Environmental Science & Technology

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Article

Assessing Additivity of Cytotoxicity Associated with Disinfection Byproducts in Potable Reuse and Conventional Drinking Waters

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Cite This: *Environ. Sci. Technol.* 2020, 54, 5729–5736

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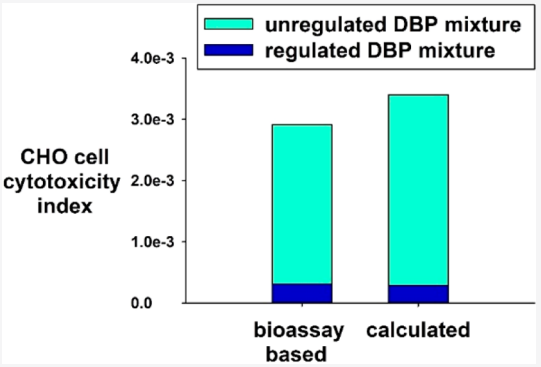
ABSTRACT: Recent studies used the sum of the measured concentrations of individual disinfection byproducts (DBPs) weighted by their Chinese hamster ovary (CHO) cell cytotoxicity LC_{50} values to estimate the DBP-associated cytotoxicity of disinfected waters. This approach assumed that cytotoxicity was additive rather than synergistic or antagonistic. In this study, we evaluated whether this assumption was valid for mixtures containing DBPs at the concentration ratios measured in authentic disinfected waters. We examined the CHO cell cytotoxicity of defined DBP mixtures based on the concentrations of 43 regulated and unregulated DBPs measured in eight drinking and potable reuse waters. The hypothesis for additivity was supported using three experimental approaches. First, we demonstrated that the calculated additive toxicity (CAT) and bioassay-based calculated additive toxicity (BCAT) of the DBP mixtures agree within 12% on a median basis. We also found an additive toxicity response (CAT ≈ BCAT) between the regulated and unregulated DBP classes. Finally, the empirical biological cytotoxicity of the DBP subset mixtures, independent of the calculated toxicity, was additive. These results support the validity of using the sum of cytotoxic potency-weighted DBP concentrations as an estimate of the CHO cell cytotoxicity associated with known DBPs in real disinfected waters.



INTRODUCTION

Disinfection byproducts (DBPs), formed from the reactions of disinfectants with organic matter and halides in drinking water sources, have been a health concern since trihalomethanes (THMs) were discovered in chlorinated waters in 1974.[1,2] Many DBPs are cytotoxic and genotoxic;[3] epidemiological studies have linked the consumption of chlorinated water to bladder, colon, and rectal cancers.[4−6] Although >700 DBPs have been identified, fewer than 30 have been tested for carcinogenicity.[7] *In vivo* toxicological evidence is important for the development of regulations;[8] however, with the total number of DBPs in disinfected drinking waters likely exceeding 1000,[9] it is impractical to conduct animal testing on every compound. Thus, cell-based *in vitro* assays are used for prioritizing DBPs.

Although a number of *in vitro* assays have been employed,[10] the most extensive toxicological database (with >100 DBPs quantitatively analyzed) is based on Chinese hamster ovary (CHO) cell cytotoxicity and genotoxicity.[11] Unregulated, nitrogen-containing DBPs (e.g., haloacetonitriles, HANs) are more cytotoxic and genotoxic than the four trihalomethanes (THM4) and five haloacetic acids (HAA5) that are regulated in the U.S.[11] However, many unregulated DBPs occur at lower concentrations than THM4 or HAA5.[7] To prioritize DBPs likely to contribute the most toward the overall toxicity of DBP mixtures in real waters, previous researchers weighted the measured concentrations of individual DBPs by metrics of toxic potency and then compared these individual toxic potency-weighted concentrations to the sum of the toxic potency-weighted concentrations across all known DBPs.[12−16] These calculations suggested that unregulated DBP classes, particularly HANs and haloacetamides (HAMs), may be more important drivers of the cytotoxicity of disinfected waters than THM4 or HAA5. Using the sum of toxic potency-weighted concentrations as described above assumes that the cytotoxicity of DBPs in mixtures is additive. However, interactions among chemicals in a mixture may be additive, synergistic, or antagonistic.[17] The additivity of DBP cytotoxicity has not been explicitly tested, and if the additivity assumption is invalid, the toxic potency-weighting calculations might not be useful for prioritizing DBPs.

Previous *in vivo* and *in vitro* studies employing defined DBP mixtures to evaluate DBP interactions have reached differing

Received: February 18, 2020
Revised: April 3, 2020
Accepted: April 10, 2020
Published: April 10, 2020

Table 1. Concentrations of DBPs (in μg/L) Measured in Conventional Drinking Water (DW) and Potable Reuse Water (PRW)[a]

	DW1	DW2	DW3	PRW1	PRW2	PRW3	PRW4	PRW5
acetaldehyde[b]	0.05				2.80	0.60		2.40
TCM	6.37	6.36	2.00	11.00	1.85	5.57	2.30	0.95
BDCM	3.46	6.56	1.49	5.50	2.90	4.21	4.04	8.30
DBCM	1.02	3.60	0.36	1.40	1.99	1.81	2.50	27.91
TBM	0.10	0.91	0.03		0.67	0.21	2.06	37.86
DCIM		1.72					3.58	
BCIM		0.22			0.50		2.75	0.17
DBIM		0.03			0.52		2.15	0.07
CDIM					0.44		1.84	
BDIM					0.60		0.96	
TIM					1.13			
TCAL	5.25	2.85	0.77	0.79		3.98		0.13
BDCAL	1.39	0.70	0.85			1.88	0.49	1.38
DBCAL			0.28			0.88	0.50	0.92
TBAL								0.25
TCNM	2.04	0.79	0.26		0.02	2.46	0.27	
1,1-DCP[b]				0.54	0.36			
1,1,1-TCP[b]	2.98					1.90		0.10
TCAN					0.01			
DCAN	0.75	1.67	0.37	3.30	0.33	1.01	0.49	0.28
BCAN	0.41	1.25	0.05	0.43	0.60	0.90	1.00	1.32
DBAN	0.11	0.57			0.38	0.29	0.63	3.57
DCAM	0.15	2.56	0.30	2.20	0.20	0.10	0.64	0.07
BCAM	0.04	0.69				0.04	0.47	
TCAM								
DBAM		0.46					0.42	0.69
CAA	3.48	2.99	0.42		1.04	2.84	1.89	0.50
BAA	0.40	0.27			0.50	0.50	0.12	1.47
DCAA	10.42	11.34	4.30	4.80	0.22	7.24	1.86	1.27
TCAA	2.22	3.93	1.71	3.10	0.25	4.60	0.30	0.66
BCAA	2.01	4.40	0.97	1.00		2.45	1.82	4.11
DBAA	0.46	1.55	0.21	0.56	0.22	0.78	1.57	10.38
BDCAA	0.67	1.01				1.62		
CDBAA	0.21	0.35				0.43		2.18
TBAA		0.10						1.81
IAA		0.11			0.01		0.21	
NDMA[b]	1.84×10^{-4}	1.39×10^{-2}		9.00×10^{-3}	3.10×10^{-3}	1.88×10^{-4}	6.92×10^{-3}	2.40×10^{-3}
NMOR	1.03×10^{-4}					4.88×10^{-4}		3.90×10^{-3}

[a]TCM = chloroform; BDCM = bromodichloromethane; DBCM = dibromochloromethane; TBM = bromoform; DCIM = dichloroiodomethane; BCIM = bromochloroiodomethane; DBIM = dibromoiodomethane; CDIM = chlorodiiodomethane; BDIM = bromodiiodomethane; TIM = iodoform; TCAL = chloral hydrate; BDCAL = bromodichloroacetaldehyde; DBCAL = dibromochloroacetaldehyde; TBAL = tribromoacetaldehyde; TCNM = chloropicrin; 1,1-DCP = 1,1-dichloropropanone; 1,1,1-TCP = 1,1,1-trichloropropanone; TCAN = trichloroacetonitrile; DCAN = dichloroacetonitrile; BCAN = bromochloroacetonitrile; DBAN = dibromoacetonitrile; DCAM = dichloroacetamide; BCAM = bromochloroacetamide; TCAM = trichloroacetamide; DBAM = dibromoacetamide; CAA = chloroacetic acid; BAA = bromoacetic acid; DCAA = dichloroacetic acid; TCAA = trichloroacetic acid; BCAA = bromochloroacetic acid; DBAA = dibromoacetic acid; BDCAA = bromodichloroacetic acid; CDBAA = chlorodibromoacetic acid; TBAA = tribromoacetic acid; IAA = iodoacetic acid; NDMA = nitrosodimethylamine; NMOR = nitrosomorpholine. [b]These DBPs measured in the real waters were included in the defined mixtures but were not included in the CAT calculations because the LC_{50} values are not available (Table S1).

conclusions. However, these studies (1) typically evaluated a limited number of DBPs (frequently only of one class), (2) often featured a low power to resolve small deviations from additivity (particularly *in vivo* studies), and (3) evaluated endpoints that were different from the CHO cell cytotoxicity endpoint employed in toxic potency-weighting calculations. Additivity was observed for developmental effects on rat embryos with mixtures of three HAAs[18] and for hepatotoxicity in mice with THM4,[19] but antagonism was observed for renal cancer in rats with mixtures containing bromate, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), chloroform, and bromodichloromethane.[20] Synergism was found for cytotoxicity in rat kidney cells with mixtures of chlorite, bromate, and bromochloroacetic acid[21] and for genotoxicity in bacterial and mammalian cells with mixtures of MX and microcystins-LR.[22] Both additivity and synergism were observed for hepatic tumor promoting activity in mice[23] and for oxidative stress in mouse livers[24] with binary mixtures of HAAs. In a previous and more limited study using different conditions and depending on the total DBP concentrations, either additivity or antagonism was found for CHO cell cytotoxicity with mixtures of nine HAAs.[25] Additivity,

https://dx.doi.org/10.1021/acs.est.0c00958
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synergism, or antagonism was observed for bacterial growth inhibition with binary mixtures of aromatic halogenated DBPs.[26] Several *in vivo* studies examined the reproductive and developmental effects of mixtures of regulated DBPs in rats;[27−29] however, these studies could not resolve additivity. A recent study employing three reporter gene assays based on human cancer cell lines and one bacterial assay, the most relevant study in terms of evaluating mixtures of multiple DBPs from various classes, found additive effects of equipotent concentrations of DBP mixtures; HANs, haloketones (HKs), and monohalogenated HAAs were the toxicity drivers.[30] Previously, we demonstrated a high correlation in the activation of the antioxidant response element reporter gene induced by the monohalogenated HAAs in nontransformed human cells and the CHO cell cytotoxicity of these monohalogenated HAAs,[31] suggesting that the additivity observed with reporter gene assays in human cancer cell lines may apply to CHO cell cytotoxicity. However, whether DBP-associated CHO cell cytotoxicity, the toxicological endpoint frequently used in the toxic potency-weighting approach,[12−16] is additive when DBPs are present at the concentration ratios measured in real disinfected waters has not been evaluated.

Although previous research has evaluated the toxicity of whole disinfected drinking waters,[32−35] wastewater effluents,[36−38] and swimming pool waters,[39−41] such studies did not address DBP interaction effects. As known DBPs account for only ∼30% (on a median basis) of the total organic halogen (TOX) in chlorinated waters,[9,42] DBP mixtures extracted from whole disinfected waters are poorly defined and different DBP classes cannot be isolated by selective removal. Characterization of the toxicological effects of DBPs using defined mixtures is an appropriate approach to test the hypothesis of DBP mixture additivity.

