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1 **Mixture effects of drinking water disinfection by-products:**

2 **implications for risk assessment†**

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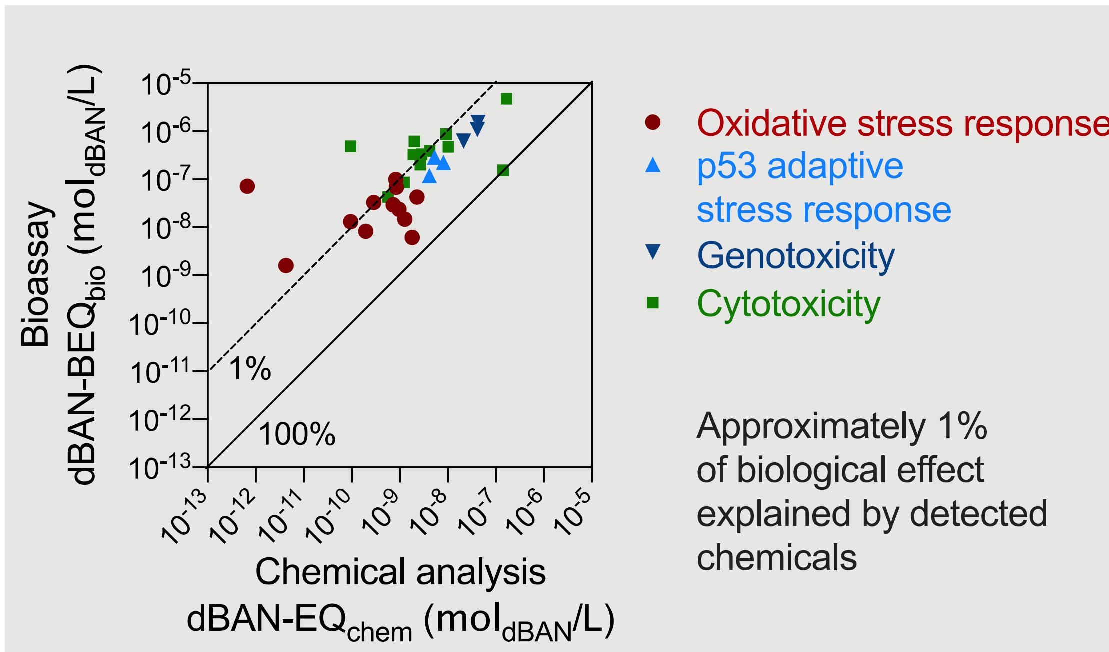
TOC Entry



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* bioassays[12-14] 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 triochloroacetic 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

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

91 Zhang et al.[29] found synergistic effects of sodium chlorite on bromate-induced renal cell
92 death in an in vitro bioassay using rat kidney cells with the most likely explanation that
93 $NaClO_2$ partially reversed the bromate induced and cell protective G2/M arrest. Greater
94 than additive effects of a binary DBP mixture were also found with several in vitro assays.[30]
95 In contrast, Simmons et al.[31] found an antagonistic departure from additivity for various
96 mixtures of five HAAs in a Chinese Hamster Ovary cell chronic cytotoxicity assay. Simmons et
97 al.[32] demonstrated that the mixing ratio had a significant impact on the toxicity of mixtures
98 of 10 HAA in the same assay.

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

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

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

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

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

126 After confirming CA with equipotent mixtures, we calculated the effect contribution of
127 individual DBPs in known DBP mixtures detected in various drinking water samples in
128 literature[38-43] to evaluate which detected DBPs dominate mixture effects. Finally, the
129 mixture effects of known and detected chemicals were compared to the effect of the
130 extract of the entire water sample measured previously in the same bioassays.[43]

