Mixture effects of drinking water disinfection by-products: implications for risk assessment†

Daniel Stalter,^{a,b,‡,*} Elissa O'Malley,^a Urs von Gunten,^{b,c} Beate I. Escher^{a,d,e}

^aQueensland Alliance for Environmental Health Sciences, The University of Queensland,
Brisbane, Australia

^bEawag, Swiss Federal Institute of Aquatic Science and Technology, Duebendorf, Switzerland

^cSchool of Architecture, Civil and Environmental Engineering (ENAC), Ecole Polytechnique
Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

^dDepartment of Cell Toxicology, UFZ – Helmholtz Centre for Environmental Research,
Leipzig, Germany

^eEnvironmental Toxicology, Center for Applied Geoscience, Eberhard Karls University,
Tübingen, Germany

*Corresponding author: Beate I. Escher, beate.escher@ufz.de

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‡ Now at: German Federal Institute for Risk Assessment, Department of Chemical and Product
Safety, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany

TOC Entry

Abstract

Disinfection by-products (DBPs) in drinking water have been associated with increased cancer risk but single DBPs cannot explain epidemiological cancer occurrences. To test if combined effects of DBPs are plausible to explain epidemiological evidence for adverse health effects, we assessed if mixture effects of DBPs can be predicted using the concentration addition (CA) model. We prepared 12 mixtures of DBPs (trihalomethanes, halonitromethanes, haloacetonitriles, haloketones, haloacetic acids, chloral hydrate, haloacetamides, 3-chloro-4-(dichloromethyl)-5-hydroxy-5H-furan-2-one) in equipotent concentration ratios. We determined effect concentrations with three reporter gene bioassays (AREc32, ARE-bla, and p53-bla) based on human cell lines and one bacterial assay (Microtox). The experimental effect concentrations agreed well with the effect concentrations predicted with the CA model, which suggests that the CA model is applicable

for reactive DBPs despite different molecular mechanisms because the reporter gene assays are only detecting one mechanism each. Modelling of mixture effects of DBPs in ratios detected in drinking water revealed that haloacetonitriles, haloketones, and mono-haloacetic acids contributed the most to the total effect indicating a higher health relevance of these DBP groups. In drinking water samples the sum of the detected DBPs explained <6% of effect in most cases. The CA model could be applied to prioritize DBPs for further risk assessments to potentially close the gap between toxicological cancer risk predictions and epidemiological findings.

Keywords

Disinfection byproducts, DBPs; mixture toxicity; concentration addition; independent action; tap water; risk assessment

1. Introduction

Despite its critical importance for public health,¹ disinfection of drinking water has raised concerns because of the formation of disinfection by-products (DBPs).^{2, 3} DBPs are formed by the reaction between disinfectants (commonly chlorine or chloramine) and natural organic matter (NOM) as well as inorganic precursors (e.g., bromide).² Epidemiological studies suggested an increased risk of bladder cancer after life-long ingestion of chlorinated drinking water pointing toward adverse health effects of DBPs.⁴⁻⁶ Based on such epidemiological studies the US-EPA calculated that 2 – 17% of bladder cancer cases could be avoided if the exposure to DBPs were ceased.⁷ However, the causation of urinary bladder cancer and other diseases by DBP exposure has not been conclusively proven.⁶ Additionally, known DBPs cannot explain epidemiological risk estimates despite approximately 700 DBPs identified within the last decades.^{2, 4, 8-11} The majority of all identified DBPs is not yet quantifiable, let alone toxicologically characterized. Some of the known DBPs have been characterized by various *in vitro* bioassays¹²⁻¹⁴ and such tools have also been applied to evaluate drinking water quality.¹⁵

Given that more than 50% of total organic halogens are unknown,^{16, 17} and that there is additionally a diverse set of unknown non-halogenated compounds formed during disinfection,¹⁸ it seems plausible that unknown DBPs contribute to adverse health effects.¹⁰ Unknown DBPs occur—most likely—at low concentrations and thus single DBPs would have to have an extreme potency if they were to close the gap between toxicological risk assessment estimates and epidemiologic estimates. Therefore, unknown and known DBPs with similar mechanisms of action or common adverse outcomes acting together as mixtures might cause the observed epidemiological evidence.