In the present study, we addressed two questions: (1) Is the CHO cell cytotoxicity associated with known DBPs additive? (2) Do unregulated DBPs contribute more toward the overall cytotoxicity of mixtures than regulated DBPs? Our approach involved spiking DBP standards directly into small volumes of organic solvent based on the relative concentrations of those compounds reported[12,15] for eight different conventional drinking waters and potable reuse waters. These waters were chosen for their differences in specific DBP classes. These defined DBP mixtures were assessed for CHO cell chronic cytotoxicity. We compared the observed cytotoxicity of these mixtures with the sum of individual toxic potency-weighted DBP concentrations to assess additivity. We selectively included specific DBP classes and excluded others to investigate which DBP classes contributed the most toward the observed cytotoxicity of the mixture.

MATERIALS AND METHODS

Preparation of Defined DBP Mixtures. Authentic DBP standards were used to prepare mixtures by spiking them directly into 1 mL of either acetonitrile, methyl *tert*-butyl ether (MtBE), or a mixture of MtBE and methanol. Names and vendor information for the DBP standards are listed in Table S1. The concentrations of DBPs in the mixtures were based on those reported[12,15] for conventional drinking waters (three mixtures, DW1−DW3) and potable reuse waters (five mixtures). The supplies for all three of the drinking waters were surface waters. DW1 was treated by ozonation and biofiltration followed by chlorine disinfection. DW2 was treated by chlorine, chlorine dioxide, filtration, and chloramination. DW3 was treated by clarification, ozonation, filtration, chlorination, and then chloramination. Two potable reuse waters (PRW1 and PRW2) were from treatment trains based on microfiltration (ultrafiltration for PRW2), reverse osmosis, and the UV/hydrogen peroxide advanced oxidation process (MF/RO/AOP), while the other three (PRW3−PRW5) were from treatment trains based on oxidation, biofiltration, and/or granular activated carbon (Ox/BAF/GAC). The secondary effluent at PRW3 was treated by filtration, ozonation, biological activated carbon (BAC), ozonation, and biofiltration. The secondary effluent at PRW4 was treated by riverbank filtration, softening, the UV/hydrogen peroxide AOP, BAC, and GAC. The secondary effluent at PRW5 was treated by sedimentation, ozonation, BAC, and GAC. The effluents from PRW1−PRW4 were chloraminated, while the effluent from PRW5 was chlorinated. Additional details of the treatment trains and disinfectants employed, as well as the references for the individual waters, are presented in Text S1.

Individual DBP standards were spiked directly into 1 mL of organic solvent to achieve concentrations that were 10^6-fold higher than those measured previously[12,15] in the water samples. This step was necessary so that the DBP-spiked solvent could be diluted into the cell culture media to avoid cytotoxicity of the solvent to CHO cells (Text S2). With this approach, we preconcentrated the samples for bioassay analysis without any DBP loss associated with extraction from water and concentration by nitrogen gas blowdown. The DBP concentrations in the original waters are compiled in Table 1.

CHO Cell Chronic Cytotoxicity Analyses. CHO cell cytotoxicity captures a wide array of toxic insults and adverse biological impacts. This assay measures the reduction in cell density after exposure of CHO cells (CHO cell line K1; AS52, clone 11−4−8) to a defined DBP mixture for 72 h (a chronic exposure encompassing 3−4 cell divisions) compared to that in untreated concurrent controls.[11] Detailed descriptions of the CHO cell line, this assay, and the statistical analyses of the data were published.[11,43] This CHO cell line, which had been used in previous research to develop cytotoxicity LC_{50} values (Table S1), did not feature cytochrome P450 monooxygenases. However, the DBPs evaluated (Table 1), except for nitrosamines, are direct-acting agents that do not require activation by cytochrome P450 monooxygenases.

The CHO cells were maintained in a Hams F12 medium containing 5% fetal bovine serum (FBS), 1% antibiotics (0.25 μg/mL amphotericin B, 100 μg/mL streptomycin sulfate, and 100 units/mL sodium penicillin G in 0.85% saline), and 1% L-glutamine in a mammalian cell incubator (37 °C) with a humidified atmosphere (5% CO_2).[44]

The assay evaluates a series of concentrations of the DBP mixture samples for each experimental group using a 96-well flat-bottomed microplate. One column of eight microplate wells contained 200 μL of F12 + 5% FBS medium as blank controls. Another column containing 3×10^3 CHO cells plus F12 + FBS medium served as the concurrent negative control. The remaining wells contained 3×10^3 CHO cells, F12 + FBS, and a known concentration of the defined DBP mixture sample at a total volume of 200 μL. A sheet of sterile Alumna Seal covered the wells to prevent volatile cross contamination of adjacent wells. The microplate was placed on a rocking platform at 37 °C for two 5 min periods (the plate was turned 90° after the first 5 min) to ensure an even distribution of cells.

https://dx.doi.org/10.1021/acs.est.0c00958
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The cells were placed in the mammalian cell incubator (37 °C, 5% CO_2) for 72 h.

After 72 h, the medium from each well was aspirated, and the cells were fixed in methanol for 5 min and stained for 5 min with a 1% crystal violet solution in 50% methanol. The microplate was washed in tap water. The water was removed, and 50 μL of DMSO/methanol (3:1 v/v) was added to each well. The microplate was incubated at room temperature for 10 min and then analyzed at 595 nm with a SpectraMax microplate reader. The absorbency of each well was recorded and stored in a spreadsheet file. There was a direct relationship between the absorbency of the crystal violet dye associated with the cell density and the number of viable cells, as demonstrated by previous calibrations.[45] The averaged absorbency of the blank wells was subtracted from the absorbency data from each well. The mean blank-corrected absorbency value of the negative control was set at 100%. The absorbency for each of the other wells was converted into a percentage of the negative control. This procedure normalized the data, generated error terms for the control and each concentration, and allowed the combination of data from multiple microplates for use in generating a concentration–response curve for each defined DBP mixture sample.

The range in summed molar concentrations was selected to span from concentrations that did not induce cytotoxicity to concentrations that reduced cell density in the microplate well. A cytotoxicity concentration–response curve for each defined DBP mixture was generated from summary data that included a range-finding experiment plus 2 repeated experiments with 4–24 independent replicates per concentration. Regression analysis was applied to each defined DBP mixture sample concentration–response curve in order to calculate the LC_{50}. The LC_{50} is the calculated total molar concentration of the DBPs in a defined DBP mixture sample that reduced the cell density to 50% of the negative control. For each defined DBP mixture, a one-way analysis of variance (ANOVA) test was conducted to determine the lowest summed molar concentration that induced a statistically significant level of cytotoxicity as compared to their concurrent negative control ($P \leq 0.05$). The power of the ANOVA test was maintained at >0.8 at $\alpha = 0.05$.[46] If an experimental series did not achieve this statistical power, additional experiments were conducted to increase the degrees of freedom in the dataset. Bootstrap statistics were used to generate a mean and a standard error of the mean for the LC_{50} value for each defined DBP mixture sample.[47,48] A detailed discussion of the statistical methods was published.[11]

Toxicity Index Calculations. For each mixture, the calculated additive toxicity (CAT) index was computed by summing the individual toxic potency-weighted DBP concentrations according to eq 1, where the molar concentration of each DBP in the original water sample is divided by the molar concentration of that DBP that is associated with a 50% reduction in CHO cell density compared to untreated controls (i.e., cytotoxicity LC_{50} values); the LC_{50} values used to calculate CAT were obtained from published literature[11] and are provided in Table S1. The inverse of each LC_{50}-weighted DBP concentration represents the concentration factor needed for the DBP concentration in the original water to exert a 50% reduction in CHO cell density. The bioassay-based calculated additive toxicity (BCAT) index (eq 2) was computed as the ratio of the summed molar concentration of DBPs measured in the water sample to the summed molar concentration of DBPs that exerted a 50% reduction in the cell density. Similar to CAT, BCAT represents the inverse of the concentration factor (CF_{50}) needed for the defined mixture of DBP concentrations in the original water to exert a 50% reduction in CHO cell density. The percent difference, computed according to eq 3, was used to compare the values of CAT and BCAT for each mixture.

$$\mathrm{CAT} = \sum_{i=1}^{n} \left(\frac{[\mathrm{DBP}]_i}{\mathrm{LC}_{50_i}} \right) \tag{1}$$

$$\mathrm{BCAT} = \frac{\left(\sum_{i=1}^{n} [\mathrm{DBP}]_i\right)_{\mathrm{initial}}}{\left(\sum_{i=1}^{n} [\mathrm{DBP}]_i\right)_{\mathrm{LC}_{50}}} = \left(\frac{1}{\mathrm{CF}_{50}} \right) \tag{2}$$

$$\text{percent difference} = \frac{|\mathrm{CAT} - \mathrm{BCAT}|}{((\mathrm{CAT} + \mathrm{BCAT})/2)} \times 100\% \tag{3}$$

RESULTS AND DISCUSSION

Figure 1 illustrates the concentration–response curves for PRW3, including mixtures containing subsets of DBP classes.

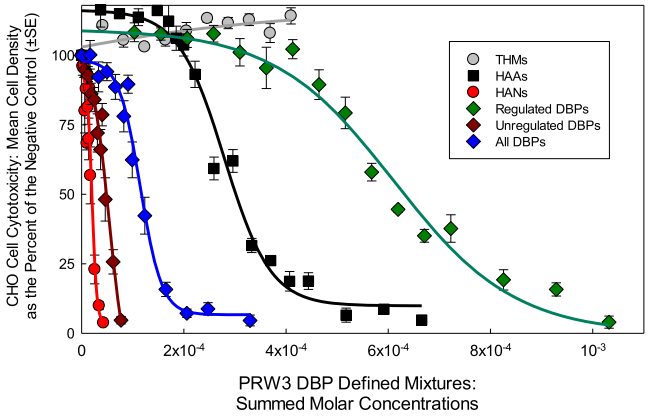

Figure 1. Concentration–response curves comparing the CHO cell cytotoxicity of the PRW3 defined DBP mixture samples. The LC_{50} represents the total molar concentration of the DBPs associated with a 50% reduction in the cell density compared to the untreated controls, as determined by regression from these concentration–response curves. Knowing the total molar concentration of DBPs in the original water sample, we can convert this LC_{50} value into the concentration factor of the original water sample associated with the LC_{50} (i.e., the CF_{50}).