131 # 2. Materials and Methods

132 ## 2.1 Chemicals

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

142 ## 2.2 Bioassays

143 We applied four bioassays for this mixture study. The bacterial cytotoxicity assay using
144 *Aliivibrio fischeri* (formerly termed *Vibrio fischeri*) bioluminescence inhibition was selected
145 as non-specific cytotoxicity screen because of its sensitivity to DBPs.[41, 42, 45] The human
146 MCF7 cell-based AREc32 assay targets the activation of the oxidative stress response
147 pathway NRf2-ARE.[36] The activation of the Nrf2-ARE stress response has been demonstrated
148 in previous studies to be an important adaptive stress response pathway of mono-HAAs[35, 46]
149 and appears to play a central role for the toxicity of many more DBPs.[14, 47] The ARE-bla
150 assay[48] was employed in addition to the AREc32 assay to detect oxidative stress response
151 because it is based on a different cell line (HepG2 liver cells), which might reveal cell-specific
152 differences in the response. Additionally, different reporter gene constructs may lead to a
153 different responsiveness of assays depending on promoter/enhancer construction, ARE

154 orientation and other factors.[49] The p53-bla assay,[45, 50] derived from HCT-116 human colon
155 carcinoma cells, was applied because activation of p53 has been discussed as marker for
156 genotoxic properties of chemicals[51] and because many DBPs activate the p53 adaptive stress
157 response.[14] For all mixtures the bioassays were performed free of headspace[52] or with
158 reduced headspace for the ARE-bla and p53-bla to reduce the loss of volatile DBPs.[14] Each
159 mixture was analysed in 8-step 2-fold dilutions in two to four independent experiments to
160 derive full concentration-response curves. All replicates were evaluated together. If two
161 independent repeats aligned closely, they were not further repeated, if there were
162 differences up to four experiments were performed. The concentrations of the stock
163 solutions were >300 times higher than the EC-values and complete concentration-effect
164 curves with constant concentration ratios were derived. We used methanol as solvent at a
165 concentration of 1% in the bioassays because it showed the lowest effect in the AREc32
166 assay compared to DMSO, ethanol and MTBE.[53] The methanol control did not exhibit effects
167 different from the medium control. The bioassays were performed according to the same
168 protocols as in the previous study on single chemicals and the effect concentrations of the
169 chemicals included in this study are reprinted in the ESI, Table S2.[14]

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

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

182 In addition to the four applied in the experimental mixture study, we also included the
183 bacterial umuC assay[57] in the modelling part of this study. The umuC assay detects the
184 activation of the cellular SOS-response, a global response to DNA damage to induce DNA
185 repair mechanisms, and hence indirectly detects genotoxicity.

186 **2.3 Mixture design**

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

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

196 **2.4 Mixture toxicity prediction**

197 We compared the experimentally derived effect concentrations ($EC_{mix,\ exp}$) with ECs
198 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 ($\Sigma p_i =$

200 1) from the individual effect concentrations EC_i of all mixture components i.

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

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

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

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

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

216 ## 2.5 Mixture predictions using literature data

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

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

223 The dBAN-EQ of the DBPs chemically quantified in a sample ($dBAN\text{-}EQ_{chem}$) was calculated
224 from the concentration C_i and the relative effect potency REP_i (eqn (5)) of all previously
225 detected DBPs i (eqn (6)).[60] The REP_i for all bioassays stem from the EC values derived in our
226 previous study[14] and are reprinted for convenience in the ESI, Table S2.

227 $$REP_i = \frac{EC_{dBAN}}{EC_i} \quad (5)$$

228 $$dBAN\text{-}EQ_{chem} = \sum_{i=1}^{n} C_i \cdot REP_i \quad (6)$$

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

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

236 $$\text{contribution of dBAN-EQ}_i \text{ to dBAN-EQ}_{chem} = \frac{\text{dBAN-EQ}_i}{\text{dBAN-EQ}_{chem}} = \frac{C_i \cdot REP_i}{\text{dBAN-EQ}_{chem}}$$

237 (7)

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

240 $$\text{dBAN-EQ}_{bio} = \frac{EC_{dBAN}}{EC_{sample}} \quad (8)$$

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

243

244 ## 2.6 Effect contribution of DBPs in a known mixture

245 We calculated the dBAN-EQ_{chem} (eqn (6)) in 20 known DBP mixtures based on literature data
246 of DBP concentrations from 16 different disinfected drinking water samples[38-43] plus four
247 hypothetical mixtures. Concentrations and literature source of data are listed in Table S5,
248 ESI).