66 Our working hypothesis is that DBPs act in mixtures according to the model of concentration
67 addition (CA), which is a widely used model to describe the effects of mixtures with the
68 same mode of action.^{19, 20} The model of CA was derived initially for receptor-mediated
69 effects but was shown to be applicable also to apical effects provided the chemicals had
70 similar modes of action.^{21, 22} While strictly valid only for mixtures with components that
71 have the same mode of action, empirical evidence points to CA being a realistic worst case
72 even if chemicals of diverse modes of action act together and apical endpoints such as
73 mortality or growth inhibition are assessed.²³

74 Mixture studies with DBPs have been performed previously with somewhat conflicting
75 results. Narotsky et al.²⁴ demonstrated that five HAAs and four trihalomethanes (THMs)
76 contributed to DBP-induced pregnancy loss in rats. However, a characterization of the type
77 of interaction of the nine component chemicals was not possible. Hooth et al.²⁵ evaluated
78 mixtures of bromate, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX),
79 chloroform, and bromodichloromethane in a rat model for hereditary renal cancer. The
80 authors found that the mixtures were not more carcinogenic than the most potent mixture
81 constituent and concluded that application of the CA model may overestimate the
82 carcinogenic effect of DBP mixtures. In contrast, Andrews et al.²⁶ found that the CA model
83 adequately predicted the observed developmental toxicity of three haloacetic acids (HAAs)
84 in a rat whole embryo assay. In a binary mixture study of two HAAs (dichloroacetic acid and
85 trichloroacetic acid) Hassoun et al.²⁷ found additive or slightly greater than additive effects
86 on oxidative stress induction in hepatic tissue of mice. Parvez et al.²⁸ proposed a method to
87 evaluate the contribution of unknown DBPs to mixtures for the endpoint puberty acquisition
88 in rats by comparing whole mixtures of disinfected water with defined mixtures of regulated

DBPs found in the whole mixture and concluded that the nine regulated DBPs could explain most of the effect.

Zhang et al.²⁹ found synergistic effects of sodium chlorite on bromate-induced renal cell death in an in vitro bioassay using rat kidney cells with the most likely explanation that NaClO₂ partially reversed the bromate induced and cell protective G2/M arrest. Greater than additive effects of a binary DBP mixture were also found with several in vitro assays.³⁰ In contrast, Simmons et al.³¹ found an antagonistic departure from additivity for various mixtures of five HAAs in a Chinese Hamster Ovary cell chronic cytotoxicity assay. Simmons et al.³² demonstrated that the mixing ratio had a significant impact on the toxicity of mixtures of 10 HAA in the same assay.

These examples demonstrate uncertainties regarding mixture effects of DBPs. Conflicting outcomes could be a result of differing mixture designs and differing biological endpoints. It is critical to use equipotent concentrations for testing the hypothesis whether CA applies. In equipotent mixtures all components are mixed in concentration ratios of their effect concentrations, i.e., more potent chemicals have a lower concentration but equal contribution to the mixture effect as low-potency chemicals. Otherwise a combination of very abundant and/or very potent compounds may dominate the mixture effect.³³ The use of equipotent concentrations is clearly stated only in one of the mentioned studies.²⁶ More often the applied concentration ratios were equimolar or based on environmental concentrations.^{24, 31, 32}

DBPs act via different molecular initiating events and cellular toxicity pathways^{34, 35} but this knowledge on the toxicity pathways remains incomplete. Most DBPs trigger reactive mechanisms and converge into the same adaptive stress response pathways, in particular

oxidative stress response.¹⁴ We applied reporter gene assays that target one mechanism only. Previous work has demonstrated that in reporter gene assays, CA is a suitable mixture model unless the specific mechanism targeted is masked by cytotoxicity.^{36, 37}

To test our hypothesis that DBPs act together in a concentration additive manner, we prepared mixtures with DBPs found in drinking water from three representative water treatment plants and additional DBPs, which are known to be particularly potent, such as mono-HAAs, haloacetamides (HAcAms) and MX (3-chloro-4-(dichloromethyl)-5-hydroxy-5H-furan-2-one).¹⁴ Mixtures were prepared in equipotent concentration ratios for the AREc32 and p53-bla assay.

