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Time-dependence in mixture toxicity prediction

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Abstract

The value of time-dependent toxicity (TDT) data in predicting mixture toxicity was examined. Single chemical (A and B) and mixture (A + B) toxicity tests using Microtox® were conducted with inhibition of bioluminescence (*Vibrio fischeri*) being quantified after 15, 30 and 45-min of exposure. Single chemical and mixture tests for 25 sham (A_1:A_2) and 125 true (A:B) combinations had a minimum of seven duplicated concentrations with a duplicated control treatment for each test. Concentration/response (*x*/*y*) data were fitted to sigmoid curves using the five-parameter logistic minus one parameter (5PL-1P) function, from which slope, EC_{25}, EC_{50}, EC_{75}, asymmetry, maximum effect, and r^2 values were obtained for each chemical and mixture at each exposure duration. Toxicity data were used to calculate percentage-based TDT values for each individual chemical and mixture of each combination. Predicted TDT values for each mixture were calculated by averaging the TDT values of the individual components and regressed against the observed TDT values obtained in testing, resulting in strong correlations for both sham (r^2 = 0.989, n = 25) and true mixtures (r^2 = 0.944, n = 125). Additionally, regression analyses confirmed that observed mixture TDT values calculated for the 50% effect level were somewhat better correlated with predicted mixture TDT values than at the 25 and 75% effect levels. Single chemical and mixture TDT values were classified into five levels in order to discern trends. The results suggested that the ability to predict mixture TDT by averaging the TDT of the single agents was modestly reduced when one agent of the combination had a positive TDT value and the other had a minimal or negative TDT value.

Keywords

Microtox®; Soft electrophiles; Acute toxicity; Time-dependent toxicity

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Conflict of interest
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1. Introduction

Recent mixture toxicity research has been wide-ranging. Such research has included in vitro and in vivo studies (Boyd et al., 2013; Cedergreen et al., 2012; Coors et al., 2012), toxicity assessment using combined effects models (Crépet et al., 2013; Hertzberg et al., 2013; Moser et al., 2012; Rider et al., 2008; Webster, 2013), evaluating stressor impacts environmentally (Allan et al., 2012; Florian et al., 2013; Løkke 2010), risk assessment studies (Johnson et al., 2013; Løkke et al., 2013; Meek, 2013; Moore and Teed, 2013) and examining chemical reactivity within complex mixtures (Goel et al., 2013). Although the specifics of such research vary, the common goal is improving the ability to predict the effects of exposure to chemical mixtures.

The Microtox® acute test utilizes bioluminescence in the marine bacterium *Vibrio fischeri* to assess the toxicity of organic chemicals, effluents, and chemical mixtures. When at a sufficient concentration, toxicants will inhibit bioluminescence, which is read by a calibrated light meter, and the effect is determined relative to light emitted by the control samples. Since reduced bioluminescence can be the result of inhibition of bacterial metabolism, bacterial death, or both, it is effective for evaluating reversible and irreversible toxic effects that may be caused by a single toxicant or mixture of chemicals. The system allows the operator to read light levels for all vials prior to, during and at the end of chemical exposure.

The toxicity of a given chemical or chemical mixture to a living organism may increase, decrease, or remain the same over exposure time; any such change is referred to as time-dependent toxicity (TDT). With Microtox® one can determine effects of a chemical on bacterial luminescence at up to three exposure times to observe such changes in toxicity. Those changes can be quantified and converted to a percentage basis to allow for comparison.

Log-linear plots of concentration/response (*x*/*y*) data collected over multiple exposure times allow one to visualize the magnitude of toxicity change for a given chemical (e.g., Dawson et al., 2011 -Fig. 2). In such plots a high level of TDT (e.g., 80%) shows that the concentration-response curve for a longer exposure duration is left-shifted from that of a shorter one. With a lower TDT level (e.g., 30%), while there is still a left-shift for the longer duration curve vs. the shorter one, the curves will be closer together. A chemical with no change in toxicity over exposure time (e.g., TDT = 0%) has *x*/*y* curves for the longer and shorter exposures that overlap. Chemicals with negative TDT values show reduced toxicity (i.e., some recovery) with increased exposure time, resulting in the curve for a longer exposure duration being slightly right-shifted from the shorter duration curve.

Initial examination of chemical mixture toxicity using Microtox®, as conducted herein, included a chemical reactivity perspective developed from results of work by Schultz et al. (2005). The initial study highlighted the importance of assessing changes in toxicity over exposure time (Dawson et al., 2006). Subsequent mixture studies evaluated TDT and mixture toxicity for: (a) selected soft electrophiles with a non-reactive chemical (Gagan et al., 2007), (b) Michael acceptors with varying levels of electro(nucleo) philic reactivity (Dawson et al., 2008), and (c) chemicals reactive by the bimolecular nucleophilic

substitution (SN2) mechanism (Dawson et al., 2010, 2011, 2014). Chemical selection in these studies considered relative reactivity levels (e.g., very fast to very slow or no reactivity) and TDT in order to assess combined effects against the dose-addition (e.g., Chen et al., 2001) and independence (Bliss, 1939) models of combined effects. These studies were conducted to examine whether the actual mixture toxicity observed (e.g., greater-than dose-additive, dose-additive, less-than dose-additive) might be related to the agents having common or different reaction mechanisms, such as Michael addition, aliphatic substitution, aromatic substitution, or to a lack of reactivity. To date no clear mixture toxicity patterns have emerged from these studies, perhaps in part due to some chemicals having reversible toxicity at lower concentrations and irreversible toxicity at higher concentrations and/or to some chemicals being more rapidly reactive than others.

One feature of these studies, though, was the finding that the *x*/*y* data were well-fitted by a logistic function that incorporated four parameters: slope, asymmetry, EC_{50} and maximum effect. This approach involved modifying a 5-parameter logistic function by removing the minimum effect parameter. Therefore, this curve-fitting technique was referred to as the five-parameter logistic minus one parameter (5PL-1P) function (Dawson et al., 2012). The study showed that the 5PL-1P function typically gave improved fitting of *x*/*y* data vs. the standard four-parameter logistic function (which employed the minimum effect parameter but not the asymmetry parameter) and suggested that changes in slope, asymmetry, and toxicity over exposure time could be useful in predicting mixture toxicity.

A second feature of this Microtox® research was the testing of sham combinations. In mixture toxicity, a sham combination is defined as a test in which two separate stock solutions of a single chemical are tested as a mixture. This was done to assess whether the “sham mixture” would cause toxicity consistent with that predicted by the dose-addition model of combined effect (e.g., Dawson et al., 2010). In dose-addition the toxic effect can be predicted based simply on the level of increase in the dose applied. When a sham combination produces a dose-additive combined effect at both shorter and longer exposure durations, one can hypothesize that the TDT of the “mixture” should be about the average of the TDT values of the individual “components”. By extension then, it can be suggested that the same might hold for a “true” mixture showing dose-addition. If that is the case, then other questions arise, such as would this also be expected for: (1) a mixture that was not dose-additive and (2) a mixture in which one component had large changes in toxicity over time while the other component had minimal change in TDT or showed some recovery from the toxic effects (i.e., negative TDT)?

Therefore, in this report the data from published (Dawson et al., 2006, 2008, 2010, 2011, 2014; Gagan et al., 2007) and unpublished Microtox® mixture toxicity experiments were compiled solely to assess the potential value of TDT determinations in predicting mixture toxicity. While combined effects were determined for all the combinations evaluated in this study, those results were not included herein because they were outside the scope of this paper. The specific question addressed in this study was: can TDT of binary mixtures (A + B) be well-predicted by averaging the individual TDT values of the mixture components, i.e., A and B?

