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Mixture Toxicity of S_N2-Reactive Soft Electrophiles: 3. Evaluation of Ethyl α-Halogenated Acetates with α-Halogenated Acetonitriles

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Abstract

Mixture toxicity for each of four ethyl α-halogenated acetates (ExACs) with each of three α-halogenated acetonitriles (xANs) was assessed. Inhibition of bioluminescence in *Vibrio fischeri* was measured after 15, 30 and 45-min of exposure. Concentration-response curves were developed for each chemical at each exposure duration and used to develop predicted concentration-response curves for the dose-addition and independence models of combined effect. Concentration-response curves for each mixture and each exposure duration were then evaluated against the predicted curves, using three metrics per model: 1) EC₅₀-based additivity quotient (AQ) or independence quotient (IQ) values, 2) mean AQ (mAQ) or mean IQ (mIQ) values, calculated by averaging the EC₂₅, EC₅₀ and EC₇₅ AQ or IQ values, and 3) deviation values from additivity (DV-A) or independence (DV-I). Mixture toxicity for ethyl iodoacetate (EIAC) was dose-additive with each of the xANs at all exposure durations and was often consistent with independence as well. The same was true for mixture toxicity of ethyl bromoacetate (EBAC) with each xAN. However, for the two more slowly reactive chemicals ethyl chloroacetate (ECAC) and ethyl fluoroacetate (EFAC) mixture toxicity with each xAN only became consistent with dose-addition upon increasing exposure duration. Consistency with independence for both ECAC and EFAC with the xANs was essentially limited to the EC₅₀-IQ metric; thereby demonstrating the utility of calculating the mean quotient (mAQ, mIQ) and deviation value (DV-A, DV-I) metrics. Upon review of these findings with those from the first two papers in the series, the results suggest that instances in which mixture toxicity was not consistent with dose-addition relate: 1) to differences in the capability of the chemicals to form strong H-bonds with water and 2) to differences in relative reactivity and time-dependent toxicity levels of the chemicals.

Chemical mixture toxicity is frequently assessed by comparing experimental results against predictions from two combined effects models: dose-addition (i.e., concentration addition) and independent action (i.e., independence). Implicit in the former is the idea that the chemicals in the mixture have the same mechanism of action and differ only by having varying potencies (Calabrese 1991; Pösch 1993; Kortenkamp et al. 2009). In this approach, the concentrations of the individual chemicals are scaled to put them on an equivalent-potency basis and added together to estimate the toxicity of the mixture (SCHER et al. 2012). In contrast, independence is a simple, probability-based combined effects model (Bliss 1939) for chemical or physical factors that induce similar toxic effects but at different sites of action within the organism. Due to the difference in sites of action, the resulting toxicity is unlikely to be due to a single, common mechanism of action (Ariëns et al. 1956; Berenbaum 1981; Pösch and Holzmann 1980/1981; Pösch et al. 1990; Pösch 1993; Kortenkamp et al. 2009; SCHER et al. 2012). This mechanistic distinction between dose-addition and independence, then, has the potential to be useful in systematic examinations of mixture toxicity, especially when coupled with evaluations of relative reactivity and time-dependent toxicity of soft electrophiles (Dawson et al. 2010).

Electron deficient chemicals are termed electrophiles, as they tend to react with electron-rich chemicals (i.e., nucleophiles) during a chemical reaction. In toxicology, exogenous electrophiles, upon getting inside the cell, may react with endogenous nucleophiles, such as the N and O atoms of amino acids or nucleic acids, to form a covalent bond. Such reactions can involve addition of an atom or molecule to the nucleophile or a substitution between the electrophile and nucleophile. Depending on the softness or hardness of the exogenous chemical, a variety of toxic insults may then result, such as enzyme inhibition or mutation.

A simple substitution reaction is the S_N2 type, in which one group in the reaction is directly displaced at a carbon atom by another group (Jacobs 1997). S_N2 electrophiles include chemicals capable of forming strong hydrogen bonds with water (Hansch and Leo 1979) and chemicals lacking such capability. The former are termed S_N2 -H-polar chemicals and are exemplified by the ethyl α -halogenated acetates (ExACs) $[X-CH_2-CO(=O)-C_2H_5; X = \text{halogen}]$ (Roberts et al. 2010). The latter include the α -halogenated acetonitriles (xANs) $[X-CH_2-C\equiv N; X = \text{halogen}]$.

Earlier works have demonstrated the utility of incorporating time-dependent toxicity evaluations (e.g., Gagan et al. 2007) and an asymmetry parameter in concentration-response curve-fitting (Dawson et al. 2012) when evaluating mixture toxicity. Two recent studies examining toxicity of xAN-containing (Dawson et al. 2010) and ExAC-containing binary mixtures (Dawson et al. 2011) included: 1) both sham (i.e., A:A) and true combinations (i.e., A:B) for each chemical group and 2) combinations of each of those chemicals with a model nonpolar narcotic, 3-methyl-2-butanone (3M2B). In this paper, results of ExAC:xAN combinations are presented and the results of the three studies are summarized.

Materials and Methods

Chemicals

Four ethyl α -halogenated acetates (ExACs) and three α -halogenated acetonitriles (xANs; x represents a halogen in both groups) were purchased from Sigma-Aldrich and used without further purification (abbreviation, Chemical Abstract Service registry number [purity]): ethyl iodoacetate (EIAC, 623-48-3 [98%]), ethyl bromoacetate (EBAC, 105-36-2 [98%]), ethyl chloroacetate (ECAC, 105-39-5 [99%]), ethyl fluoroacetate (EFAC, 459-72-3 [98%]), iodoacetonitrile (IAN, 624-75-9 [98%]), bromoacetonitrile (BRAN, 590-17-0 [97%]), and chloroacetonitrile (CLAN, 107-14-2 [99%]). Single-chemical stock solutions were prepared in Microtox[®] diluent (a 2% NaCl solution) or in DMSO (maximum concentration in testing 0.1%) with subsequent dilution in diluent. Test solutions were prepared just before testing and held in the dark at 15°C in closed glass vials.