Table 2 provides statistics for PRW3. Cytotoxicity increased (LC_{50} values decreased) in the order haloacetonitriles (HANs) > other unregulated DBPs ≫ all-DBPs ~ HAAs. The THM mixture was not cytotoxic. Cytotoxicity for the HAN and unregulated DBP mixtures occurred at lower total molar DBP concentrations in these mixtures than for the all-DBP mixture; because of the relatively low cytotoxicity of the regulated THMs and HAAs, a higher total molar DBP concentration was needed to observe cytotoxicity in the all-DBPs mixture. Additivity between DBP classes is discussed below. The average relative standard error of the LC_{50} values for PRW3 was 3%. Concentration–response curves for individual DBP mixtures for the other water samples are presented in Figures S2–S8; corresponding statistical analyses are presented in Tables S2–S8. For each mixture, the mean LC_{50} ± SE; the

https://dx.doi.org/10.1021/acs.est.0c00958
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Table 2. Summary of CHO Cell Cytotoxicity Statistics for PRW3 Defined DBP Mixtures

PRW3 DBP mixture sample	lowest cytotoxic conc. ($\sum$M)[a]	mean LC_{50} value ± SE ($\sum$M)[b]	r^2[c]	ANOVA test statistic[d]
THM4	NS[e]	NA[e]	NA	NS
HAAs	2.58×10^{-4}	$3.01 \pm 0.03 \times 10^{-4}$	0.97	$F_{18, 81} = 164.5$; $P \leq 0.001$
HANs	1.18×10^{-5}	$1.94 \pm 0.11 \times 10^{-5}$	0.92	$F_{13, 90} = 41.06$; $P \leq 0.001$
regulated DBPs	5.16×10^{-4}	$6.09 \pm 0.06 \times 10^{-4}$	0.98	$F_{16, 87} = 79.72$; $P \leq 0.001$
unregulated DBPs	1.84×10^{-5}	$4.78 \pm 0.22 \times 10^{-5}$	0.97	$F_{12, 91} = 64.62$; $P \leq 0.001$
all-DBPs	8.23×10^{-5}	$1.17 \pm 0.04 \times 10^{-4}$	0.98	$F_{12, 91} = 104.2$; $P \leq 0.001$

[a]Lowest cytotoxic concentration was the lowest summed molar concentration ($\sum$M) of the PRW3 defined DBP mixture samples that induced a statistically significant reduction in cell density as compared to their concurrent negative controls. [b]The LC_{50} value is the concentration factor of the water sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The mean and the standard error (SE) were derived from multiple regression analyses using bootstrap statistics. [c]The r^2 is the coefficient of determination for the regression analysis of the concentration−response data upon which the LC_{50} value was calculated. [d]This is the degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value. [e]NS = not significant; NA = not applicable.

values for CF_{50}, BCAT, CAT, and the percent difference between BCAT and CAT are presented in Tables S9−S16.

Comparison of BCAT and CAT. The median percent difference between BCAT and CAT for the DBP mixtures listed in Table 1 was 12% (maximum 39% for PRW5, Table 3). The agreement between BCAT and CAT indicates that the cytotoxicity of individual DBPs in these mixtures was additive (Figure 2).

Table 3. Median and Maximum Values for the Percent Difference between CAT and BCAT and the Percent Contribution of BCAT Values[a]

	difference between CAT and BCAT (%)			contribution to BCAT for all-DBP mix (%)		
water	all-DBPs	reg[b] DBPs	unreg[b] DBPs	reg DBPs	unreg DBPs	reg + unreg
DW1	16	39	10	22	93	115
DW2	14	7	18	10	88	98
DW3	9	89	18	9	88	98
PRW1	5	NA[c]	16	0	117	117
PRW2	1	67	15	12	89	101
PRW3	10	29	4	12	91	103
PRW4	23	76	32	1	55	57
PRW5	39	39	18	12	110	122
Median Value						
	12	39	17	11	90	102
Maximum Value						
	39	89	32	22	117	122

[a]The percent contribution of the BCAT values are for the contributions of the regulated and unregulated DBP subset mixtures to the BCAT for the all-DBPs mixtures. [b]The abbreviation reg = regulated, and unreg = unregulated. [c]NA = not applicable.

Toxicity Drivers in DBP Mixtures. For each water, we prepared separate mixtures containing subsets of DBPs at the same concentrations as those in the all-DBPs mixture. We compared the regulated DBPs (THM4, HAA5, and NDMA) or all of the unregulated DBPs to the full DBP mixtures (Figure 3). The median percent difference between CAT and BCAT was 39% (maximum 89% for DW3) for the regulated DBPs and 17% (maximum 32% for PRW4) for the unregulated DBPs (Table 3). The greater percent difference for the regulated DBPs may reflect the experimental uncertainty associated with low BCAT values. The median BCAT values for the regulated DBP mixtures were nearly an order of magnitude lower than those for the unregulated DBP mixtures as well as those for the all-DBPs mixtures; BCAT was not detectable for PRW1. Nonetheless, the low percentage difference between CAT and BCAT values for the regulated

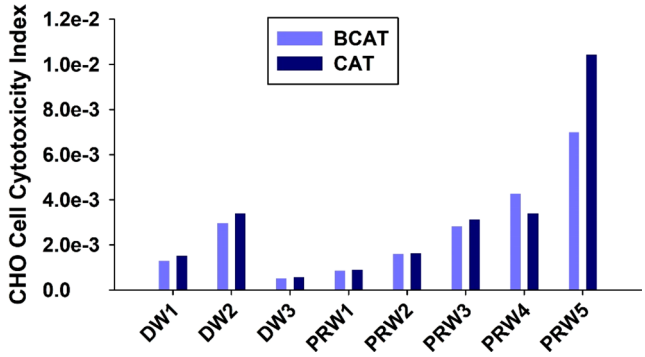

Figure 2. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices obtained from CHO cell chronic cytotoxicity assays for defined mixtures containing DBPs measured in three surface water-derived drinking waters (DW) and five potable reuse waters (PRW). DW1 employed ozone, biofiltration, and chlorine. DW2 employed chlorine, chlorine dioxide, filtration, and chloramines. DW3 employed ozone, filtration, chlorine, and chloramines. PRW1 and PRW2 are based on treatment trains employing microfiltration, reverse osmosis, and advanced oxidation processes (MF/RO/AOP), while PRW3−PRW5 are based on treatment trains employing oxidation, biofiltration, and/or granular activated carbon (Ox/BAF/GAC).

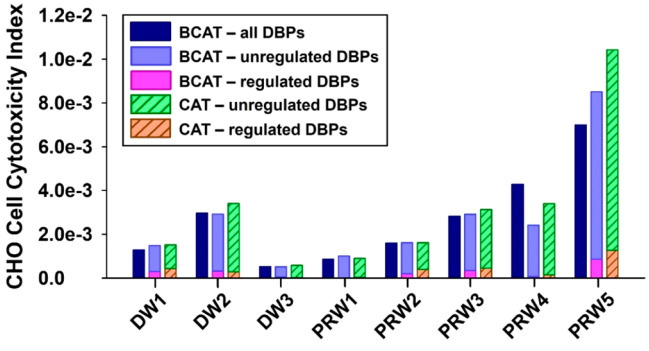

Figure 3. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices obtained from CHO cell chronic cytotoxicity assays for defined mixtures containing all of the DBPs listed in Table 1, unregulated DBPs only, or regulated DBPs (THM4, HAA5, and NDMA) only.

https://dx.doi.org/10.1021/acs.est.0c00958
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and unregulated DBP mixtures provides validation that the cytotoxicity of these DBPs is additive (Figures S9−S14).

The BCAT of the regulated DBP subset was 11% of the BCAT of the all-DBPs mixture on a median basis (22% maximum for DW1, Table 3). The BCAT of the unregulated DBP subset was 90% of the BCAT of the all-DBPs mixture on a median basis (117% maximum for PRW1). The unregulated DBPs were substantially greater forcing factors of cytotoxicity at the concentrations at which they occur in conventional drinking waters and potable reuse waters. Using a bioassay-based approach, the sum of BCAT values for the regulated and unregulated DBP subsets for each water was 102% of the BCAT values for the all-DBPs mixtures on a median basis (maximum 122% for PRW5). These results demonstrate that the cytotoxicity of the subset DBP mixtures was additive as measured by the CHO cell bioassay, independent of the calculated toxicity (CAT values).

For five of the waters, additional subset mixtures were analyzed to evaluate specific DBP classes for toxicity. Figure 4

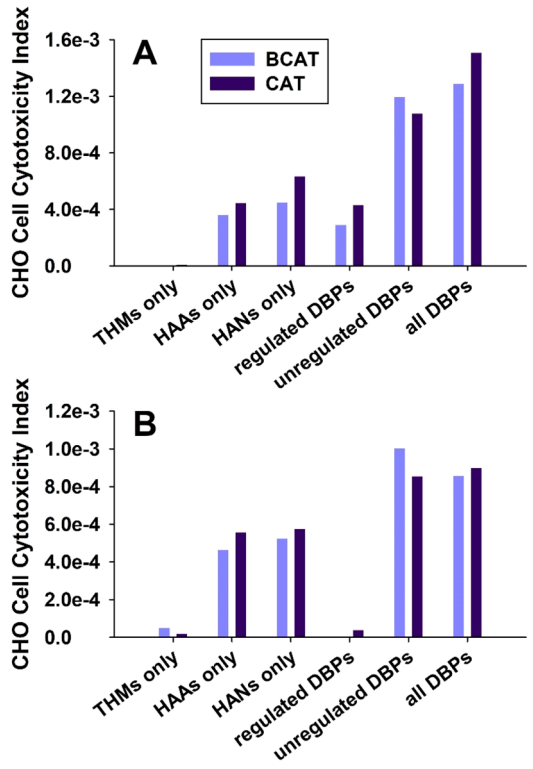

Figure 4. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices obtained from CHO cell chronic cytotoxicity assays for defined mixtures of various DBP classes in (a) DW1 and (b) PRW3.

presents results for DW1 and PRW3; Figures S11, S12, and S14 provide results for the other waters. For both BCAT and CAT, the contribution of THM4 to cytotoxicity was negligible for DW1 and PRW3. For both waters, HAA5 dominated the cytotoxicity of the regulated DBP subset. Haloacetonitriles accounted for a large fraction of the cytotoxicity of the unregulated DBP subset. Although we did not isolate the contributions of other unregulated DBP classes by bioassay analysis, CAT calculations indicated that haloacetamides and haloacetaldehydes may also contribute to the cytotoxicity. Similar results were observed in the other waters.

There has been substantial interest in the potential contribution of iodinated DBPs to toxicity.[34,49,50] We measured the BCAT associated with the mixture of THM4 and iodinated trihalomethanes (I-THMs) in PRW2 (Figure S12) and of I-THMs alone in PRW5 (Figure S14). The BCAT for the mixture of THM4 and I-THMs in PRW2 was not detectable, while the BCAT for I-THMs in PRW5 was 0.4% of the BCAT of the all-DBPs mixture. These results suggest that I-THMs are relatively unimportant contributors to cytotoxicity at the concentrations at which they occur in many conventional drinking waters and potable reuse waters.

Implications. Using defined mixtures of DBPs with the same relative concentrations as those measured in disinfected drinking and potable reuse waters, we demonstrated that the cytotoxicity indices measured by the CHO cell cytotoxicity bioassay (BCAT) agree with those calculated by summing the concentrations of individual DBPs weighted by their published cytotoxicity LC_{50} values (CAT). The agreement between BCAT and CAT also held for mixtures of subsets of the DBPs, and the sum of the BCAT values for subset mixtures matched the BCAT values for the all-DBP mixtures. Previous estimates of CHO cell cytotoxicity associated with DBP mixtures in disinfected waters, obtained using toxic potency-weighted DBP concentrations, assumed that the cytotoxicity of individual DBPs in mixtures was additive. Our study tested this assumption and found it to be valid.

Our results show that CAT calculations can be used to indicate the relative importance of specific DBP classes to CHO cell cytotoxicity. Furthermore, our results concur with previous CAT estimates that unregulated DBP classes, such as haloacetonitriles,[12−16,30,51] may be more important contributors to cytotoxicity than the regulated DBPs, at least with respect to mammalian cells. Given the limited *in vivo* analyses, we suggest that CAT calculations can help prioritize DBPs, either individually or within mixtures. The contribution of the known DBPs to cytotoxicity (as estimated by CAT) should be compared to the BCAT of whole waters (which predominantly represents the unknown DBP fraction) to evaluate whether the known DBPs that are of current research interest are important relative to the unknown DBP fraction. It is important to note that additivity for DBPs with other toxicity endpoints remains uncertain, although we are currently evaluating additivity with respect to CHO cell genotoxicity. The results of such studies can help identify potential toxicity drivers for further evaluation in *in vivo* toxicity studies and for associations with adverse health effects in future epidemiological studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c00958.