2. Materials and methods

2.1. Chemicals and reagents

Chemicals selected for toxicity testing (Table 1) were obtained from Sigma–Aldrich (Milwaukee, WI) at 95% to 99+% purity and used as received. Microtox® supplies (bacterial reagent, reconstitution solution and diluent) were obtained from Modern Water, Inc. (New Castle, DE).

2.2. Toxicity testing

A Microtox® 500 analyzer was used to determine inhibition of bioluminescence in the marine bacterium *Vibrio fischeri* following established procedures (Dawson et al., 2014). The experimental protocol used herein defines an experiment as consisting of three toxicity tests: chemical A-alone (A), chemical B-alone (B) and a mixture test (A + B). Some experiments were designed to be "sham" experiments, in which a single chemical was tested twice singly, from two separate preparations (A_1 and A_2), and as a "mixture" ($A_1 + A_2$).

Generally for an A + B combination, the chemical designated A had greater toxic potency than B, but this could vary due to differing levels of time-dependent toxicity of the individual chemicals over exposure time. Concentration selection for each agent, made based on results of preliminary testing, was intended to obtain an approximately equitoxic potency ratio (i.e., 1:1) after 30-min of exposure.

While seven, eight or nine concentrations were tested among the experiments reported herein; within an experiment each test always had the same number of duplicated concentrations and a duplicated control treatment. Concentrations tested were nominal, density corrected and prepared via serial dilution. Within an experiment a single dilution factor was used; being one of the following: 1.6, 1.75, 1.867 or 2.0. The dilution factor was selected to most effectively calculate EC_{25}, EC_{50} and EC_{75} values, based on preliminary test results. The EC_{50} is the half-maximal effective concentration. The EC_{25} and EC_{75} are the one-quarter and three-quarters-maximal effective concentrations, respectively. Initial light readings were taken before chemical exposure. During exposure, readings were taken at 15, 30 and 45-min. Microtox® Omni software automatically calculated % effect values for each concentration replicate at each exposure duration.

2.3. Curve fitting

With three exposure durations, each experiment produced nine concentration-response (x/y) curves (i.e., three each for A, B and A + B). These data were input to SigmaPlot® (v. 11.0; Systat Software, Chicago, IL) and fitted to sigmoid curves using the 5PL-1P function described previously (Dawson et al., 2012). This approach utilized four parameters: EC_{50}, slope, maximum effect and asymmetry; the minimum effect parameter had been removed from the original 5PL function within the software.

Curve fitting was performed using:

$$y = \max \div [1 + (xb \div x)^{\text{slope}}]^s$$

wherein y = % effect, max = maximum effect, x = concentration, s = asymmetry. The variable xb was determined using:

$$xb = EC_{50} \times 10^{[(1 \div \text{slope}) \times \log(2^{(1 \div s)} - 1)]}$$

Initial parameters for regressions were automatically estimated while employing three constraints: (a) $EC_{50}>0$; (b) $0.1<s<10$; and (c) max < 100. For any given test, when the initially calculated maximum effect values at 15,30 and 45-min differed by more than 2.5%, the mean of those maximum effect values was used as the constraint for max, thereby giving consistency in calculating TDT values (see below). For all single-chemical x/y data, EC_{25}, EC_{50}, EC_{75}, slope, asymmetry and maximum effect values were calculated for each exposure duration. The quality of data fitting to the 5PL-1P function was assessed by calculating the coefficient of determination (r^2).

For mixture x/y data, concentrations of chemical B were converted to concentration equivalents of chemical A. The conversion factor used to calculate those equivalent concentrations was determined by dividing the concentration of chemical A by the concentration of chemical B (Dawson et al., 2010). This permitted the total chemical concentration of the mixture to be made relative to those of chemical A alone, while allowing the plot of the mixture curve, at a given exposure duration, to be shown along with the actual concentrations used for chemical A and chemical B individually. The same curve-fitting methods used for the individual chemicals were used for the mixture tests.

2.4. Calculation of TDT values

Calculations of TDT values quantified changes in toxicity over exposure time. Such calculations can be made using data from any exposure time series for which toxicity has been determined and at any effect levels selected. In this study, owing to toxicity measurements being taken at 15, 30 and 45min of exposure and to toxicity being calculated at the 25%, 50% and 75% effect levels, a variety of TDT values were calculated. In essence the TDT values calculated formed a 3 by 3 matrix across exposure time series (15–30, 30–45, 15–45 min) and effect level (25%, 50%, 75%). Calculation of the various TDT values was based on the rationale and approach developed by Haber (1924) using the methodology described below.

Since toxicity was measured at three exposure durations, it was possible to calculate TDT values for each of three time series: (1) 15–30 min, (2) 30–45 min and (3) 15–45 min, when employing the appropriate time factor for the time series being assessed (Gagan et al., 2007). Three time factors were needed, one for each time series. These factors were calculated by dividing the difference between the later time (t_2) and earlier time (t_1) by t_2. Hence, the factor for the 15–30 min series was 15/30 or 0.5, the factor for 30–45 min series was 15/45 or 0.333, and the factor for the 15–45 min series was 30/45 or 0.667.

The following set of equations was then used to calculate TDT:

$$d = EC_{x_{t1}} - EC_{x_{t2}}$$

$$e = d \div (EC_{x_{t1}} \times f_{t1:t2})$$

$$TDT = e \times 100$$

in which EC_x is the effect level, t_2 is the later time of the exposure time series, t_1 is the earlier time of that time series, and $f_{t1:t2}$ is the appropriate factor (see above) for the time series under consideration. Using the 50% effect level for the 15–45 min time series to exemplify the TDT calculation process, the steps were: (a) subtraction of the 45-min EC_{50} from the 15-min EC_{50}; (b) dividing that difference by the product of the 15-min EC_{50} value and 0.667; and (c) multiplying that quotient by 100 to put it on a percentage basis. Hence, for a hypothetical chemical with a 15-min EC_{50} of 15 mg/L and a 45-min EC_{50} of 5 mg/L, the TDT at 50% effect for the 15–45 time series was 100%, as shown:

a. 15 mg/L – 5 mg/L =10 mg/L

b. 10 mg/L ÷ (15 mg/L × 0.667 = 1)

c. 1 × 100 = 100%

Values for TDT_{25} and TDT_{75} were calculated similarly using the respective time factor values (noted above) and EC25 or EC_{75} data, respectively.

Four sets of TDT values are reported herein: (1) the mean $TDT_{15–45}$ values, which were calculated by adding the values at the 25%, 50% and 75% effect levels for the 15–45 min time series and taking the average, and the: (2) mean TDT_{25}, (3) mean TDT_{50}, and (4) mean TDT_{75} values. In each of the latter three cases these were calculated by averaging the $TDT_{15–30}$, $TDT_{30–45}$, and $TDT_{15–45}$ values at the appropriate effect level. Note that for simplicity and readability throughout, that mean $TDT_{15–45}$ values are referred to simply as mean TDT, whereas the mean TDT_{25}, mean TDT_{50} and mean TDT_{75} values are referred to as TDT_{25}, TDT_{50} and TDT_{75} values, respectively.