Toxicity Assay Procedures

Inhibition of bioluminescence was the toxicity endpoint measured using Microtox[®] (Modern Water Inc., New Castle, DE). For each chemical combination there was a test of each chemical alone (chemical A, chemical B) and a mixture test (i.e., A:B). Each test had at least seven duplicated concentrations and a duplicated control treatment. Chemical concentrations were nominal, density corrected and prepared by serial dilution using a 1.867 dilution factor. The concentrations selected for testing were designed to effectively calculate EC₂₅, EC₅₀ and EC₇₅ values. The EC₅₀ is defined as the half-maximal effective concentration. The EC₂₅ and EC₇₅ are similarly defined as the one-quarter and three-quarter-maximal effective concentrations, respectively.

Freeze-dried bacterial reagent (*Vibrio fischeri*) was reconstituted prior to test initiation and held at 5 + 0.1°C for 15-20 min. Initial light readings were quantified for each treatment vial prior to the chemical or mixture being added and thereafter readings were taken at 15, 30, and 45-minutes of exposure. Treatment vials were held at 15 ± 0.2°C during testing.

Procedures for Generating Single-Chemical Concentration-Response Curves

MicrotoxOmni[®] software converted light readings to percent effect values. Those data were input into SigmaPlot (v. 11.0, Systat Software, Chicago, IL) worksheets and evaluated within user-designed program files. Experimental data were fitted to sigmoid curves using a five-parameter logistic function that had been modified by removing the minimum effect parameter, as it can unduly influence curve-fitting thereby adversely affecting calculation of accurate experimental and predicted concentration-response curves (Dawson et al. 2012). The four remaining parameters in this function were EC₅₀, slope, maximum effect and asymmetry (s). The modified function is termed the five-parameter logistic minus one-parameter (5PL-1P) function, to distinguish it from the standard four and five parameter logistic functions.

Curve fitting was performed using:

$$y = \max / (1 + (xb/x)^{\text{Hillslope}})^s \quad (1)$$

in which y = % effect (effect = inhibition of bioluminescence), $\max$ = maximum effect, x = concentration, s = asymmetry and $^{\wedge}$ indicates exponential form (i.e., to the power of). The variable xb was determined using:

$$xb = EC_{50} * 10^{((1/\text{Hillslope}) * \log(2^{(1/s-1)}))}. \quad (2)$$

Within SigmaPlot regressions were run with automatic estimation of the initial parameters. Three constraints used for data fitting were: a) $EC_{50} > 0$; b) $0.1 < s < 10$; and c) $\max < 100$. For concentration-response data, EC_{25} , EC_{50} , EC_{75} , slope, asymmetry and maximum effect values for each chemical alone were calculated for each of the exposure times.

Procedures for Generating Mixture Concentration-Response Curves

For mixture data, total chemical concentrations (i.e., chemical A and chemical B combined) were expressed in terms of concentration equivalents of chemical A. The conversion factor was: $[B] = [A] / [B]$ (Dawson et al. 2010). Experimental EC_{25} , EC_{50} , EC_{75} , slope, asymmetry and maximum effect values for each mixture were calculated by the methods and equations used for single-chemical curves (see previous section).

Time-Dependent Toxicity Value Calculation

Time-dependent toxicity (TDT) values were calculated based on the equation $E = c * t$ (c = concentration, t = time) (Gagan et al. 2007). TDT values were initially determined for three exposure periods 15-30 min, 30-45 min and 15-45 min for each individual chemical. The only TDT values reported herein are for the 15-45 min exposure period and those were then averaged across replicate tests for each chemical alone. The 15-45 min TDT values were calculated as the 15-min EC_{50} minus the 45-min EC_{50} , with that difference then being divided by the product of the 15-min EC_{50} and 0.667. The resulting value was then multiplied by 100 to give it a percentage basis (Gagan et al., 2007).

Theoretical Dose-Addition and Independence Curve Calculations from Single-Chemical Data

Theoretical concentration-response curves for both dose-addition and independence were developed as follows (Dawson et al., 2011). In concept, when agents A and B are dose-additive, the EC_{50} for their mixture is shifted to the left by a dose-ratio (DR) factor = 2 when the agents are equieffective. This is shown below:

$$\text{Add50} = a50 / DR50 \quad (3)$$

in which Add50 is the EC_{50} for dose-addition, $a50$ represents the EC_{50} of the more potent agent and $b50$ the EC_{50} of the less potent agent. The DR50 was determined using:

$$DR_{50} = 1 + (a_{50}/b_{50}) \quad (4)$$

So, when $a_{50} = b_{50}$ (i.e., equieffective agents) the $DR_{50} = 1 + (1) = 2$. As a result the $Add_{50} = a_{50}/2$, giving the EC_{50} for the theoretical dose-addition curve of the mixture. In similar fashion, calculation of the EC_{25} and EC_{75} values for the predicted dose-addition curve was performed. Taken together the predicted dose-addition values for the EC_{25} , EC_{50} and EC_{75} and the dose-additive maximum effect (calculated as $\max = a_{50} * 100\%$), allow for the calculation of the theoretical dose-addition curve by applying the same curve fitting procedure used to generate the single chemical curves (see Procedures for Generating Single-Chemical Concentration-Response Curves, above).

Theoretical curves for the independence model of combined effect were developed using:

$$y_A + (y_B * (100 - y_A) / 100) \quad (5)$$

where y_A is the percent effect for agent A and y_B is the percent effect for agent B.

Dose-Additivity and Independence Quotient, Mean Quotient, and Quotient Deviation Values

For each combination, EC_{25} , EC_{50} and EC_{75} values were calculated at each time-point for each chemical alone, for the mixture and for the predicted dose-addition and independence curves. Additivity quotient (AQ) values were calculated as $AQ = \text{experimental value} / \text{predicted value for dose-addition}$, while independence quotient values (IQ) were calculated as $IQ = \text{experimental value} / \text{predicted value for independence}$.

