INVESTIGATIONS IN FISH CONTROL

67. Method for Assessment of Toxicity or Efficacy of Mixtures of Chemicals

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by

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ABSTRACT

The individual toxic contributions of poisons were summed, and the additive toxicity was defined by a linear index for two chemicals in combination. This index expresses the toxicity quantitatively: zero indicates additive toxicity, negative values indicate less than additive toxicity, and positive values indicate greater than additive toxicity. We selected examples from the literature and conducted tests in the laboratory to assess the additive toxicity of selected chemical mixtures to fish. The values ranged from -1.37 for zinc and cyanide to 7.20 for malathion and Delnav (R) . The method quantifies additive toxicity or efficacy, and assists in evaluating the advantages as well as environmental hazards resulting from chemical mixtures.

INTRODUCTION

The effect of mixtures of two or more chemicals is commonly referred to as additive, synergistic, or antagonistic depending on the relation of the toxicity of the mixtures to that of the individual components. Because these terms are abiguous and nonquantitative (Fingl and Woodbury 1965), a better system of terminology and quantification is needed.

British researchers have investigated methods of predicting the toxic effects of chemical mixtures in water by adding up "toxic units" of individual toxic materials (Lloyd 1961; Herbert and Shurben 1964; Herbert and Vandyke 1964; Brown et al. 1968; Brown et al. 1969; and Brown and Dalton 1970). Brown (1968) stated his reservations about the use of this technique and cautioned that actual bioassays of polluted water are to be preferred. Sprague and Ramsey (1965) used the "toxic unit" method to predict the toxicity of copper and zinc mixtures to Atlantic salmon *(Salmo salar).*

Other techniques for evaluating the toxicity of mixtures of chemicals to mammals have been advanced (Keplinger and Deichmann 1967; Smyth et al. 1969); most of which follow the mathematical model for additive joint toxicity that yields the harmonic mean of the LD50's for the components (Finney 1952). This model tests the hypothesis that the toxicity of chemical mixtures is simply additive. Smyth et al. (1969) normalized the values obtained from Finney's equation with a frequency distribution curve and adjusted the values to indicate additive toxicity with zero. Smyth et al. (1970) derived values in terms of adjusted ratios for mixtures of industrial organic chemicals fed to rats.

The objective of this paper is to adapt current methods and terminology to quantitatively describe additive toxicity of chemicals in water and to assign significance to the additive toxicity index.

MATERIALS AND METHODS

For toxicity tests, hatchery-reared rainbow trout *(Salmo gairdneri)* and bluegill *(Lepomis macrochirus)* were maintained as described by Hunn et al. (1968) and acclimated to test waters. The toxicities of individual chemicals to fish were determined according to standard laboratory procedures (Lennon and Walker 1964; Marking 1969a). The toxicities of chemicals in mixtures were determined similarly except that two chemicals were added in a fixed ratio to the test vessels. Toxicity was defined by the LC50's (concentrations producing 50% mortality) and their 95% confidence intervals (Litchfield and Wilcoxon 1949).

The additive indices of mixtures of chemicals were derived as follows. The effective contributions of each chemical (A and B) in a mixture are represented by the formula: $\frac{A_m}{A_i}+\frac{B_m}{B_i}=S$, where A and B are chemicals, i and m are the toxicities (LC50's) of the individual chemicals and the mixtures, respectively, and S is the sum of the biological activity. If the sum of toxicity of the chemicals is simply additive, S = 1.0; sums that are less than 1.0 indicate greater than additive toxicity, and sums greater than 1.0 indicate less than additive toxicity (Fig. 1). This sum alone could function as a quantitative indication of additive toxicity, except that values greater than 1.0 are not linear with values less than 1.0.