For each experiment, mean TDT values for A and B were averaged to obtain a predicted mixture mean TDT value. Linear regression analyses were then conducted to evaluate predicted mixture mean TDT values against those obtained experimentally (i.e., observed mixture mean TDT values). Separate regressions were run for the 25 sham (A_1+A_2) and 125 true (A + B) combinations.

Likewise, separate regressions of predicted vs. observed TDT were performed for the TDT_{25}, TDT_{50}, and TDT_{75} data; again considering sham and true mixtures separately.

As a quality control measure, test-to-test variability of TDT values was assessed on individual chemicals for which at least five separate tests had been conducted. Mean, standard error, and 95% confidence intervals for those TDT values were determined.

2.5. TDT classification

After determining mean TDT values for A, B and A + B in each experiment, those values were grouped into one of five TDT classes: (a) full (TDT > 100%), (b) high (TDT = 70–100%), (c) moderate (TDT = 30–69%), (d) low (TDT = 0–29%), or (e) negative (TDT < 0%).

3. Results

3.1. Logistic curve-fitting

For all experiments, the 5PL-1P function was used for initial fitting of *x*/*y* data, with the quality of fitting being assessed by the r^2 value for each test (A, B, A + B) at each exposure duration (15, 30, 45-min). For all *x*/*y* curves, whether single chemical or mixture or whether part of a sham or true combination, the r^2 values are independent of each other. Overall, 98.5% of r^2 values were ≥0.9900 and 92.4% were ≥0.9950. Fully 40% (540/1350) of *x*/*y* curves had an r^2 of 0.9990 or higher with 5PL-1P fitting. The overall mean r^2 (±s.d.) was 0.9980 (±0.0022) and the median value was 0.9988 (Table 2). The mean r^2 values were not normally distributed, as they could fall much farther below the mean than they could rise above it. Therefore, the Kruskal–Wallis One Way Analysis of Variance on Ranks test was used to determine significance across median values, for each exposure duration. For A, B and A + B, the median r^2 values were not statistically different from each other at any common exposure duration (Table 2).

3.2. TDT

As noted above, toxicity that changes over time is time-dependent toxicity. Log-linear plots of *x*/*y* data collected over multiple exposure times allow one to visualize the magnitude of toxicity change for a given chemical. For example, in Fig. 1 typical *x*/*y* curves derived from the 5PL-1P function were compared for two chemicals, bromoacetonitrile (BRAN) and 3-chloro-2-butanone (3C2B). The 15, 30 and 45-min curves for BRAN showed greater separation than those for 3C2B; with the curves for the longer exposure durations being left-shifted from the 15-min curve. Differences in separation of the 15- and 45-min curves were quantified as a higher TDT value for BRAN (106%) than for 3C2B (67%).

3.3. Mixture toxicity - regression of observed TDT vs. predicted TDT

In this study, both sham (A_1 +A_2) and true (A + B) combinations were evaluated and mean TDT values for each single chemical and mixture test were determined (Tables 3 and 4). Separate linear regressions of predicted mixture mean TDT vs. observed mixture mean TDT values for both sham and true mixtures resulted in r^2 values of 0.989 and 0.944, respectively (Table 5, rows 1 and 2; Fig. 2a, b). In addition to the regressions, separate linear regressions (predicted TDT vs. observed TDT) were performed on the TDT_{25}, TDT_{50} and TDT_{75} values; with the sham and true mixtures being analyzed separately. The best correlations were obtained for TDT_{50} (sham = 0.988, true = 0.950; Table 5, rows 10 and 11). Respective comparisons of predicted vs. observed TDT for TDT_{25} (Table 5, rows 6 and 7) and TDT_{75} (Table 5, rows 14 and 15) values showed somewhat lower r^2 values.

Consistency of mean TDT values among chemicals for which at least five separate tests had been conducted (n = 24) resulted in standard error values ranging from ±0.3 to ±7.7 (mean = 2.0; median = 1.6) and 95% confidence intervals between ±0.7% and ±17.1% (mean = 4.7%; median = 3.6%) (Table 6).

3.4. TDT classification

Single chemical mean TDT values obtained for each of the sham and true combinations were separated into five classes: mean TDT: (a) >100% or full TDT, (b) 70–100% or high TDT, (c) 30–69% or moderate TDT, (d) 0–29% or low TDT, and (e) <0% or negative TDT. For the mixtures, a comparison of the mean deviation (%) of observed TDT vs. predicted TDT by TDT level, separated by sham and true combinations, showed that the larger average deviations tended to be associated with chemicals that had mean TDT values that were the furthest apart, e.g., a chemical having full TDT being paired with a chemical having low TDT, and, especially, when a chemical with a positive TDT value was combined with one having a negative TDT (Table 7).

As a consequence of this finding an additional set of regression analyses was performed on predicted TDT vs. observed TDT data for the true mixtures only. True mixtures were separated into two groups: (1) when A and B had the same TDT classification (e.g., full vs. full) or (2) when A and B had different TDT classifications (e.g., full vs. low). Regressions were run for each group. These regressions were conducted separately, for mean TDT, TDT_{25}, TDT_{50} and TDT_{75} values (Table 5, rows 3 and 4, 8 and 9, 12 and 13 and 16 and 17, respectively). In each case the r^2 values were higher when chemicals A and B in the pairing had the same TDT classification, with TDT_{50} values producing the best correlations (same TDT class = 0.984, different TDT class = 0.919; Table 5). The n values for the same and different TDT classifications are different among the mean TDT, TDT_{25}, TDT_{50} and TDT_{75} analyses, because the TDT classification for some agents were different at the various effect levels.

As stated above, higher average differences between predicted TDT and observed TDT were noted when a chemical having a positive TDT value was paired with one having a negative TDT value. To evaluate this finding more fully, 27 true mixtures that contained either 3-methyl-2-butanone (3M2B) or dibromoacetonitrile (DBRAN) were removed from the regression analyses. Both of those chemicals had low or negative TDT values. Since 3M2B had been paired with chemicals having mean TDT values across all five TDT classifications, removal of those pairing was deemed an effective way to assess how much impact the positive-negative TDT pairings had on the predictability of mixture TDT. For DBRAN, mean TDT values were always between −14 and 6% (Table 4), but those values are misleading because DBRAN was so highly reactive that toxicity was produced very quickly and did not change much after 15 min. Together, removal of these 27 data sets from the regression improved the correlation between predicted mixture TDT and observed mixture TDT (r^2 = 0.964; Table 5 - row 5, Fig. 2c).

Finally, to further compare the regressions for the sham, true and true minus 3M2B- and DBRAN-containing mixtures, 95% prediction intervals were calculated. For the sham mixtures (Fig. 2a) at an observed mean TDT of 50.0%, the regression line gave a predicted TDT value of 49.1% with the 95% prediction interval being 37.4–60.8%. For all 125 true mixtures (Fig. 2b) at an observed mean TDT of 50.2%, the predicted mixture TDT was 46.9% and the 95% interval was 29.1–64.6%. For the 98 true mixtures remaining once 3M2B- and DBRAN-containing mixtures were excluded (Fig. 2c), at an observed mean

TDT of 50.2%, the predicted mixture TDT was 47.2%, with the 95% prediction interval being 33.8–60.5%.

