SafeCREW ANALYTICAL PROTOCOL #3

TEST PROTOCOL FOR EFFECT-BASED IN-VITRO TOXICITY ASSESSMENT OF DISINFECTION BY-PRODUCTS

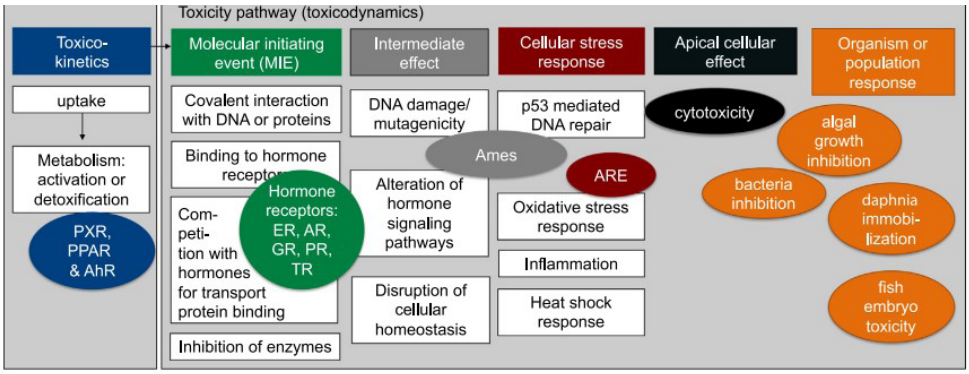

Figure 1 Non-animal and effect-based methods (NAM/EBM) for water testing (Escher et al. 2018)

Introduction

The European Commission aims for zero pollution in air, water, and soil by 2050, with the European Green Deal and Chemicals Strategy promoting a toxic-free, safer environment. The purpose of the guideline for effect-based methods (EBM) is to enhance the assessment of water quality and safety by providing a more comprehensive approach to evaluating the potential adverse effects of chemical mixtures. EBM allows for the detection of mixture effects that cannot be addressed by chemical analysis alone, thereby improving the accuracy and reliability of water quality assessments (see e.g., Figure 1 from Escher et al. 2018 as well as GWRC 2023). The guidelines aim to support regulatory agencies and the water sector in making informed decisions regarding water quality management and safety planning (GWRC, 2023; Wernersson et al. 2015, Enault et al. 2023).

EBM aim to characterize chemicals by their biological activity or toxicity, and not by their chemical structure. EBM are used to determine the potency of chemicals, chemical mixtures, or water samples to cause adverse effects on whole organisms (in vivo, for example with fish, crustaceans or algae), or cells, cultured tissues, or isolated enzymes (in vitro). Compared with in vivo assays, the advantages of in vitro bioassays are multiple: they generally show less variability, are easier to implement, faster, cheaper, have a higher capacity due to high-through-put screening (HTPS) set-up and lower ethical barriers. In vitro bioassays can also provide information about specific toxic key events, such as estrogenic activity or genotoxicity. Many EBM are used in different fields of water monitoring for many kinds of pollutants (some examples are described in Alygizakis et al 2023, Phan et al 2023).

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Target Audience

Effect-based methods (EBMs) are often used in a variety of disciplines, including environmental science, policy-making, and risk assessment, to evaluate the outcomes or effects of interventions, actions, or events rather than merely focusing on the specific inputs or processes. When it comes to guidelines for using effect-based methods, the users or stakeholders typically fall into several categories depending on the context in which the methods are applied such as a) environmental policymakers and regulators, b) water utilities, c) industrial sector (such as adsorbent materials producers), d) NGOs and advocacy groups, e) researchers and academics and f) international organizations and shall pave the way for the adoption of advanced water treatment solutions to ensure safe drinking water in a climate change scenario.

In terms of practical implementation, many of these guidelines and methodologies are described in national and international frameworks, such as the European Water Framework Directive, or the OECD Guidelines for the Testing of Chemicals.