Description of water samples, CHO cell cytotoxicity concentration−response curves, LC_{50} tables, and BCAT vs CAT plots for DBP subset mixtures (PDF)

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https://dx.doi.org/10.1021/acs.est.0c00958
Environ. Sci. Technol. 2020, 54, 5729−5736

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by funding from the National Science Foundation (CBET 1706154 and CBET 1706575).

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https://dx.doi.org/10.1021/acs.est.0c00958
Environ. Sci. Technol. 2020, 54, 5729−5736

Supporting Information for

Assessing additivity of cytotoxicity associated with disinfection byproducts in potable reuse and conventional drinking waters

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Contains 28 pages, 16 tables, and 14 figures

Table S1. Names, vendor information, and Chinese hamster ovary (CHO) cell LC_{50} values for individual DBPs used to prepare defined mixtures

Compound	Abbreviation	CHO LC_{50} (M) [a]	Vendor
Acetaldehyde	—	N/A [b]	Sigma-Aldrich
Trichloromethane (chloroform)	TCM	9.62×10^{-3}	Fisher Scientific
Bromodichloromethane	BDCM	1.15×10^{-2}	Sigma-Aldrich
Dibromochloromethane	DBCM	5.36×10^{-3}	Sigma-Aldrich
Tribromomethane (bromoform)	TBM	3.96×10^{-3}	Sigma-Aldrich
Dichloroiodomethane	DCIM	4.13×10^{-3}	CanSyn Chem. Corp.
Bromochloroiodomethane	BCIM	2.42×10^{-3}	CanSyn Chem. Corp.
Dibromoiodomethane	DBIM	1.91×10^{-3}	CanSyn Chem. Corp.
Chlorodiiodomethane	CDIM	2.41×10^{-3}	CanSyn Chem. Corp.
Bromodiiodomethane	BDIM	1.40×10^{-3}	CanSyn Chem. Corp.
Triiodomethane (iodoform)	TIM	6.60×10^{-5}	Sigma-Aldrich
Trichloroacetaldehyde (chloral hydrate)	TCAL	1.16×10^{-3}	Sigma-Aldrich
Bromodichloroacetaldehyde	BDCAL	2.04×10^{-5}	CanSyn Chem. Corp.
Dibromochloroacetaldehyde	DBCAL	5.15×10^{-6}	CanSyn Chem. Corp.
Tribromoacetaldehyde	TBAL	3.58×10^{-6}	Sigma-Aldrich
Trichloronitromethane (chloropicrin)	TCNM	5.36×10^{-4}	Chem Service
1,1-Dichloropropanone	1,1-DCP	N/A	Supelco
1,1,1-Trichloropropanone	1,1,1-TCP	N/A	Sigma-Aldrich
Trichloroacetonitrile	TCAN	1.60×10^{-4}	Acros Organics
Dichloroacetonitrile	DCAN	5.73×10^{-5}	Sigma-Aldrich
Bromochloroacetonitrile	BCAN	8.46×10^{-6}	AccuStandard
Dibromoacetonitrile	DBAN	2.85×10^{-6}	Matrix Scientific
Dichloroacetamide	DCAM	1.92×10^{-3}	Alfa Aesar
Bromochloroacetamide	BCAM	1.71×10^{-5}	CanSyn Chem. Corp.
Trichloroacetamide	TCAM	2.05×10^{-3}	Sigma-Aldrich
Dibromoacetamide	DBAM	1.22×10^{-5}	CanSyn Chem. Corp.
Chloroacetic acid	CAA	8.10×10^{-4}	Fluka
Bromoacetic acid	BAA	9.60×10^{-6}	Sigma-Aldrich
Dichloroacetic acid	DCAA	7.30×10^{-3}	Alfa Aesar
Trichloroacetic acid	TCAA	2.40×10^{-3}	Alfa Aesar
Bromochloroacetic acid	BCAA	7.78×10^{-4}	Sigma-Aldrich
Dibromoacetic acid	DBAA	5.90×10^{-4}	Supelco
Bromodichloroacetic acid	BDCAA	6.85×10^{-4}	Sigma-Aldrich
Chlorodibromoacetic acid	CDBAA	2.02×10^{-4}	Sigma-Aldrich
Tribromoacetic acid	TBAA	8.50×10^{-5}	Acros Organics
Iodoacetic acid	IAA	2.95×10^{-6}	Sigma-Aldrich
N-Nitrosodimethylamine	NDMA	N/A	Chem Service
N-Nitrosomorpholine	NMOR	1.11×10^{-2}	Sigma-Aldrich

[a] CHO LC_{50} values from ref. 1

[b] N/A = not available

Text S1. Description of waters

We selected three conventional drinking waters, two potable reuse waters treated by MF/RO/AOP-based treatment trains, and three potable reuse waters treated by Ox/BAF/GAC-based treatment trains for which the concentrations of regulated and unregulated DBPs have been reported previously. Table S2 provides the concentrations of the DBPs in each water. These waters covered a range of water types and exhibited a range of different DBP concentrations. Descriptions of the different waters are provided below.

DW1

This water is the third sampling event for the conventional drinking water associated with Utility 1 in ref. 2. This water is a surface water treated by ozonation, flocculation, and biofiltration. The biofiltration effluent was adjusted to pH 8 in the laboratory and then treated with free chlorine to attain a residual of ~1 mg/L as Cl_2 after 24 h contact at room temperature.

DW2

This water is the second sampling event for the conventional drinking water associated with Utility 5 in ref. 2. This water is a surface water treated with alum, chlorine, chlorine dioxide, flocculation, and filtration. The filter effluent was treated with preformed monochloramine (5 mg/L as Cl_2) in the laboratory at pH 8 for 3 days at room temperature, sufficient to leave a residual of at least 1 mg/L as Cl_2 after that time.

DW3

This water is the third sampling event for the conventional drinking water associated with Utility 3 in ref. 2. This water is a surface water treated by coagulation, clarification, ozonation, filtration, chlorination, and chloramination for maintaining a distribution system residual. The sample was collected directly from the clearwell.

PRW1

The DBP concentrations represent the average values measured over two sampling events for the simulated distribution system sample for the RO/AOP-based potable reuse train associated with Utility B in ref. 3. Secondary effluent was treated by chloramination, microfiltration, RO, and UV/H_2O_2 AOP. The AOP effluent was treated with preformed monochloramine (2.5 mg/L as Cl_2) in the laboratory at pH 8 for 3 days at room temperature, sufficient to leave a residual of at least 1 mg/L as Cl_2 after that time.

PRW2

This water is the first sampling event for the RO/AOP-based potable reuse train associated with Utility 3 in ref. 2. Secondary effluent was treated by chloramination, ultrafiltration, RO, and UV/H_2O_2 AOP. However, this sample was collected from the RO effluent and was treated with

preformed monochloramine (5 mg/L as Cl_2) in the laboratory at pH 8 for 3 days at room temperature, sufficient to leave a residual of at least 1 mg/L as Cl_2 after that time.

PRW3

This water is the third sampling event for the Ox/BAF/GAC-based potable reuse train associated with Utility 1 in ref. 2. Secondary effluent was treated by coagulation, filtration, ozonation, BAC, ozonation, and then additional treatment in a pilot unit by ozone, flocculation, and biofiltration. The biofiltration effluent was treated with preformed monochloramine (5 mg/L as Cl_2) in the laboratory at pH 8 for 3 days at room temperature, sufficient to leave a residual of at least 1 mg/L as Cl_2 after that time.

PRW4

This water is the second sampling event for the Ox/BAF/GAC-based potable reuse train associated with Utility 5 in ref. 2. Secondary effluent was treated by riverbank filtration, softening, UV/H_2O_2 AOP, BAC, and GAC. The GAC effluent was treated with preformed monochloramine (5 mg/L as Cl_2) in the laboratory at pH 8 for 3 days at room temperature, sufficient to leave a residual of at least 1 mg/L as Cl_2 after that time.

PRW5

This water is the third sampling event for the Ox/BAF/GAC-based potable reuse train associated with Utility 3 in ref. 2. Secondary effluent was treated by chloramination followed by flocculation and settling, ozonation, BAC, and GAC. The GAC effluent was adjusted to pH 8 in the laboratory and then treated with free chlorine to attain a residual of ~1 mg/L as Cl_2 after 24 h contact at room temperature.

Text S2. Solvent toxicity

Organic solvents can cause cytotoxicity in CHO cells when present at sufficiently high concentrations in cell culture media. The solvents used to prepare the eight defined DBP mixture samples were acetonitrile (DW1, PRW1, PRW2, and PRW3), methyl *tert*-butyl ether (MtBE) (DW3 and PRW5), and an azeotrope of MtBE and methanol azeotrope (containing 70% MtBE and 30% methanol; DW2 and PRW4). In order to determine the maximum solvent concentration that would not cause artifacts in CHO cell chronic cytotoxicity assays, we exposed CHO cells to various volume percentages of the three solvents without addition of any DBPs.

Concentration-response curves obtained for the three solvents are shown in Figure S1. For acetonitrile, > 98% of CHO cells were viable at a solvent concentration of 0.55%. For MtBE and MtBE-methanol mixtures, CHO cell viability began to decrease at a solvent concentration above 0.6% (Figure S1). Accordingly, we maintained solvent concentrations below their toxic levels while generating the dilution series from defined DBP mixtures in cell culture medium in order to prevent artifacts due to solvent toxicity.

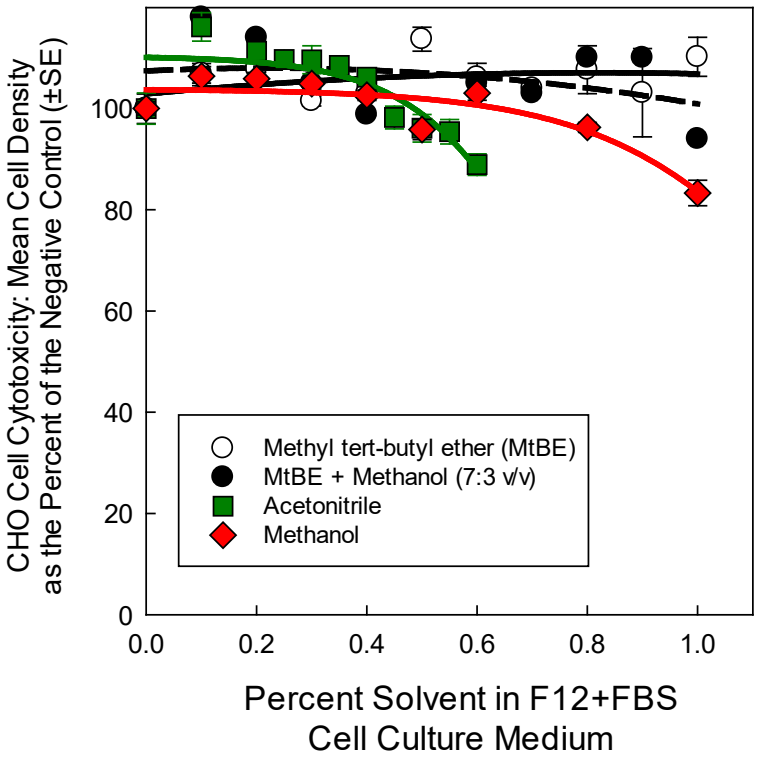

Figure S1. Concentration-response curves illustrating the CHO cell cytotoxicity analyses of the solvents, methyl *tert*-butyl ether (MtBE), MtBE-methanol mixture, acetonitrile, and methanol in terms of the volume percentage of solvent in cell culture media.

