Toxicity of Binary Mixtures of Reactive Toxicants

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This study evaluated the toxicity of binary mixtures of reactive toxicants using the Microtox test. Greater than additive effects were quite frequently observed (18%) among chemicals with different mechanisms of toxicity, and some of them were severely synergistic. The concentration-addition model, therefore, may not be appropriate for estimating the multiple toxicity of mixtures containing reactive toxicants. The slope of a chemical's concentration–response curve was found to play an important role in determining the mode of joint actions, which could be related to the complex joint action identified earlier. The results of this study have been summarized into several relationships that can be used to estimate the potential risks for mixtures with unknown toxicity.

INTRODUCTION

The joint effects of organic mixtures have been studied by scientists in the past few decades. Early pioneering works focused on establishing the groundwork for this particular area of interests such as identification of the major modes of joint action and the construction of mathematical models to illustrate and to predict these joint actions. Starting from 1980, with the development of the quantitative structure–activity relationships (QSARs), a considerable number of studies have been given to estimating the toxicity of organic mixtures based on the QSAR relationships. Most of the studies have focused on the combined effects of nonreactive toxicants defined by narcosis QSARs. Little was known about the joint effects of reactive toxicants.

In analyzing the combined effects of organic mixtures, several studies showed that the concentration addition model has successfully described the response of *Daphnia magna* or fish to mixtures of nonreactive chemicals (Konemann, 1981; Hermens et al., 1984a; Hermens et al., 1984b; Hermens et al., 1985; Broderius and Kahl, 1985). Hermens and Leeuwangh (1982) found that even for chemicals with diverse reaction modes, the combined effects were near concentration addition. Both lethal and sublethal types of response were evaluated by the above studies. A more recent work (Nirmalakhandan et al., 1994) has reached a similar conclusion using the respirometric technique. By systematically examining the toxicity of binary mixtures of organic chemicals, a distinct type of joint action (the complex joint action) was identified, which indicated that even for chemicals from the same QSAR group, strong antagonistic effects could be expected if the two chemicals had apparently different concentration–response curves (Chen and Chiou, 1995). The mechanism of such a joint action is not yet clear. The contradistinction of the results obtained by Chen and Chiou, as compared to the previous discussions, is probably due to the difference in the number of chemicals tested each time. Chen and Chiou's tests were based on binary mixtures, while other tests contained 10–50 chemicals at equitoxic ratios. As the number of chemicals increased to n, the effects of antagonistic or synergistic joint actions could be reduced sharply because the concentration of each toxicant was only $1/n$ of its LC_{50} value.

Through the study of QSARs, many organic compounds were screened out for their excess toxicity, which could not be fitted into the narcosis QSAR.

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TABLE I. Toxicity data[a]

Classifications	Chemical	n	EC_{50} (mg/L)	SD (mg/L)	CV (%)	EC_{50_m} (mmol/L)	$-\log EC_{50_m}$	Slope
Electrophilic nonelectrolytes	(a) Michael-type addition							
	Acrolein	5	0.14	0.036	25.70	0.00250	2.60	1.57
	Acrylamide	7	9,910	560	5.65	140	−2.14	2.07
	Methyl vinyl ketone	6	1.06	0.16	15.09	0.0151	1.82	1.55
	(b) Schiff base formation							
	Formaldehyde	5	6.46	0.96	14.86	0.215	0.67	1.69
	Acetaldehyde	9	340	74	21.76	7.72	−1.95	1.76
	Propionaldehyde	7	228	35	15.35	3.92	−0.59	1.79
	Butyraldehyde	5	150	33	22	2.07	−0.40	1.15
	Glutardialdehyde	8	3.95	1.05	26.58	0.0394	1.40	1.51
Proelectrophilic nonelectrolytes	Allyl alcohol	4	739	100	13.53	12.7	−1.10	1.70
	Propargyl alcohol	4	2,070	680	32.85	36.9	−1.57	1.78
	2-Butyn-1,4-diol	5	772	89	11.53	8.97	−0.95	1.66
	3-Butyn-1-ol	4	3,590	710	19.78	51.3	−1.71	2.08
Cyanogenic nonelectrolytes	Acetonitrile	4	15,900	980	6.16	388	−2.59	4.16
	Malononitrile	7	221	46	20.81	33.34	−0.52	1.55
Multiple mechanisms	*p*-Nitroso-*n*,*n*-dimethyl-aniline	4	0.096	0.012	12.5	0.00064	3.19	2.15
	Ethylene chlorohydrin	6	11,800	1,400	11.86	146	−2.16	2.94

[a] n: number of data; SD: standard deviation of EC_{50}; CV: percent coefficient of variation of EC_{50}; EC_{50_m}: EC_{50} in mmol/L.