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

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

257

258 2.6 Predicted effects versus measured effects in whole samples

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

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

271

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]

294 For the AREc32 assay on oxidative stress response 6 of the 12 mixtures were equipotent

295 ($mix1_{AREc32}$ to $mix6_{AREc32}$). For all 12 mixtures, the experimental concentration-effect curves

296 (Figure S3) matched the CA prediction with −0.34 ≤ IPQ ≤ 0.84 (Figure 1 and Table S2, ESI).

297 For ARE-bla (Figure S4) the agreement was only slightly lower with −0.83 ≤ IPQ ≤ 1.03

298 (Figure 1 and Table S2, ESI). The AREc32 and ARE-bla assays are reporter gene assays and

299 hence there is no effect observed for chemicals not triggering this particular response. Thus,

300 CA in mixtures can be expected and has been observed previously for diverse

301 micropollutants.[36] We observed a similar level of agreement between modelled and

302 experimental effects for the ARE-bla assay on oxidative stress with −0.83 ≥ IPQ ≤ 1.03

303 (Figure 1 and Table S7, ESI).

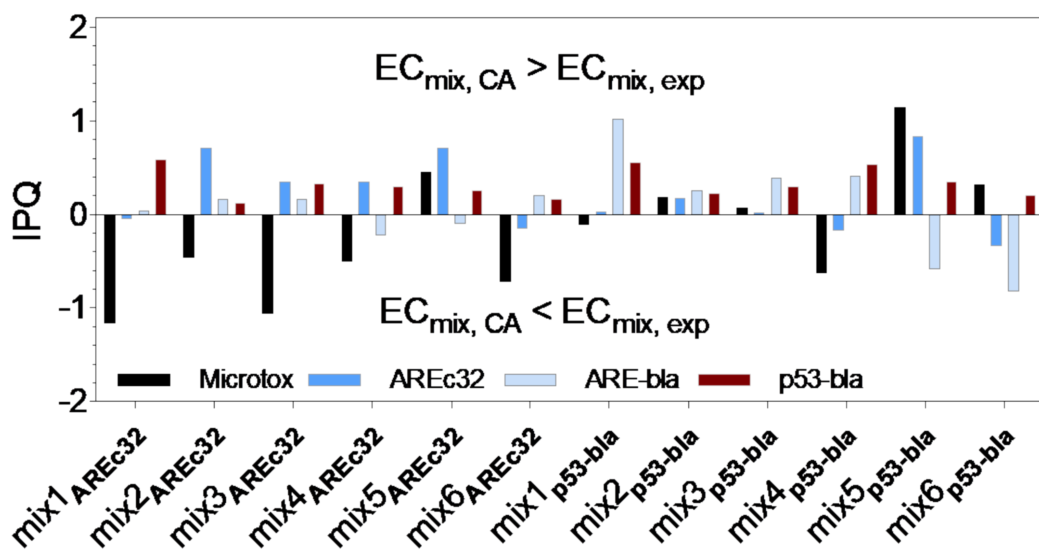
304 For the p53-bla assay on adaptive stress response to genotoxicity (Figure S5) 6 of the 12

305 mixtures were equipotent ($mix1_{p53\text{-}bla}$ to $mix6_{p53\text{-}bla}$) and we observed a systematic deviation

306 from zero for all mixtures (experimental potency was higher than predicted by CA (Table S6

307 and Figure S2, ESI). However, the IPQs for p53-bla were ≤ 0.62, and hence variations were

308 relatively small (Figure 1, Table S7 and Figure S2).