All mixtures were exposed in three human cell-based bioassays for activation of oxidative stress response (AREc32, ARE-bla) and genotoxicity (p53-bla) and one bacterial assay on cytotoxicity (Microtox). We compared the full experimental concentration-effect curves from the bioassays with the calculated concentration-effect curves predicted with the CA model to evaluate also the dependence of mixture interaction on the effect level.

After confirming CA with equipotent mixtures, we calculated the effect contribution of individual DBPs in known DBP mixtures detected in various drinking water samples in literature³⁸⁻⁴³ to evaluate which detected DBPs dominate mixture effects. Finally, the mixture effects of known and detected chemicals were compared to the effect of the extract of the entire water sample measured previously in the same bioassays.⁴³

2. Materials and Methods

2.1 Chemicals

For the mixture experiments we selected 22 DBPs that were detected in drinking water from three water treatment plants (WTPs) of the greater Brisbane area, Australia.^{43, 44} Additionally, we selected 10 HAcAms, MX, and three mono-HAAs because of their high toxicity.¹⁴ Prior to this study 50 DBPs, including the 36 of the present study, were toxicologically characterized with various cell-based bioassays,¹⁴ and these single-chemical effect data were used for the mixture effect predictions. Details about the tested chemicals, including abbreviations, supplier and purity are compiled in Table S1 of the Electronic Supplementary Information (ESI). Methanolic stock solutions of all DBPs and DBP mixtures were stored at –80°C.

2.2 Bioassays

We applied four bioassays for this mixture study. The bacterial cytotoxicity assay using *Aliivibrio fischeri* (formerly termed *Vibrio fischeri*) bioluminescence inhibition was selected as non-specific cytotoxicity screen because of its sensitivity to DBPs.^{41, 42, 45} The human MCF7 cell-based AREc32 assay targets the activation of the oxidative stress response pathway Nrf2-ARE.³⁶ The activation of the Nrf2-ARE stress response has been demonstrated in previous studies to be an important adaptive stress response pathway of mono-HAAs^{35, 46} and appears to play a central role for the toxicity of many more DBPs.^{14, 47} The ARE-bla assay⁴⁸ was employed in addition to the AREc32 assay to detect oxidative stress response because it is based on a different cell line (HepG2 liver cells), which might reveal cell-specific differences in the response. Additionally, different reporter gene constructs may lead to a different responsiveness of assays depending on promoter/enhancer construction, ARE

orientation and other factors.⁴⁹ The p53-bla assay,^{45, 50} derived from HCT-116 human colon carcinoma cells, was applied because activation of p53 has been discussed as marker for genotoxic properties of chemicals⁵¹ and because many DBPs activate the p53 adaptive stress response.¹⁴ For all mixtures the bioassays were performed free of headspace⁵² or with reduced headspace for the ARE-bla and p53-bla to reduce the loss of volatile DBPs.¹⁴ Each mixture was analysed in 8-step 2-fold dilutions in two to four independent experiments to derive full concentration-response curves. All replicates were evaluated together. If two independent repeats aligned closely, they were not further repeated, if there were differences up to four experiments were performed. The concentrations of the stock solutions were >300 times higher than the EC-values and complete concentration-effect curves with constant concentration ratios were derived. We used methanol as solvent at a concentration of 1% in the bioassays because it showed the lowest effect in the AREc32 assay compared to DMSO, ethanol and MTBE.⁵³ The methanol control did not exhibit effects different from the medium control. The bioassays were performed according to the same protocols as in the previous study on single chemicals and the effect concentrations of the chemicals included in this study are reprinted in the ESI, Table S2.¹⁴

For the Microtox assay the assessment endpoint was the effect concentration (EC) that caused 50% inhibition of bioluminescence (EC₅₀). The EC₅₀ was derived from a log-logistic concentration-effect curve as described in Escher et al.⁵⁴ The concentrations of the mixtures were expressed as the sum of molar concentrations of all components. For the oxidative stress response, p53 pathway activation and activation of the SOS response we used the induction ratio (IR) as the measure of effect. The IR is defined as the ratio of effect of the sample divided by the average effect observed in the solvent control (medium with 1%

methanol). The effect concentration $EC_{IR1.5}$ that elicits an IR of 1.5 (i.e., 50% effect increase compared to the negative control) was the assessment endpoint for these assays, calculated by use of linear regression of all experimental data points up to IR 4 with a fixed intercept at IR 1, which is the IR of the negative controls.⁵⁵ The standard error $\sigma EC_{IR1.5}$ was derived by error propagation as outlined by Escher et al.^{36, 56}

In addition to the four applied in the experimental mixture study, we also included the bacterial umuC assay⁵⁷ in the modelling part of this study. The umuC assay detects the activation of the cellular SOS-response, a global response to DNA damage to induce DNA repair mechanisms, and hence indirectly detects genotoxicity.