These outlier chemicals exerted their toxicity through mechanisms other than narcosis reaction, and were called reactive toxicants. Lipnick (1991) has provided a clear classification for these chemicals, based on the molecular mechanisms of toxicity. In our study, joint effects of chemicals from the same mechanistic group or different groups were evaluated according to Lipnick's classification. The objective of our work was to establish basic criteria to estimate the potential risks for organic mixtures containing reactive toxicants.

EXPERIMENTAL METHODS

Sixteen organic compounds, as listed in Table I, were selected according to the classification by Lipnick (1991). These chemicals were mainly classified into four groups—i.e., electrophilic nonelectrolytes, proelectrophilic nonelectrolytes, cyanogenic nonelectrolytes, and multiple mechanisms. The electrophilic nonelectrolytes could be divided further into two groups (Michael-type addition and Schiff base formation) according to their chemical mechanisms. All chemicals used were of reagent grade.

Microtox testing was performed using a Microtox model M500 analyzer and standard procedures recommended by the Microbics Corporation. Fifteen-minute test results were analyzed using the probit model (Finney, 1971) to obtain EC_{50} values and the dose–response relationships. The type of joint action for a specific binary mixture of toxicants was determined by the sum of toxic unit (M):

$$M = \frac{z_1}{Z_1} + \frac{z_2}{Z_2} \tag{1}$$

where z_i is the toxicant concentration and Z_i is the EC_{50} value. The combination of z_1 and z_2 resulted in exactly 50% response. Simple addition is characterized by $M = 1$. $M > 1$ represents antagonism and $M < 1$ indicates synergism. By testing the toxicity of a organic mixture, M and its 95% confidence interval (CI) at 50% response could be determined. Mixtures that resulted in 95% CI for M that overlapped 1 were judged to be additive; 95% CI that did not overlap 1 were either antagonistic or synergistic in toxicity.

RESULTS AND DISCUSSION

The individual toxicity for the 16 reactive toxicants are given in Table I, in terms of their EC_{50} values. Acrolein, methyl vinyl ketone, formaldehyde, glutardialdehyde, and *p*-nitroso-*n*,*n*-dimethylaniline were found to be the most toxic among the 16 chemicals. Variations of EC_{50}

TABLE II. Effects of mixtures of toxicants from the same mechanistic group[a]

Toxicant	Toxicant	M	95%	CL	Effect	S_1/S_2[b]
Acrolein	Methyl vinyl	1.28	1.23	1.34	A	1.01
Acrolein	Acrylamide	1.23	1.02	1.53	A	1.32
Methyl vinyl ketone	Acrylamide	1.19	1	1.44	A	1.34
Butyraldehyde	Glutardialdehyde	0.9	0.87	0.94	S	1.31
Propionaldehyde	Acetaldehyde	1.14	1	1.3	+	1.02
Glutardialdehyde	Acetaldehyde	0.92	0.74	1.16	+	1.17
Glutardialdehyde	Propionaldehyde	0.98	0.87	1.1	+	1.19
2-Butyn-1,4-diol	Allyl alcohol	1	0.97	1.02	+	1.02
Formaldehyde	Acetaldehyde	1.22	1.11	1.35	A	1.04
Formaldehyde	Propionaldehyde	1.44	1.27	1.62	A	1.06
Butyraldehyde	Formaldehyde	1.25	1.09	1.42	A	1.47
Glutardialdehyde	Formaldehyde	1.25	1.1	1.42	A	1.12
Butyraldehyde	Acetaldehyde	1.15	1.02	1.29	A	1.53
Propargyl alcohol	Allyl alcohol	1.31	1.21	1.42	A	1.05
2-Butyn-1,4-diol	Propargyl alcohol	1.2	1.13	1.29	A	1.07
Allyl alcohol	3-Butyn-1-ol	0.83	0.71	1	+	1.22
Propargyl alcohol	3-Butyn-1-ol	1.04	0.92	1.2	+	1.16
2-Butyn-1,4-diol	3-Butyn-1-ol	1.03	0.79	1.41	+	1.25
Malononitrile	Acetonitrile	1.84	1.8	1.89	A	2.00
p-Nitroso-*n*,*n*-dimethylaniline	Ethylene chlorohydrin	1.54	1.44	1.64	A	1.37