4. Discussion

4.1. Curve-fitting

Logistic regression is commonly used to evaluate fitting of toxicity data to a sigmoid curve. Herein, the 5PL-1P function typically gave high quality curve-fitting of the *x*/*y* data, as only 19/1350 curves had an r^2 below 0.9900. Of those nineteen, eighteen were single chemical or mixture curves that contained one of three chemicals: 1-bromo-2,4-dinitrobenzene (BDNB), 1-chloro-2,4-dinitrobenzene (CDNB) and ethyl fluoroacetate (EFAC). However, tests of each of these chemicals alone more often gave 5PL-1P-derived curves with r^2 values ≥0.9900 (BDNB – 30/33 = 90.9%, CDNB – 20/27 = 74.1%, EFAC – 21/27 = 77.8%). The results confirm the finding from an earlier report (Dawson et al., 2012) that the 5PL-1P function is well-suited to fitting Microtox®-derived toxicity data to sigmoid curves.

4.2. TDT

Mixture toxicity studies that include assessing changes in toxicity over time are becoming recognized as being important (Broerse and van Gestel, 2010; Cetojevic-Simin et al., 2013; Schnug et al., 2013; Tarantini et al., 2011). The previous Microtox® studies noted earlier typically reported TDT values for the 15–30, 30–45 and 15–45 min exposure durations; but generally just using EC_{50} data (i.e., TDT_{50}) in TDT calculations (e.g., Dawson et al., 2008). In this study, TDT values were also calculated at the 25% (TDT_{25}) and 75% (TDT_{75}) effect levels, as a means of incorporating data from a larger segment of the *x*/*y* curves. With those additional TDT values being available, mean TDT values were assessed to include TDT information from a range of effect levels along the *x*/*y* curves rather than at just the midpoint.

4.3. Mixture toxicity - regression of observed TDT vs. predicted TDT

In this study, after the visual observation that mean TDT values for mixtures appeared to be about midway between those for chemicals A and B alone, the predicted mean TDT for a given mixture was calculated by taking the average of the mean TDT values for the individual components of the mixture. The mixture toxicity data were divided into two sets: “sham” mixtures (as a control) and “true” mixtures. They were separated because it was hypothesized that TDT for a sham mixture would be close to the average of its individual components, as sham combinations typically show dose-additive toxicity irrespective of TDT level (Dawson et al., 2010, 2011). The high r^2 value obtained herein for observed vs. predicted TDT of sham combinations (0.989) is consistent with that hypothesis.

To determine whether such a correlation existed for the true mixtures, linear regression analysis of observed mean TDT vs. predicted mean TDT was then conducted. This analysis also showed a strong correlation (r^2 = 0.944). These mixtures included combinations that were dose-additive, greater-than dose-additive and less-than dose-additive (e.g., Dawson et al., 2010, 2011, 2014 and unpublished data), so the high correlation was not the result of all the mixtures having the same category of combined effect.

Additionally, TDT values were evaluated by effect level (25, 50 and 75%). While TDT_{50} values gave better predicted vs. observed TDT values for mixture toxicity, high quality (i.e., r^2 values >0.900) equations were typically obtained for regression of TDT_{25} and TDT_{75} data. There was no pattern to TDT values across these effect levels, for example TDT_{25} values were not always lower than those for the higher effect levels, or vice versa, for the single chemical and mixture *x*/*y* curves as a whole. This is likely a result of the chemicals tested having different rates of chemical reactivity and some of the chemicals being non-reactive. For individual chemicals with intermediate levels of TDT, it is possible that toxic effects are due to both inhibition of metabolism and cell death.

4.4. TDT classification

The finding that mixture TDT can be well-predicted by averaging the mean TDT of the individual components was examined more fully by sorting TDT values for the chemicals into five TDT classes. One might expect that two chemicals having the same relative level of TDT would show mixture TDT at about the average of the single chemical TDT values. In contrast, it might be expected that when a chemical with full TDT (i.e., >100%) was tested with one having negative TDT (i.e., TDT <0%) the difference between observed and predicted TDT for the mixture would show more variability. This was the case. From Table 7 it can be seen that when two chemicals had the same relative mean TDT level (e.g., high TDT with high TDT, low TDT with low TDT) the average difference between observed and predicted TDT was low, typically being less than ±5%. The greatest average difference was observed for the situation in which a chemical having full TDT was given with one having negative TDT (±16.2%). As a result of this finding, the average difference between predicted mean TDT and observed mean TDT for the mixtures was evaluated further by examining whether the individual chemicals in a given mixture: (a) had the same TDT level (e.g., high TDT with high TDT) or (b) different TDT levels (e.g., high TDT with low TDT) (Table 5). The same comparative analyses were also done for TDT at three effect levels (i.e., TDT_{25}, TDT_{50} and TDT_{75}; Table 5).

These latter regressions showed that better correlations were obtained when the agents in the combination had the same TDT level. In fact, the mean TDT analysis for true mixtures containing agents that had the same TDT level resulted in a better correlation ($r^2 = 0.993$; Table 5, row 3) than did the sham combinations ($r^2 = 0.989$; Table 5, row 1). While predictability of mean TDT was lower for true mixtures containing chemicals from different TDT classes, it was still good (i.e., $r^2 \geq 0.900$). Results for the TDT_{50} regression for true mixtures showed a very high level of predictability ($r^2 = 0.984$; Table 5, row 12). The same general pattern was observed at the TCT_{25} and TDT_{75}, although predictability was somewhat lower for mixtures containing chemicals from different TDT classes (TDT_{25}, $r^2 = 0.844$) and (TDT_{75}, $r^2 = 0.861$).

Data from Table 7 showed that the largest average differences between predicted mixture TDT and observed mixture TDT were found in true mixtures in which one chemical had a positive mean TDT and the other chemical had a negative mean TDT. To evaluate this finding further, combinations of true mixtures containing either 3M2B or DBRAN were removed from the regression (Table 5, row 5). The results strongly suggested that predicting

mean TDT for mixtures by taking the average of the mean TDT of the individual chemicals in the mixture will be the most challenging when a positive TDT chemical (i.e., a reactive toxicant) is combined with a chemical having negative TDT (such as chemicals which are toxic solely by disrupting membrane integrity for a time before being accommodated by the organism and beginning recovery).

When one knows the TDT of the individual chemicals in the mixture, calculating TDT of the mixture should most often be a straightforward process. The major issue for this approach comes when one does not have TDT information for the chemicals. Preliminary analyses (not shown) suggest that curve-fitting parameters such as slope, asymmetry or the difference between slope and asymmetry can be used to help classify chemicals by TDT level. While more data is needed to assess this idea, it is suggested that such information, when coupled with physico-chemical parameters and/or reactivity rate constant data for the individual chemicals could help to classify each agent by its TDT level without having to conduct time-course studies. Such studies when conducted using a rapid, lower-cost assay like Microtox® offer potential to further advance the field of mixture toxicity prediction.

4.5. Further study

An aspect of this research that requires additional study is environmental relevance, such as evaluating the observed TDT correlations at concentrations of chemicals actually present in an aquatic setting. The primary purpose of developing the current data set was to discern any relationships between TDT, combined effects and organic chemical reaction mechanisms (and/or lack of chemical reactivity). Studies examining environmental relevance will require a different experimental design than used herein. It should be possible, however, to calculate TDT at lower effect levels (e.g., TDT_5 or TDT_{10}) for chemicals A, B and A + B and determine whether observed vs. predicted mixture TDT values are still well-correlated. While such an evaluation may be useful, it should be noted that examining the effects of including and excluding the asymmetry parameter in curve-fitting the *x*/*y* data may be needed, since that parameter frequently affects fitting at lower effect levels.