2.3 Mixture design

Since the single DBPs exhibit differing relative effect potencies in the tested bioassays, an equipotent mixture in one bioassay may not be equipotent in another bioassay. Therefore, we designed the equipotent mixture according to the EC values in two bioassays (AREc32 and p53-bla) but measured each mixture in all bioassays.

For six mixtures, 3 to 24 DBPs were mixed in equipotent concentration ratios, where the ratios of concentration were according to the ratios of effect concentrations in the AREc32 assay (mix1_{AREc32} to mix6_{AREc32}; Table S3). Additionally, six mixtures of 3 to 21 DBPs were mixed in equipotent concentration ratios derived from effect concentrations in the p53-bla assay (mix1_{p53-bla} to mix6_{p53-bla}; Table S4).

2.4 Mixture toxicity prediction

We compared the experimentally derived effect concentrations ($EC_{mix, exp}$) with ECs predicted by use of the CA model.^{19, 20, 58} The $EC_{mix, CA}$ of the mixture predicted with the

199 model of CA can be calculated with eqn (1) for i components present in the fraction p_i ($\sum p_i =$
 200 1) from the individual effect concentrations EC_i of all mixture components i.

$$201 \quad EC_{mix,CA} = \frac{1}{\sum_{i=1}^n \frac{p_i}{EC_i}} \quad (1)$$

202 The error of the CA prediction ($\sigma EC_{mix, CA}$) was propagated from experimental standard
 203 deviations of the effect concentration of each mixture component (σEC_i) assuming no error
 204 in the fractions p_i (eqn (2)).

$$205 \quad \sigma EC_{mix,CA} = \sqrt{\sum_{i=1}^n \left(\left(\frac{\delta EC_{mix,CA}}{\delta EC_i} \right)^2 \cdot (\sigma EC_i)^2 \right)} = \sqrt{\sum_{i=1}^n \left(\left(\frac{EC_{CA}^2 \cdot p_i^2}{EC_i^2} \right)^2 \cdot (\sigma EC_i)^2 \right)} \quad (2)$$

206 The $EC_{mix, CA}$ can be calculated for all effect levels to construct predicted concentration-
 207 response curves of the mixtures depicted in Figures S1-S4.

208 We used the index on prediction quality (IPQ, eq. 3 and 4) as a measure for the deviation
 209 between experimental ($EC_{mix, exp}$) and predicted mixture effect ($EC_{mix, CA}$).^{36, 59}

$$210 \quad \text{For } EC_{mix,CA} > EC_{mix,exp}: \quad IPQ = \frac{EC_{mix,CA}}{EC_{mix,exp}} - 1 \quad (3)$$

$$211 \quad \text{For } EC_{mix,CA} < EC_{mix,exp}: \quad IPQ = 1 - \frac{EC_{mix,CA}}{EC_{mix,exp}} \quad (4)$$

212 If the ratio between $EC_{mix, exp}$ and $EC_{mix, CA}$ is 1, then the IPQ is 0. A ratio of 2 results in an IPQ
 213 of 1 (if $EC_{mix, CA}$ is greater than $EC_{mix, exp}$) or -1 (if $EC_{mix, exp}$ is greater than $EC_{mix, CA}$), a ratio of 3
 214 yields an IPQ of ± 2 , and so on.

215

2.5 Mixture predictions using literature data

We calculated bioanalytical equivalent concentrations (BEQ)^{45, 60} to identify the mixture risk drivers among known chemicals and to compare bioanalytical results of water samples with predicted effects based on DBPs quantified in the extracts.

Dibromoacetonitrile (dBAN) was selected as common reference compound for all bioassays due to its high potency and because it was active in all bioassays.¹⁴ Hence, BEQs were expressed as dBAN equivalent concentrations (dBAN-EQ) in units of mol_{dBAN}/L.