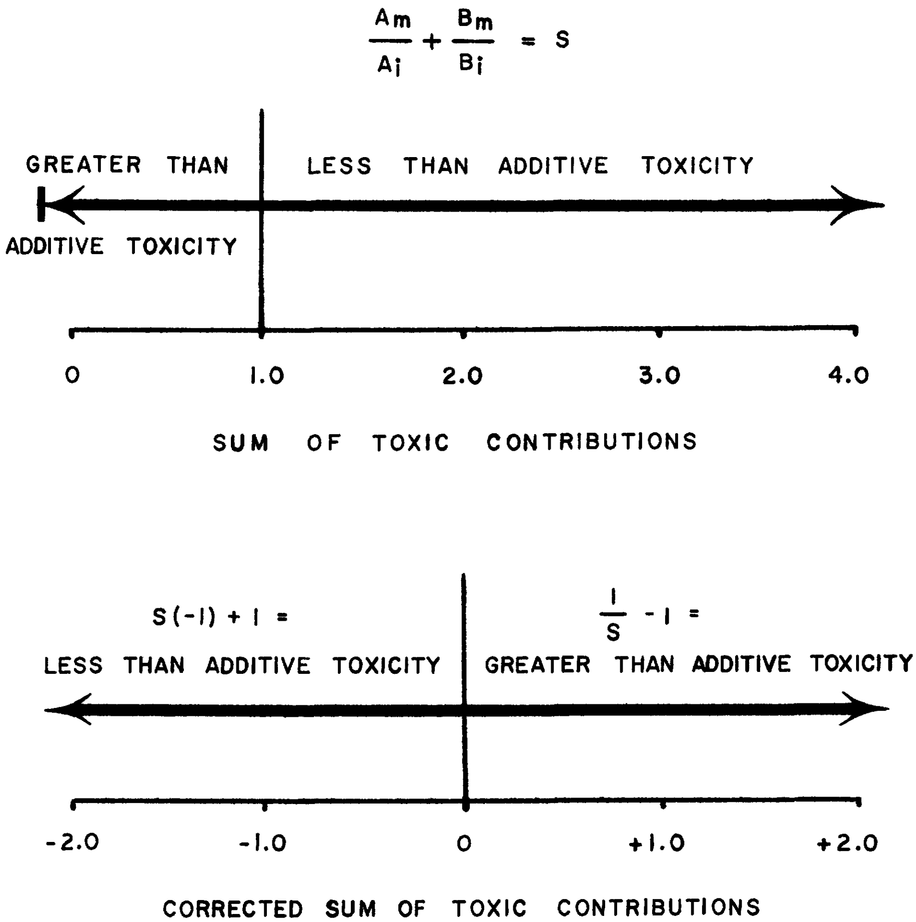

Figure 1.—Sums (S) of toxic contributions for a chemical mixture, which are nonlinear for less than additive and greater than additive toxicity (upper illustration) were corrected for linearity and direction of plus and minus values (lower illustration).

A system in which the index represents simple additive, greater than additive, and less than additive effects by zero, positive, and negative values, respectively, would be desirable. Such a system can be developed by establishing linearity and by assigning a reference point of zero for simple additive toxicity (Fig. 1). We established linearity by using the reciprocal of the values of S which were less than 1.0, and a zero reference point was achieved by subtracting 1.0 (the expected sum for simple additive toxicity) from the reciprocal $\left[\left(\frac{1}{S}\right)-1\right]$. Thus greater than additive toxicity is represented by index values greater than zero. Index values representing less than additive toxicity were obtained by multiplying the values of S which were greater than 1.0 by -1 to make them negative, and a zero reference point was achieved by adding 1.0 to this negative value [S(-1)+1]. Thus, less than additive toxicity is represented by negative index values. The sum of effective contributions (S) is modified by one of two procedures: either the additive index $= \frac{1}{S} - 1$ for $S \leq 1.0$ (greater than additive toxicity) or $S(-1) + 1$ for $S \geq 1.0$ (less than additive toxicity). A sum (S) of 1 yields an index value of zero by either modifying procedure and represents simple additive toxicity. A summary of the procedure follows.

$$\frac{A_m}{A_i} + \frac{B_m}{B_i} = S, \text{ the sum of biological effects}$$

$$\text{Additive index} = \frac{1}{S} - 1.0 \text{ for } S \leq 1.0 \text{ and}$$

$$\text{Additive index} = S(-1) + 1.0 \text{ for } S \geq 1.0$$

We assessed the significance of additive indices close to zero in our data by substituting values from the 95% confidence intervals into the formula to determine whether the range for additive indices overlapped zero (simple additive toxicity). The range was derived by selecting values of the 95% confidence interval yielding the greatest deviation from the additive index. The lower limits of the individual toxicants (A_i and B_i) and the upper limits of the mixtures (A_m and B_m) were substituted for LC50's to determine the lower limit of the index. Correspondingly, the upper limits of the individual toxicants (A_i and B_i) and the lower limits of the mixtures (A_m and B_m) were substituted into the formula to determine the upper limit of the index.