Mean additivity quotient (mAQ) values for each exposure-duration were calculated by adding the individual EC_{25} -AQ, EC_{50} -AQ and EC_{75} -AQ values for a given exposure duration and dividing by three. Likewise, mean independence quotient (mIQ) values were calculated using the appropriate IQ values for the combination. Mean AQ and IQ values offer the advantage of assessing data fit to the models over a wider range of the concentration-response curve (i.e., from 25 to 75% effect) than using just the midpoint (i.e., 50% effect).

In interpreting mixture toxicity though, sometimes calculated mAQ and mIQ values can be misleading; as is also true for EC_{50} -AQ or EC_{50} -IQ values. For example, an actual mixture concentration-response curve can appear left-shifted from the predicted dose-addition (or independence) curve at lower concentrations, cross it at middle concentrations and be right-shifted from it at higher concentrations (or vice versa). As a result the mAQ (or mIQ) value can be close to 1.0, suggesting that actual mixture toxicity data fit the model well when it does not. As a hypothetical example, a mixture with $mAQ = 1.04$ – reflecting dose-addition – would result from AQ values of 0.83 at the EC_{25} , 1.01 at the EC_{50} and 1.27 at the EC_{75} . To address this possibility, the additivity quotient deviation value (DV-A) and the independence quotient deviation value (DV-I) were developed. Calculating the DV-A, using the example values noted above, involves taking the absolute value of the difference between the actual AQ value and 1.00 for each effect level, summing them and dividing by

three (e.g., $|1.00 - 0.83| + |1.00 - 1.01| + |1.00 - 1.27| / 3 = 0.45 / 3 = 0.15$). In contrast, a mixture with EC₂₅, EC₅₀ and EC₇₅ AQ values of 1.02, 1.04 and 1.06 has the same mAQ value (1.04) but a smaller DV-A value (0.03); thereby indicating that the deviation of the EC₂₅, EC₅₀ and EC₇₅ values from that predicted dose-addition is low between 25 and 75% effect. Calculation of the DV-I is done the same way, using appropriate IQ values. In this approach, when EC₅₀-AQ and mAQ values are between 0.90 – 1.10 (inclusive) the effect is considered to be consistent with dose-addition when the DV-A for the mixture is 0.10. The EC₅₀-IQ, mIQ and DV-I values are similarly considered to be consistent with independence in those same ranges. Mixture toxicity for some two-chemical combinations can, on occasion, be consistent with both dose-addition and independence.

Data Quality Determination

Concentration-response data quality was examined by calculating the coefficient of determination (r^2) for each single-chemical and mixture curve. Test-to-test consistency of each chemical alone was assessed by calculating coefficient of variation (CV) values for the EC₅₀ and slope parameters at each time-point. Use of CV rather than standard error values is preferred when data result from work carried out by multiple operators (Steel and Torrie 1980).

Results

Toxicity of Single-Chemicals

Toxicity data (Table 1) are presented as mean values; each Ex AC was tested alone three times (once each with IAN, BRAN and CLAN) and each xAN was tested alone four times (once each with EIAC, EBAC, ECAC and EFAC). Mean EC₅₀ values show that toxicity increased (i.e., the EC₅₀ declined) over time for each chemical (also reflected by mean TDT values of around 100%), except for EFAC, which showed minimal TDT. Mean asymmetry values tended to decrease with increasing exposure duration, while slope values tended to increase over exposure time. For all seven chemicals, concentration-response data were well fitted to sigmoid curves by the 5PL-1P function, with mean r^2 values exceeding 0.990 and typically being above 0.997.

Toxicity of ExAC:xAN Mixtures

For the twelve ExAC:xAN combinations, results showed that mixture toxicity was generally consistent with dose-addition (Table 2 – dose-additive values are shown in bolded typeface; Fig. 1). However, some exceptions were noted at the shorter exposure durations: 1) toxicity was less-than that predicted for dose-addition for each of the three ECAC:xAN combinations at 15-min of exposure (Fig. 2) and with BRAN at 30-min and 2) toxicity was greater-than that expected for dose-addition for EFAC with IAN at 15-min. After 45-min of exposure all EC₅₀-AQ, mAQ and DV-A values reflected a combined effect consistent dose-addition. Irrespective of the actual combined effect observed for each combination and exposure duration, the mAQ and DV-A values produced a consistent combined effect determination.

Mixture toxicity data assessed for consistency with independence gave more varied results (Table 3 – values consistent with independence are shown in bolded, italicized typeface). All EIAC:xAN combinations gave EC₅₀-IQ, mIQ and DV-I values consistent with independence. The EBAC:xAN combinations tended to as well, but EBAC:BRAN and EBAC:CLAN gave mIQ and DV-I values outside the independence range at 45-min. For ECAC:xAN combinations all mIQ and DV-I values were outside the respective ranges for independence and all but the 45-min EC₅₀-IQ value for ECAC:IAN were as well. For EFAC:xAN combinations, generally the combined effects fell outside independence. One notable exception – EFAC:IAN at 15-min – gave EC₅₀-IQ and mIQ values consistent with independence, but the DV-I value (0.19) fell outside the independence range; thereby highlighting the need for including quotient deviation values (e.g., DV-I) in the analysis. For this combination and exposure duration the EC₂₅-IQ (0.83), EC₅₀-IQ (0.93) and EC₇₅-IQ (1.32) gave the mIQ of 1.03, but the DV-I of 0.19 indicated that the mixture concentration response curve did not overlap the predicted independence curve well, except in the range from about 50 to 60% effect (Fig. 3).

