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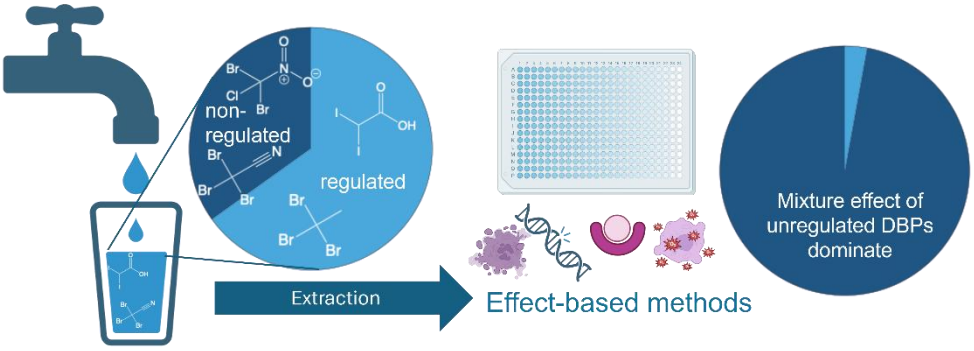
Graphical Abstract



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**Neurotoxicity was evaluated for the first time in drinking water in this study, with samples also tested in oxidative stress response and cytotoxicity assays. The oxidative stress response assay was the most responsive, with iceberg modelling indicating that unregulated DBPs, namely (brominated) haloacetonitriles, were the main contributors to the observed oxidative stress response.*

[12] Choi Y, Lee Y, Liou SY, Son H, Lee Y: *In vitro* bioanalytical assessment of the occurrence and variation of nine bioactivities in a drinking water treatment plant in Korea. Sci Total Environ 2025, 971, 179070.

**This study applied a comprehensive battery of bioassays covering nine endpoints to samples collected from different stages of a full-scale drinking water plant, with the measured effect compared with effect-based trigger values. The study also used iceberg modelling to demonstrate that volatile DBPs only had a small contribution to the oxidative stress response*

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[28] Lau SS, Bokenkamp K, Tecza A, Wagner ED, Plewa MJ, Mitch WA: Toxicological assessment of potable reuse and conventional drinking waters. Nat Sustain 2023, 6, 39-46.

**Cytotoxicity was evaluated in several potable reuse treatment trains. Regulated DBPs had a smaller contribution to cytotoxicity than unregulated DBPs, though unmeasured DBPs and other micropollutants accounted for most of the total cytotoxicity. Cytotoxicity in the influent was also measured before and after disinfection to determine the contribution of DBPs to cytotoxicity.*

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**This study applied the calculated additive toxicity approach to drinking water samples with different source water and disinfectant types over two seasons. Unregulated DBPs, namely haloacetonitriles and iodoacetic acid, were the main contributors to the predicted oxidative stress response and cytotoxicity. Predicted toxicity varied with season and source water type.*

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**This study follows on from previous work by Lau et al. [38] who showed that DBP mixtures act in a concentration additive manner in a cytotoxicity assay. Using five defined mixtures of regulated and unregulated DBPs, this study found that genotoxicity in mixtures was less than additive. This study suggested that genotoxicity predicted based on calculated additive toxicity should be considered an initial upper-bound estimate.*

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Applying effect-based methods to evaluate the mixture effects of disinfection by-products and other chemicals in disinfected water

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Abstract

Effect-based methods (EBM) are increasingly used to assess disinfected water quality as they can detect the mixture effects of all bioactive chemicals, including disinfection by-products (DBPs). In this review, we discuss the application of EBM to extracts of disinfected water, with bioassays for cytotoxicity, genotoxicity and oxidative stress response being most responsive to DBPs. The contribution of known DBPs to the mixture effect can be predicted from single DBPs' effects using established mixture models. Regulated DBPs typically have a minor contribution to the observed effect. Effect-based trigger values can be used to determine if the treated water quality is acceptable.