A second aspect of this work requiring further study is development of a more detailed mechanistic rationale for the value of TDT in toxicity prediction for mixtures. It is likely that the larger deviations between observed and predicted TDT noted above are associated with pairs of agents that are likely to exert toxicity via different modes/mechanisms of action. The working hypothesis for this idea is that those differences appear to be reflected by differences in slope of the *x*/*y* curves, especially for combinations that produce mixture toxicity that is not dose-additive.

5. Conclusions

Time-dependent toxicity assessment of individual chemicals provided information useful for predicting mixture toxicity. Mixture TDT was well-predicted simply by averaging the TDT values of the individual components of binary combinations. While this finding requires confirmation with additional sets of toxicity data and/or evaluation using other toxicity assays, it appears to provide a simple way of estimating mixture toxicity when the TDT of the individual chemicals is known. Scientists interested in obtaining the concentration-

response data used to generate the TDT values presented herein are invited to contact the corresponding author.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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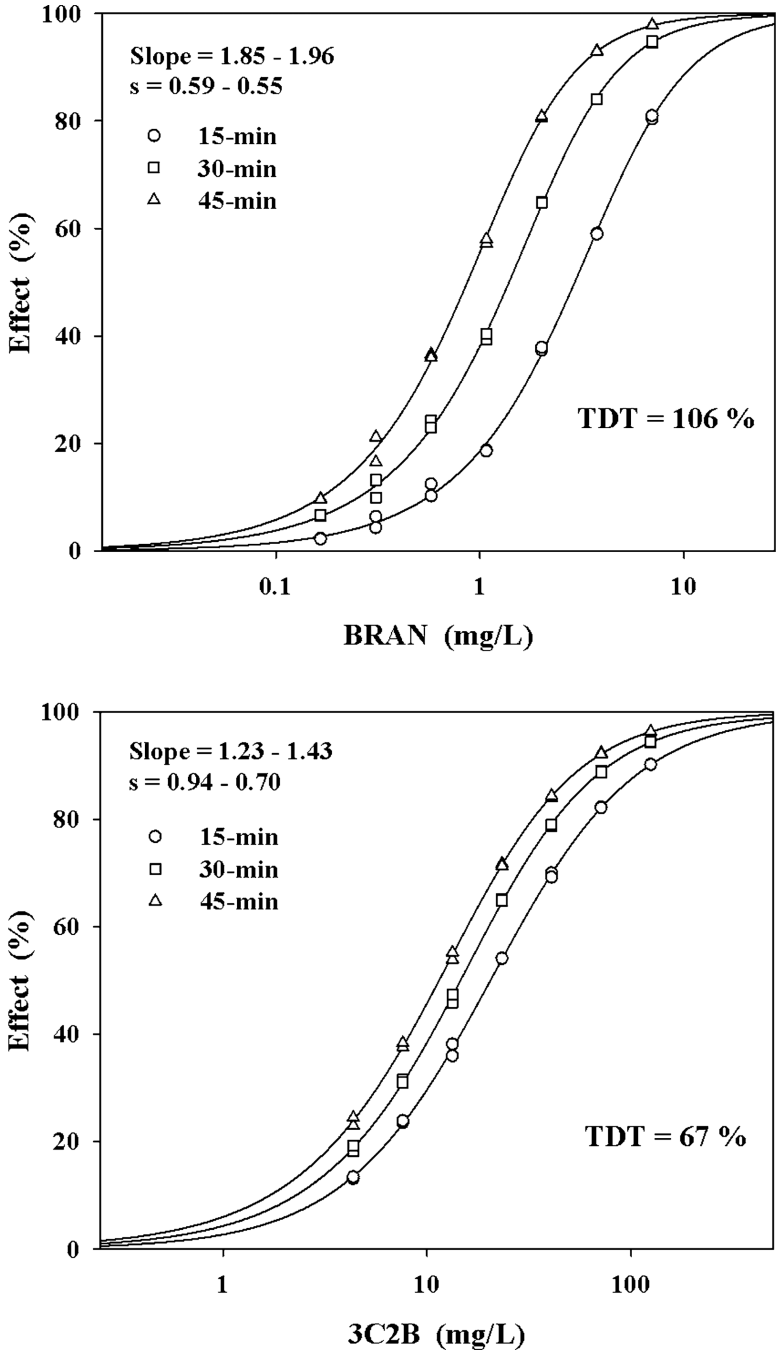

Fig. 1.

Toxicity data for bromoacetonitrile alone (BRAN – upper graph) and 3-chloro-2-butanone alone (3C2B – lower graph) plotted at 15, 30 and 45-min of exposure. Each graph shows the change in toxicity over time, with BRAN having a greater left-shift of the curves upon increased exposure time vs. that for 3C2B. The differences in time-dependent toxicity (TDT) and concentration-response curve parameters between the two chemicals are shown by the larger TDT value, steeper slope values and lower asymmetry (*s*) values (given for 15 and 45-min) for BRAN vs. 3C2B.

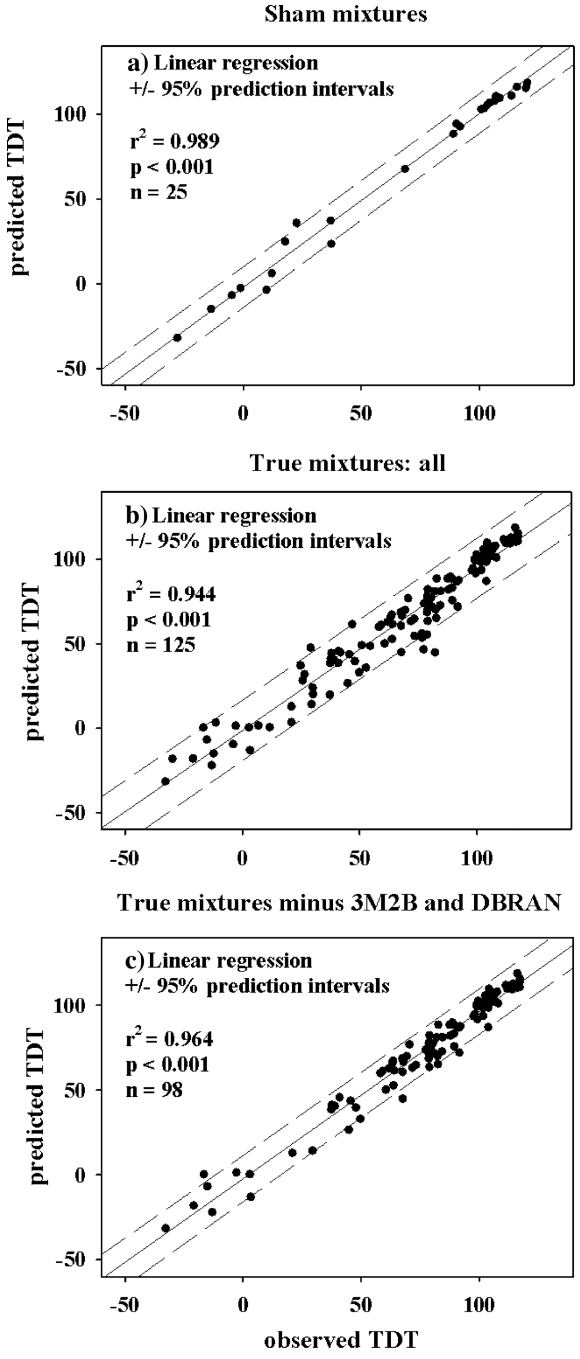

Fig. 2.