The dBAN-EQ of the DBPs chemically quantified in a sample (dBAN-EQ_{chem}) was calculated from the concentration C_i and the relative effect potency REP_i (eqn (5)) of all previously detected DBPs i (eqn (6)).⁶⁰ The REP_i for all bioassays stem from the EC values derived in our previous study¹⁴ and are reprinted for convenience in the ESI, Table S2.

$$REP_i = \frac{EC_{dBAN}}{EC_i} \quad (5)$$

$$dBAN-EQ_{chem} = \sum_{i=1}^n C_i \cdot REP_i \quad (6)$$

Eqn (6) is based on the assumption that CA is valid and that the relative effect potency is independent of the effect level. Accordingly, BEQs are considered a special case of CA where the log sigmoidal concentration-effect curves of all mixture components are assumed to be parallel or linear concentration-effect curves but apply for all effect levels in case of linear concentration-effect curves.⁵⁶

The contribution of each mixture component i , BEQ_i , to the total effect (BEQ_{chem}) was calculated by eqn (7).

$$\text{contribution of dBAN-EQ}_i \text{ to dBAN-EQ}_{\text{chem}} = \frac{\text{dBAN-EQ}_i}{\text{dBAN-EQ}_{\text{chem}}} = \frac{C_i \cdot \text{REP}_i}{\text{dBAN-EQ}_{\text{chem}}} \quad (7)$$

The measured dBAN equivalent concentration in a sample (dBAN-EQ_{bio}) was calculated by dividing the EC of the reference compound by the EC of the sample (eqn (8)).

$$\text{dBAN-EQ}_{\text{bio}} = \frac{\text{EC}_{\text{dBAN}}}{\text{EC}_{\text{sample}}} \quad (8)$$

The ratio between BEQ_{chem} and BEQ_{bio} (i.e., dBAN-EQ_{chem} and dBAN-EQ_{bio}) yields the fraction of effect that can be explained by the quantified DBPs.⁴⁵

2.6 Effect contribution of DBPs in a known mixture

We calculated the dBAN-EQ_{chem} (eqn (6)) in 20 known DBP mixtures based on literature data of DBP concentrations from 16 different disinfected drinking water samples³⁸⁻⁴³ plus four hypothetical mixtures. Concentrations and literature source of data are listed in Table S5, ESI).

These mixtures had not been toxicologically profiled, we just use the analytical data to predict the mixture effect and the contribution of the components to the overall predicted mixture effect. The hypothetical mixtures were included because comprehensive DBP occurrence data are rare and most studies focus on a limited number of DBP groups. Thus, some highly toxic DBPs are often not included, such as iodinated DBPs, haloacetamides, mono-HAAs or MX, wherefore we included them in the hypothetical mixtures. Hypothetical

mixture component concentrations were selected based on occurrence data of the respective compounds (Table S5).

2.6 Predicted effects versus measured effects in whole samples

We used results of water samples analysed in a previous study⁴³ to calculate the fraction of effect of whole mixtures that stem from unknown or undetected DBPs. We had sampled drinking water from three different taps (TW 1 – 3, two sampling campaigns) and three water treatment plants (WTP1: chloramination, WTP2: chloramination, WTP3: chlorination) in the greater Brisbane area (Queensland, Australia).⁴³ The distribution system is connected with all three treatment plants, and thus the tap waters could be mixtures originating from different drinking water treatment plants with differing disinfection methods.

We had used solid phase extraction to enrich the non-volatile DBP fraction.⁴³ For samples TW1 – 3, we additionally enriched the volatile fraction with a purge and trap method⁴³ before applying solid phase extraction. The extracts had been tested with the AREc32, p53-bla, umuC and Microtox assays and analysed for DBPs.⁴³ For convenience the concentrations detected⁴³ are reprinted in Table S6.

3. Results and Discussion

3.1 Does concentration addition predict mixture effects of DBPs?

The mixtures were not equipotent with respect to the Microtox assay but the comparison between experimental and CA-predicted concentration-effect curves showed a good agreement (Figure S1, ESI) especially at higher effect levels. The IPQ (eqn (3) and (4)) is a quantitative measure of the agreement between experiments and prediction model and ranged from -1.16 to 1.15 (Table S7, ESI). In the Microtox assay the IPQ showed a larger range than in the other bioassays (Figure S2, ESI).