Keywords: drinking water; effect-based trigger value; iceberg modelling; *in vitro* bioassays

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1. Introduction

Disinfection by-products (DBPs), which are formed from the reaction of disinfectants (e.g., chlorine, chloramine) with organic and inorganic precursors in water, can be present in disinfected drinking water at concentrations up to 1000 times higher than some other organic micropollutants, such as per- and polyfluoroalkyl substances (PFAS), pesticides and pharmaceuticals [1]. With over 6000 DBPs reported in the literature since 1974, including 651 confirmed DBPs [2], chemical analysis of a targeted suite of DBPs will only detect a small fraction of the DBPs potentially present in water. While this chemical-by-chemical approach is the norm for water quality monitoring, there is increasing interest in combining chemical analysis with effect-based methods (EBM) (e.g., cell-based *in vitro* bioassays and well plate-based *in vivo* assays with whole organisms) to assess disinfected water quality [e.g., 3, 4-6]. This is because EBM can detect the mixture effects of all active chemicals in a sample, including unknown chemicals and chemicals present below their analytical detection limit [7]. EBM are risk-scaled, meaning more potent chemicals will have a greater response than less potent chemicals. This is relevant for DBPs because the frequently measured regulated DBPs, such as trihalomethanes (THMs) and haloacetic acids (HAAs), are often less toxic than other unregulated DBP classes, such as halonitromethanes (HNMs) and haloacetonitriles (HANs) [8-10]. Contrary to common belief, numerous studies have indicated that EBM are sensitive, robust, reliable and cost-effective for assessing drinking water quality provided that appropriate extraction techniques are applied [11]. EBM alone cannot identify the individual DBPs that explain the effect, so the complementary use of EBM and chemical analysis is recommended.

In this review we discuss the application of EBM to disinfected water treated with both conventional treatment processes (e.g., coagulation, flocculation, sedimentation) and advanced treated processes (e.g., activated carbon, advanced oxidation, biofiltration, membrane filtration). We also identify approaches to determine which DBPs are contributing to the observed effect in disinfected water and discuss how to determine if the chemical water quality is acceptable or not. This review includes studies from the last 10 years, with studies published since 2020 prioritised.

2. Application of EBM to disinfected water samples

Disinfected water samples have been tested in EBM covering different stages of the cellular toxicity pathway (Figure 1). This includes assays indicative of xenobiotic metabolism (e.g., activation of the aryl hydrocarbon receptor (AhR) [6, 12-17] and binding to peroxisome proliferator-activated receptors (PPAR) [12, 15, 16]), receptor-mediated effects (e.g., activation of the estrogen receptor (ER) [5, 6, 12, 14-22] and neurotoxicity [4]), reactive toxicity (e.g., genotoxicity [5, 13, 14, 17, 22-25]) and adaptive stress responses (e.g., oxidative stress response [6, 12-17, 20, 25, 26] and p53 response [12, 20, 23, 26]). Further, several studies have applied the Chinese hamster ovary (CHO)

cell chronic cytotoxicity assay to assess non-specific toxicity [3, 27-29]. While an effect at the cellular level may not lead to an adverse effect at a higher level, cell-based *in vitro* bioassays are sensitive chemical indicators and therefore can be viewed as surrogates for chronic effects [7].

Some studies have applied comprehensive test batteries with bioassays covering many endpoints to disinfected water [12, 20], but for routine water quality monitoring of disinfected water a practical test battery covering activation of AhR, activation of ER, oxidative stress response and genotoxicity or mutagenicity, as well as cytotoxicity, is recommended [7] (Figure 1). Both activation of AhR and the oxidative stress response are often detected in treated drinking water [e.g., 6, 12, 14], while estrogenic activity is less frequently detected after treatment [e.g., 21, 30] but is relevant to assure that human contaminants in source water are removed. While many DBPs are responsive in oxidative stress response assays [8], the inclusion of an assay indicative of either genotoxicity or mutagenicity is recommended to capture direct genotoxicity and DNA damage.