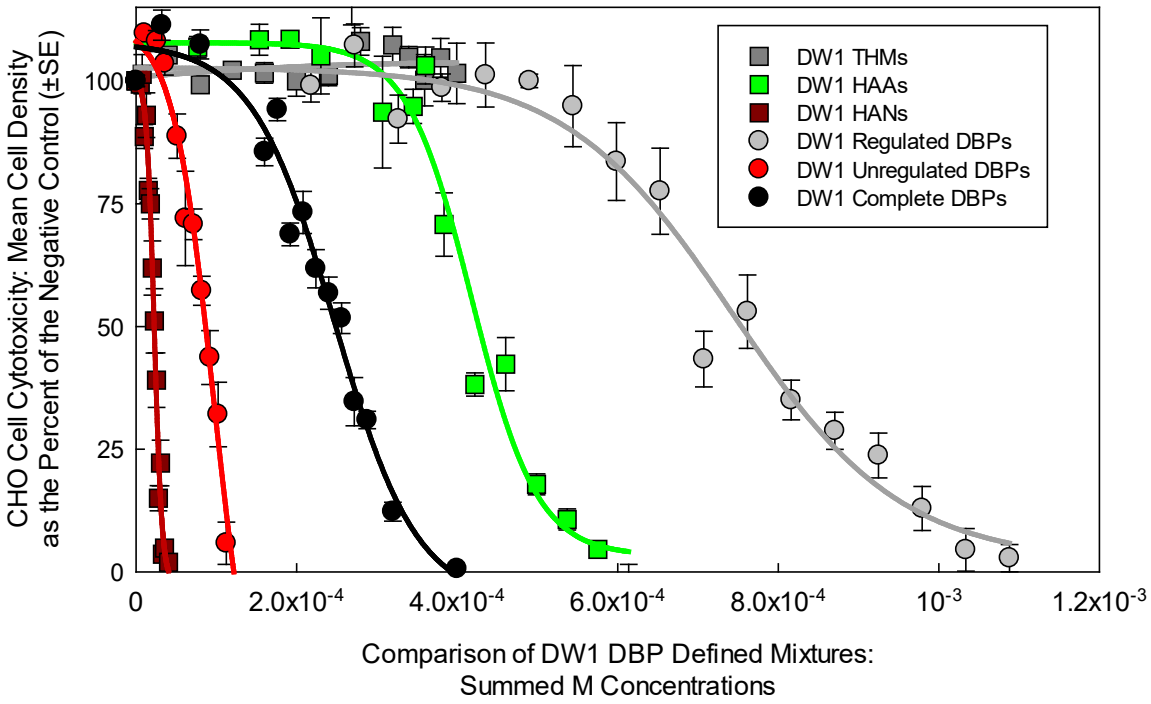

Figure S2. Concentration-response curves comparing the CHO cytotoxicity of DW1 DBP defined mixtures. The y-axis indicates the summed molar concentration of DBPs in the CHO cell culture medium. The LC_{50} represents the summed molar concentration of DBPs associated with a 50% reduction in cell density compared to the untreated controls, as determined by regression from these concentration-response curves. Knowing the summed molar concentration of DBPs in the original water sample, this LC_{50} value can be converted into the concentration factor of the original water sample associated with the LC_{50} (i.e., the CF_{50}).

Table S2. Summary of CHO cell cytotoxicity statistics for DW1 defined DBP mixtures

DW1 DBP Mixture Sample	Lowest Cytotoxic Conc. (ΣM) [a]	Mean LC_{50} Value ± SE (ΣM) [b]	r^2 [c]	ANOVA Test Statistic [d]
THM4	NS [e]	NA [e]	NA	NS (not toxic)
HAAs	3.83×10^{-5}	$4.26 \pm 0.05 \times 10^{-4}$	0.96	$F_{17, 90} = 93.99$; $P < 0.001$
HANs	1.51×10^{-5}	$2.25 \pm 0.07 \times 10^{-5}$	0.98	$F_{14, 97} = 86.94$; $P < 0.001$
Regulated DBPs	5.98×10^{-4}	$7.52 \pm 0.12 \times 10^{-4}$	0.97	$F_{17, 84} = 50.40$; $P < 0.001$
Unregulated DBPs	6.13×10^{-5}	$8.54 \pm 0.18 \times 10^{-5}$	0.98	$F_{11, 76} = 54.92$; $P < 0.001$
All DBPs	1.60×10^{-4}	$2.48 \pm 0.02 \times 10^{-4}$	0.97	$F_{14, 136} = 160.7$; $P < 0.001$

[a] Lowest cytotoxic concentration was the lowest summed molar concentration (ΣM) of the DW1 defined DBP mixture samples that induced a statistically significant reduction in cell density as compared to their concurrent negative controls.

[b] The LC_{50} value is the concentration factor of the water sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The mean and the standard error (SE) were derived from multiple regression analyses using bootstrap statistics.

[c] The r^2 is the coefficient of determination for the regression analysis of the concentration-response data upon which the LC_{50} value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

[e] NS = not significant; NA = not applicable.

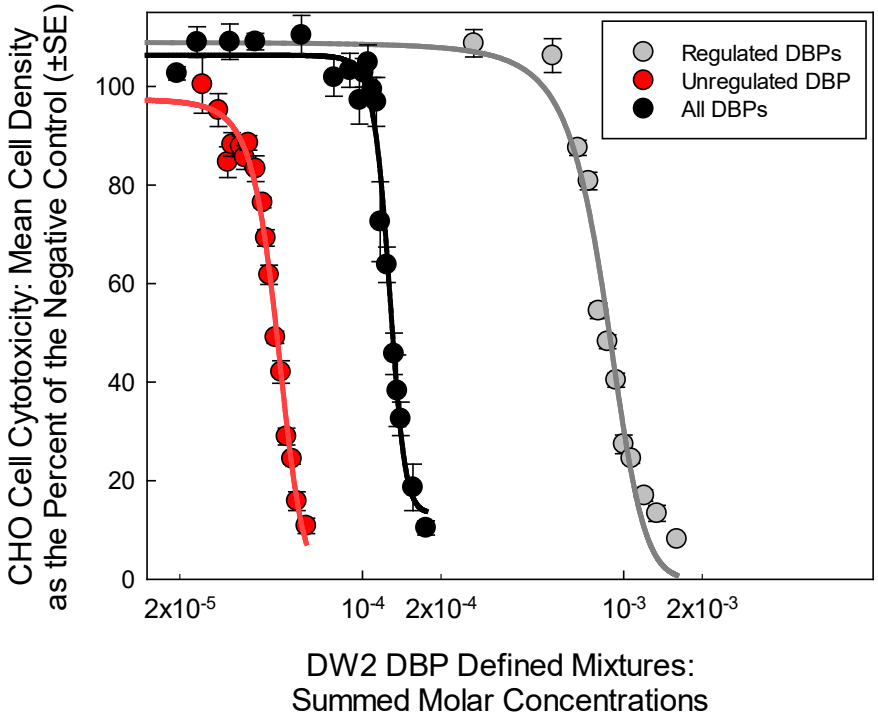

Figure S3. Concentration-response curves comparing the CHO cytotoxicity of DW2 defined DBP mixture samples. The LC_{50} represents the summed molar concentration of DBPs associated with a 50% reduction in cell density compared to the untreated controls, as determined by regression from these concentration-response curves. Knowing the total molar concentration of DBPs in the original water sample, this LC_{50} value can be converted into the concentration factor of the original water sample associated with the LC_{50} (i.e., the CF_{50}).

Table S3. Summary of CHO cell cytotoxicity statistics for DW2 defined DBP mixtures

DW2 DBP Mixture Sample	Lowest Cytotoxic Conc. (ΣM) [a]	Mean LC_{50} Value ± SE (ΣM) [b]	r^2 [c]	ANOVA Test Statistic [d]
Regulated DBPs	6.68×10^{-4}	$8.60 \pm 0.02 \times 10^{-4}$	0.98	$F_{12,138} = 565.3$; $P \leq 0.001$
Unregulated DBPs	3.05×10^{-5}	$4.68 \pm 0.02 \times 10^{-5}$	0.98	$F_{17,138} = 250.4$; $P \leq 0.001$
All DBPs	1.17×10^{-4}	$1.31 \pm 0.01 \times 10^{-4}$	0.98	$F_{19,124} = 38.8$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest summed molar concentration (ΣM) of the DW2 defined DBP mixture samples that induced a statistically significant reduction in cell density as compared to their concurrent negative controls.

[b] The LC_{50} value is the concentration factor of the water sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The mean and the standard error (SE) were derived from multiple regression analyses using bootstrap statistics.

[c] The r^2 is the coefficient of determination for the regression analysis of the concentration-response data upon which the LC_{50} value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

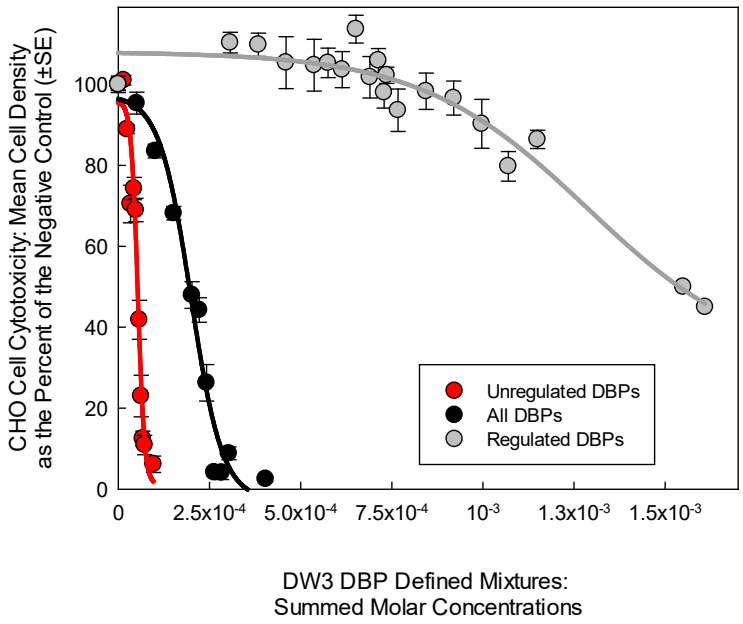

Figure S4. Concentration-response curves comparing the CHO cytotoxicity of DW3 defined DBP mixtures. The LC_{50} represents the summed molar concentration of DBPs associated with a 50% reduction in cell density compared to the untreated controls, as determined by regression from these concentration-response curves. Knowing the total molar concentration of DBPs in the original water sample, this LC_{50} value can be converted into the concentration factor of the original water sample associated with the LC_{50} (i.e., the CF_{50}).

Table S4. Summary of CHO cell cytotoxicity statistics for DW3 defined DBP mixtures

DW3 DBP Mixture Sample	Lowest Cytotoxic Conc. (ΣM) [a]	Mean LC_{50} Value ± SE (ΣM) [b]	r^2 [c]	ANOVA Test Statistic [d]
Regulated DBPs	1.15×10^{-3}	$1.56 \pm 0.002 \times 10^{-3}$	0.73	$F_{17,93} = 3.07$; $P \leq 0.001$
Unregulated DBPs	2.40×10^{-5}	$5.19 \pm 0.11 \times 10^{-5}$	0.96	$F_{10,66} = 211.3$; $P \leq 0.001$
All DBPs	1.01×10^{-4}	$1.92 \pm 0.05 \times 10^{-4}$	0.97	$F_{10,62} = 385.8$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest summed molar concentration (ΣM) of the DW3 defined DBP mixture samples that induced a statistically significant reduction in cell density as compared to their concurrent negative controls.