[a] A: antagonistic; +: additive; S: synergistic. 95% CL: 95% confidence level of M.
[b] S_1, S_2: are the slopes of the two toxicants acting jointly, $S_i > S_2$.

were about 10–20%, which were slightly higher than the case of nonreactive toxicants (Chen and Chiou, 1995). Generally speaking, it can be seen that toxicity is inversely related to the magnitude of slope. The only exception in this study was *p*-nitroso-*n*,*n*-dimethylaniline, which even though it had a steep slope, was nevertheless very toxic. The same relationship could be found from the toxicity data of nonreactive toxicants (Chen and Chiou, 1995).

Joint Actions Between Chemicals from the Same Mechanistic Group

The combined effects of mixtures of chemicals from the same mechanistic group are shown in Table II. Among the 20 cases of joint toxicity, 12 (60%) yielded antagonism and 7 showed additive effects (35%). Only the butyraldehyde–glutardialdehyde pair, with the sum of toxic units equal to 0.9, was slightly synergistic. For mixtures demonstrating additivity, their slope ratios (s_1/s_2, where s_i is the slope of the dose–response curve of the test chemical) were close to 1, which indicated parallel dose–response curves. When the slope ratio was greater than 1.3, the less than additive effect was the most likely result to be expected, with the exception of the butyraldehyde–glutardialdehyde case. The case of malononitrile and acetonitrile, with the slope ratio increased to 2, showed strong antagonistic effects, suggesting a complex joint action was predominant. We should also take note that there were 5 cases of antagonism with a slope ratio that was less than 1.3. It means that toxicants of the same group with parallel concentration–response curves may not necessarily be additive.

For reactive toxicants acting via the same toxicity mechanism, we may conclude that either simple addition or antagonism will be the possible mode of joint actions. It is not likely to encounter significant synergistic phenomenon in this case. The butyraldehyde–glutardialdehyde case should be more practically considered as "near concentration addition." Those mixtures that demonstrate additivity must have parallel dose–response curves. However, parallel dose–response curves do not necessarily guarantee strict additivity. It seems that as the slopes of the two chemicals became more apparently different, antagonistic effects would gradually show up, and eventually, took the predominant role of the joint action.

Joint Actions Between Chemicals from Different Mechanistic Groups

Table III lists 67 cases of combined effects of reactive toxicants having different mechanisms. This table was

TABLE III. Effects of reactive toxicants from different mechanistic group[a]