309

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

313 Generally, discrepancies between CA-prediction and experimental data were small and,
314 apart from p53-bla, not systematic as indicated by the distribution of IPQs. In 44 out of 48
315 comparisons between experimental and modelled ECs (i.e., 90%; Table S7), we found $-1 <$
316 $IPQ < 1$ (Figure 1) and the highest IPQ deviation from IPQ = 0 (i.e., perfect agreement with
317 CA) was −1.16 (Table S7, Figure 1). This is in agreement with a previous study where 303
318 mixture effect data where analysed from literature and 88% fell within $-1 < IPQ < 1$.[65] A
319 similar level of agreement was also found by Escher et al.[36] and Tang et al.[33] for mixtures of
320 micropollutants. Thus, our results demonstrate that the CA model satisfactorily predicts the
321 mixture effects of DBPs for adaptive stress responses and cytotoxicity despite differences in
322 molecular initiating events triggering the toxicity pathway.

323

324 ## 3.2 Identifying the risk drivers in a known mixture

325 Given the good agreement between the experimental mixture effects and the CA model, we
326 can calculate the contribution of each mixture component to the total effect (eqn (6)). We
327 selected literature data of DBP concentrations from 16 different samples[38-43] and used four
328 hypothetical mixtures that we considered to be representative for drinking water samples
329 (Table S5, ESI) to calculate the contribution of each mixture component to the total effect of
330 the detected DBPs (BEQ_{chem}). The hypothetical mixtures were used to include some highly
331 toxic DBPs, such as iodinated DBPs, haloacetamides, mono-HAAs, or MX, which are often
332 not included in DBP monitoring studies.