[b] The LC_{50} value is the concentration factor of the water sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The mean and the standard error (SE) were derived from multiple regression analyses using bootstrap statistics.

[c] The r^2 is the coefficient of determination for the regression analysis of the concentration-response data upon which the LC_{50} value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

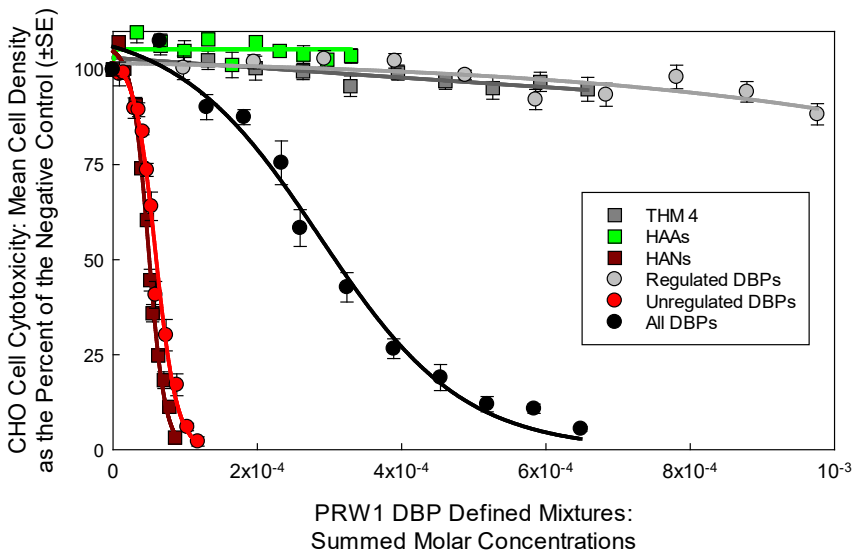

Figure S5. Concentration-response curves comparing the CHO cytotoxicity of PRW1 defined DBP mixture samples. The LC_{50} represents the summed molar concentration of DBPs associated with a 50% reduction in cell density compared to the untreated controls, as determined by regression from these concentration-response curves. Knowing the total molar concentration of DBPs in the original water sample, this LC_{50} value can be converted into the concentration factor of the original water sample associated with the LC_{50} (i.e., the CF_{50}).

Table S5. Summary of CHO cell cytotoxicity statistics for PRW1 defined DBP mixtures

PRW1 DBP Mixture Sample	Lowest Cytotoxic Conc. (ΣM) [a]	Mean LC_{50} Value ± SE (ΣM) [b]	r^2 [c]	ANOVA Test Statistic [d]
THM4	NS [e]	NA [e]	NA	NS
HAAs	NS	NA	NA	NS
HANs	3.13×10^{-6}	$5.02 \pm 0.04 \times 10^{-5}$	0.99	$F_{11, 80} = 410.9$; $P \leq 0.001$
Regulated DBPs	NS	NA	NA	NS
Unregulated DBPs	2.94×10^{-5}	$5.87 \pm 0.08 \times 10^{-5}$	0.98	$F_{12, 87} = 186.1$; $P \leq 0.001$
All DBPs	1.29×10^{-4}	$3.03 \pm 0.06 \times 10^{-4}$	0.99	$F_{11, 81} = 172.8$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest summed molar concentration (ΣM) of the PRW1 samples that induced a statistically significant reduction in cell density as compared to their concurrent negative controls.

[b] The LC_{50} value is the concentration factor of the water sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The mean and the standard error (SE) were derived from multiple regression analyses using bootstrap statistics.

[c] The r^2 is the coefficient of determination for the regression analysis of the concentration-response data upon which the LC_{50} value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

[e] NS = not significant; NA = not applicable.

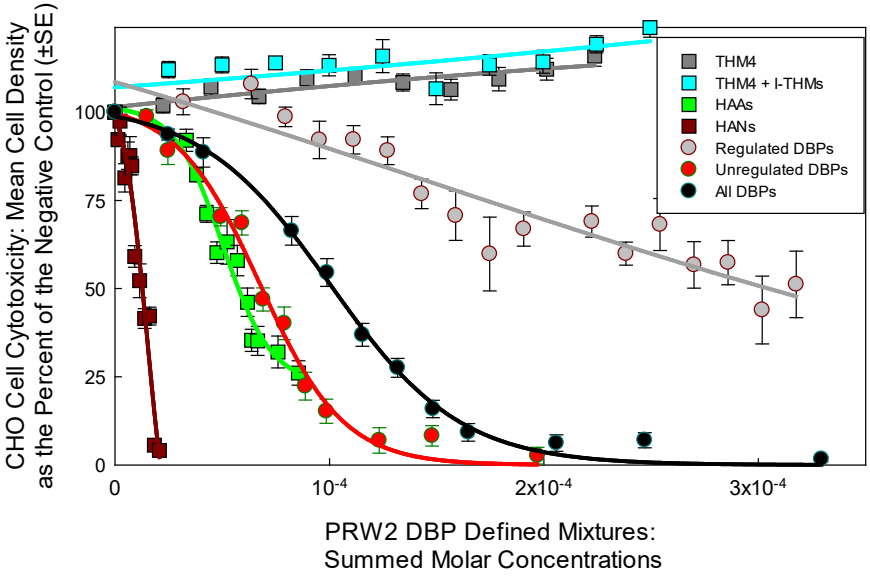

Figure S6. Concentration-response curves comparing the CHO cytotoxicity of PRW2 defined DBP mixture samples. The LC_{50} represents the summed molar concentration of DBPs associated with a 50% reduction in cell density compared to the untreated controls, as determined by regression from these concentration-response curves. Knowing the total molar concentration of DBPs in the original water sample, this LC_{50} value can be converted into the concentration factor of the original water sample associated with the LC_{50} (i.e., the CF_{50}).

Table S6. Summary of CHO cell cytotoxicity statistics for PRW2 defined DBP mixtures

PRW2 DBP Mixture Sample	Lowest Cytotoxic Conc. (ΣM) [a]	Mean LC_{50} Value ± SE (ΣM) [b]	r^2 [c]	ANOVA Test Statistic [d]
THM4	NS [e]	NA [e]	NA	NS
THM4 + I-THMs	NS	NA	NA	NS
HAAs	3.33×10^{-5}	$5.76 \pm 0.20 \times 10^{-5}$	0.96	$F_{12, 92} = 60.65$; $P \leq 0.001$
HANs	4.59×10^{-6}	$1.28 \pm 0.02 \times 10^{-5}$	0.95	$F_{12, 69} = 105.5$; $P \leq 0.001$
Regulated DBPs	1.43×10^{-4}	$3.25 \pm 0.15 \times 10^{-4}$	0.96	$F_{17, 93} = 14.57$; $P \leq 0.001$
Unregulated DBPs	2.46×10^{-5}	$6.94 \pm 0.16 \times 10^{-5}$	0.99	$F_{11, 94} = 160.6$; $P \leq 0.001$
All DBPs	4.12×10^{-5}	$1.03 \pm 0.03 \times 10^{-4}$	0.94	$F_{11, 96} = 221.7$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest summed molar concentration (ΣM) of the PRW2 samples that induced a statistically significant reduction in cell density as compared to their concurrent negative controls.

[b] The LC_{50} value is the concentration factor of the water sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The mean and the standard error (SE) were derived from multiple regression analyses using bootstrap statistics.

[c] The r^2 is the coefficient of determination for the regression analysis of the concentration-response data upon which the LC_{50} value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

[e] NS = not significant; NA = not applicable.

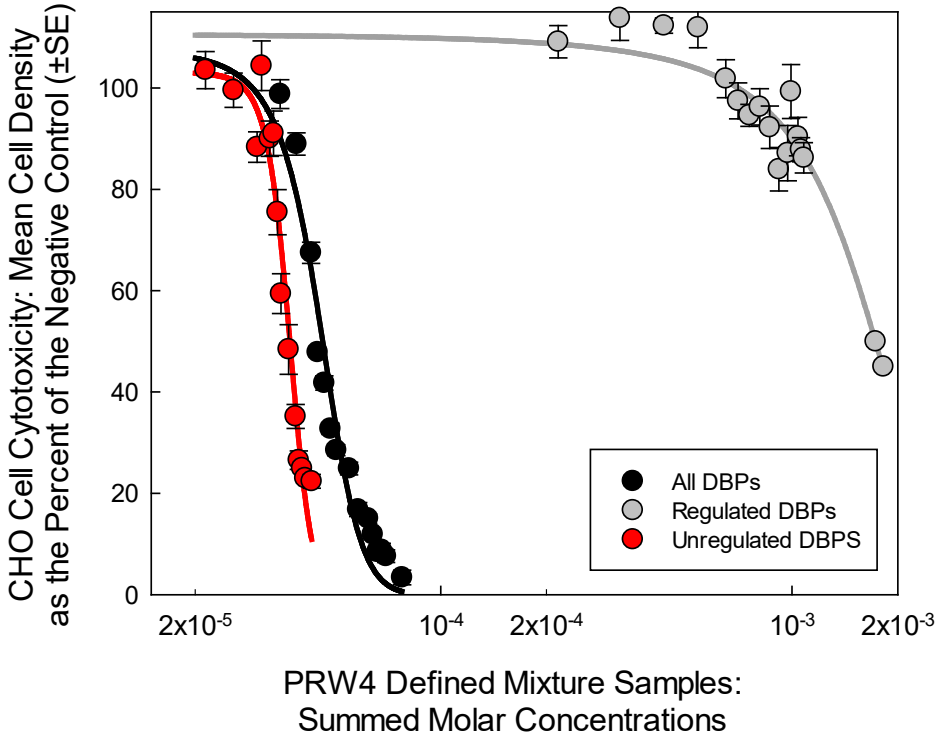

Figure S7. Concentration-response curves comparing the CHO cytotoxicity of PRW4 defined DBP mixture samples. The LC_{50} represents the summed molar concentration of DBPs associated with a 50% reduction in cell density compared to the untreated controls, as determined by regression from these concentration-response curves. Knowing the total molar concentration of DBPs in the original water sample, this LC_{50} value can be converted into the concentration factor of the original water sample associated with the LC_{50} (i.e., the CF_{50}).

Table S7. Summary of CHO cell cytotoxicity statistics for PRW4 defined DBP mixtures

PRW4 DBP Mixture Sample	Lowest Cytotoxic Conc. (ΣM) [a]	Mean LC_{50} Value ± SE (ΣM) [b]	r^2 [c]	ANOVA Test Statistic [d]
Regulated DBPs	9.18×10^{-4}	$1.72 \pm 0.004 \times 10^{-3}$	0.70	$F_{16,90} = 8.03$; $P \leq 0.001$
Unregulated DBPs	3.43×10^{-5}	$3.64 \pm 0.03 \times 10^{-5}$	0.96	$F_{17,118} = 75.6$; $P \leq 0.001$
All DBPs	3.88×10^{-5}	$4.54 \pm 0.01 \times 10^{-5}$	0.98	$F_{15,140} = 586$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest summed molar concentration (ΣM) of the PRW4 defined DBP mixture samples that induced a statistically significant reduction in cell density as compared to their concurrent negative controls.