In addition to DBPs, disinfected water can also contain organic micropollutants, such as pharmaceuticals, pesticides and industrial compounds [32, 33]. Further, transformation products can also form from the reaction of organic micropollutants with disinfectants [34, 35]. The contribution of formed DBPs to the observed effect can be evaluated by assessing the effect before and after disinfection [e.g., 20, 28]. For example, formed DBPs explained between 17 to 58% of the oxidative stress response in chlorinated drinking water distribution systems in France [26]. A more variable picture emerged for influent from advanced water treatment plants for potable water reuse after chloramine disinfection [24, 28]. For example, chloramination reduced cytotoxicity by 85% and genotoxicity by 87% in influent (i.e., secondary wastewater effluent) from an agricultural area, indicating that disinfection degraded cytotoxic and genotoxic organic contaminants [24, 28]. In contrast, cytotoxicity increased by 61% and genotoxicity increased over 87% in disinfected water that used source water from riverbank filtration, showing that DBPs were important contributors to the observed effect [24, 28].

Despite the effects detected in disinfected water, many studies have shown that raw drinking water typically has a greater response than disinfected water [e.g., 3, 15, 20], putting the toxicity of formed DBPs into context with organic micropollutants present in untreated water.

3. Which DBPs contribute to the mixture effects?

EBM alone cannot identify which chemicals contribute to the mixture effect in water. However, the effect can be predicted using the measured concentration of each detected DBP from target analysis scaled by their potency in the studied assay. Comparing the observed effect and the predicted effect reveals how much can be explained by known chemicals and how much is due to unknown chemicals (i.e., undetected chemicals or chemicals without potency data), highlighting the utility of combining

both chemical analysis and EBM. This approach has been applied to disinfected waters and is referred to as iceberg or mixture toxicity modelling [e.g., 4, 26], the "TIC-Tox" method [e.g., 27, 36] or calculated additive toxicity [e.g., 37, 38]. Despite the different terminology, the approaches are all similar and assume that all chemicals in the mixture act in a concentration additive manner. This assumption of concentration addition was confirmed for DBP mixtures by Stalter et al. [39] who tested mixtures of up to 24 DBPs in assays indicative of bacterial toxicity, oxidative stress response and p53 response. Over 90% of the mixture predictions fell within a factor of 2 of the measured effect, indicating that concentration addition is suitable for predicting mixture toxicity, despite DBPs having diverse modes of action. Lau et al. [38] also found that DBP mixtures acted in a concentration additive manner in the CHO cell cytotoxicity assay, though DBP mixtures were less than additive in the CHO cell acute genotoxicity assay [40].

Despite commonly regulated DBPs, namely THM_4 (i.e., chloroform, bromodichloromethane, dibromochloromethane, bromoform) and HAA_5 (i.e., monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic acid, dibromoacetic acid), being found at higher concentrations in disinfected water, the predicted effect is dominated by unregulated DBPs, especially HANs and HMNs [e.g., 27, 29, 36, 37, 41, 42]. This is illustrated in Figure 2, where regulated DBPs contributed around 65% on average of the sum molar concentration of DBPs detected in chlorinated water, but only 0.6 to 8% on average of the predicted cytotoxicity, genotoxicity and oxidative stress response [27, 36, 43]. Even when analysing both regulated and unregulated DBPs, only <5 to 17% of the measured cytotoxicity could be explained by detected DBPs in drinking water and potable reuse water [28, 29], while detected DBPs explained up to 27% of measured genotoxicity [24]. Further, detected DBPs explained between 0.16 to 460% of the oxidative stress response in treated drinking water, with the presence of potent HANs contributing to high fraction explained [4, 26]. The fact that EBM account for chemical potency as well as concentration provides a better understanding of the risk of DBP mixtures, highlighting a key advantage of EBM.

The predicted effect calculations rely on single chemical effect data, with two studies, Stalter et al. [8] and Wagner and Plewa [9], fingerprinting the effects of DBPs in a range of assays. This is summarised in Figure 3, with available single chemical effect data for individual DBPs provided in Table S1 of the Supplementary Material. The availability of single chemical effect data will affect the predicted effect calculations, with McKenna et al. [44] showing that the suite of DBPs included or omitted affected predicted cytotoxicity and genotoxicity.