Linear regression plots of observed mixture time-dependent toxicity (TDT) values (%) vs. predicted mixture TDT values for (a) sham and (b) true mixtures and for (c) true mixtures excluding those that contained either 3-methyl-2-butanone (3M2B) or dibromoacetonitrile (DBRAN).

Table 1

Selected chemicals for mixture toxicity studies using Microtox®.

Chemical name	Abbr.	CAS #	Chemical name	Abbr.	CAS #
2,3-Butanone	23B	431-03-8	Ethyl acrylate	EA	140-88-5
2,6-Dichloro-4-nitropyridine	26D4NP	25194-01-8	Ethyl acetate	EAC	141-78-6
2-Chloro-4-nitropyridine	2C4NP	23056-36-2	Ethyl bromoacetate	EBAC	105-36-2
2-Chloropyrimidine	2CP	1722-12-9	Ethyl chloroacetate	ECAC	105-39-5
2,4-Dichloropyrimidine	2DCP	3934-20-1	Ethyl fluoroacetate	EFAC	459-72-3
2-Hydroxyethylacrylate	2HEA	818-61-1	Ethyl iodoacetate	EIAC	623-48-3
3-Chloro-2,4-pentanedione	3C24P	1694-29-7	Ethyl propiolate	EP	623-47-2
3-Chloro-2-butanone	3C2B	4091-39-8	Eugenol	EUG	97-53-0
3-Methyl-2-butanone	3M2B	563-80-4	Ethyl vinyl ketone	EVK	1629-58-9
4-Nitrobenzyl bromide	4NBB	100-11-8	Geraniol	GER	106-24-1
4-Vinylpyridine	4VP	100-43-6	Hydroxypropyl methacrylate	HPM	27813-02-1
1-Bromo-2,4-dinitrobenzene	BDNB	584-48-5	Iodoacetonitrile	IAN	624-75-9
Butyl glycidyl ether	BGE	2426-08-6	Isoeugenol	IEG	97-54-1
(+)-Borneol	BOR	464-43-7	Linalool	LIN	78-70-6
Bromoacetonitrile	BRAN	590-17-0	Methyl-2-bromobutyrate	M2BB	69043-96-5
Carvacrol	CAR	499-75-2	Methyl-2-bromopropionate	M2BP	5445-17-0
1-Chloro-2,4-dinitrobenzene	CDNB	97-00-7	Methyl-2-chloroacetoacetate	M2CA	4755-81-1
4-Chloro-3,5-dinitrobenzotrifluoride	CDNT	393-75-9	Methyl crotonate	MC	623-43-8
Chloroacetonitrile	CLAN	107-14-2	Methyl tiglate	MT	6622-76-0
Dibromoacetonitrile	DBRAN	3252-43-5	Methyl vinyl ketone	MVK	78-94-4
Dichloroacetonitrile	DCLAN	3018-12-0	Propionitrile	PN	107-12-0
Diethyl maleate	DEM	141-05-9	Trichloroacetonitrile	TCLAN	545-02-2
Diethyl sulfate	DES	64-67-5	γ-Terpinene	TER	99-85-4
Dimethyl sulfate	DMS	77-78-1	Thymol	THY	89-83-8

Table 2

Summary[a] of coefficient of determination (r^2) values from fitting concentration-response data using the 5PL-1P[b] function.

	A-15min	B-15min	MX-15min	A-30 min	B-30 min	MX-30min	A-45 min	B-45 min	MX-45 min	All
Mean (±s.d.)	0.9976 ± 0.0024	0.9974 ± 0.0031	0.9979 ± 0.0019	0.9981 ± 0.0019	0.9978 ± 0.0025	0.9983 ± 0.0016	0.9984 ± 0.0018	0.9981 ± 0.0021	0.9984 ± 0.0017	0.9980 ± 0.0022
Median	0.9984[c]	0.9984[c]	0.9986[c]	0.9987[c]	0.9988[c]	0.9988[c]	0.9990[c]	0.9988[c]	0.9990[c]	0.9988
Minimum	0.9857	0.9773	0.9879	0.9883	0.9853	0.9916	0.9884	0.9871	0.9879	0.9773
Maximum	0.9998	0.9999	0.9999	0.9998	0.9998	0.9999	0.9998	0.9999	0.9999	0.9999

[a] r^2 values for - A: chemical A alone, B: chemical B alone, MX: mixture (A + B); exposure durations were 15, 30, and 45-min.

[b] 5-parameter logistic minus 1 parameter function; results summarize 1350 concentration-response curves from 150 experiments.

[c] Within each exposure duration, median values were not significantly different (Kruskal–Wallis One Way Analysis of Variance on Ranks test, $p = 0.516, 0.771, 0.156$, respectively).

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Table 3

Time-dependent toxicity (TDT)[a] values for 25 sham combinations.

Sham Combination A_1:A_2	Obs. TDT A_1	Obs. TDT A_2	Obs. TDT mixture	Pred. TDT mixture	Mixture TDT obs.–pred.
2HEA:2HEA	104.0	101. 7	100.8	102.9	−2.1
3M2B:3M2B	−21.3	−8.6	−13.7	−15.0	1. 3
4NBB:4NBB	93.7	94.8	90.2	94.3	−4.1
BDNB:BDNB	111 .8	109.9	113.6	110.8	2.8
BGE:BGE	12.9	−0.6	12.1	6.1	6.0
BRAN:BRAN	105.6	105.2	103.3	105.4	−2.1
CDNB:CDNB	116.6	115.5	116.0	116.1	−0.1
CLAN:CLAN	107.7	10 7. 9	106.6	107.8	−1.2
CLAN:CLAN	109.2	109.7	108.6	109.5	−0.9
DBRAN:DBRAN	2.6	−10.1	9.9	−3.7	13.6
DEM:DEM	37.3	37.0	37.0	37.2	−0.2
EAC:EAC	−37.9	−26.4	−28.0	−32.1	4.1
EBAC:EBAC	119.9	11 7.2	120.3	118.5	1. 8
ECAC:ECAC	88.6	87.9	89.0	88.3	0.7
EFAC:EFAC	21.3	28.3	17.7	24.8	−7.1
EIAC:EIAC	115.0	115.7	119.8	115.3	4.5
EP:EP	108.8	105.0	104.5	106.9	−2.4
HPM:HPM	−1.4	−3.8	−1.2	−2.6	1. 4
IAN:IAN	103.2	103.5	102.0	103.4	−1.4
LIN:LIN	−13.4	−0.4	−4.9	−6.9	2.0
M2BP:M2BP	68.1	67.1	68.5	67.6	0.9
M2CA:M2CA	111 .9	109.2	107.0	110.5	−3.5
MC:MC	18.4	28.5	37.2	23.4	13.8
MVK:MVK	95.4	89.9	91.9	92.7	−0.8
TCLAN:TCLAN	36.2	35.4	22.5	35.8	−13.3

[a] See text Section 2.5 for TDT calculation methods; obs.: observed, pred.: predicted.

Table 4

Time-dependent toxicity (TDT)[a] values for 125 true combinations.