Scope and Objectives

A wide range of non-animal-based / effect-based monitoring (NAM/EBM) tools have been used for water assessment since several decades, significantly enhancing the detection of harmful chemical mixtures (e.g. Wernersson et al 2015; GWRC, 2023). Several of these bioassays have been validated in international studies (e.g. ISO 19040, ISO 24295, OECD TG455, OECD TG 458) Within the EU project SafeCREW, we focus on disinfection by-products (DBPs) formed when disinfectants such as chlorine, chloramines, chlorine dioxide, or ozone react with organic or inorganic matter. Commonly known DBPs in drinking water include chlorate, chlorite, bromate, trihalomethanes (THMs), and haloacetic acids. To assess the impact of these substances and their mixtures, we apply an extensive panel of human cell–based biological detection methods (e.g., CALUX®assays) to screen for key toxicity pathways, including cytotoxicity, genotoxicity, oxidative stress, endocrine activity, PAH-like effects, obesity-related mechanisms, and TTR–thyroid hormone displacement (PFAS-like toxicity). These analyses are conducted at drinking water treatment plants (DWTPs) in Milan, Italy, and Tarragona, Spain.

This set of guidelines is designed to

- Creating and applying a wide range of non-animal based toxicity methods for a toxic-free Green Deal strategy.
- Holistic assessment of chemical mixtures, not limited to known individual substances.
- Rapid, cost-effective, and animal-free testing methods.
- Identification of both regulated and unregulated contaminants, including unknowns.
- Validation of existing effect-based trigger values (EBTs).
- Improved competitiveness for EU SMEs and innovation ecosystems.

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Guideline for non-animal- and effect-based methods (NAM/EBM)

This guideline provides a procedure to evaluate a comprehensive panel of human cell-based biological detection methods (i.e. CALUX® assay) to assess the impact of disinfection by-products (DBPs), related chemicals and chemical mixtures on a range of key types of toxicity pathways (e.g. cytotoxicity, genotoxicity, oxidative stress, endocrine effects, PAH, obesity and competition for TTR-thyroid hormone binding/PFAS-like toxic activities) in all kinds of waters such as a) in contact with pipe materials, b) treated ground- and river water from different drinking water treatment facilities.

Such effect-based bioanalysis allows for detection of known/unregulated/unknown chemicals and to evaluate a safer and more sustainable monitoring of total toxicity of water samples (such as µg PFOA-BEQ/L water levels), including mixture effects.

Steps contain 1) Materials and Methods as well as 2) CALUX reporter gene assays bioanalysis:

1) Materials and methods for water samples collection and extraction

Drinking water effluent samples are collected during dry weather and under normal operating conditions. Samples for analyses of organic substances are stored and transported frozen (-200 C) for bioanalysis and upon arrival, the samples are processed immediately. For application of bioassays, 1000 ml water samples are loaded on a solid phase extraction (SPE) cartridge (OASIS HLB SPE; 500 mg, 6 cc, Waters 186000115), washed with 6 ml 5% methanol in water and finally eluted with 10 ml of methanol followed by 10 ml of acetonitrile. Both fractions are pooled and evaporated under a gentle stream of nitrogen. The final extracts are re-dissolved in 100 µl of DMSO after which serial dilution in DMSO are prepared.

2) CALUX reporter gene assays

CALUX reporter gene assays are performed as described earlier (e.g. Phan et al 2023; Alygizakis et al. 2023). In short, stably transfected human U2OS-cell lines for cytotoxicity, ERα-, TTR TR_{β}-, PXR- and Nrf2-CALUX bioassays are cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF, Gibco) supplemented with 7.5 % fetal calf serum (FCS) and 200 µg/ml G418. The stable transfected H4IIE-cell line for PAH CALUX is cultured in α-MEM medium supplemented with 10% FCS. Cells are seeded into 96-well plates in medium supplemented with hormone-stripped serum. The next day, the medium is replaced with medium containing serial dilutions of the water extract in DMSO (0.1% (ERα and PXR CALUX); 1.0% (cytotox and Nrf2); 0.8% (PAH). After 24 hours exposure, the medium is removed and the cells are lysed. The substrate luciferin is added to the wells to quantify the amount of luciferase produced by the cells by measuring the amount of light using a luminometer. For equivalents calculation, a dose-response curve of the reference compound is included in the analysis.

The reported reference compounds equivalents used for the different bioassays are: PFOA-, 17ß-estradiol, B[a]P, curcumin and nicardipine for the PFAS, ERα, PAH, Nrf2 and PXR CALUX, respectively. To test for possible cytotoxic effects of the samples analyzed, the cytotox CALUX activity is also determined. Cytotox CALUX cells constitutively express luciferase. In case cytotox CALUX cells are exposed to sample extracts causing cytotoxicity, a decrease in luminescence is observed.