4. Extraction of disinfected water samples for EBM

Sample extraction is typically required prior to EBM to enrich the micropollutant and DBP concentration and to remove metals and other inorganics present in water [7]. Different sample

extraction methods are applied to extract DBPs prior to chemical analysis and bioanalysis, with solid-phase extraction (SPE) using XAD resin or other polymeric sorbents often used for bioanalysis [45]. SPE is able to extract non-volatile and hydrophobic DBPs, but does not effectively capture volatile and semi-volatile DBPs, with less than 30% recovery by XAD and between 30 to 60% recovery by SPE [46]. Further, solvent blow-down methods during sample processing also affect DBP recovery [47]. More complex extraction methods using purge and cold trap can capture more volatile DBPs [43, 48], with Stalter et al. [43] finding 50-100% recovery of THMs and HANs. However, several studies have shown that (semi)volatile DBPs only have a minor contribution to cytotoxicity [28, 48], oxidative stress [4, 12, 26, 43] and genotoxicity [24], suggesting that the loss of these compounds during extraction will not significantly underestimate the DBP mixture effects.

5. Is the treated water quality acceptable?

The cell-based *in vitro* bioassays recommended for monitoring disinfected water quality are very sensitive and even highly treated water may still induce a response in an assay. This response does not necessarily mean that the water quality is unacceptable, with effect-based trigger values (EBTs) used to differentiate between acceptable and unacceptable chemical water quality. EBTs are similar to guideline values for individual chemicals, with EBTs available for different assays and protection goals (e.g., human health for drinking water and ecological health for environmental waters) [7]. Several studies have derived human health-relevant EBTs for drinking water and potable reuse water [e.g., 49, 50, 51], or a process to derive interim EBTs where needed [52]. Treated drinking water is typically below the proposed human EBTs for hormone receptor-mediated effects, such as estrogenic activity and androgenic activity [e.g., 12, 14, 18, 20]. Further, the oxidative stress response in treated drinking water is often below the proposed human EBT [4, 14, 20, 26], though five out of ten tested Australian tap water samples in Stalter et al. [25] exceeded the human EBT by up to 2.2 times (Figure 4).

While human EBTs are available for most endpoints, EBTs are not readily available for relevant endpoints including genotoxicity (e.g., CHO cell acute direct genotoxicity assay, umuC assay, *in vitro* micronucleus test) and mutagenicity (e.g., Ames assay), though Pronk et al. [51] derived a preliminary EBT for an assay indicative of the p53 response for genotoxicity. Future work should focus on the development of robust EBTs for these endpoints, though interim EBTs can be estimated using the approach in Neale et al. [52] in the meantime. Using the example of the umuC assay, Stalter et al. [25] reported an effect concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) of 20.9 µg/L for assay reference compound 4-nitroquinoline 1-oxide (4NQO). Applying the median EC to EBT ratio of 0.7 for human EBTs [52], this yielded an interim human EBT of 30 µg/L 4NQO

equivalents for the umuC assay. The response in the umuC assay for all tap water samples in Stalter et al. [25] was 42 to 93 times lower than the interim human EBT.

Overall, comparison with proposed human EBTs demonstrates that in most cases the chemical water quality of treated drinking water is acceptable.

6. Conclusions

EBM can assess the mixture effects of all active chemicals in a sample and, when combined with chemical analysis, can provide a more comprehensive picture of the chemical burden in water. Disinfected water has been shown to be active in bioassays covering a range of endpoints including activation of AhR, oxidative stress response, genotoxicity and cytotoxicity. However, the effect in treated water is often lower than raw water, with hormone receptor-mediated effects and the oxidative stress response in disinfected drinking water typically below human EBTs. While regulated DBPs make up the majority of the formed DBPs in disinfected water, unregulated DBPs dominate the predicted cytotoxicity, genotoxicity and oxidative stress response. This highlights a key advantage of EBM as more potent and thus more toxicologically relevant DBPs will have a greater response in the assay than less potent DBPs.