Combination A:B	Obs. TDT A	Obs. TDT B	Obs. TDT mixture	Pred. TDT mixture	Mixture TDT obs.–pred.
26D4NP:BDNB	33.8	110.3	91.7	72.0	19.7
2C4NP:BDNB	57.9	115.9	91.4	86.9	4.5
2C4NP:3M2B	51.4	−3.3	30.0	24.1	5.9
2DCP:2CP	103.4	−3.0	60.6	50.2	10.4
2HEA:23B	97.3	89.9	97.8	93.6	4.2
3C24P:BDNB	91.4	110.1	104.0	100.8	3.2
3C24P:CDNB	94.1	117.6	102.7	105.9	−3.2
3C24P:ECAC	88.9	88.0	89.5	88.4	1.1
3C24P:MC	94.6	39.9	63.6	67.2	−3.6
3C2B:3M2B	59.0	−19.1	37.3	19.9	17.4
3C2B:MC	56.3	31.2	45.6	43.7	1.9
3M2B:EA	−4.4	61.0	25.7	28.3	−2.6
3M2B:EAC	−10.8	−19.2	−12.2	−15.0	2.8
3M2B:HPM	−7.2	−11.8	−3.9	−9.5	5.6
3M2B:MC	−20.8	23.9	6.8	1.5	5.3
4VP:3M2B	19.1	−12.1	20.9	3.5	17.4
4VP:DEM	9.4	56.7	49.8	33.0	16.8
4VP:HPM	13.7	12.2	20.9	12.9	8.0
BDNB:3C2B	107.8	58.6	89.4	83.2	6.2
BDNB:3M2B	111.7	−2.3	73.3	54.7	18.6
BDNB:CDNB	116.1	121.7	116.2	118.9	−2.7
BDNB:M2BP	112.9	70.8	99.4	91.8	7.6
BRAN:3M2B	106.1	−10.9	29.1	47.6	−18.5
BRAN:CLAN	106.1	107.9	106.8	107.0	−0.2
BRAN:DEM	107.4	41.5	79.4	74.5	4.9
BRAN:EFAC	107.0	19.9	79.0	63.5	15.5
BRAN:MC	102.3	37.7	69.3	70.0	−0.7
BRAN:PN	106.3	27.1	68.0	66.7	1.3

Combination A:B	Obs. TDT A	Obs. TDT B	Obs. TDT mixture	Pred. TDT mixture	Mixture TDT obs.–pred.
CAR:THY	−21.0	−15.0	−20.9	−18.0	−2.9
CDNB:3C2B	112.9	66.6	88.7	89.8	−1.1
CDNB:3M2B	111.3	−14.0	54.4	48.7	5.7
CDNB:M2BP	121.1	68.4	98.2	94.7	3.5
CDNT:BDNB	101.6	114.6	107.7	108.1	−0.4
CDNT:CDNB	103.9	119.6	111.7	111.7	0.0
CLAN:3M2B	10 7. 5	15.6	46.8	61.6	−14.8
CLAN:DEM	104.2	43.6	77.9	73.9	4.0
CLAN:EFAC	111.6	18.9	82.6	65.3	17.3
CLAN:PN	106.6	19.9	71.8	63.3	8.5
DBRAN:BRAN	5.5	107.0	76.5	56.2	20.3
DBRAN:CLAN	3.6	107.2	78.7	55.4	23.3
DBRAN:DCLAN	−1.1	72.8	52.7	35.9	16.8
DBRAN:IAN	−13.7	103.4	82.1	44.9	37.2
DCLAN:CLAN	62.5	112.8	92.1	87.6	4.5
DES:3M2B	−21.4	−14.9	−29.8	−18.2	−11.6
DMS:3M2B	21.8	−15.2	−11.3	3.3	−14.6
DMS:DES	18.7	−18.0	−16.6	0.4	−17.0
EA:MC	68.6	22.8	41.0	45.7	−4.7
EAC:PN	−20.2	23.1	−2.8	1.4	−4.2
EBAC:2HEA	125.9	95.6	117.3	110.8	6.5
EBAC:3C24P	115.6	86.6	108.1	101.1	7.0
EBAC:3C2B	119.1	55.2	103.9	87.2	16.7
EBAC:3M2B	120.7	−13.3	76.6	53.7	22.9
EBAC:BRAN	120.6	107.5	117.4	114.1	3.3
EBAC:CLAN	117.5	108.1	114.5	112.8	1.7
EBAC:EAC	119.3	−29.3	67.7	45.0	22.7
EBAC:ECAC	120.2	91.3	106.4	105.8	0.6
EBAC:EFAC	123.1	30.7	70.6	76.9	−6.3
EBAC:IAN	118.5	102.4	116.4	110.4	6.0
EBAC:M2BP	125.1	72.1	104.0	98.6	5.4

Combination A:B	Obs. TDT A	Obs. TDT B	Obs. TDT mixture	Pred. TDT mixture	Mixture TDT obs.–pred.
EBAC:M2CA	115.6	109.1	114.7	112.4	2.3
ECAC:3M2B	89.4	−12.0	40.8	38.7	2.1
ECAC:BRAN	93.0	107.0	103.9	100.0	3.9
ECAC:CLAN	88.1	108.8	101.8	98.4	3.4
ECAC:EA	92.3	64.0	78.8	78.1	0.7
ECAC:EAC	89.6	−36.5	44.9	26.6	18.3
ECAC:EFAC	102.3	29.8	63.2	66.1	−2.9
ECAC:IAN	86.1	101.5	101.8	93.8	8.0
ECAC:MC	90.9	32.5	64.0	61.7	2.3
ECAC:MC	93.2	44.3	78.7	68.7	10.0
ECAC:PN	93.1	28.3	67.6	60.7	6.9
EFAC:3M2B	23.3	−22.2	11.6	0.5	11.1
EIAC:3M2B	114.1	−20.9	77.2	46.6	30.6
EIAC:BRAN	115.0	104.0	114.0	109.5	4.5
EIAC:CLAN	116.0	107.3	112.7	111.6	1.1
EIAC:EAC	119.9	−14.2	63.8	52.8	11.0
EIAC:EBAC	113.6	117.7	117.3	115.6	1.7
EIAC:ECAC	114.1	85.9	102.6	100.0	2.6
EIAC:EFAC	119.5	21.0	78.9	70.2	8.7
EIAC:IAN	118.0	102.6	113.3	110.3	3.0
EIAC:PN	117.7	22.8	82.4	70.2	12.2
EP:3C24P	107.6	92.5	98.9	100.1	−1.2
EP:3C2B	94.0	61.1	80.5	77.5	3.0
EP:3M2B	108.5	−10.0	50.9	49.2	1.7
EP:BRAN	110.0	106.1	104.4	108.1	−3.7
EP:EA	99.9	62.6	84.5	81.2	3.3
EP:ECAC	109.1	95.4	106.3	102.2	4.1
EUG:DEM	−9.7	38.1	29.5	14.2	15.3
EUG:IEG	26.4	−52.5	3.3	−13.0	16.3
EVK:3M2B	92.8	−18.6	24.8	37.1	−12.3
EVK:EA	92.1	59.6	89.5	75.8	13.7