	Toxicant	Toxicant	*M*	95%	CL	Effect	S_1/S_2[b]
Part 1	Acrolein	Formaldehyde	0.61	0.55	0.68	S	1.08
	Acrolein	Propargyl alcohol	0.84	0.76	0.93	S	1.13
	Formaldehyde	Propargyl alcohol	0.9	1.01	0.8	S	1.05
	Malononitrile	Acrolein	0.35	0.17	0.55	S	1.01
	Malononitrile	Methyl vinyl ketone	0.67	0.59	0.76	S	1.00
	Malononitrile	Formaldehyde	0.03	0.02	0.05	S	1.09
	Malononitrile	Acetaldehyde	0.13	0.13	0.14	S	1.14
	Malononitrile	Butyraldehyde	0.37	0.34	0.4	S	1.35
	Malononitrile	Glutardialdehyde	0.68	0.58	0.81	S	1.03
	Glutardialdehyde	Acrolein	1.03	0.92	1.15	+	1.04
	Formaldehyde	Methyl vinyl ketone	1	0.96	1.04	+	1.09
	Glutardialdehyde	Methyl vinyl ketone	0.98	0.81	1.19	+	1.03
	Acrolein	Allyl alcohol	1.13	0.96	1.13	+	1.08
	Methyl vinyl ketone	Allyl alcohol	1.17	0.88	1.63	+	1.10
	Formaldehyde	Allyl alcohol	1.13	0.98	1.3	+	1.01
	Allyl alcohol	Acetaldehyde	1.08	1	1.18	+	1.04
	Propionaldehyde	Propargyl alcohol	1.16	1	1.35	+	1.01
	2-Butyn-1,4-diol	Propionaldehyde	1.42	0.97	1.87	+	1.08
	Glutardialdehyde	Allyl alcohol	1.08	0.88	1.32	+	1.13
	Acrolein	Acetaldehyde	1.22	1.14	1.31	A	1.12
	Acrolein	Propionaldehyde	1.6	1.43	1.79	A	1.14
	Butyraldehyde	Acrolein	1.35	1.17	1.55	A	1.37
	Methyl vinyl ketone	Acetaldehyde	1.12	1.06	1.18	A	1.14
	Methyl vinyl ketone	Propionaldehyde	1.27	1.12	1.44	A	1.15
	Butyraldehyde	Methyl vinyl ketone	1.21	1.04	1.4	A	1.35
	Acrolein	2-Butyn-1,4-diol	1.16	1.14	1.17	A	1.08
	Methyl vinyl ketone	Propargyl alcohol	1.29	1.25	1.34	A	1.15
	Methyl vinyl ketone	2-Butyn-1,4-diol	1.23	1.12	1.35	A	1.07
	Propargyl alcohol	Acetaldehyde	1.39	1.08	1.86	A	1.01
	2-Butyn-1,4-diol	Acetaldehyde	1.68	1.37	2.08	A	1.06
	Propionaldehyde	Allyl alcohol	1.23	1.08	1.41	A	1.05
Part 2	Formaldehyde	Acrylamide	0.67	0.64	0.71	S	1.22
	Glutardialdehyde	*p*-Nitroso-*n*,*n*-dimethylaniline	0.66	0.62	0.7	S	1.42
	Malononitrile	*p*-Nitroso-*n*,*n*-dimethylaniline	0.76	0.7	0.83	S	1.39
	Acetaldehyde	Acrylamide	1.13	0.82	1.69	+	1.18
	Propionaldehyde	Acrylamide	1.18	0.84	1.8	+	1.16
	Butyraldehyde	Acrylamide	1.01	0.81	1.28	+	1.8
	Allyl alcohol	Acrylamide	1.15	0.96	1.41	+	1.22
	2-Butyn-1,4-diol	Acrylamide	1.16	0.99	1.37	+	1.25
	Acrolein	*p*-Nitroso-*n*,*n*-dimethylaniline	0.85	0.73	1	+	1.37
	Formaldehyde	3-Butyn-1-ol	0.79	0.63	1.02	+	1.23
	Acetaldehyde	3-Butyn-1-ol	0.91	0.91	1.23	+	1.18
	Formaldehyde	*p*-Nitroso-*n*,*n*-dimethylaniline	0.99	0.92	1.06	+	1.27
	Acetaldehyde	Ethylene chlorohydrin	0.98	0.89	1.07	+	1.67
	Butyraldehyde	Ethylene chlorohydrin	1.06	0.8	1.52	+	2.56
	Glutardialdehyde	Ethylene chlorohydrin	1.11	0.95	1.31	+	1.95

TABLE III. (*Continued*)

