Bull. Environ. Contam. Toxicol. (1991) 46:550–553

Bulletin of Environmental Contamination and Toxicology

Microtox Assay of Trinitrotoluene, Diaminonitrotoluene, and Dinitromethylaniline Mixtures

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Contamination of waste waters, soils, and sediments with 2,4,6-trinitrotoluene (TNT) from the operation of munitions manufacturing, loading, and assembling and packing facilities, is an environmental hazard. TNT has been identified in the soil, surface water, and ground water after leaching from disposal sites. TNT is very toxic to, and causes a variety of toxicities in organisms in several phylla. Aquatic surveys of streams showed that ammunition wastes caused changes in, or losses to, downstream biological communities (Putnam et al. 1981). These studies were not able to attribute a cause-effect relationship to TNT since its byproducts and other contaminants either were also being discharged or were forming in streams after TNT was discharged. Therefore, assessment of the risk to the environment from TNT contamination needs to consider whether the toxicity of these mixtures is additive or is described by more complex interactions.

This research used the Microtox test to evaluate the toxicity of aqueous solutions of 2,4,6-trinitrotoluene (TNT), 2,6-diamino-4-nitrotoluene (DANT) and 2,6-dinitro-4-methylaniline (DNMA) alone and as binary mixtures in 3^2 factorial combinations. The Microtox test is an instrumented bioassay which measures the reduction in chemiluminescence when the marine bacterium *Photobacterium phosphoreum* is exposed to toxic chemical(s). The response is concentration dependent; the EC_{50} is the concentration of a compound which inhibits 50% of the bacterial bioluminescence.

MATERIALS AND METHODS

The standard Microtox (Microbics Inc., Carlsbad, CA) procedure (Microbics 1988) was followed except that reconstitution of the bacteria was accomplished by rapidly adding chilled reagent to a vial of bacteria maintained in ice on a vortex mixer. The vortex mixer increased the initial light output by a factor of 5-10. Bioluminescence intensity at 15 C was determined at 0 min in the blank ($I_{0(b)}$) and sample ($I_{0(x)}$), and in each at 15 min ($I_{t(b)}$) and $I_{t(x)}$) after addition of toxicants to the test medium. Light output is expressed as

$$\log_e \Gamma = \log_e [(1 - \beta) / \beta],$$

where $\beta = I_{t(x)} / (I_{0(x)}.BR)$ and $BR = I_{t(b)} / I_{0(b)}$. $\log_e \Gamma$ is a logit (Finney

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1971). EC_{50}s were computed using the trimmed logit method (Sanathanan et al. 1987). DANT and DMNA were purchased from Aldrich (Milwaukee, Wisconsin). TNT was obtained from the U.S. Army.

Schaeffer (1987) showed that if one compound, **A**, in a mixture was used as a "reference" and the other(s) were the "matrix" **B**, then analysis of covariance (ANCOVA) (Eq. 1) could be used to test for parallelism of slopes between pure **A** and **A** in a given concentration of **B**. In the model, **B** takes on values of 1 or 2 depending on whether the concentration of **B** is 0 or > 0. The continuous covariate, **A**, is the logarithm of the concentration of **A**. When the interaction term **AB** was significant, the ANCOVA assumption of parallel of slopes was not met and the hypothesis that a mixture of **A** + **B** acted by simple similar action was rejected. Otherwise, the covariance analysis was carried out by reanalyzing the data without this term. The data were analyzed a second time with the compounds assigned to **A** and **B** reversed.

$$\text{Log}_e \Gamma = \text{constant} + \mathbf{B} + \mathbf{A} (+ \mathbf{AB}) \qquad (1)$$

If ANCOVA (1) held, the regression model $\ln \Gamma = b_0 + b_1[\mathbf{A}] + b_2\mathbf{B}$ was fitted. Here, b_0 is the average intercept, b_1 is the pooled slope, b_2 is the displacement in the intercepts between the pure **A** and the mixture, **B** = 1 for the pure reference and **B** = 2 for the sample. The equivalent toxicity, ET, is:

$$ET = \exp[(-b_0 - b_2) / b_1] - \exp[(-b_0 - 2\,b_2) / b_1]. \qquad (2)$$

For example, for DANT in 20 mg/L DNMA, $b_0 = -6.46$, $b_1 = 1.15$, $b_2 = 1.19$, so ET = exp[(6.46-1.19) / 1.15) - exp[{6.46-2(1.19)} / 1.15)] = 64.31 mg/L.

RESULTS AND DISCUSSION

Table 1 summarizes the statistical analyses for each mixture. The table is organized into "sets" corresponding to a given reference toxicant curve. Data for a binary combination was statistically analyzed twice: once with one component as **A** and the other as **B**, and then vice versa. This gave results sets A,B; C,D; and E,F (Table 1). Additional concentrations were used to define the slope for the pure compounds, so the data used in the two analyses was similar but not necessarily identical. For example, the pure DNMA curve in the DNMA+DANT mixtures was obtained using a point at 43.2 mg/L DNMA but this concentration was not included in a binary mixture because the combined toxicity would have been too high.

The first row in each set gives the EC_{50} and 95% confidence bounds, and the slope for the reference toxicant. The "Equality of Slopes" column tests the applicability of the covariance model. Samples marked "NS" had a slope for the reference toxicant which did not differ significantly from the slope for the pure reference toxicant. For these samples, the "Intercept" column shows if the lines were coincident ("NS") or significantly separated but parallel. When the test for the equality of slopes was significant, analysis of covariance could not be used to test the equality of intercepts.