Combination A:B	Obs. TDT A	Obs. TDT B	Obs. TDT mixture	Pred. TDT mixture	Mixture TDT obs.–pred.
EVK:M2CA	95.9	108.2	100.7	102.0	−1.3
GER:BOR	−15.3	−28.6	−13.0	−22.0	9.0
GER:TER	−15.8	−47.5	−32.8	−31.6	−1.2
GER:LIN	−8.8	−4.8	−15.1	−6.8	−8.3
IAN:3M2B	103.0	−13.9	38.0	44.5	−6.5
IAN:BRAN	103.9	105.2	104.5	104.5	0.0
IAN:CLAN	103.7	108.1	104.5	105.9	−1.4
IAN:DCLAN	103.7	73.6	87.5	88.6	−1.1
IAN:DEM	105.2	50.2	78.8	77.7	1.1
IAN:EAC	100.8	−21.3	47.9	39.7	8.2
IAN:EFAC	108.9	28.6	67.5	68.7	−1.2
IAN:PN	105.0	20.9	61.9	62.9	−1.0
M2BB:M2BP	5.5	77.1	37.7	41.3	−3.6
M2BB:MT	−0.4	1.1	2.9	0.4	2.5
M2BP:2HEA	71.2	91.0	82.0	81.1	0.9
M2BP:3M2B	75.9	−35.6	30.1	20.2	9.9
M2BP:BGE	74.7	2.8	37.4	38.7	−1.4
M2BP:DMS	70.5	11.0	38.9	40.7	−1.8
M2BP:ECAC	71.1	93.5	79.0	82.3	−3.3
M2BP:MC	87.5	42.2	73.3	64.8	8.5
M2CA:3C2B	109.9	67.3	82.8	88.6	−5.8
M2CA:BDNB	110.8	109.0	104.3	109.9	−5.6
M2CA:CDNB	107.5	116.3	111.3	111.9	−0.6
M2CA:DMS	109.1	11.0	58.2	60.0	−1.8
MVK:3M2B	99.8	−10.3	41.8	44.8	−3.0
MVK:BRAN	99.4	106.1	99.7	102.7	−3.0
MVK:EA	96.4	51.4	77.4	73.9	3.5
MVK:EBAC	99.7	119.8	112.1	109.7	2.4
MVK:ECAC	99.3	99.4	99.2	99.3	−0.1
MVK:EP	96.5	105.5	102.3	101. 0	1.3
MVK:MC	89.2	33.3	59.2	61.3	−2.1

Combination A:B	Obs. TDT A	Obs. TDT B	Obs. TDT mixture	Pred. TDT mixture	Mixture TDT obs.–pred.
TCLAN:BRAN	58.1	106.2	87.8	82.2	5.6
TCLAN:DBRAN	60.4	3.5	26.5	32.0	−5.5
TCLAN:DCLAN	65.0	78.8	81.0	71.9	9.1
TCLAN:IAN	40.7	104.5	84.3	72.8	11.5

[a] See text Section 2.5 for TDT calculation methods; obs.: observed, pred.: predicted.

Table 5

Regression results for various predicted TDT vs. observed TDT analyses.

Row #	Regression	Equation	*n*	s.e.[a]	*p*	r^2
	Mean TDT					
1	Sham	pred. TDT = −2.105 + (1.024 × obs. TDT)	25	0.0226	<0.001	0.989
2	True	pred. TDT = −1.420 + (0.963 × obs. TDT)	125	0.0211	<0.001	0.944
3	Same TDT class[b]	pred. TDT = 0.131 + (0.988 × obs. TDT)	32	0.0153	<0.001	0.993
4	Different TDT class[c]	pred. TDT = −0.311 + (0.928 × obs. TDT)	93	0.0325	<0.001	0.900
5	3M2B/DBRAN removed[d] TDT_{25}	pred. TDT = −2.262 + (0.985 × obs. TDT)	98	0.0194	<0.001	0.964
6	Sham	pred. TDT = −0.658 + (1.001 × obs. TDT)	25	0.0302	<0.001	0.979
7	True	pred. TDT = 3.640 + (0.896 × obs. TDT)	125	0.0258	<0.001	0.907
8	Same TDT class	pred. TDT = −0.270 + (0.964 × obs. TDT)	27	0.0241	<0.001	0.985
9	Different TDT class	pred. TDT = 7.110 + (0.840 × obs. TDT)	98	0.0368	<0.001	0.844
	TDT_{50}					
10	Sham	pred. TDT = 0.317 + (0.996 × obs. TDT)	25	0.0229	<0.001	0.988
11	True	pred. TDT = −0.291 + (0.966 × obs. TDT)	125	0.0199	<0.001	0.950
12	Same TDT class	pred. TDT= 1.099 + (0.983 × obs. TDT)	30	0.0240	<0.001	0.984
13	Different TDT class	pred. TDT = 0.521 + (0.940 × obs. TDT)	95	0.0290	<0.001	0.919
	TDT_{75}					
14	Sham	pred. TDT= 1.433 + (0.991 × obs. TDT)	25	0.0237	<0.001	0.987
15	True	pred. TDT= 1.891 + (0.949 × obs. TDT)	125	0.0251	<0.001	0.921
16	Same TDT class	pred. TDT = 1.895 + (0.987 × obs. TDT)	33	0.0292	<0.001	0.974
17	Different TDT class	pred. TDT = 4.483 + (0.893 × obs. TDT)	92	0.0379	<0.001	0.861

[a] Standard error.

[b] True mixtures in which the individual chemicals had the same level of TDT (see text for complete description).

[c] True mixtures in which the individual chemicals had different levels of TDT (see text).

[d] True mixtures from which all 3M2B- and DBRAN-containing mixtures were removed from the analyses.

Table 6

Average time-dependent toxicity (TDT) values for 24 chemicals tested singly at least five times.

Chemical	*n*	Average TDT	±std. err.	±95% CI
2HEA	5	97.9	2.3	6.4
3C24P	6	91.4	1.3	3.3
3C2B	7	60.6	1.8	4.4
3M2B	24	−12.8	1.9	4.0
BDNB	11	111.8	0.8	1.9
BRAN	17	105.9	0.3	0.7
CDNB	9	117.0	1.2	2.7
BRAN	15	108.3	0.5	1.1
DBRAN	7	−1.3	2.8	7.0
DEM	7	43.5	2.8	6.9
EA	6	61.2	2.3	6.0
EAC	8	−25.6	3.0	7.1
EBAC	16	119.7	0.7	1.6
ECAC	17	91.5	1.1	2.3
EFAC	9	24.6	1.6	3.6
EIAC	11	123.5	7.7	17.1
EP	9	105.4	1.8	4.0
IAN	15	103.7	0.5	1.0
M2BP	12	72.9	1.6	3.5
M2CA	7	109.6	0.5	1.3
MC	10	32.4	2.8	6.3
MVK	9	96.2	1.4	3.2
PN	6	23.7	1.4	3.5
TCLAN	6	49.3	5.4	14.0

Table 7

Average difference in observed time-dependent toxicity (TDT) vs. predicted TDT by TDT level for 150 mixture tests (# of tests/pairing).

TDT level	TDT >100%	TDT 70–100%	TDT 30–69%	TDT 0–29%	TDT <0%
TDT >100	sh[a] – 2.1 (11) tr[b] – 2.2 (17)	tr – 3.7 (18)	tr – 6.4 (15)	tr – 9.9 (13)	tr – 16.2 (12)
TDT 70–100		sh – 1.9 (3) tr – 1.9 (5)	tr – 5.4 (11)	tr – 3.4 (4)	tr – 10.4 (6)
TDT 30–69			sh – 4.8 (3) tr – 1. 9 (1)	tr – 9.0 (3)	tr – 10.3 (4)
TDT 0–29				sh – 10.5 (2) tr – 8.0 (1)	tr – 10.8 (10)
TDT <0					sh – 2.2 (4) tr – 5.9 (7)

[a] sh: sham combinations.

[b] tr: true combinations.