Agreement with CA is consistent with previous mixture studies on organic micropollutant in the Microtox assay,³³ where most of the tested chemicals were classified as baseline toxicants. In contrast, most DBPs were classified as reactive toxicants in the Microtox assay.¹⁴ Concentration additive mixture effects of reactive soft electrophiles have been observed before in the Microtox assay.^{61, 62} The endpoint of the Microtox assay is bioluminescence inhibition, which is related to energy depletion, e.g., caused by impaired supporting physiological pathways (for example the respiratory chain) or non-specifically via narcosis. Thus, bioluminescence inhibition can be regarded as integrative endpoint and many chemicals act as baseline toxicants in the Microtox assay.⁶³ Mixtures of soft electrophiles have resulted in at least close to concentration additive effects in the Microtox assay.⁶¹ In a previous study, a cytotoxicity assay based on *Escherichia coli* growth inhibition was able to differentiate between soft and hard electrophiles. Within each group electrophiles acted according to CA but the model for independent action (IA) was valid between the groups.⁶⁴

For the AREc32 assay on oxidative stress response 6 of the 12 mixtures were equipotent (mix1_{AREc32} to mix6_{AREc32}). For all 12 mixtures, the experimental concentration-effect curves (Figure S3) matched the CA prediction with $-0.34 \leq \text{IPQ} \leq 0.84$ (Figure 1 and Table S2, ESI). For ARE-bla (Figure S4) the agreement was only slightly lower with $-0.83 \leq \text{IPQ} \leq 1.03$ (Figure 1 and Table S2, ESI). The AREc32 and ARE-bla assays are reporter gene assays and hence there is no effect observed for chemicals not triggering this particular response. Thus, CA in mixtures can be expected and has been observed previously for diverse micropollutants.³⁶ We observed a similar level of agreement between modelled and experimental effects for the ARE-bla assay on oxidative stress with $-0.83 \geq \text{IPQ} \leq 1.03$ (Figure 1 and Table S7, ESI).

For the p53-bla assay on adaptive stress response to genotoxicity (Figure S5) 6 of the 12 mixtures were equipotent (mix1_{p53-bla} to mix6_{p53-bla}) and we observed a systematic deviation from zero for all mixtures (experimental potency was higher than predicted by CA (Table S6 and Figure S2, ESI). However, the IPQs for p53-bla were ≤ 0.62 , and hence variations were relatively small (Figure 1, Table S7 and Figure S2).

Figure 1. Index on prediction quality (IPQ) for all equipotent mixtures in four bioassays (Table S7). A ratio of predicted ($EC_{mix, CA}$) and experimental EC ($EC_{mix, exp}$) of 1 resulted in an IPQ of 0, a ratio of 2 yields an IPQ of ± 1 .

Generally, discrepancies between CA-prediction and experimental data were small and, apart from p53-bla, not systematic as indicated by the distribution of IPQs. In 44 out of 48 comparisons between experimental and modelled ECs (i.e., 90%; Table S7), we found $-1 < IPQ < 1$ (Figure 1) and the highest IPQ deviation from $IPQ = 0$ (i.e., perfect agreement with CA) was -1.16 (Table S7, Figure 1). This is in agreement with a previous study where mixture effect data were analysed from literature and 88% fell within $-1 < IPQ < 1$.⁶⁵ A similar level of agreement was also found by Escher et al.³⁶ and Tang et al.³³ for mixtures of micropollutants. Thus, our results demonstrate that the CA model satisfactorily predicts the mixture effects of DBPs for adaptive stress responses and cytotoxicity despite differences in molecular initiating events triggering the toxicity pathway.

3.2 Identifying the risk drivers in a known mixture

Given the good agreement between the experimental mixture effects and the CA model, we can calculate the contribution of each mixture component to the total effect (eqn (6)). We selected literature data of DBP concentrations from 16 different samples³⁸⁻⁴³ and used four hypothetical mixtures that we considered to be representative for drinking water samples (Table S5, ESI) to calculate the contribution of each mixture component to the total effect of the detected DBPs (BEQ_{chem}). The hypothetical mixtures were used to include some highly toxic DBPs, such as iodinated DBPs, haloacetamides, mono-HAAs, or MX, which are often not included in DBP monitoring studies.

