In vitro bioassays to assess drinking water quality

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25 **Abstract:** *In vitro* assays indicative of different stages of cellular toxicity pathways have been
26 applied to both source water and drinking water. The majority of studies showed a decrease in
27 receptor-mediated effects after drinking water treatment due to the removal of micropollutants,
28 while reactive toxicity typically increased after chlorination due to the formation of disinfection by-
29 products. Using both chemical analysis and bioanalysis, iceberg modelling can be applied to
30 determine which chemicals are contributing to the observed effect, though one limitation is that
31 typical sample pretreatment for bioanalysis fails to capture volatile chemicals. Bioassays are
32 increasingly sensitive and effects can be detected in clean samples, thus effect-based trigger values
33 can be applied to determine whether an effect in drinking water is acceptable.

34
35 **Keywords:** bioanalysis; drinking water treatment; effect-based trigger value; estrogenic activity;
36 iceberg modelling; reactive toxicity

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38

1. Introduction

There is increasing concern about the presence of micropollutants, such as pharmaceuticals, pesticides and industrial compounds, in drinking water, with micropollutants detected in both source water and treated drinking water around the world [1-4]. During water treatment, disinfection by-product (DBPs) can form from the reaction of disinfectants, such as chlorine, chloramine and ozone, with organic and inorganic matter naturally present in source water [5], with DBPs commonly detected in disinfected drinking water [6, 7]. Further, micropollutant transformation products (TP) can also form during water treatment with disinfectants and during other advanced oxidation processes [8]. Consequently, drinking water can contain a complex mixture of micropollutants, TPs and DBPs (Figure 1). While targeted chemical analysis is often used for monitoring drinking water quality, it is unlikely to comprehensively capture the diversity of chemicals potentially present in drinking water, especially as many micropollutants and their TPs will be present at low nanogram per litre concentrations. Instead, *in vitro* bioassays can be applied complementary to chemical analysis as they can incorporate the mixture effects of all active chemicals in a sample without the identification of single compounds [9, 10]. They are also risk-scaled, meaning that more potent chemicals will have a greater contribution to the mixture effect than less potent chemicals at similar concentrations. In the current study we review the application of high-throughput *in vitro* assays to drinking water, with a focus on studies that have been published in the last two years. In addition, we also discuss the application of iceberg modelling for drinking water, sample preparation considerations and the need for effect-based trigger values.

2. Application of *in vitro* bioassays to drinking water

In vitro bioassays indicative of different stages of cellular toxicity pathways, including induction of xenobiotic metabolism [11, 12], hormone receptor-mediated effects [1, 13, 14], reactive modes of action [15, 16], adaptive stress responses [11, 17, 18] and cytotoxicity [19, 20], have been applied to evaluate effects in drinking water. While some of the assays utilized in the cited studies are not currently high-throughput (e.g. run in 96 or 384 well plate), they all have the potential to be high-throughput (e.g. Ames plate-incorporation test using agar plates versus the Ames fluctuation test in 384 well plates).

The effect in a bioassay is often expressed as a bioanalytical equivalent concentration (BEQ_{bio}), which relates the effect of a sample to the concentration of a reference compound that would elicit the same response as the mixture of chemicals in the tested water sample. For example, estrogenic activity is often expressed as an estradiol equivalent concentration (EEQ).

Estrogenic activity has been widely studied in treated drinking water using a range of *in vitro* assays [11-14, 21-25], with an overview of reported activity provided in Figure 2. Many studies also measure estrogenic activity in source water and compare BEQ_{bio} before and after treatment, with 39 to 99% removal of estrogenic activity reported [12, 13, 23-25], which indicates the removal or degradation of causative compounds. In many cases, estrogenic activity was below the assay detection limit after treatment. While less studied, other types of hormonal activity, such as activation of the androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR) and thyroid receptor (TR), have not been detected in drinking water [1, 11, 12, 26-28], with the exception of low androgenic activity in one drinking water sample from the Netherlands [29].

Reactive toxicity, specifically genotoxicity [16, 18, 19] and mutagenicity [15, 25, 30], is also commonly studied in drinking water. The majority of studies typically showed an increase in reactive toxicity after water treatment with disinfectants [15, 16, 18, 25, 30], which is attributed to the formation of DBPs. In contrast, while some source water samples induced a response in the micronucleus test for genotoxicity and the Ames fluctuation test for mutagenicity, Shi et al. [12] found that none of the corresponding treated water samples had an effect after conventional treatment (coagulation, sedimentation and sand filtration) with chlorination. The effect in the source water was attributed to the presence of micropollutants, such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls, as mutagenicity in the source water was only observed after metabolic activation using rat liver S9.