Toxicant	Toxicant	M	95%	CL	Effect	S_1/S_2[b]
Malononitrile	Ethylene chlorohydrin	1.12	0.91	1.4	+	1.90
Malononitrile	3-Butyn-1-ol	0.94	0.78	1.14	+	1.34
Acrylamide	*p*-Nitroso-*n*,*n*-dimethylaniline	1.06	0.92	1.23	+	1.04
Acrylamide	Ethylene chlorohydrin	1.07	0.97	1.18	+	1.42
3-Butyn-1-ol	Ethylene chlorohydrin	1.05	0.89	1.24	+	1.41
Glutardialdehyde	Acrylamide	1.2	1.04	1.39	A	1.37
Propargyl alcohol	Acrylamide	1.16	1.05	1.3	A	1.16
Acrolein	Acetonitrile	1.63	1.58	1.69	A	2.65
Malononitrile	Acrylamide	1.33	1.03	2.87	A	1.34
Acrolein	Ethylene chlorohydrin	1.23	1.09	1.38	A	1.87
Methyl vinyl ketone	Ethylene chlorohydrin	1.48	1.01	2.3	A	1.90
Formaldehyde	Acetonitrile	1.72	1.66	1.79	A	2.46
Formaldehyde	Ethylene chlorohydrin	1.3	1.13	1.52	A	1.74
Acetaldehyde	Acetonitrile	1.62	1.60	1.65	A	2.36
Acetaldehyde	*p*-Nitroso-*n*,*n*-dimethylaniline	1.22	1.1	1.35	A	1.22
Butyraldehyde	Acetonitrile	1.59	1.44	1.76	A	3.62
Butyraldehyde	*p*-Nitroso-*n*,*n*-dimethylaniline	1.76	1.59	1.91	A	1.87
Glutardialdehyde	Acetonitrile	1.55	1.37	1.77	A	2.75
Acrylamide	Acetonitrile	1.43	1.34	1.52	A	2.01
Actonitrile	*p*-Nitroso-*n*,*n*-dimethylaniline	1.66	1.61	1.71	A	1.93
Acetonitrile	Ethylene chlorohydrin	1.29	1.25	1.32	A	1.41

[a] A: antagonistic; +: additive; S: synergistic; 95% CL: 95% confidence level of M.
[b] S_1, S_2 are the slopes of the two toxicants acting jointly, $S_1 > S_2$.

divided into two parts according to the magnitude of toxicant's slope. Part 1 lists those mixtures of chemicals with smaller slopes (less than 2.0), and Part 2 lists mixtures associated with at least one steep slope chemical (slope > 2.0). The numbers of tests showing synergism, simple addition, or antagonism mode of action are 12 (18%), 27 (40%), and 28 (42%), respectively. No obvious relationship can be found between M values and slope ratios. However, the effects of slope emerge from the summary statistics of Table III. Table IV summarizes the numbers of different modes of joint actions for chemicals of different mechanisms. In Part 1, 29% of the tests show synergistic effects. With the participation of steep-slope chemicals, this figure decreases to only 8% in Part 2. Moreover, in Table III, most of the antagonistic effects in Part 1 were quite moderate while those in Part 2 were much stronger. For practical purposes, we define the condition of $M > 1.4$ as significantly antagonistic, and $M < 1.4$ as moderately antagonistic. In Table III, there are 12 cases that are significantly antagonistic (as listed in Table V), and 75% of them are associated with slope ratios greater than 1.87. The slope ratios in Part 1 generally lie between 1 to 1.15, while Part 2 has much greater ratios. As the complex joint action, which will cause strong less than additive effects, may occur between two chemicals having nonparallel dose–response curves, it is quite reasonable that stronger antagonistic effects and less synergistic phenomena have been observed in Part 2. Similar conclusion, as to be discussed below, could be drawn from our previous work (Chen and Chiou, 1995). It was found that the slopes of nonreactive toxicants fell into two distinct categories: those between 1.14 and 1.79 and those within 2.89 to 3.73. The joint actions between these two groups of chemicals, with an average slope ratio equal to 2.29, were

TABLE IV. Summary of the combined effects shown in Table III

	Overall		Part 1		Part 2	
	No. of cases	%	No. of cases	%	No. of cases	%
Synergism	12	18	9	29	3	8
Addition	27	40	10	32	17	47
Antagonism	28	42	12	39	16	45
Total	67	100	31	100	36	100