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A reduction of 20% in luminescence is considered as a cytotoxic response. For TTR-T4 competition studies [TTR-TRβ (PFAS) CALUX], serial dilutions of sample extracts in DMSO are incubated with a fixed concentration of TTR and T4 overnight at 40º C (3.2% DMSO). Next, TTR-bound T4 was separated from free T4 using a bio gel column. The eluate containing the TTR-bound T4 is added to DMEM-medium (not containing FCS), added to seeded TR_{β} CALUX cells. After 24 hours on incubation, the amount of luciferase produced by the cells by measuring the amount of light using a luminometer. PFOA is used as reference compound. In Figure 2 the water sampling, extraction and measurement of a wide range effect-based methods (EBM) called CALUX bioassays are described.

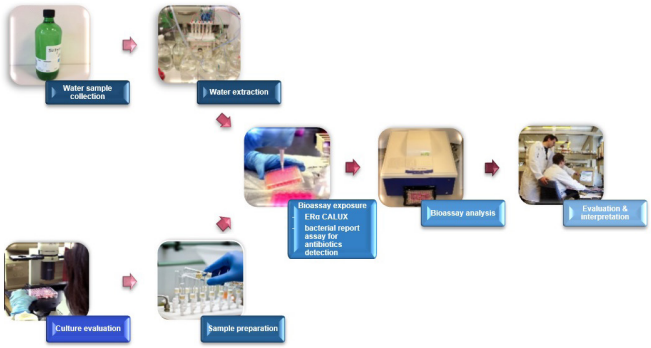

Figure 2 Water sampling, extraction and measurement of a wide range effect-based methods (EBM) called CALUX bioassays

3) Effect-based trigger values (EBT) for water

Several studies have evaluated and developed effect-based trigger values for the interpretation of the results obtained by a panel of EBMs (see in Table 1 an overview).

The EBTs (based on 80% percentile) obtained in SafeCREW are in the same magnitude as these earlier reported values and hereby prove the usefulness of such guidance values for water safety assessments.

Effect-based trigger values (EBTs)		Escher et al 2018	van der Oost et al 2017	Brand et al 2013	Besselink et al. 2017	SafeCrew 2026
ERa CALUX	(ng 17β-estradiol eq./l samp	0,1	0,5	3,8	1,6	0,088
anti-AR CALUX	(µg Flutamide eq./l sample)	14,4	25	---	10	
Nrf2 CALUX	(µg Curcumine eq./l sample)	21	10	---	93	58
PAH CALUX	(ng B[a]P eq./l sample)	6,2	150	---	320	59
PXR CALUX	(µg Nicardipine eq./l sample	54	3	---	66	12
TTR-TRb CALUX	(µg PFOA eq./l sample)	---	---	---	---	2,5

Figure 3 Comparison of a wide range of effect-based trigger values (EBT) obtained by CALUX bioassays

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Conclusion

Procedures following the above guidelines were tested on several relevant disinfection-by-products (DBPs) by the CALUX panel for cytotoxicity, genotoxicity, oxidative stress, endocrine effects, PAH and PFAS-like properties as well as obesity to obtain relative potency factors (RPFs).For this first round of analyses, model and real water samples of natural organic matter (NOM) proxies for ground and surface waters were selected to evaluate the response of the innovative biotests on different disinfection methods with their potential different disinfection-by-products.

Additional water treatment using innovative treatment technologies (oxidation, advanced oxidation) improved water quality significantly as determined by a panel of effect-based CALUX bioassays. Results from effect-based CALUX bioassays can be used to evaluate the efficiency of treatment technologies in removing bioactive substances.

Our applied effect-based trigger values (EBTs) for assessing the water quality and implementing effect-based bioassays in regulatory water frameworks for risk assessment are discussed (see Phan et al. 2023 and Alygizakis et al. 2023 for an overview from earlier assessments).

The action plan based on EBT for water treatment plant operators presented here enhances the applicability of effect-based bioassay for the assessment of water quality and regulatory acceptance.

Effect- and non-animal based (EBM/NAM) offer a safe and sustainable monitoring of known und yet unknown chemicals (such as PFAS and DBPs) in the water cycle of water treatment plants.

The guidelines proved useful and the results of the testing are described in Deliverable D1.3 Test protocol for effect-based in-vitro toxicity assessment of disinfection by-products. Also, more details about the test protocols are described in this deliverable.

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