Figure 2 shows the DBP concentrations (A) in comparison with the resulting BEQ_{chem} and the contribution of each included DBP for the AREc32 assay (B), ARE-bla (C), p53-bla (D), umuC (E), and Microtox assay (F). The concentrations are clearly dominated by trihalomethanes (THMs, Figure 2A) but they hardly contribute to the mixture effects (Figures 3B-F). For the mammalian cell assays (Figures 3B-D), the results suggest not only a minor effect contribution of THMs, but also of di- and tri-haloacetic acids, halonitromethanes (HNMs), chloral hydrate, haloacetamides, and MX (i.e., % effect contribution <5%, except for bromochloroacetic acid BCAA). In contrast, haloacetonitriles (HANs), haloketones (HKs, AREc32 only), and mono-HAAs, if present in a sample, are expected to make a large contribution to the mixture effect even if present in low concentrations due to their high relative effect potency (Figures 3B-D). HKs were not active in the ARE-bla assay and hence did not contribute to the total effect for oxidative stress response activation in this assay (Figure 2C).

The bacterial assays delivered a very different pattern (Figures 3E, F). Mono-HAAs did not contribute to the total effect in the umuC assay because cytotoxic effects masked genotoxic effects and hence we could not derive ECs for these compounds.¹⁴ Additionally, genotoxic effects in the umuC assay were largely dominated by MX (>80%), which is known to be more potent in bacterial assays than in mammalian cell-based assays.^{14, 66} In the Microtox assay, effects were dominated by HNMs (<70%), whenever present in a sample (Figure 2F), because HNMs have a high REP in this assay.¹⁴

Figure 2. (A) DBP concentrations from 16 samples based on literature data and four hypothetical mixtures that include more potent DBPs, which are often not quantified in studies (see Table S5, ESI, for numerical values of the concentrations). (B – F) Contribution of all DBPs present in a sample to the bioanalytical equivalent concentrations ($\text{dBAN-EQ}_{\text{chem}}$, units of $\text{mol}_{\text{dBAN}}/\text{L}$) based on DBP concentrations and the relative effect potencies in the respective bioassays. B, C: adaptive stress response to oxidative stress in human cells (AREc32 and ARE-BLA). D: adaptive stress response to genotoxicity in human cells (p53-BLA). E: bacterial genotoxicity (umuC). F: bacterial cytotoxicity (Microtox).

It needs to be emphasized that the effect contribution of compounds, which require metabolic activation, such as *N*-nitrosamines, may be underestimated with the applied bioassays.¹⁴ Treating DBPs or DBP mixtures with rat liver S9 fractions prior to dosing into the bioassays could help to evaluate the role of metabolic activation for toxicity. Because of the limited data base available compared to the wide range of DBPs identified in real water samples,⁸ these samples do not allow for a comprehensive risk comparison but serve as blueprint on how to use DBP occurrence data and the CA model to assess which DBPs are most relevant in a mixture of known DBPs. Our approach is similar to the TIC-Tox approach proposed recently by Plewa et al.⁶⁷ They also demonstrated that the THM, which dominate the concentrations (expressed as peak area of the chromatogram, total ion current TIC), are no drivers of the mixture cytotoxicity on Chinese hamster ovary (CHO) cells but that haloacetonitriles and haloacetamides are dominating the mixture effect, just like in the present study evidenced for the mammalian reporter gene assays.

3.3 Effect-contribution of unknown DBPs in whole mixtures

As opposed to the approach based on mixtures with known components, whole mixture approaches reflect the real-world scenario because the major fraction of DBPs in drinking water is unknown. Calculating the % contribution of known components in a drinking water sample to the total effect expressed as bioanalytical equivalents enables one to estimate how relevant the known DBPs are compared to the unknown DBP fraction. It also allows one to estimate if newly discovered DBPs would be able to lessen the gap between observed effect in a sample and predicted mixture effect based on the known components.