While most studies focus on surface water as a source of drinking water, several studies have applied *in vitro* assays to assess reactive toxicity of drinking water from groundwater sources [20, 30, 31]. In all studies, there was negligible toxicity in treated water, with Pellacani et al. [20] observing a decrease in genotoxicity and cytotoxicity after treatment despite disinfection being the only form of treatment. The negligible toxicity after treatment was attributed to the low concentrations of DBP precursor natural organic matter in groundwater.

As all active chemicals will induce a response in a bioassay, it is difficult to distinguish between the effect of micropollutants, TPs and DBPs in the assay. In an attempt to differentiate the contribution of micropollutants and DBPs to the oxidative stress response in samples from drinking water distribution systems, Hebert et al. [17] compared BEQ_{bio} before and after treatment (Equation 1) and found that DBPs could contribute up to 58% of the oxidative stress response. A limitation of this approach is that it does not account for removal of micropollutants during treatment and thus may potentially underestimate the contribution from DBPs.

$$BEQ_{bio, DBP} = BEQ_{bio, after treatment} - BEQ_{bio, before treatment}$$

(1)

3. Which chemicals are driving the observed effects in drinking water?

Several studies have applied both bioanalysis and chemical analysis to assess drinking water quality [12, 17, 30] and iceberg modelling can be used to determine the contribution of detected chemicals to the observed effect [32]. In iceberg modelling BEQ_{bio} is compared to bioanalytical equivalent concentrations from chemical analysis (BEQ_{chem}), which is calculated using the detected chemical concentration and the relative effect potency (REP_i) of the detected chemical to the assay reference compound (Figure 3). Comparison of BEQ_{bio} and BEQ_{chem} can reveal if a certain chemical or group of chemicals can explain the majority of the effect or whether the effect is predominately triggered by unknown chemicals. Iceberg modelling has been applied to both source water and treated drinking water recently, with natural and synthetic hormones found to explain the majority of observed estrogenic activity [12, 13, 23]. In contrast, the detected chemicals could only explain between 0.2 to 6.5% of the dioxin-like response in the EROD assay in source water [12]. This is in line with previous observation in surface water [33]. A similar approach is the TIC-Tox metric, which aims to determine the forcing agents in disinfected water [34]. Using semi-quantitative total ion current (TIC) data from Jeong et al. [19] and cytotoxicity data (Tox) from Wagner and Plewa [35], haloacetonitriles and haloacetamides were found to be the main drivers of toxicity in drinking water extracts [34].

The effect of the individual detected chemicals is required for iceberg modelling in order to calculate REP_i . Recently, the effect of conventional and emerging DBPs have been fingerprinted in bioassays commonly used for water quality monitoring [e.g. 6, 35-39]. While technically *in vivo*, early life-stage whole organism assays, such as the fish embryo toxicity (FET) assay, can also be run in high-throughput mode and are considered legally as *in vitro*, with a recent study fingerprinting the effects of individual DBPs in the FET assay [40]. In addition, the US EPA ToxCast database (<https://actor.epa.gov/dashboard/>) contains effect data for over 9000 chemicals, including many DBPs and other micropollutants detected in drinking water. It has already been utilized for iceberg modelling in surface water and wastewater [33, 41].

4. Sample pretreatment for bioanalysis

As micropollutants are typically present in drinking water at low concentrations, sample enrichment is required prior to applying *in vitro* bioassays, with several studies enriching drinking water

142 samples 10,000 times or more [12, 19, 31, 42]. The majority of studies apply solid phase extraction
143 (SPE) [e.g. 12, 13, 14] or XAD resins [e.g. 15, 19, 31] to enrich drinking water. While these
144 extraction methods will extract non-volatile and semi-volatile chemicals, they are unable to capture
145 volatile chemicals. This is particularly pertinent for DBPs, as many are volatile and thus typical
146 enrichment techniques may not capture all of the toxicologically relevant chemicals. To investigate
147 this further, Stalter et al. [43] applied both SPE and purge and cold-trap methods to capture both
148 non-volatile and volatile DBPs from disinfected drinking water samples. For the bacterial Microtox
149 assay the volatile fraction induced a greater effect than the non-volatile fraction in some samples,
150 while the non-volatile fraction induced the majority of the oxidative stress response in the AREc32
151 assay. Similarly, using iceberg modelling, Hebert et al. [17] found that volatile DBPs only had a
152 minor contribution to the observed oxidative stress response in samples collected from French
153 drinking water distribution systems. As these examples only focus on two assays, further work is
154 required to understand the contribution of volatile and non-volatile DBPs in other assays commonly
155 applied to water extracts, such as the Chinese hamster ovary (CHO) cell line [19] and the Ames
156 assay [15, 25].