[b] The LC_{50} value is the concentration factor of the water sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The mean and the standard error (SE) were derived from multiple regression analyses using bootstrap statistics.

[c] The r^2 is the coefficient of determination for the regression analysis of the concentration-response data upon which the LC_{50} value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

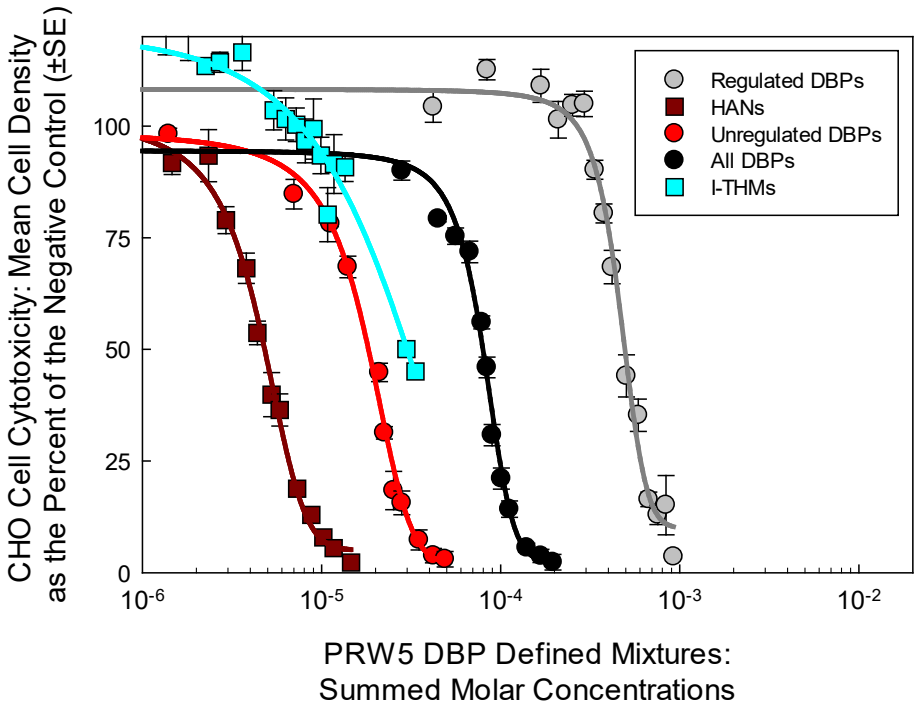

Figure S8. Concentration-response curves comparing the CHO cytotoxicity of PRW5 defined DBP mixture samples. The LC_{50} represents the summed molar concentration of DBPs associated with a 50% reduction in cell density compared to the untreated controls, as determined by regression from these concentration-response curves. Knowing the total molar concentration of DBPs in the original water sample, this LC_{50} value can be converted into the concentration factor of the original water sample associated with the LC_{50} (i.e., the CF_{50}).

Table S8. Summary of CHO cell cytotoxicity statistics for PRW5 defined DBP mixtures

PRW5 DBP Mixture Sample	Lowest Cytotoxic Conc. (ΣM) [a]	Mean LC_{50} Value ± SE (ΣM) [b]	r^2 [c]	ANOVA Test Statistic [d]
Regulated DBPs	3.35×10^{-4}	$4.92 \pm 0.07 \times 10^{-4}$	0.99	$F_{15,82} = 156$; $P \leq 0.001$
I-THMs	1.08×10^{-5}	$2.98 \pm 0.02 \times 10^{-5}$ [e]	0.77	$F_{21,82} = 18.4$; $P \leq 0.001$
HANs	1.46×10^{-6}	$4.75 \pm 0.09 \times 10^{-6}$	0.99	$F_{13,90} = 283$; $P \leq 0.001$
Unregulated DBPs	7.00×10^{-6}	$1.83 \pm 0.02 \times 10^{-5}$	0.99	$F_{11,80} = 379$; $P \leq 0.001$
All DBPs	2.80×10^{-5}	$8.00 \pm 0.06 \times 10^{-5}$	0.98	$F_{12,79} = 428$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest summed molar concentration (ΣM) of the PRW5 defined DBP mixture samples that induced a statistically significant reduction in cell density as compared to their concurrent negative controls.

[b] The LC_{50} value is the concentration factor of the water sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The mean and the standard error (SE) were derived from multiple regression analyses using bootstrap statistics.

[c] The r^2 is the coefficient of determination for the regression analysis of the concentration-response data upon which the LC_{50} value was calculated.

[d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

[e] LC_{50} value generated by extrapolation because of the solvent concentration limit.

Table S9. Results of CHO cell chronic cytotoxicity analyses for DW1

DW1 Mixture	Mean LC_{50} ± SE (M)	CF_{50}	BCAT	CAT	% Difference Between BCAT and CAT
THM4 only	Not cytotoxic	N/A	N/A	8.42×10^{-6}	N/A
HAAs only	$4.26 \pm 0.05 \times 10^{-4}$	2777	3.60×10^{-4}	4.47×10^{-4}	21%
HANs only	$2.25 \pm 0.07 \times 10^{-5}$	2230	4.48×10^{-4}	6.32×10^{-4}	34%
Regulated DBPs	$7.52 \pm 0.12 \times 10^{-4}$	3455	2.89×10^{-4}	4.31×10^{-4}	39%
Unregulated DBPs	$8.54 \pm 0.18 \times 10^{-5}$	836	1.20×10^{-3}	1.08×10^{-3}	10%
All DBPs	$2.48 \pm 0.02 \times 10^{-4}$	776	1.29×10^{-3}	1.51×10^{-3}	16%

Table S10. Results of CHO cell chronic cytotoxicity analyses for DW2

DW2 Mixture	Mean LC_{50} ± SE (M)	CF_{50}	BCAT	CAT	% Difference Between BCAT and CAT
Regulated DBPs	$8.60 \pm 0.02 \times 10^{-4}$	3217	3.11×10^{-4}	2.89×10^{-4}	7%
Unregulated DBPs	$4.68 \pm 0.02 \times 10^{-5}$	384	2.60×10^{-3}	3.11×10^{-3}	18%
All DBPs	$1.31 \pm 0.01 \times 10^{-4}$	337	2.97×10^{-3}	3.40×10^{-3}	14%

Table S11. Results of CHO cell chronic cytotoxicity analyses for DW3

DW3 Mixture	Mean LC_{50} ± SE (M)	CF_{50}	BCAT	CAT	% Difference Between BCAT and CAT
Regulated DBPs	$1.56 \pm 0.002 \times 10^{-3}$	20338	4.92×10^{-5}	1.89×10^{-5}	89%
Unregulated DBPs	$5.19 \pm 0.11 \times 10^{-5}$	2159	4.63×10^{-4}	5.55×10^{-4}	18%
All DBPs	$1.92 \pm 0.05 \times 10^{-4}$	1906	5.25×10^{-4}	5.74×10^{-4}	9%

Table S12. Results of CHO cell chronic cytotoxicity analyses for PRW1

PRW1 Mixture	Mean LC_{50} ± SE (M)	CF_{50}	BCAT	CAT	% Difference Between BCAT and CAT
THM4 only	Not cytotoxic	N/A	N/A	1.37×10^{-5}	N/A
HAAs only	Not cytotoxic	N/A	N/A	2.52×10^{-5}	N/A
HANs only	$5.02 \pm 0.04 \times 10^{-5}$	1608	6.22×10^{-4}	8.23×10^{-4}	28%
Regulated DBPs	Not cytotoxic	N/A	N/A	3.83×10^{-5}	N/A
Unregulated DBPs	$5.87 \pm 0.08 \times 10^{-5}$	997	1.00×10^{-3}	8.54×10^{-4}	16%
All DBPs	$3.03 \pm 0.06 \times 10^{-4}$	1168	8.56×10^{-4}	8.98×10^{-4}	5%

Table S13. Results of CHO cell chronic cytotoxicity analyses for PRW2

PRW2 Mixture	Mean LC_{50} ± SE (M)	CF_{50}	BCAT	CAT	% Difference Between BCAT and CAT
THM4 only	Not cytotoxic	N/A	N/A	5.52×10^{-6}	N/A
THM4 + I-THMs	Not cytotoxic	N/A	N/A	1.45×10^{-5}	N/A
HAAs only	$5.76 \pm 0.20 \times 10^{-5}$	3023	3.31×10^{-4}	4.16×10^{-4}	23%
HANs only	$1.28 \pm 0.02 \times 10^{-5}$	1395	7.17×10^{-4}	1.19×10^{-3}	50%
Regulated DBPs	$3.25 \pm 0.15 \times 10^{-4}$	5110	1.96×10^{-4}	3.94×10^{-4}	67%
Unregulated DBPs	$6.94 \pm 0.16 \times 10^{-5}$	705	1.42×10^{-3}	1.22×10^{-3}	15%
All DBPs	$1.03 \pm 0.03 \times 10^{-4}$	625	1.60×10^{-3}	1.62×10^{-3}	1%

Table S14. Results of CHO cell chronic cytotoxicity analyses for PRW3

PRW3 Mixture	Mean LC_{50} ± SE (M)	CF_{50}	BCAT	CAT	% Difference Between BCAT and CAT
THM4 only	Not cytotoxic	N/A	N/A	8.99×10^{-6}	N/A
HAAs only	$3.01 \pm 0.02 \times 10^{-4}$	2036	4.91×10^{-4}	4.81×10^{-4}	2%
HANs only	$1.94 (\pm 0.11) \times 10^{-5}$	1143	8.75×10^{-4}	1.37×10^{-3}	44%
Regulated DBPs	$6.09 (\pm 0.06) \times 10^{-4}$	2951	3.39×10^{-4}	4.52×10^{-4}	29%
Unregulated DBPs	$4.78 (\pm 0.02) \times 10^{-5}$	389	2.57×10^{-3}	2.67×10^{-3}	4%
All DBPs	$1.17 (\pm 0.04) \times 10^{-4}$	355	2.82×10^{-3}	3.12×10^{-3}	10%

Table S15. Results of CHO cell chronic cytotoxicity analyses for PRW4

PRW4 Mixture	Mean LC_{50} ± SE (M)	CF_{50}	BCAT	CAT	% Difference Between BCAT and CAT
Regulated DBPs	$1.72 \pm 0.004 \times 10^{-3}$	15856	6.31×10^{-5}	1.40×10^{-4}	76%
Unregulated DBPs	$3.64 \pm 0.03 \times 10^{-5}$	425	2.36×10^{-3}	3.26×10^{-3}	32%
All DBPs	$4.54 \pm 0.01 \times 10^{-5}$	234	4.28×10^{-3}	3.40×10^{-3}	23%

Table S16. Results of CHO cell chronic cytotoxicity analyses for PRW5

PRW5 Mixture	Mean LC_{50} ± SE) (M)	CF_{50}	BCAT	CAT	% Difference Between BCAT and CAT
Regulated DBPs	$4.92 \pm 0.07 \times 10^{-4}$	1173	8.53×10^{-4}	1.27×10^{-3}	39%
I-THMs only	$2.98 \pm 0.02 \times 10^{-5}$ [a]	33063	3.02×10^{-5}	3.99×10^{-7}	195%
HANs only	$4.75 \pm 0.09 \times 10^{-6}$	163	6.15×10^{-3}	7.38×10^{-3}	18%
Unregulated DBPs	$1.83 \pm 0.02 \times 10^{-5}$	131	7.66×10^{-3}	9.16×10^{-3}	18%
All DBPs	$8.00 \pm 0.06 \times 10^{-5}$	143	7.00×10^{-3}	1.04×10^{-2}	39%

[a] LC_{50} value generated by extrapolation because of solvent concentration limit.