Discussion

This paper is the third from this group evaluating mixture toxicity of S_N2-reactive chemicals. The toxicity of xAN-containing combinations (Dawson et al. 2010) and ExAC-containing combinations (Dawson et al. 2011) has been reported. In both studies sham combinations (e.g., IAN:IAN, EBAC:EBAC), true combinations (e.g., IAN:BRAN, EBAC:ECAC) and S_N2-reactive:nonpolar narcotic (S_N2:NPN) combinations were tested. To facilitate discussion of the current work alongside results of those previous studies, concentration-response data from the xAN study (Dawson et al. 2010) were re-evaluated using the 5PL-1P curve-fitting function (instead of the standard 4PL used therein). Also, mAQ, mIQ, DV-A and DV-I values were calculated for all combinations from both previous studies. Those results appear in the Appendix (Tables 4-7).

Toxicity of the Single-Chemicals

Mean toxicity values for each chemical tested alone in this study (Table 1) were included with those published previously (Dawson et al. 2010 Dawson et al. 2011) for comparison (see Table 4 - Appendix). Coefficient of variation (CV) values for EC₅₀ and slope of the single chemicals tested in this study were always <25, typically <15 (95.2%) and frequently <10 (78.6%); thereby comparing similarly with the single chemical data combined from all three studies [i.e., <25 (100%), <15 (89.6%), and <10 (68.8%); Table 4 – see Appendix]. Such CV values have been reported to reflect low to very low levels of variability across multiple tests of the same chemical and are within acceptable limits for toxicity bioassays (Bantle et al. 1994; Parkhurst et al. 1992). Mean asymmetry (s) and coefficient of determination (r²) values for chemicals tested in this study (Table 1) and for the three studies combined (Table 4 – Appendix) are presented for comparative and archival purposes.

Single chemical toxicity was also quantified on a time-dependent basis, giving TDT values. Mean TDT values between 15 and 45 min (Table 1) were consistent with those obtained earlier (Dawson et al. 2010 Dawson et al. 2011: Table 4 – Appendix) and indicated that the

toxicities of EIAC, EBAC, IAN, BRAN and CLAN alone were fully time-dependent, while that for ECAC and EFAC showed high and minimal time-dependency, respectively. Earlier papers have suggested that TDT values reflect relative levels of chemical electro(nucleo)philic reactivity (Dawson et al. 2010 Dawson et al. 2011) when coupled with glutathione-reactivity results (Schultz et al. 2005). This information has been used to help characterize results of these mixture toxicity tests.

Toxicity of ExAC:xAN Mixtures with Review of the Two Previous Studies

For the various ExAC:xAN combinations, mixture toxicity was generally consistent with dose-addition (Table 2) and with independence (Table 3) for combinations in which both chemicals showed fully time-dependent toxicity (i.e., EIAC or EBAC with IAN, BRAN or CLAN). When a chemical with a lower TDT level (i.e., ECAC, EFAC) was tested with one of the xANs, toxicity consistent with dose-addition was more commonly observed after 30 and 45-min exposures than at 15-min. However, the ECAC:xAN and EFAC:xAN combinations did not show improved consistency with independence as exposure time increased. In those instances where consistency with independence was observed it was typically only with EC₅₀-IQ values and not with the mIQ or DV-I metrics.

Sham combination results showed that mixture toxicity for all xAN-shams (e.g., BRAN:BRAN), ExAC-shams (e.g., EIAC:EIAC), and the 3M2B-sham mixture were consistent with (or in one instance bordered on) the dose-addition range at each exposure duration (Appendix – Table 5, upper section). In contrast, only the xAN-sham mixtures showed across-exposure duration consistency with independence. The ExAC-sham and 3M2B-sham mixtures showed little or no consistency with independence (Appendix – Table 5, lower section). These results suggest some difference, perhaps in molecular bonding, reactivity or toxic action, between ExACs and xANs.

Results for non-sham xAN:xAN combinations (e.g., IAN:BRAN) were fully consistent with dose addition and typically consistent with independence (Appendix – Table 6, top three lines of upper and lower sections). The DV-A values for these combinations (0.01-0.04) were generally lower than the corresponding DV-I values.

Non-sham ExAC:ExAC combinations (e.g., EIAC-EBAC) showed more varied results (Appendix – Table 6). Once again combinations that included ECAC or EFAC showed fewer instances of consistency with dose-addition. Consistency with independence was limited to the EC₅₀-IQ metric at the 15-min exposure duration only. All other metrics were inconsistent with a combined effect expected for independence (Appendix – Table 6, lower section lines 4-9). With increasing exposure duration, mIQ and DV-I values increased, thereby becoming more distant from consistency with independence.

Each chemical in the two sets of S_N2-reactive chemicals (xANs, ExACs) was tested with the model nonpolar narcotic 3-methyl-2-butanone (3M2B). The latter chemical had negative time-dependent toxicity values and lacked electro(nucleo)philic reactivity (Dawson et al. 2010; Dawson et al 2011). These tests were performed to provide a common frame of reference for evaluating mixture toxicity between the xANs and the ExACs. For the xAN:3M2B combinations, some AQ metric values were consistent with dose-addition at the early

exposure durations, but the values increased thereafter so that by 45-min none reflected dose-addition (Appendix - Table 7, upper section lines 1-3). This was also the case for xAN:3M2B mixtures evaluated against independence (Appendix - Table 7, lower section lines 1-3). For the ExAC:3M2B mixtures, mAQ and DV-A values were both consistent with dose-addition only with EFAC (the S_N2 chemical with minimal TDT) at 15-min but not thereafter (Appendix - Table 7, upper section line 7). The EFAC:3M2B mixture always had mIQ and DV-I values that were outside the independence range. The other three combinations frequently had EC₅₀-IQ and mIQ values consistent with independence, but the DV-I values were usually above 0.10 (Appendix - Table 7, lower section line 4-6).