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CRediT Statement

Peta Neale: Conceptualisation, Investigation, Writing – original draft, Visualization; **Beate Escher**: Conceptualisation, Writing – review and editing; **Frederic Leusch**: Conceptualisation, Writing – review and editing

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Figure 1

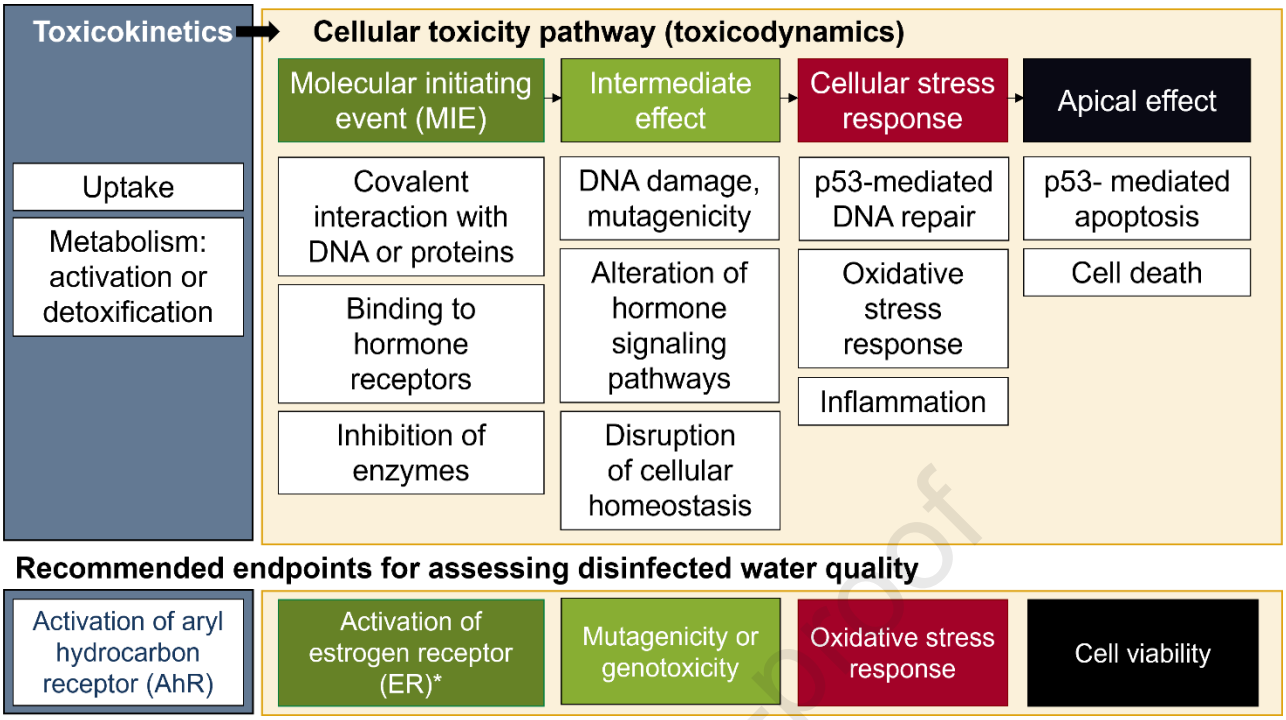

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Figure 2

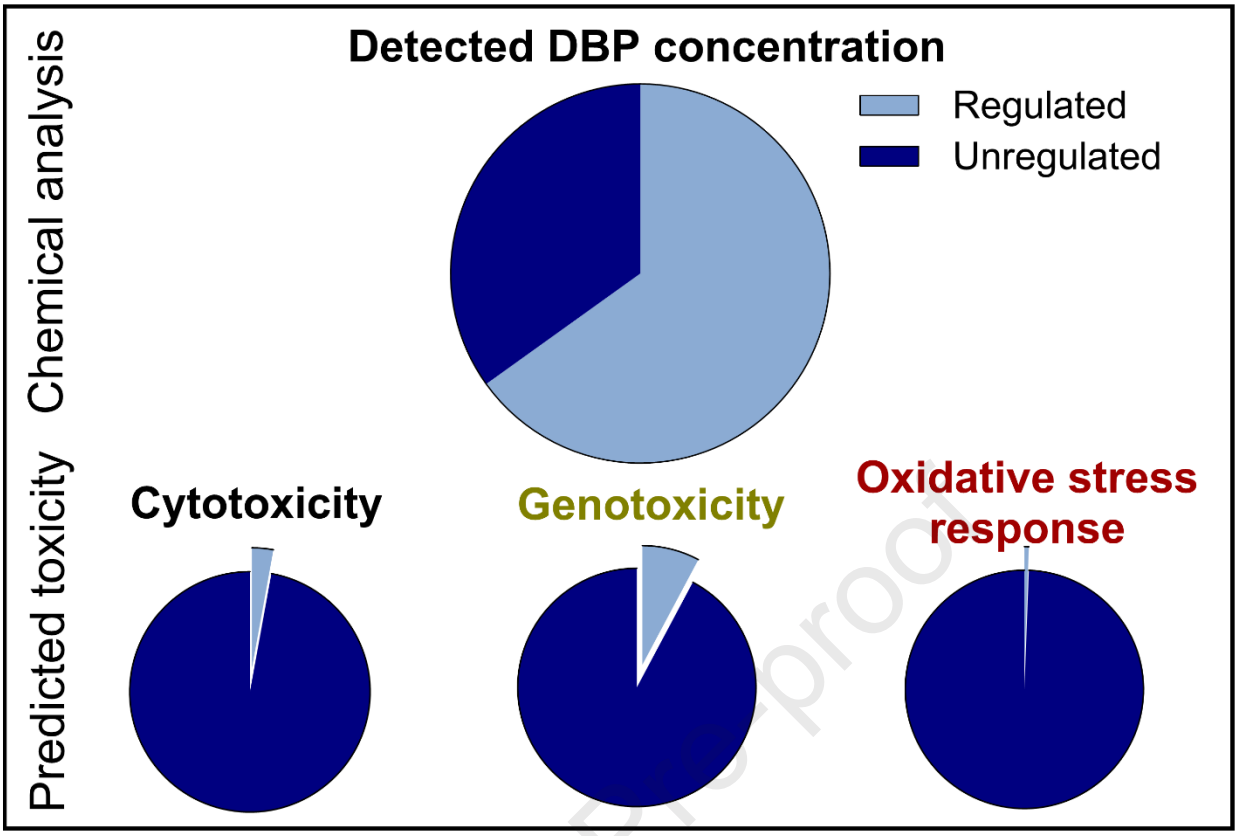

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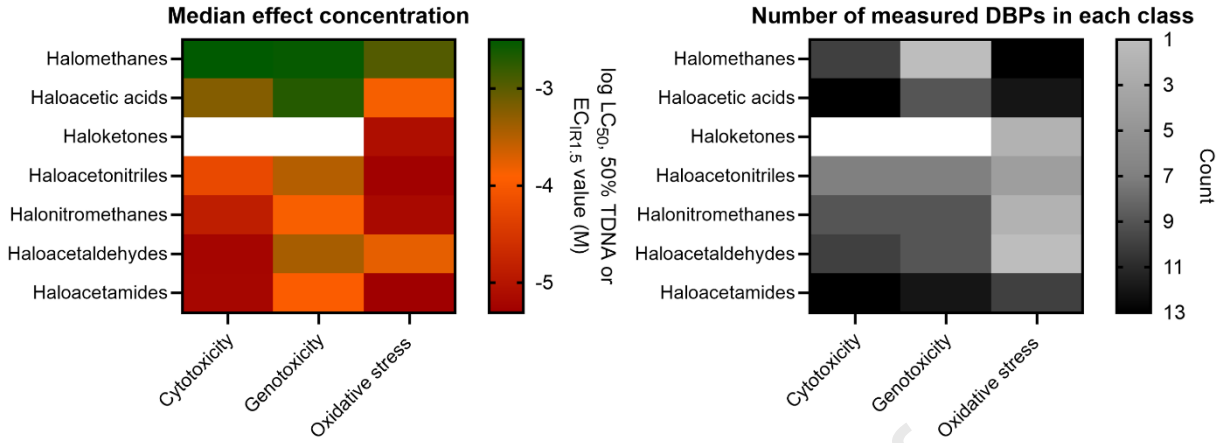
Figure 3