Applications and Discussion

To test the method on existing information, we selected toxicity data from published papers in which the author had provided the toxicity for the individual components and for the mixtures. In some instances observed mortality was reported and used in place of statistically derived LC50's. The significance of additive indices derived from data in the literature was not assigned because 95% confidence intervals were usually not reported.

Doudoroff (1952) studied the toxic activity of mixtures of zinc and copper, two suspected metal ion contaminants in some effluents. He showed extraordinary toxic activity against fathead minnows *(Pimephales promelas)* in 8-h tests in which survival was recorded rather than median lethal concentrations (LC50's). The additive index is

$$\frac{1.0}{8.0} + \frac{0.025}{0.20} = 0.250; \left(\frac{1}{0.250}\right) - 1 = 3,$$

a value which definitely supports Doudoroff's conclusions (Table 1). Lloyd (1961) and Sprague and Ramsey (1965) found less or no potentiation in zinc and copper mixtures, but they defined the toxicity with LC50's for longer exposures; however, they did report that lethal mixtures of those metal ions act 2 to 3 times as fast as the metals singly.

Cairns and Scheier (1968) reported "slight antagonistic interaction" of zinc and cyanide to fathead minnows, in contrast to the opposite activity of zinc and copper. They attributed the effect to complexation of these ions. The additive index (-1.37) as computed in the present study indicates considerable antagonism. Chen and Selleck (1969) also considered the zinc-cyanide combination to be very antagonistic, but they did not quantify the toxicity.

Howland (1969) reported additive effects for mixtures of two fish toxicants, antimycin and rotenone, whereas our index is -0.39. Although our value suggests slightly less than additive toxicity, the significance of the value should be defined before the results are interpreted.

Antimycin could be applied to water with a fluorescent tracer, rhodamine B. The tracer

Table 1. Toxicity or efficacy of chemicals applied individually and in combination against fishes and the calculated additive index

Chemical mixtures	Toxic unit	96-h LC50 or EC50 of chemical		Additive index	Reference
		Individually	In combination		
Zinc[a]	mg/l	8.0	1.0		
and				3.00	Doudoroff 1952
Copper[a]	mg/l	0.2	0.025		
Zinc	mg/l	4.2	3.90		
and				-1.37	Cairns and Scheier 1968
Cyanide	mg/l	0.18	0.26		
Antimycin	µg/l	0.032	0.027		
and				-0.39	Howland 1969
Rotenone	µg/l	57.0	31.0		
Antimycin	µg/l	0.048	0.047		
and				0.00	Marking 1969b
Rhodamine B	mg/l	217	5.0		
MS-222	mg/l	80	30		
and				0.29	Berger 1969
$QdSO_4$	mg/l	25	10		
Malachite green	mg/l	0.2	0.05		
and				0.83	Leteux and Meyer 1972
Formalin[b]	mg/l	50	15		

[a] An 8-h time response, based on survival rather than LC50.
[b] Concentrations effective against parasites.

interacts little, if any, with the toxicant (additive index = 0.00), and the suitability of this tracer was supported (Marking 1969b).

The additive index method can be used for characteristics other than toxicity. For instance, the efficacy of MS-222 (tricaine methanesulfonate) and $QdSO_4$ (quinaldine sulfate), two fish anesthetics, shows the rapid and sustaining anesthetic qualities, respectively, and an improved safety factor when the two chemicals are mixed rather than applied separately. The index of 0.29 suggests greater than additive efficacy and agrees with Berger's (1969) interpretation that the interaction was synergic. Schoettger and Steucke (1970) discussed advantages of the anesthetic mixture, including a cost reduction of 60 to 80%. Mixtures of some fish therapeutants are more effective than individual disease treatment chemicals. For example, the additive index for the efficacy of malachite green and formalin is 0.8 (Leteux and Meyer 1972).

Several pairs of toxic chemicals were chosen for determining additive toxicity in our laboratory (Table 2). When the fish toxicants antimycin and TFM (lampricide) were tested individually and in combination against bluegills, the additive index was 0.343 and the range computed from the 95% confidence interval was -0.189 to 1.17. Since the range overlaps zero, the toxicity for these two chemicals in this particular test was merely additive.