For all three studies, the results can be generally summarized as: 1) sham combinations of all chemicals were consistent with dose-addition, irrespective of their relative reactivity and TDT levels, 2) xAN-shams were also consistent with independence, but ExAC- and 3M2B-shams were not and became even less so with increased exposure time, 3) non-sham mixtures of S_N2-reactive chemicals that showed rapid relative reactivity and had toxicity that was fully time-dependent (i.e., EIAC, EBAC, IAN, BRAN, CLAN) were generally consistent with dose-addition, irrespective of whether the combinations were xAN:xAN, ExAC:ExAC or ExAC:xAN, 4) non-sham mixtures of xANs were also consistent with independence but those for ExACs were not, 5) the more slowly reacting, lower TDT-level ExACs (i.e., ECAC, EFAC) when given with an xAN produced toxicity that became consistent with dose-addition as exposure duration increased; but this was typically not the case when they were tested with the fast-reacting ExACs (i.e., EIAC, EBAC), 7) xAN:3M2B combinations tended to have toxicity less-than that predicted for both dose-addition and independence, especially as exposure duration increased, and 8) ExAC:3M2B mixtures tended to have toxicity slightly greater-than that predicted for dose-addition across exposure durations.

Conclusions

Across the three studies, mixture toxicity was sometimes consistent with and sometimes inconsistent with the dose-addition and/or independence models. The areas of inconsistency appear to be related to: 1) differences in the capability of the two groups of S_N2-reactive chemicals to form strong H-bonds with water and 2) differences in relative reactivity and time-dependent toxicity levels of the chemicals.

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Appendix

Table 4
Microtox-derived mean single-chemical toxicity data
with coefficient of variation (CV) values

Agent ^a	t ^b	EC ₅₀ ^c (CV)	slope ^d (CV)	s ^e	r ²	TDT ^f
EIAC	15	0.31 (16.2)	1.45 (5.6)	0.65	0.9982	
	30	0.13 (19.2)	1.83 (6.0)	0.36	0.9989	
	45	0.08 (20.3)	2.00 (5.4)	0.27	0.9992	113
EBAC	15	1.09 (6.7)	1.59 (7.9)	0.51	0.9990	
	30	0.43 (10.0)	1.86 (7.0)	0.32	0.9994	
	45	0.24 (12.3)	2.07 (9.2)	0.23	0.9994	118
ECAC	15	88.6 (7.8)	0.80 (6.7)	1.61	0.9982	
	30	55.9 (7.2)	0.91 (7.4)	1.14	0.9979	
	45	37.5 (7.3)	1.09 (7.4)	0.64	0.9982	87
EFAC	15	1207 (12.5)	0.64 (7.7)	2.86	0.9943	
	30	1143 (12.0)	0.85 (12.9)	0.94	0.9915	
	45	1129 (15.1)	1.16 (24.3)	0.53	0.9908	9
IAN	15	3.04 (7.7)	1.66 (6.6)	0.76	0.9989	
	30	1.49 (5.7)	1.91 (9.0)	0.58	0.9994	
	45	0.96 (5.2)	2.07 (9.0)	0.49	0.9995	102
BRAN	15	2.82 (2.7)	1.80 (8.5)	0.62	0.9983	
	30	1.34 (2.9)	1.92 (5.3)	0.55	0.9992	
	45	0.83 (3.1)	1.94 (3.9)	0.54	0.9993	106
CLAN	15	159.4 (4.6)	1.82 (10.1)	0.62	0.9989	
	30	73.2 (4.0)	1.92 (5.4)	0.52	0.9994	
	45	44.8 (3.8)	1.93 (7.0)	0.50	0.9995	108
3M2B ^g	15	38.9 (13.2)	0.84 (12.3)	2.08	0.9989	
	30	41.9 (13.2)	0.83 (6.7)	1.99	0.9988	
	45	44.0 (13.9)	0.85 (8.5)	1.74	0.9988	- 16

^a includes data from this study and Dawson et al. 2010; 2011

^b exposure duration (min)

^c mean EC₅₀ – in mg/L (CV)

^d mean slope of concentration-response curve (CV)

^e asymmetry parameter value

^f mean time-dependent toxicity value: 15-45 min exposure (%)

^g 3M2B – 3-methyl-2-butanone, a nonpolar narcotic (NPN)

Table 5
Dose-addition and independence metrics for toxicity of
sham combinations^a

Agent A	Agent B	15-min			30-min			45-min		
		EC ₅₀ -AQ ^b	mAQ ^c	DV-A ^d	EC ₅₀ -AQ	mAQ	DV-A	EC ₅₀ -AQ	mAQ	DV-A
3M2B	3M2B	0.97^e	0.95	0.05	0.98	0.97	0.03	1.02	1.00	0.01
IAN	IAN	1.02	1.03	0.03	1.01	1.01	0.01	1.02	1.03	0.03
BRAN	BRAN	1.00	1.00	0.01	1.01	1.00	0.01	1.05	1.05	0.05
CLAN	CLAN	0.99	0.98	0.02	0.99	0.99	0.01	1.01	1.01	0.01
CLAN	CLAN	0.95	0.95	0.05	0.94	0.93	0.07	0.98	0.96	0.04
EIAC	EIAC	1.00	1.00	0.02	0.93	0.93	0.07	0.90	0.89	0.11
EBAC	EBAC	1.02	1.04	0.04	1.05	1.06	0.06	1.00	1.02	0.03
ECAC	ECAC	1.01	1.00	0.02	1.01	1.02	0.03	0.98	0.99	0.02
EFAC	EFAC	0.94	0.94	0.06	1.00	0.98	0.02	1.02	1.00	0.02
Agent A	Agent B	EC ₅₀ -IQ ^f	mIQ ^g	DV-I ^h	EC ₅₀ -IQ	mIQ	DV-I	EC ₅₀ -IQ	mIQ	DV-I
3M2B	3M2B	1.18	1.21	0.27	1.22	1.27	0.28	1.26	1.32	0.32
IAN	IAN	0.90ⁱ	0.92	0.09	0.91	0.93	0.07	0.95	0.96	0.04
BRAN	BRAN	0.92	0.94	0.06	0.94	0.95	0.05	0.99	1.00	0.01
CLAN	CLAN	0.92	0.93	0.09	0.94	0.96	0.04	0.97	0.99	0.03
CLAN	CLAN	0.88	0.91	0.09	0.89	0.90	0.10	0.92	0.93	0.07
EIAC	EIAC	1.04	1.07	0.09	1.07	1.09	0.09	1.15	1.14	0.14
EBAC	EBAC	1.14	1.19	0.19	1.31	1.33	0.33	1.42	1.43	0.43
ECAC	ECAC	1.29	1.35	0.35	1.30	1.37	0.37	1.32	1.38	0.38
EFAC	EFAC	1.31	1.46	0.49	1.46	1.52	0.52	1.56	1.56	0.56