TABLE V. The relationship between antagonism and slope ratios

Toxicant	Toxicant	M	S_1/S_2
2-Butyn-1,4-diol	Propionaldehyde	1.42	1.08
Acrolein	Propionaldehyde	1.6	1.14
2-Butyn-1,4-diol	Acetaldehyde	1.68	1.06
Acrolein	Acetonitrile	1.63	2.65
Methyl vinyl ketone	Ethylene chlorohydrin	1.48	1.90
Formaldehyde	Acetonitrile	1.72	2.46
Acetaldehyde	Acetonitrile	1.62	2.36
Butyraldehyde	Acetonitrile	1.59	3.62
Butyraldehyde	*p*-Nitroso-*n*,*n*-dimethylaniline	1.76	1.87
Glutardialdehyde	Acetonitrile	1.55	2.75
Acrylamide	Acetonitrile	1.43	2.01
Acetonitrile	*p*-Nitroso-*n*,*n*-dimethylaniline	1.66	1.93

significantly antagonistic. Also, in a total of 79 binary mixtures, there were 5 synergistic joint actions observed, but none of them was associated with steep slope chemicals. It shows that the complex joint action has played an important role in determining the mode of mixture toxicity for both reactive and nonreactive toxicants. It is also possible that the slope of a dose–response curve might be related to certain mechanisms of toxicity.

Among the 12 synergistic actions listed in Table III, 7 of them are related to malononitrile. It has been well documented that malononitrile is very chemically reactive and thus can react with many organic compounds under specific conditions. Quite often, several products will be formed when it reacts with one particular compound (Freeman, 1969). It is very likely that the strong synergistic effects related to malononitrile was due to the formation of certain toxic compounds through biologically mediated interactions (under the Microtox test environment) between the two test chemicals. The reaction product (or products), which was probably linked with one or several cyanide groups, must be very reactive in attacking microbial cells and was much more toxic than malononitrile itself. It is also possible that the excess toxicity was simply due to the fact that more cyanide ions were available to microorganisms, as a result of certain chemical reactions. Table VI lists all the joint actions related to malononitrile. It can be seen that several mixtures containing malononitrile yielded strong greater than additive effects. Also, for aldehydes, the length of the carbon chain seems to be inversely proportional to the synergistic effects. Formaldehyde, acetaldehyde, and acrolein are extremely dangerous when they react with malononitrile. Several isobolograms of the above joint actions are shown in Fig. 1. The diagrams were constructed by examining five different ratios of the two test chemicals. The sum of toxic units giving 50% response and its 95% confidence intervals were indicated for each specific ratio. The joint action between malononitrile and formaldehyde, with the mixture toxicity increased 33 times compared to their individual toxicity ($M = 0.03$), cannot be plotted because most experimental points converged at a location near the origin. As referred to in Table VI, the first three isobolograms that showed synergistic effects had a slope ratio ranging from 1.14 to 1.35, while the slope ratios for the other three cases indicating addition or antagonism were within 1.34–1.90. One may also find that the slope of the dose–response curve for acetaldehyde, butyraldehyde, and methyl vinyl ketone is between 1.15 and 1.76. On the other hand, 3-butyn-1-ol, ethylene chlorohydrin, and acrylamide all have a slope greater than 2.0. As indicated by Table VI and Fig. 1, malononitrile yielded apparent greater than additive effects when reacted with other chemicals associated with a small slope. However, the reactions with steep slope chemicals were mainly additive or antagonistic. The joint effect of malononitrile and *p*-nitroso-*n*,*n*-dimethylaniline, although was still synergistic, is much milder compared with other synergistic effects in Table VI. This suggests that there were at least two factors governing the behavior of chemical's joint action: one is the chemical interactions between the two test compounds, and the other is the complex joint action, which is due to the difference in slopes. For malononitrile, the result of chemical interactions could be the formation of some very toxic products, and thus is synergistic. On the other hand, complex joint action that is antagonistic would still exist be-

TABLE VI. Joint effects of malononitrile with another reactive toxicant

Toxicant	Slope	M	Effect	S_1/S_2
Acrolein	1.57	0.35	S	1.01
Methyl vinyl ketone	1.55	0.67	S	1.00
Formaldehyde	1.69	0.03	S	1.09
Acetaldehyde	1.76	0.13	S	1.14
Butyraldehyde	1.15	0.37	S	1.35
Glutardialdehyde	1.51	0.68	S	1.03
p-Nitroso-*n*,*n*-dimethylaniline	2.15	0.76	S	1.39
Ethylene chlorohydrin	2.94	1.12	+	1.90
3-Butyn-1-ol	2.08	0.94	+	1.34
Acrylamide	2.07	1.33	A	1.34