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158 **5. Is drinking water quality acceptable?**

159 Many *in vitro* assays, particularly reporter gene assays, are very sensitive and can detect an effect in
160 clean water samples with sufficient enrichment, including highly treated drinking water and bottled
161 water [17]. However, just because a water sample induces a response in a bioassay does not mean
162 that the water quality is necessarily unacceptable. Consequently, effect-based trigger values (EBT)
163 can be applied to differentiate between acceptable and unacceptable water quality [44], with
164 drinking water EBTs developed for a number of *in vitro* assays [29, 45, 46]. Estrogenic activity in
165 drinking water was compared to available EBTs for the ER-CALUX, E-Screen and yeast estrogen
166 screen (YES) assays (Figure 2). In the majority of cases, the reported activity was far below the
167 corresponding EBT, with the exception of two treated drinking water samples from China [12].
168 Further, the oxidative stress response in drinking water from France and Australia was compared to
169 the proposed oxidative stress EBT from Escher et al. [46] (Figure 4). While there was a margin of
170 safety of 2 to 16 for treated drinking water from France [17], drinking water sampled in Australia
171 often exceeded the EBT [18, 26, 47]. EBTs are important tools for interpreting bioassay results,
172 though further work is required to derive EBTs for more assays used for drinking water quality
173 monitoring.

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177 **6. Conclusions**

178 *In vitro* bioassays are a valuable tool to complement to chemical analysis for drinking water quality
179 monitoring as they are able to integrate the effects from a complex mixture of micropollutants, TPs
180 and DBPs. While a range of effects, including estrogenic activity, oxidative stress response, reactive
181 toxicity and cytotoxicity, have been detected in treated drinking water, the effects are generally low
182 and are mostly below available EBTs. Although volatile DBPs are not captured by common sample
183 pretreatment processes, iceberg modelling has suggested that volatile DBPs only have a minor
184 contribution to the oxidative stress response, though this remains to be seen for other biological
185 endpoints. To better understand the contribution of micropollutants and DBPs to the observed effect
186 in drinking water a bioassay test battery covering different stages of cellular toxicity pathways is
187 recommended. Based on the current review, a suitable test battery for drinking water may include
188 assays indicative of activation of the estrogen receptor, genotoxicity, oxidative stress response and
189 cytotoxicity.

190

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194

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371 water samples, with the EBTs generally shown to be able to differentiate between drinking water
372 and more chemically contaminated water samples.*

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377 using the AREc32 reporter gene assay indicative of the oxidative stress response pathway, *J.*
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381 **Figure 1:** Overview of chemicals potentially present in source water and treated drinking water
382 with the solid arrows indicating the formation of new chemicals after treatment. Comparison of
383 bioanalytical equivalent concentrations before treatment ($BEQ_{\text{before treatment}}$) and after treatment
384 ($BEQ_{\text{after treatment}}$) can shed light on treatment efficiency and disinfection by-product (DBP)
385 formation.

389 **Figure 2:** Estradiol equivalent concentrations (EEQ) measured in treated drinking water from
 390 around the Netherlands^a, China^{b,d,e,f}, Taiwan^{c,g}, United States^h, South Africaⁱ and Sweden^j using
 391 different *in vitro* bioassays for binding to the estrogen receptor. The solid coloured lines indicate
 392 effect-based trigger values (EBT) from Escher et al. [45], while the dotted red line indicates the ER
 393 CALUX EBT from Brand et al. [29]. No EBT has been developed for the T47D-KBluc or
 394 VM7Luc4E2 assays.
 395 ^aBrand et al. [29] (2/3 samples above limit of detection (LOD)), ^bShi et al. [12] (2/7 samples above
 396 LOD), ^cGou et al. [22] (maximum EEQ shown only), ^dLv et al. [23] (4/4 sample above LOD), ^eXiao
 397 et al. [24] (22/36 samples above LOD), ^fXiao et al. [25] (8/54 samples above LOD), ^gChou et al.
 398 [21] (average EEQ of 5 samples shown), ^hConley et al. [13] (3/24 samples above LOD), ⁱVan Zijl et
 399 al. [14] (33/80 samples above LOD), ^jRosenmai et al. [11] (3/3 samples around LOD)

403 **Figure 3:** Application of iceberg modelling to drinking water samples.

407 **Figure 4:** Oxidative stress response, expressed as toxic units (TU), measured in treated drinking
 408 water in Australia^{a,b,c} and France^d using the AREc32 and Nrf2-CALUX assays. The solid red line
 409 indicates the EBT for the AREc32 assay from Escher et al. [46]. No EBT is available for the Nrf2-
 410 CALUX assay. TU calculated from effect concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$)
 411 in units of relative enrichment factor (REF).
 412 ^aEscher et al. [47], ^bNeale et al. [18], ^cEscher et al. [26], ^dHebert et al. [17]

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