^aData from Dawson et al. 2010; 2011; data from the former were re-evaluated by 5PL-1P curve-fitting analysis

^bEC₅₀-AQ – EC₅₀-additivity quotient = experimental EC₅₀ / predicted dose-addition EC₅₀

^cmAQ – mean additivity quotient = EC₅₀-AQ + EC₅₀-AQ + EC₅₀-AQ / 3

^dDV-A – mean additivity quotient deviation value (see text for details)

^evalues in bolded typeface are consistent with dose-addition

^fEC₅₀-IQ – EC₅₀-independence quotient = experimental EC₅₀ / predicted independence EC₅₀

^gmIQ – mean independence quotient = EC₂₅-IQ + EC₅₀-IQ + EC₇₅-IQ / 3

^hDV-I – mean independence quotient deviation value (see text for details)

ⁱvalues in bolded, italicized typeface are consistent with independence

Table 6
Dose-addition and independence metrics for toxicity of
non-sham combinations^a

Agent A	Agent B	15-min			30-min			45-min		
		EC ₅₀ -AQ ^b	mAQ ^c	DV-A ^d	EC ₅₀ -AQ	mAQ	DV-A	EC ₅₀ -AQ	mAQ	DV-A
IAN	BRAN	1.00^e	0.99	0.02	1.01	1.00	0.02	1.00	0.99	0.01
IAN	CLAN	0.96	0.96	0.04	0.96	0.96	0.04	0.99	0.99	0.01

Agent A	Agent B	15-min			30-min			45-min		
		EC ₅₀ -AQ ^b	mAQ ^c	DV-A ^d	EC ₅₀ -AQ	mAQ	DV-A	EC ₅₀ -AQ	mAQ	DV-A
BRAN	CLAN	1.04	1.04	0.04	1.04	1.03	0.03	1.05	1.04	0.04
EIAC	EBAC	1.02	1.04	0.04	1.02	1.04	0.04	1.00	1.02	0.02
EIAC	ECAC	1.02	1.00	0.02	1.07	1.08	0.09	1.07	1.08	0.08
EIAC	EFAC	1.12	1.18	0.18	1.26	1.33	0.33	1.55	1.58	0.58
EBAC	ECAC	1.07	1.08	0.10	1.09	1.11	0.11	1.08	1.11	0.11
EBAC	EFAC	0.86	0.90	0.10	0.95	1.02	0.11	1.13	1.23	0.23
ECAC	EFAC	0.82	0.85	0.15	0.83	0.90	0.10	0.84	0.94	0.08
		EC ₅₀ -IQ ^f	mIQ ^g	DV-I ^h	EC ₅₀ -IQ	mIQ	DV-I	EC ₅₀ -IQ	mIQ	DV-I
IAN	BRAN	<i>0.91</i> ⁱ	0.92	<i>0.08</i>	<i>0.93</i>	<i>0.94</i>	<i>0.06</i>	<i>0.94</i>	<i>0.95</i>	<i>0.05</i>
IAN	CLAN	0.87	0.89	0.11	0.88	<i>0.90</i>	<i>0.10</i>	<i>0.92</i>	<i>0.94</i>	<i>0.07</i>
BRAN	CLAN	<i>0.96</i>	<i>0.97</i>	<i>0.04</i>	<i>0.96</i>	<i>0.97</i>	<i>0.04</i>	<i>0.97</i>	<i>0.98</i>	<i>0.04</i>
EIAC	EBAC	<i>1.10</i>	1.14	0.14	1.22	1.25	0.26	1.34	1.35	0.35
EIAC	ECAC	1.17	1.18	0.18	1.32	1.36	0.36	1.41	1.44	0.44
EIAC	EFAC	1.42	1.52	0.52	1.80	1.89	0.89	2.28	2.27	1.28
EBAC	ECAC	1.26	1.33	0.33	1.37	1.42	0.42	1.49	1.54	0.54
EBAC	EFAC	<i>1.06</i>	1.17	0.21	1.35	1.49	0.50	1.77	1.92	0.92
ECAC	EFAC	<i>1.06</i>	1.19	0.30	1.20	1.36	0.36	1.32	1.51	0.51

^aData from Dawson et al. 2010; 2011; data from the former were re-evaluated by 5PL-1P curve-fitting analysis

^bEC₅₀-AQ – EC₅₀-additivity quotient = experimental EC₅₀ / predicted dose-addition EC₅₀

^cmAQ – mean additivity quotient = EC₂₅-AQ + EC₅₀-AQ + EC₇₅-AQ / 3

^dDV-A – mean additivity quotient deviation value (see text for details)

^evalues in bolded typeface are consistent with dose-addition

^fEC₅₀-IQ – EC₅₀-independence quotient = experimental EC₅₀ / predicted independence EC₅₀

^gmIQ – mean independence quotient = EC₂₅-IQ + EC₅₀-IQ + EC₇₅-IQ / 3

^hDV-I – mean independence quotient deviation value (see text for details)

ⁱvalues in bolded, italicized typeface are consistent with independence

Table 7
Dose-addition and independence metrics for toxicity of
S_N2-reactive:nonpolar narcotic combinations^a