***Single chemical data for individual DBPs in the Supplementary Material**

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Figure 4

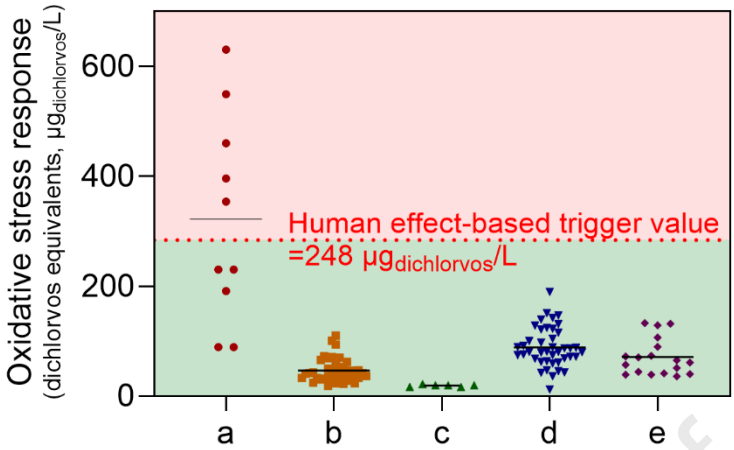

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Figure Captions

Figure 1: The cellular toxicity pathway (top panel), with recommended endpoints for assessing disinfected water quality aligning with different stages of the cellular toxicity pathway (bottom panel). Modified and reprinted with permission from Neale et al. [31]. Development of a bioanalytical test battery for water quality monitoring: Fingerprinting identified micropollutants and their contribution to effects in surface water. Water Research, 123, 734–750. © 2017 Elsevier. *The inclusion of an assay to assess estrogenic activity is important because estrogenic compounds pose a risk to human health and are indicators of wastewater contamination, not because DBPs are estrogenic.

Figure 2: The percent contribution of regulated DBPs (THM_4 and HAA_5) and unregulated DBPs to the detected DBP concentration (molar units) and predicted cytotoxicity, genotoxicity and oxidative stress response in chlorinated drinking water [27, 36, 43]. Predicted cytotoxicity, genotoxicity and oxidative stress response were calculated using single chemical effect data from Stalter et al. [8] and Wagner and Plewa [9].

Figure 3: An overview of available single chemical effect data for DBPs from the literature [8, 9], including the median effect concentration and the number of measured DBPs for each class. Available single chemical effect data are provided in Table S1 of the Supplementary Material.

Figure 4: Oxidative stress response (AREc32 assay) detected in treated drinking water from Australia[a], France[b,c], Spain[c] and Sweden[e] expressed as dichlorvos equivalents ($\mu g_{dichlorvos}/L$). The red dashed line indicates the proposed human effect-based trigger value (EBT) of 284 $\mu g_{dichlorvos}/L$ from Escher et al. [53]. The red shading indicates unacceptable chemical water quality, while the green shading indicates acceptable chemical water quality.

[a]Stalter et al. [25], [b]Hebert et al. [26], [c]Neale et al. [20], [d]Escher et al. [4], [e]Lundqvist et al. [14]

Note: Values expressed as an effect concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) were converted to dichlorvos equivalents using the dichlorvos $EC_{IR1.5}$ value from Escher et al. [53].

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Highlights

- Effect-based methods detect mixture effects of DBPs in disinfected water
- Cytotoxicity, genotoxicity and oxidative stress response found in disinfected water
- Unregulated DBPs explained most of the predicted toxicity
- Response in disinfected water typically below proposed effect-based trigger values

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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