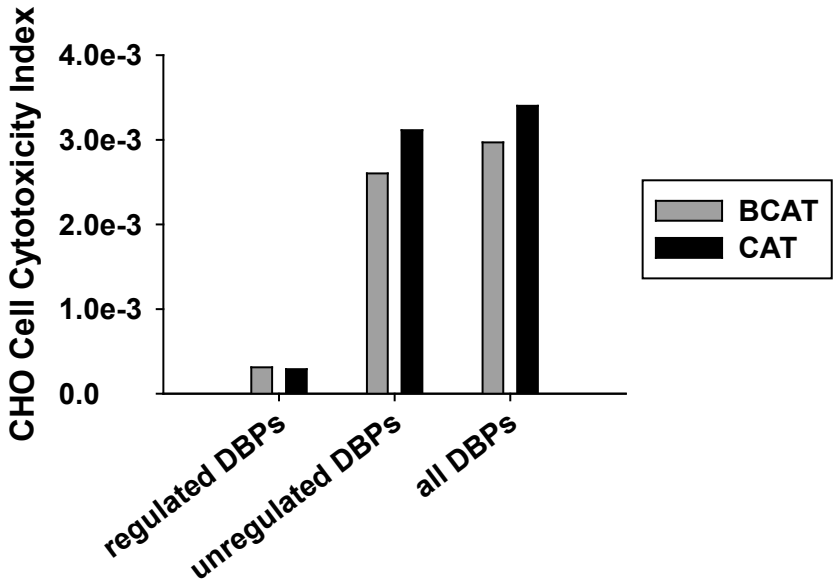

Figure S9. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices computed from CHO cell chronic cytotoxicity of defined mixtures containing various DBP classes in DW2.

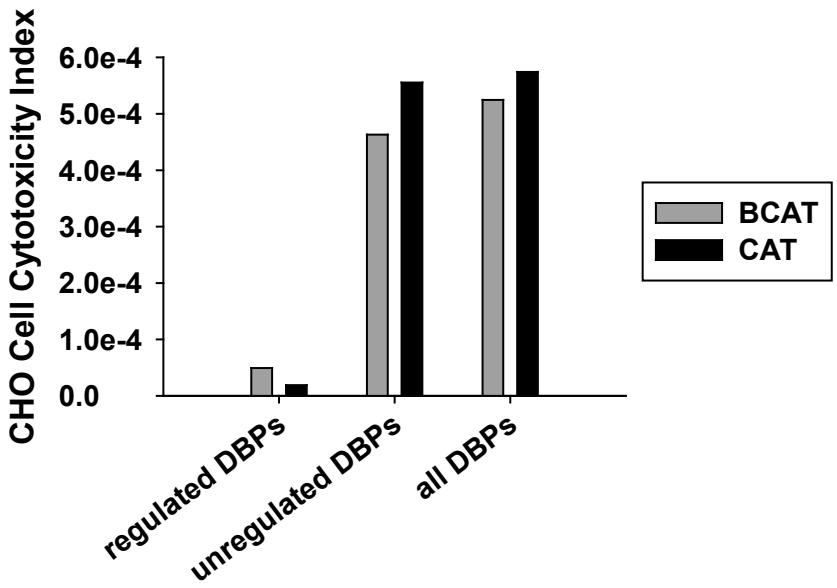

Figure S10. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices computed from CHO cell chronic cytotoxicity of defined mixtures containing various DBP classes in DW3.

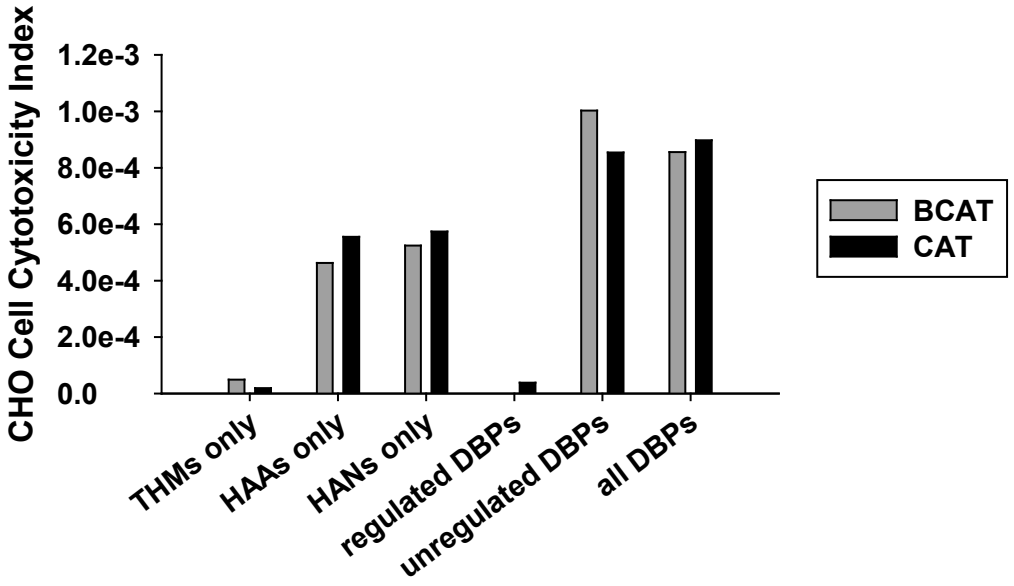

Figure S11. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices computed from CHO cell chronic cytotoxicity of defined mixtures containing various DBP classes in PRW1.

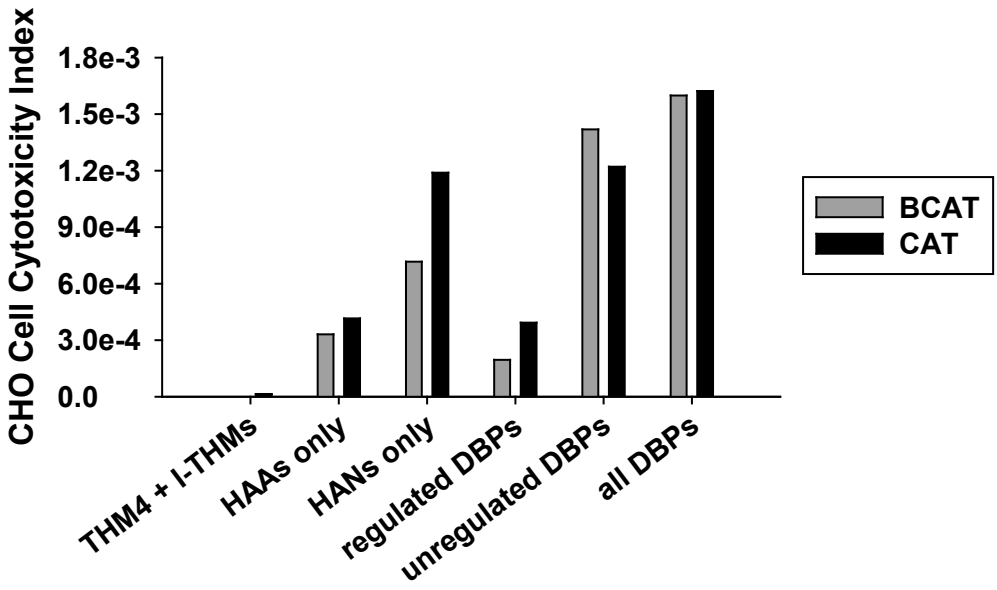

Figure S12. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices computed from CHO cell chronic cytotoxicity of defined mixtures containing various DBP classes in PRW2.

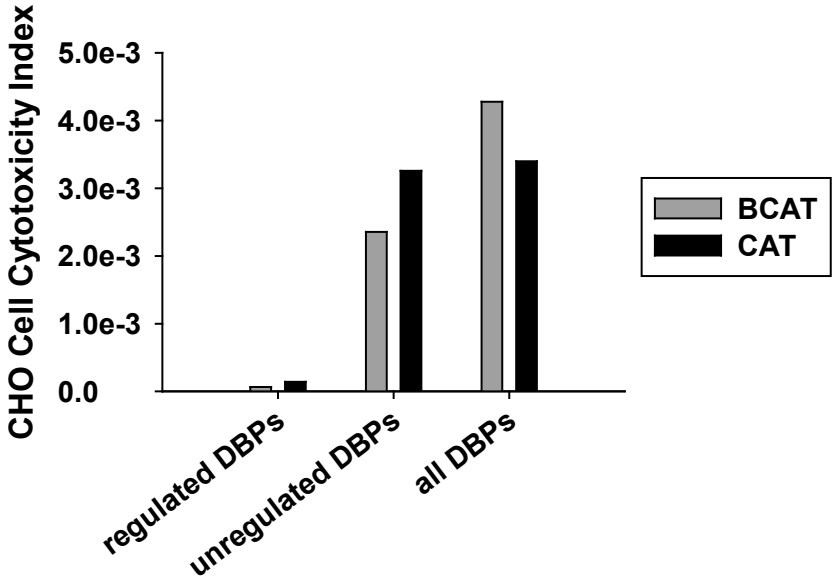

Figure S13. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices computed from CHO cell chronic cytotoxicity of defined mixtures containing various DBP classes in PRW4.

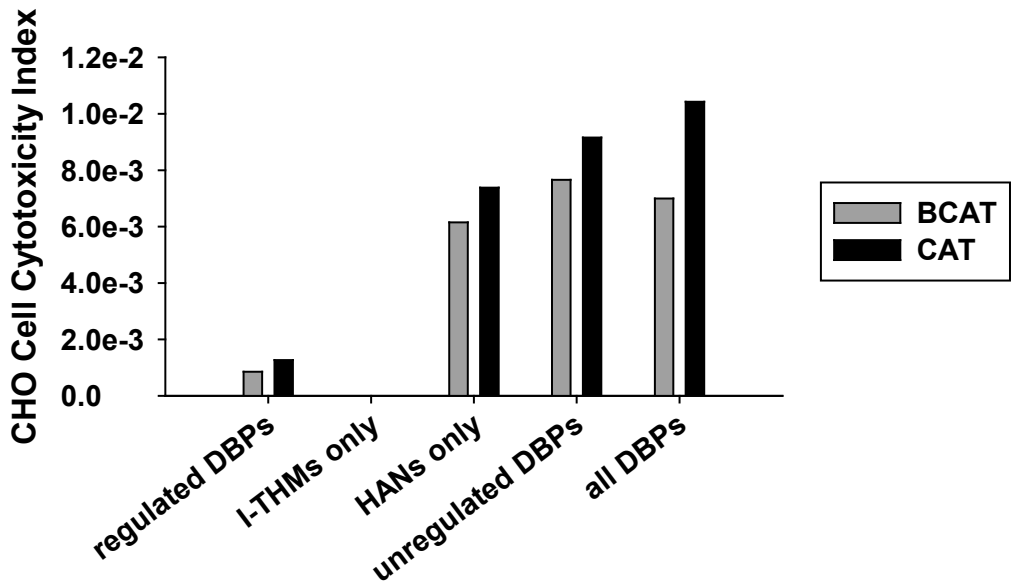

Figure S14. Bioassay-based calculated additive toxicity (BCAT) and calculated additive toxicity (CAT) indices computed from CHO cell chronic cytotoxicity of defined mixtures containing various DBP classes in PRW5.

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