Agent A	Agent B	15-min			30-min			45-min		
		EC ₅₀ -AQ ^b	mAQ ^c	DV-A ^d	EC ₅₀ -AQ	mAQ	DV-A	EC ₅₀ -AQ	mAQ	DV-A
IAN	3M2B	1.11	1.09^e	0.09	1.15	1.15	0.15	1.18	1.18	0.18
BRAN	3M2B	1.11	1.08	0.08	1.17	1.17	0.17	1.26	1.28	0.28
CLAN	3M2B	1.06	1.08	0.13	1.07	1.08	0.10	1.34	1.39	0.39
EIAC	3M2B	0.90	0.87	0.13	0.89	0.89	0.11	0.86	0.88	0.12
EBAC	3M2B	0.91	0.88	0.12	0.86	0.86	0.14	0.87	0.89	0.11
ECAC	3M2B	0.79	0.78	0.22	0.78	0.78	0.22	0.77	0.80	0.20
EFAC	3M2B	0.87	0.92	0.08	0.86	0.91	0.14	0.88	0.99	0.21

Agent A	Agent B	15-min			30-min			45-min		
		EC ₅₀ -AQ ^b	mAQ ^c	DV-A ^d	EC ₅₀ -AQ	mAQ	DV-A	EC ₅₀ -AQ	mAQ	DV-A
		EC ₅₀ -IQ ^f	mIQ ^g	DV-I ^h	EC ₅₀ -IQ	mIQ	DV-I	EC ₅₀ -IQ	mIQ	DV-I
IAN	3M2B	1.06ⁱ	1.11	0.14	1.13	1.18	0.20	1.24	1.28	0.28
BRAN	3M2B	0.97	0.99	0.07	1.15	1.20	0.20	1.26	1.32	0.32
CLAN	3M2B	1.00	1.05	0.17	1.16	1.19	0.23	1.45	1.55	0.55
EIAC	3M2B	0.97	0.99	0.12	1.04	1.07	0.16	1.03	1.07	0.13
EBAC	3M2B	0.98	0.98	0.08	1.02	1.04	0.11	1.08	1.10	0.14
ECAC	3M2B	0.97	1.00	0.13	0.96	1.01	0.10	0.99	1.05	0.06
EFAC	3M2B	1.07	1.20	0.30	1.12	1.32	0.39	1.18	1.40	0.48

^aData from Dawson et al. 2010; 2011; data from the former were re-evaluated by 5PL-1P curve-fitting analysis

^bEC₅₀-AQ – EC₅₀-additivity quotient = experimental EC₅₀ / predicted dose-addition EC₅₀

^cmAQ – mean additivity quotient = EC₂₅-AQ + EC₅₀-AQ + EC₇₅-AQ / 3

^dDV-A – additivity quotient deviation value (see text for details)

^evalues in bolded typeface are consistent with dose-addition

^fEC₅₀-IQ – EC₅₀-independence quotient = experimental EC₅₀ / predicted independence EC₅₀

^gmIQ – mean independence quotient = EC₂₅-IQ + EC₅₀-IQ + EC₇₅-IQ / 3

^hDV-I – independence quotient deviation value (see text for details)

ⁱvalues in bolded, italicized typeface are consistent with independence

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Figure 1.

Concentration-effect (inhibition of bioluminescence) curves for ethyl bromoacetate (EBAC), iodoacetonitrile (IAN) and the EBAC:IAN mixture after 15-min of exposure, along with predicted curves for the dose-addition and independence combined effects models. Actual mixture toxicity was consistent with both models up to about 80% effect where it then became separated from that of the predicted independence curve.

Figure 2.

Concentration-effect (inhibition of bioluminescence) curves for ethyl chloroacetate (ECAC), bromoacetonitrile (BRAN) and the ECAC:BRAN mixture after 15-min of exposure, along with predicted curves for the dose-addition and independence combined effects models. Actual mixture toxicity clearly deviated from that expected for both models above the 30% effect level, being less-than that expected for each model.

Figure 3.

Concentration-effect (inhibition of bioluminescence) curves for ethyl fluoroacetate (EFAC), iodoacetonitrile (IAN) and the EFAC:IAN mixture after 15-min of exposure, along with predicted curves for the dose-addition and independence combined effects models. Actual mixture toxicity was greater-than that predicted for dose-addition below about 70% effect and less-than that predicted for dose-addition above about 85% effect. Likewise, actual mixture toxicity crossed the predicted independence curve as well, but was consistent with it only between about 50 and 60% effect, thereby highlighting the value of evaluating mixture toxicity along the entire concentration-response curve.

Table 1
Microtox-derived mean single-chemical toxicity data with coefficient of variation (CV) values

Agent ^{a,b}	t ^c	EC ₅₀ ^d (CV)	slope ^e (CV)	s ^f (CV)	r ²	TD ₅₀ ^g
EIAc ^a	15	0.29 (5.7)	1.50 (4.1)	0.59 (4.1)	0.9978	
	30	0.12 (7.0)	1.80 (1.6)	0.36 (1.6)	0.9987	
	45	0.07 (6.4)	1.97 (2.4)	0.26 (2.4)	0.9992	114
EBAC ^a	15	1.09 (11.0)	1.64 (11.4)	0.49 (11.4)	0.9991	
	30	0.44 (16.0)	1.97 (2.4)	0.28 (2.4)	0.9994	
	45	0.24 (17.0)	2.19 (4.9)	0.20 (4.9)	0.9994	117
ECAC ^a	15	93.1 (5.3)	0.79 (2.9)	1.56 (2.9)	0.9979	
	30	59.3 (7.9)	0.88 (4.6)	1.30 (4.6)	0.9976	
	45	39.8 (4.3)	1.04 (4.8)	0.72 (4.8)	0.9981	98
EFAC ^a	15	1358 (4.3)	0.69 (5.2)	1.53 (5.2)	0.9937	
	30	1286 (0.3)	0.97 (1.8)	0.63 (1.8)	0.9907	
	45	1327 (3.3)	1.48 (6.9)	0.31 (6.9)	0.9908	3
IAN ^b	15	3.11 (11.3)	1.60 (7.6)	0.78 (7.6)	0.9986	
	30	1.52 (8.2)	1.82 (11.0)	0.59 (11.0)	0.9992	
	45	0.98 (7.5)	2.00 (10.8)	0.50 (10.8)	0.9993	102
BRAN ^b	15	2.81 (3.4)	1.79 (7.9)	0.59 (7.9)	0.9981	
	30	1.31 (1.9)	1.94 (6.3)	0.51 (6.3)	0.9992	
	45	0.82 (1.6)	1.93 (3.4)	0.52 (3.4)	0.9994	106
CLAN ^b	15	162.4 (5.5)	1.75 (11.6)	0.62 (11.6)	0.9985	
	30	72.9 (4.6)	1.88 (8.5)	0.52 (8.5)	0.9992	
	45	45.0 (3.3)	1.92 (12.5)	0.50 (12.5)	0.9993	108