The fraction of dBAN-EQ explained by the 34 DBPs included in the chemical analysis of which a maximum of 15 was detected (Table S6, ESI) was <6% in all real drinking water samples except for the two purge and trap extracts TW2-1_{P&T} and TW3-1_{P&T} where 29 and 92% could be explained (Figure 3). This demonstrates that unknown or undetected DBPs must account for the major fraction of effects in the samples, in particular in the non-volatile fraction. This is in concordance with the fact that known DBPs cannot explain adverse health outcomes.⁴ Among the approximately 700 DBPs reported in literature only a small fraction is routinely monitored or has been toxicologically characterized.⁸ If all of these known DBPs were included in an effect and occurrence database the calculated contribution of the known DBPs to the total effect would most likely be significantly larger.

Figure 3. Percentage of bioanalytical equivalent concentration (dBAN-EQ) in four bioassays explained by known DBPs calculated with eqn (7) detected in solid-phase extracts (SPE) and purge-and-trap (P & T) extracts of three tap water samples (TW) and in three SPE extracts from three water treatment plants (WTP). Analytical data from Stalter et al.⁴³, reprinted in Table S6; REP from Table S5. Tap water had been sampled twice from each sampling point at different time points (e.g., TW1-1 and TW1-2: sampled from the same tap at two different time points). Samples from the first sampling campaign (TW1-1, TW2-1, TW3-1) were extracted with a purge and trap method (P&T) before SPE to capture the volatile DBP fraction and samples from the second sampling campaign only with SPE.

4. Conclusions

We found good agreement between the experimental effects for biological endpoints of oxidative stress response, genotoxicity and cytotoxicity and the effects predicted with the model of concentration addition. Our findings support the conclusion by Kortenkamp et al.²⁰ that it is possible to predict the toxicity of multi-component mixtures with reasonable

413 accuracy and precision and that deviations from CA are rare and relatively small.
414 Accordingly, the concept of CA can be employed for the assessment of DBP mixtures despite
415 the inclusion of various chemical classes with different molecular mechanisms because they
416 are triggering a similar adverse outcome. This supports increasing evidence that toxicants
417 can act together in an additive manner to induce a biological effect, despite initial steps of
418 the adverse outcome pathway—including molecular initiating events and key events—may
419 differ profoundly.²⁰ Another potential issue is metabolism: many DBPs are only active after
420 metabolic activation. There is still a research gap concerning the role of metabolism in *in*
421 *vitro* assays.

422 Calculating the contribution of single compounds to BEQ_{chem} of a known mixture in drinking
423 water enables the estimation of the toxicological relevance of a compound by accounting
424 for the potency as well as concentrations. Therefore, the CA model could be a tool to
425 prioritize DBPs for further risk assessments. Compounds with a high contribution to the total
426 effect could be considered as high priority candidates for further toxicological
427 characterization. A prerequisite would be the development of a comprehensive effect
428 database derived from standardized bioassays of known DBPs while newly discovered
429 compounds need to be toxicologically characterized and continuously added to the
430 database. The difference between the results of the different reporter gene and bacterial
431 assays demonstrate the importance to consider a set of different bioassays to capture the
432 diversity of modes of action relevant for DBPs.

433 Due to the large fraction of unexplained effects in drinking water samples, further research
434 should focus on the identification of toxicologically relevant DBPs to find compounds, which
435 may explain the burden of disease reported in positive epidemiologic studies. Possible

approaches include effect directed analysis (EDA) for a targeted identification of toxic compounds,⁶⁸ which has not been applied for DBPs yet. This could allow targeted mitigation strategies during drinking water treatment to reduce potential human health hazards from DBPs.

One great challenge for future research on DBPs is to characterize all known DBPs with standardized test systems to establish an effect database to prioritize DBPs for further research to assess potential health effects. ToxCast and Tox21 have set precedence for high-throughput screening (HTS) of in vitro effects of micropollutants.⁶⁹ Due to the volatility of many DBPs, HTS approaches need to be adapted to the challenge of evaporative loss from the test system.^{43, 52}

Another and possibly more pragmatic strategy would be to apply a battery of in vitro assays as monitoring tools. Bioanalytical monitoring would not replace but complement chemical analysis of prominent DBPs. The only requirement for their application would be the definition of effect-based trigger values that can differentiate between acceptable and poor water quality. Attempts have been made to derive such thresholds for micropollutant mixtures in recycled and drinking water for a wide range of bioassays^{33, 36, 55} and similar approaches could be used for DBPs.

Conflicts of Interest

There are no conflicts to declare.

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