The index for mixtures of antimycin and Dibrom(R) against rainbow trout was -0.574 (range, -1.12 to -0.173). Since the range did not overlap zero, the toxicity of the mixture was less

Table 2. Toxicity of toxicants applied individually and in combination to fish in 96-h, standardized tests at 12°C

Species, toxicants, and toxic unit	LC50 and 95% confidence interval: Individually	LC50 and 95% confidence interval: In combination	Additive index
Bluegill			
Antimycin (μg/l)	0.0710	0.0390	
	0.0574-0.0879	0.0296-0.0506	
and			0.340
			-0.189 to 1.17
TFM (mg/l)	4.96	0.970	
	4.10-5.99	0.744-1.26	
Rainbow trout			
Antimycin (μg/l)	0.0312	0.0300	
	0.0266-0.0366	0.0272-0.0331	
and			-0.574
			-1.12 to 0.173
Dibrom (mg/l)	0.0490	0.0300	
	0.0279-0.0633	0.0272-0.0331	
Antimycin (μg/l)	0.0412	3.79	
	0.0371-0.0457	2.78-5.16	
and			-91.8
			--- ---
$KMnO_4$ (mg/l)	1.22	1.0	
	1.08-1.38	--- ---	
Malathion (μg/l)	70.0	3.44	
	59.2-82.7	2.92-4.06	
and			7.20
			5.09 to 10.0
Delnav (μg/l)	47.2	3.44	
	42.4-52.6	2.92-4.06	

than additive. Berger (1971) reported that mixtures of those toxicants are synergistically toxic to black bullhead *(Ictalurus melas)*, largemouth bass *(Micropterus salmoides)*, and yellow perch *(Perca flavescens)*. These data suggest that additive toxicity may vary for different species of fish, ratios of toxicants, or test conditions. For example, Hoff and Westman (1965) reported that a 3:2 ratio of Dibrom(R) and malathion showed promising selectivity toward bluegills and pumpkinseeds *(Lepomis gibbosus)* in the presence of largemouth bass. For these reasons, the additive index for toxicants should be determined for nontarget as well as target species.

Antimycin is readily detoxified by oxidizing agents such as potassium permanganate (Walker 1967). Both chemicals were toxic to fish, but the counteraction through oxidation greatly decreased the toxicity of the mixture. Potassium permanganate was added at 1.0 mg/l to each concentration of antimycin because that concentration of permanganate has been effective in fish management applications. As expected, the combination produced extreme antagonistic activity against rainbow trout; the additive index was -91.8 in standardized tests at pH 7.5 (Table 2). This procedure could be used to assess the effectiveness of permanganate for detoxifying antimycin under other conditions of different pH or temperature.

Mixtures of malathion and Delnav[(R)], two organic phosphates reported to be synergistic against insects, are extremely toxic to fish. The additive index is 7.20 for rainbow trout. That value is the highest for any toxicant mixture evaluated by our method and further emphasizes the potency of certain pesticide mixtures.

The 95% confidence interval influences the significance of the additive index, and significance becomes more difficult to show as the range widens because of the greater likelihood that the range will overlap zero. The 95% confidence interval is influenced by the number of concentrations and the number of test organisms per concentration. Therefore, well planned toxicity tests which result in narrow confidence intervals are the most useful in the assignment of effects of chemical mixtures.

The study of mixtures of toxic chemicals in water and the resultant benefits or hazards is fairly new, and only a few methods have been investigated. Conceivably, the additive toxicity of more than two chemicals could be evaluated by simply adding the contributions of additional chemicals according to the following formula,

$$\frac{A_m}{A_i} + \frac{B_m}{B_i} + \frac{C_m}{C_i} + \text{- - - -} = S.$$

This additive toxicity index method could be useful for assessing the economics of mixtures of chemicals, for determining toxicity advantages against target organisms, for determining hazards or disadvantages against nontarget organisms, for assessing the additive toxicity of different ratios of chemicals in a mixture, and for assessing physical influences of the environment on additive toxicity. Advantages of this method over existing methods include linearity for all index values and a procedure for determining the significance of indices.

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