^a halogenated ethyl acetates (ExACs) – mean of three tests

^b halogenated acetonitriles (xANs) – mean of four tests

^c exposure duration (min)

^d mean EC₅₀ – in mg/L (CV)

e mean slope of concentration-response curve (CV)

f asymmetry parameter value

g mean time-dependent toxicity value: 15-45 min exposure (%)

Table 2
Dose-addition metrics for the toxicity of ethyl α -halogenated acetate: α -halogenated acetonitrile mixtures

Agent A ^a	Agent B ^b	15-min			30-min			45-min		
		EC ₅₀ -AQ ^c	mAQ ^d	DV-A ^e	EC ₅₀ -AQ	mAQ	DV-A	EC ₅₀ -AQ	mAQ	DV-A
EIAC	IAN	0.95 ^f	0.96	0.04	0.96	0.97	0.04	0.95	0.97	0.04
EIAC	BRAN	0.98	0.98	0.03	0.96	0.97	0.04	0.93	0.93	0.07
EIAC	CLAN	0.95	0.93	0.07	0.97	0.96	0.04	0.96	0.94	0.06
EBAC	IAN	0.98	0.99	0.01	0.94	0.97	0.04	0.92	0.94	0.06
EBAC	BRAN	0.99	0.98	0.04	0.97	0.99	0.02	0.96	0.99	0.06
EBAC	CLAN	0.98	0.98	0.02	0.95	0.98	0.05	0.94	0.99	0.08
ECAC	IAN	1.12	1.18	0.18	1.03	1.06	0.07	0.95	0.99	0.05
ECAC	BRAN	1.14	1.18	0.18	1.12	1.15	0.15	1.06	1.09	0.09
ECAC	CLAN	1.10	1.14	0.14	1.05	1.05	0.09	1.03	1.06	0.06
EFAC	IAN	0.84	0.87	0.13	0.91	0.97	0.06	0.94	0.97	0.10
EFAC	BRAN	0.98	1.00	0.01	0.93	0.94	0.07	0.95	0.97	0.07
EFAC	CLAN	0.95	0.98	0.06	0.91	0.93	0.07	0.91	0.94	0.07

^a EIAC – ethyl iodoacetate, EBAC – ethyl bromoacetate, ECAC – ethyl chloroacetate, EFAC – ethyl fluoroacetate

^b IAN – iodoacetonitrile, BRAN – bromoacetonitrile, CLAN – chloroacetonitrile

^c EC₅₀-AQ - EC₅₀-additivity quotient = experimental EC₅₀ / predicted dose-addition EC₅₀

^d mAQ – mean additivity quotient = EC₅₀-AQ + EC₅₀-AQ + EC₅₀-AQ / 3

^e DV-A – additivity quotient deviation value (see text for calculation details)

^f values in bold typeface are consistent with dose-addition

Table 3
Independence metrics for the toxicity of ethyl α -halogenated acetate: α -halogenated acetonitrile mixtures

Agent A ^a	Agent B ^b	15-min			30-min			45-min		
		EC ₅₀ -IQ ^c	mIQ ^d	DV-I ^e	EC ₅₀ -IQ	mIQ	DV-I	EC ₅₀ -IQ	mIQ	DV-I
EIAC	IAN	0.94^f	0.97	0.07	0.98	1.01	0.02	1.02	1.04	1.04
EIAC	BRAN	0.98	0.99	0.06	1.01	1.03	0.03	0.98	0.99	0.03
EIAC	CLAN	0.93	0.93	0.07	0.97	0.98	0.03	0.99	0.99	0.04
EBAC	IAN	0.96	0.99	0.05	0.97	1.00	0.02	0.98	1.01	0.04
EBAC	BRAN	1.00	1.01	0.07	1.07	1.10	0.10	1.08	1.11	0.11
EBAC	CLAN	0.99	1.01	0.02	1.02	1.07	0.07	1.07	1.13	0.13
ECAC	IAN	1.18	1.30	0.30	1.10	1.16	0.18	1.04	1.11	0.11
ECAC	BRAN	1.20	1.30	0.31	1.20	1.27	0.28	1.17	1.23	0.23
ECAC	CLAN	1.20	1.30	0.30	1.13	1.17	0.21	1.14	1.20	0.20
EFAC	IAN	0.93	1.03	0.19	1.10	1.20	0.20	1.15	1.21	0.21
EFAC	BRAN	1.15	1.21	0.21	1.08	1.12	0.13	1.17	1.20	0.20
EFAC	CLAN	1.06	1.13	0.13	1.10	1.15	0.15	1.15	1.19	0.19

^a EIAC – ethyl iodoacetate, EBAC – ethyl bromoacetate, ECAC – ethyl chloroacetate, EFAC – ethyl fluoroacetate

^b IAN – iodoacetonitrile, BRAN – bromoacetonitrile, CLAN – chloroacetonitrile

^c EC₅₀-IQ – EC₅₀-independence quotient = experimental EC₅₀ / predicted independence EC₅₀

^d mIQ – mean independence quotient = EC₂₅-IQ + EC₅₀-IQ + EC₇₅-IQ / 3

^e DV-I – independence quotient deviation value (see text for calculation details)

^f values in bolded, italicized typeface are consistent with independence