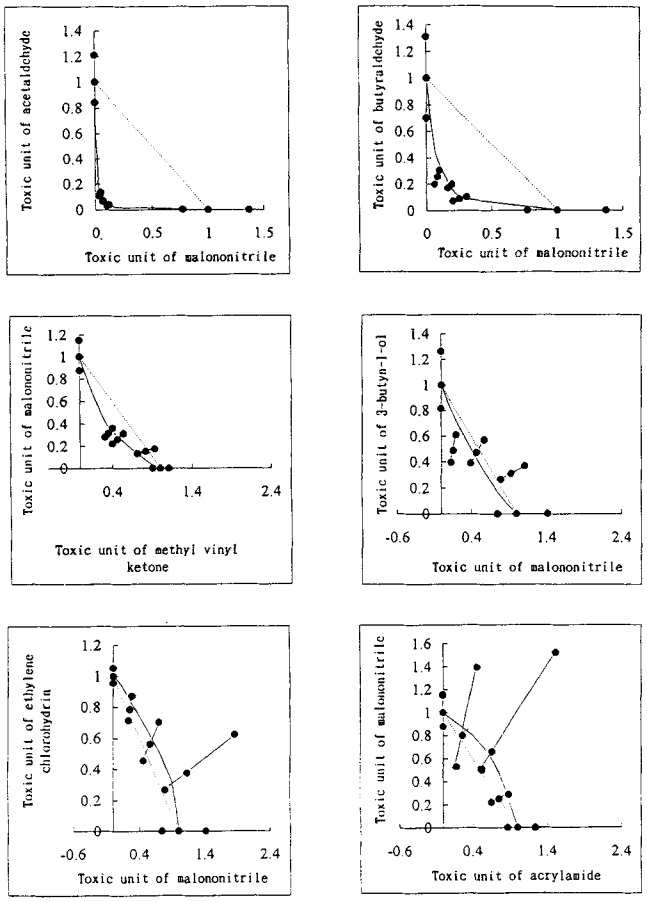

Fig. 1. Isobolograms for organic mixtures containing malononitrile.

tween the remaining portion of the two toxicants. The joint action mode or the net effect, therefore, is dependent on which factor is more significant. For malononitrile, greater than additive effects were predominant when it reacted with other toxicants with small slopes. For those cases related to steep slope chemicals, complex joint action was equally important as chemical interactions, and thus effectively reduced the synergistic phenomenon.

The aforementioned relationship between the slope ratio and the reaction mode can be observed not only from cases related to malononitrile. Formaldehyde, which was also associated with many synergistic phenomena in our study, has exhibited similar properties. Therefore, the above theory regarding mixture's net effect is valid for both cyanogenic chemicals and other reactive toxicants.

According to the above discussion, we may conclude that there are three major factors that determine the type of joint action of organic mixtures: mechanisms, chemical interaction, and complex joint action. Among them, chemical interaction is associated with great uncertainties and is most unpredictable. First, chemical reactivity (under the Microtox test environment or natural aquatic environment) between two organic compounds cannot be defined by a general rule that is applicable to all reactive toxicants. It is therefore difficult to predict whether or not a chemical interaction may occur. Second, if chemical interactions do occur, what are the equilibrium state and the % yield of the products? Third, how many end products will be formed through chemical interactions and, also, how toxic these products are? (The product can be either more toxic or less toxic than its parent compounds, which means the effect can be synergistic or antagonistic.) These uncertainties have made the prediction of mixture toxicity extremely difficult. They also provided a masking effect over the expression of complex joint action, so that the relationship between the slope ratio and the mode of joint action became less apparent. However, it should be pointed out that several phenomena observed from this study supported the existence of complex joint actions. For example, most of the significantly antagonistic effects were related to mixtures with a high slope ratio. Also, the statistics in Table IV showed that the proportion of cases showing synergism that could be related to steep slope chemicals was significantly lower than the proportion of those related to chemicals of small slopes. Furthermore, the joint actions between malononitrile and a toxicant with a steep slope were mainly additive while its effects with other toxicants of a small slope were severely synergistic. These phenomena reveal the existence of complex joint actions, and they should not be considered as a coincidence. It indicates that the slope of a toxicant's concentration–response curve is a crucial parameter that has a profound influence on multiple toxicity.