Table 1 illustrates the premise of Schaeffer's (1987) testing approach. For example, while the EC_{50} of pure DANT in set B is 50.6 mg/L, the "sample matrix" contributed the equivalent of 21.4 mg/L DANT when the DANT curve was obtained in 0.225 mg/L TNT. In set B, high concentrations of TNT

Table 1: Microtox Tests: TNT, DANT, DNMA and Their Mixtures[1].

Sample	EC_{50} mg/L	(95% CI) mg/L	Slope	Slope/ Intercept Equality	Equivalent Toxicity mg/L[2]
A. TNT in DANT					
0 DANT	1.67	(0.79, 3.51)	0.582		
20 DANT	0.57*	(0.38, 0.85)	0.403	NS/**	1.64
35 DANT	0.18*	(0.06, 0.54)	0.266	**/--	[2.77]
50 DANT	0.08*	(0.015, 0.43)	0.253	**/--	[3.04]
60 DANT	0.04*	(0.001, 1.00)	0.230	NS/**	2.53
B. DANT in TNT					
0 TNT	50.6	(40.9, 62.7)	1.263		
0.225 TNT	32.0*	(26.5, 38.7)	0.863	**/--	[21.4]
0.45 TNT	22.7*	(15.7, 33.0)	0.657	**/--	[31.0]
0.90 TNT	12.4*	(2.9, 53.8)	0.483	**/--	[33.9]
1.35 TNT	11.4*	(4.9, 26.9)	0.576	**/--	[41.3]
C. TNT in DNMA					
0.0 DNMA	0.75	(0.52, 1.08)	0.55		
0.0 DNMA	0.74	(0.54, 1.02)	0.56		
5.0 DNMA	0.84	(0.50, 1.42)	0.45	NS/**	0.24
10.0 DNMA	0.67	(0.47, 0.96)	0.67	NS/**	0.10
15.0 DNMA	0.49	(0.29, 0.83)	0.48	NS/**	-0.04
D. DNMA in TNT					
0.0 TNT	33.4	(23.1, 48.3)	0.874		
1.25 TNT	2.9*	(0.5, 18.6)	0.363	NS/**	39.60
0.0 TNT	24.6	(20.6, 29.3)	0.992		
0.25 TNT	22.1	(12.7, 38.3)	0.513	**/--	--
0.50 TNT	8.2*	(4.7, 14.3)	0.302	**/--	--
1.00 TNT	2.7*	(0.86, 8.8)	0.315	**/--	--
E. DANT in DNMA					
0.0 DNMA	49.7	(41.7, 59.3)	1.03		
5.0 DNMA	34.7*	(30.8, 39.2)	1.25	NS/**	12.4
10.0 DNMA	34.2*	(26.8, 43.8)	0.69	NS/**	17.1
20.0 DNMA	16.3*	(9.5, 28.1)	0.64	NS/**	29.9
F. DNMA in DANT					
0.0 DANT	29.3	(23.0, 37.4)	0.83		
20.0 DANT	18.2	(11.9, 27.7)	0.56	NS/**	15.5
35.0 DANT	7.1*	(5.1, 9.8)	0.54	NS/**	23.4
44.0 DANT	2.5*	(0.5, 13.6)	0.14	**/--	[58.6]

[1]TNT=2,4,6-Trinitrotoluene, DANT=2,6-Diamino-4-nitrotoluene, DNMA=2,6-Dinitro-4-methylaniline. [2]Negative value implies inhibition by the sample matrix. Computation of bracketed value assumed parallel slopes, so estimate may be low. * EC_{50} value differed from reference EC_{50} given by A = 0. ** Significant at $P < 0.05$. NS = Not Significant.

acted additively on a logarithmic scale with DANT; linear regressions of ln EC_{50} or ln ET against ln [TNT] had $R^2 \approx 0.95$.

The results for TNT as the reference toxicant in solutions of DNMA (set C) illustrate another aspect of toxicity assessment of mixtures. EC_{50} values for the mixtures did not differ statistically from the EC_{50} for TNT and the slope of each mixture line was parallel to the slope of the TNT line. However, the ET of the DNMA matrix decreased as the DNMA concentration increased. Although this apparent decrease could be an artifact of the extrapolation process, a real change would imply that the toxicity of the mixtures was not simple component additivity. Set D supports this interpretation. When DNMA was used as the reference toxicant, its slope in a mixture was not parallel to the DNMA-alone slope. The results in sets C and D show that mixture toxicity must be viewed as a complex response surface. Viewed from the perspective of TNT, the DNMA plane appeared to be flat so that the slopes for TNT alone and in mixtures were parallel. From the perspective of DNMA, the TNT surface was more complex and the mixture lines were not parallel to the pure DNMA reference line (or to each other).

The behavior of mixtures of DANT and DNMA in Sets E and F are similar to TNT and DANT in sets A and B. Whereas the regressions in Sets A and B were significant for the logarithms of the values, relationships in Sets E and F were significant for the arithmetic values (but usually not for the logarithms).

TNT was 20 to 50 times as toxic (mg/L) as DANT or DNMA. The results showed that the acute toxicity of mixtures of TNT with related compounds was often not described by simple similar addition (Finney 1971). Because TNT was more toxic than the other compounds studied, and because it is the predominant compound found in TNT-contaminated sites, preliminary risk assessments based on chemical analyses can probably proceed on the assumption of additivity. However, specific toxicity testing using several compounds found in the mixture as spikes should then be carried out.

Acknowledgments. We thank Drs. Ravi Jain and Edward Novak of the U.S. Army Construction Engineering Research Laboratory, Champaign, Illinois, and Microbics Corporation, Carlsbad, California, for support. We thank Dr. Wayne Banwart, University of Illinois, for providing the TNT.

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Received June 15, 1990; accepted September 28, 1990.