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

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

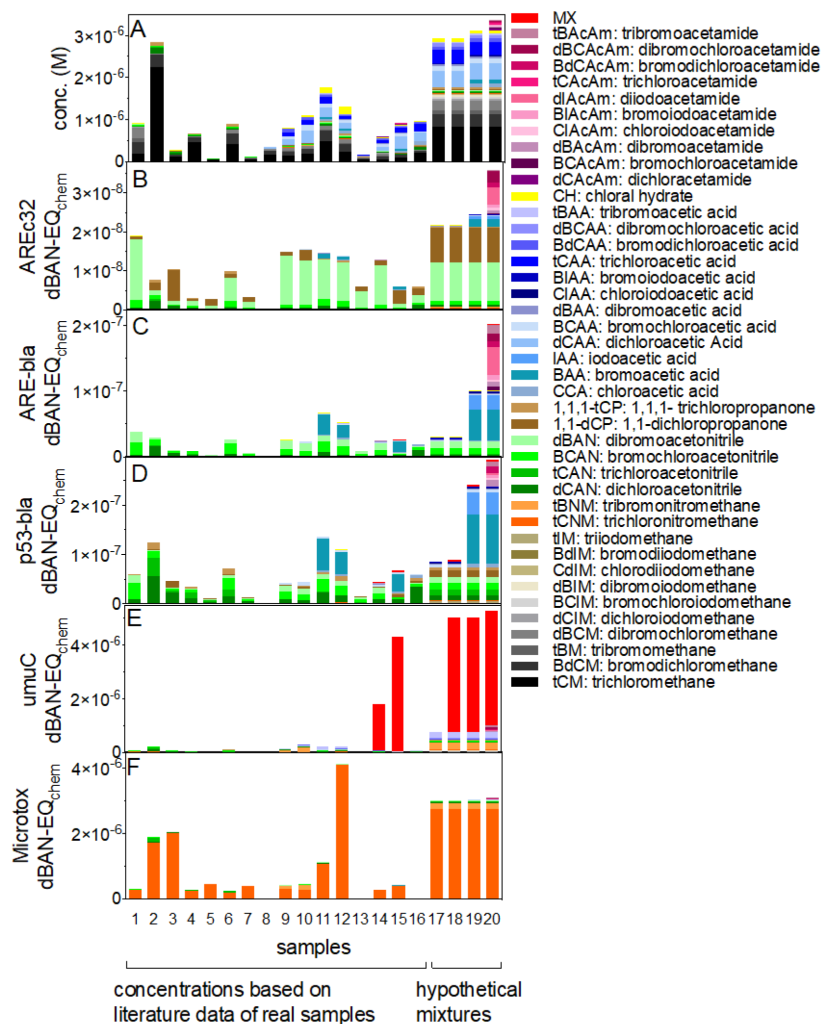


Figure 2. (A) DBP concentrations from 16 samples based on literature data and four hypothetical mixtures that include more potent DBPs, which are often not quantified in studies (see Table S5, ESI, for numerical values of the concentrations). (B – F) Contribution of all DBPs present in a sample to the bioanalytical equivalent concentrations (dBAN-EQ$_{chem}$, units of mol$_{dBAN}$/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).

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

376

377 3.3 Effect-contribution of unknown DBPs in whole mixtures

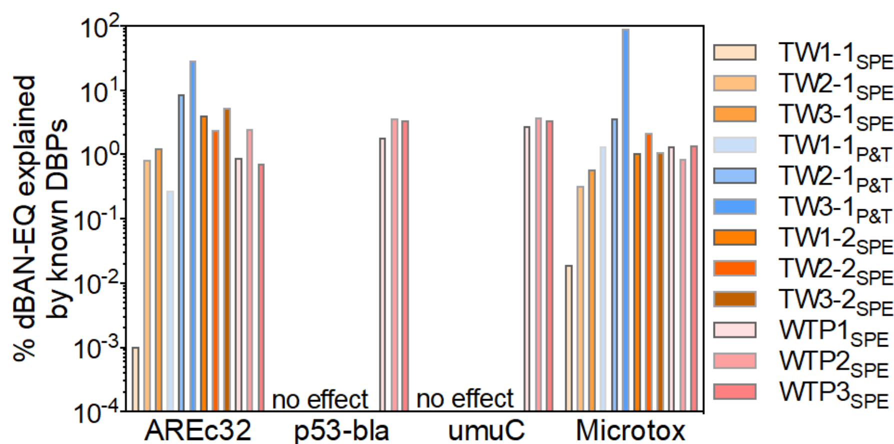
378 As opposed to the approach based on mixtures with known components, whole mixture
379 approaches reflect the real-world scenario because the major fraction of DBPs in drinking
380 water is unknown. Calculating the % contribution of known components in a drinking water
381 sample to the total effect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

385 The fraction of dBAN-EQ explained by the 34 DBPs included in the chemical analysis of
386 which a maximum of 15 was detected (Table S6, ESI) was <6% in all real drinking water
387 samples except for the two purge and trap extracts $TW2\text{-}1_{P\&T}$ and $TW3\text{-}1_{P\&T}$ where 29 and
388 92% could be explained (Figure 3). This demonstrates that unknown or undetected DBPs
389 must account for the major fraction of effects in the samples, in particular in the non-
390 volatile fraction. This is in concordance with the fact that known DBPs cannot explain
391 adverse health outcomes.[4] Among the approximately 700 DBPs reported in literature only a
392 small fraction is routinely monitored or has been toxicologically characterized.[8] If all of these
393 known DBPs were included in an effect and occurrence database the calculated contribution
394 of the known DBPs to the total effect would most likely be significantly larger.

395

396

397



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

407

408 4. Conclusions

409 We found good agreement between the experimental effects for biological endpoints of
410 oxidative stress response, genotoxicity and cytotoxicity and the effects predicted with the
411 model of concentration addition. Our findings support the conclusion by Kortenkamp et al.[20]
412 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

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

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

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

453 Conflicts of Interest

454 There are no conflicts to declare.

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