn and Ni on the other possess a similar mode of action. For the developmental assay no clear-cut conclusions could be drawn concerning parallelism of the response curves as there exists high variability around the response curve of Cu. However, the design of acute and sublethal toxicity tests only allows one to determine the probability distribution of lethal/sublethal threshold values and to estimate its central tendency (LC_{50} or EC_{50} value) and the measure of dispersion of the variables around the central tendency (standard deviation). Therefore, it is not obvious that the slope of the dose–response curve of a species to a particular chemical yields information concerning the mode of action of the chemical tested. The assumption of parallelism of slopes of the single mixture constituents is only required to obtain unbiased estimates of the expected frequencies according to the simple similar action model. Neither observed mortality responses nor developmental inhibition of juvenile *Monhystera disjuncta* could be predicted on the basis of independent dissimilar or simple similar action. Both models significantly overestimated the mortality response, except for the 2·5 mg litre^{-1} Hg + 1 mg litre^{-1} Cu mixture when the independent dissimilar action model underestimated the observed response of the bipartite metal mixture. The toxic unit mortality response curves of all single metals can be presented by one single line. Consequently, when mortality is considered, the toxic unit concept can also be used to evaluate the overall mixture response. The toxic unit concept overestimated mortality in all paired combinations. As the overall effect of the bipartite mixtures could not be determined from the dose–mortality curves of the single metals, we have concluded that all paired metal mixtures studied act in a less than additive manner. When developmental inhibition is considered, neither of the single metal toxic unit response curves overlap. This implies that the slopes of the individual dose–response curves are different. From the present developmental assays with *Monhystera disjuncta* we conclude that Zn–Ni mixtures act synergistically, that the joint action of three Zn–Cu combinations and two Cu–Ni mixtures, containing 1 mg litre^{-1} Cu, shows a similar effect and that the response type of the Hg–Cu mixture is not very clear. Similarly, as with mortality the mixture containing less copper is the most toxic. The reason why a different response type is observed for the two criteria tested is not known.

At low mixture concentrations the overall toxicity of the mixture is less than additive, which implies that the ILL in terms of toxic units of the mixture is higher when compared with the ILL of the single metals. This result is in agreement with findings reported in previous studies (Lloyd & Orr, 1969; Negilski *et al.*, 1981). Negilski *et al.* (1981) even found that mortalities were unimportant at concentrations of mixture constituents as high as one-third of the 14-day LC_{50} values.

Concerning mortality similar results were obtained by Negilski *et al.* (1981) who showed that the toxicity of Zn–Cu mixtures to the shrimp *Callianassa australiensis* was less toxic than was expected for the independent model. Mixtures of cadmium and copper (not studied in the present assays) revealed a reverse effect and were more toxic than the independent model, but less toxic than expected by simple similar action. Sprague & Ramsay (1965) found that copper–zinc mixtures acted synergistically juvenile Atlantic salmon *Salmo salar*, which is in contrast with our results. Copper–mercury mixtures showed clear synergism, towards the harpacticoid *Nitocra spinipes* (Barnes & Stanburry, 1948), the copepod *Acartia clausi* and the brine shrimp *Artemia salina* (Corner & Sparrow, 1956), which again is in variance with the present results. These and other workers (Peyfinch & Mott, 1948; Hunter, 1949) believed that Hg and Cu acted differently. They thought that the toxic action of Cu basically acts indirectly by interfering with the respiratory enzyme system and/or the osmoregulatory system while they thought that mercury was acting directly by poisoning the protoplasma. Now it is known that mercury might interfere with any enzyme containing an SH group essential for its catalytic activity (Boudou *et al.*, 1983). Further Hunter (1949) reported the same observation as we did, namely, that small amounts of Cu enhanced the toxicity of mercury towards the amphipod *Marinogammarus marinus*, whereas small amounts of mercury did not affect copper toxicity.

More recently, Bræk *et al.* (1976) found that Cu–Zn mixtures acted synergistically towards three common phytoplankton species, whereas the same mixture showed antagonism when added to cultures of *Phaeodactylum tricornutum*. It was proposed that the antagonistic effect was caused by competition between the metals for a common uptake site. This hypothesis was substantiated by the fact that Zn^{2+} toxicity increased significantly when the magnesium content of the medium was reduced, which suggests that divalent metal cations act at a common site in *P. tricornutum*. Further, Christensen *et al.* (1985) reported that the joint action of Ni and Zn on *Selenastrum caprocornutum* is of the concentration additive type. Therefore, without giving a complete literature review concerning the joint action of metals, it is clear that all possible types of action were reported in bioassays in laboratory conditions.

The main problem, however, with this type of experiment is that the biochemical pathway and the mode of action of the chemicals with regard to the test organism is very poorly known. For instance, with nematodes, only one study discusses cadmium metabolism in relation to tissue distribution and accumulation by metal-binding proteins (Howell & Smith, 1985).

At present we only can state, as is generally accepted (Babich & Stotzky, 1983), that antagonistic interactions result from competition between the chemicals for sites on the cell surfaces, whereas synergism is indicative for an increased permeability of the plasma membrane. How this knowledge can be used to explain the difference in response type between the two criteria studied, is not known.

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