CONCLUSIONS

The results of this study can be summarized as follows:

1. For reactive toxicants of the same mechanism, their joint actions were either additive or less than additive. Additivity requires that the dose–response curves of the two toxicants be parallel. No significant synergistic phenomenon occurred in this category of joint actions.
2. Twenty-nine percent of the cases for toxicants with small slopes and different mechanisms showed greater than additive effects. Some of the effects were severely synergistic. However, if the mixture contained at least one chemical that was associated with a steep slope, the percentage of synergism decreased to only 8.

3. Malononitrile, when it reacted with another toxicant of a small slope, showed strong synergistic effects. The synergistic phenomenon was significantly reduced with the participation of a steep-slope chemical, and thus the joint action modes were mainly additive or antagonistic.
4. Mixtures with a high slope ratio are most likely to be significantly antagonistic. In fact, no synergism was found from mixtures with slope ratio greater than 1.5.
5. Considering the numbers of synergistic cases related to each specific compound, malononitrile and formaldehyde have much higher tendencies to yield synergistic effects compared to others. Consequently, organic mixtures containing these two chemicals are associated with greater risks.

The above relationships can be applied to assess the potential risks for an organic mixture with unknown toxicity. For example, we may conclude that mixtures of small slope chemicals with different mechanisms of toxicity could be quite dangerous because there is almost one third of chance to yield synergistic joint actions. The risk will be even greater if the above mixtures contain malononitrile or formaldehyde. Also, as the percentage of cases of synergism is not so small as can be neglected (18%), the concentration-addition mode may not be appropriate for estimating the joint effects of mixtures containing reactive toxicants. This is especially true when the components of a mixture are not at equitoxic ratios. Lastly, our results suggest that the slope of chemical's dose–response curve could be a crucial parameter on multiple toxicity and should not be ignored.

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REFERENCES

Broderius, S. J., and M. Kakl. 1985. Acute toxicity of organic chemical mixtures to the fathead minnow. Aquat. Toxicol. **6**:307–322.

Chen, C. Y., and Y. S. Chiou. 1995. Toxicity of binary mixtures of organic chemicals. Environ. Toxicol. Water Quality **10**:97–106.

Finney, D. J. 1971. Probit Analysis (3rd edition). Cambridge: Cambridge University Press.

Freeman, F. 1969. The chemistry of malononitrile. Chem. Rev. **69**:591–624.

Hermens, J., and P. Leeuwangh. 1982. Joint toxicity of mixtures of 8 and 24 chemicals to the guppy (*Poecilia reticulata*). Ecotoxicol. Environ. Safety **6**:302–310.

Hermens, J., H. Canton, P. Janssen, and R. De Jong. 1984a. Quantitative structure-activity relationships and toxicity studies of mixtures of chemicals with anaesthetic potency: Acute lethal and sublethal toxicity to *Daphnia magna*. Aquat. Toxicol. **5**:143–154.

Hermens, J., H. Canton, N. Steyger, and R. Wegman. 1984b. Joint effects of a mixture of 14 chemicals on mortality and inhibition of reproduction of *Daphnia magna*. Aquat. Toxicol. **5**:315–322.

Hermens, J., E. Broekhuyzen, H. Canton, and R. Wegman. 1985. Quantitative structure-activity relationships and mixture toxicity studies of alcohols and chlorohydrocarbons: Effects on growth of *Daphnia magna*. Aquat. Toxicol. **6**:209–217.

Konemann, H. 1981. Quantitative structure-toxicity relationships in fish toxicity studies. Part I: Relationship for 50 industrial pollutants. Toxicology **19**:209–221.

Lipnick, R. L. 1991. Outliers: Their origin and use in the classification of molecular mechanisms of toxicity. Sci. Total Environ. **109/110**:131–153.

Nirmalakhandan, N., V. Arulgnanendran, M. Mohsin, B. Sun, and F. Cadena. 1994. Toxicity of mixtures of organic chemicals to microorganisms. Water Res. **28**:543–551.