Test protocol for effect-based *in vitro* toxicity assessment of disinfection by-products

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Abstract

For long time water disinfection is being done to inactivate or control bacteria populations in the treated water and simple chlorination is the most common method to disinfect the water. Almost certainly for over a decade chlorination is being effectively utilized for controlling water borne contamination. An assortment of disinfection by-products (DBPs) is formed like trihalomethanes (THMs), haloacetic acids (HAAs) and halogenated acetonitriles (HANs), when the naturally occurring natural organic matter (NOM) present in raw water reacts with chlorine. These DBPs may have adverse health impacts on humans. These compounds are not frequently tested due to missing regulations in many countries and there are epidemiological confirmations of close connection between its emergence and unfavourable results especially the malignant growths of cancer in fundamental organs in individuals. Due to concerns related with the prospective health hazards of DBPs, a few industrialized nations built up various guidelines (Mazhar et al., 2020).

Due to lack of toxicity data our research focuses on in vitro toxicity profiling of DBPs. This report describes the development of an integrated testing strategy to evaluate toxicological safety issues of DBPs. The strategy consists of a human CALUX® cell-based assay panel that can rapidly evaluate major types of toxicity relevant for regulatory risk assessment. (In)direct modulation of cell signalling responses by chemicals can easily be evaluated using reporter gene assays. The CALUX® reporter gene assays focus on molecular initiating and early key events that are the primary target of toxicants; each of the ~30 assays specifically responds to the modulation by the test compound of one nuclear receptor or cell signalling pathway. These processes are involved in cellular events that are key in cell growth and organismal development, and deregulation can cause developmental disorders and diseases like cancer. The CALUX activity profile therefore provides a mechanistic base for its mode-of-action, and it helps to provide input for decisions on further test requirements and risk assessment.

For the SafeCREW project, eight CALUX assays were selected based on known toxicity of DBPs. However, in order to be able to analyse the effects of DBPs, which are often volatile, several adaptations had to be made to the existing screening procedure. To create a point-of-departure, and to generate benchmark values, a list of reference compounds has been selected based on literature. These compounds were subsequently analysed on the CALUX panel. To evaluate the best bioanalytical conditions several extraction methods (liquid/liquid vs Oasis HLB SPE) and cell exposure conditions (96 well plates under sealed and non-sealed conditions) have been tested for several different water samples from two project partner locations.

In summary, both extraction methods (liquid/liquid vs Oasis HLB SPE) and cell exposure conditions (96 well plates under sealed and non-sealed conditions) have led to similar results in the different bioassays for several different water samples from two project partner locations. Therefore it is decided to use the easier, higher capacity and lower-costs approach by using Oasis HLB SPE extraction and 96 well plates under non-sealed conditions for all samples to analyse in the case studies.

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Abbreviations

AhR	Aryl hydrocarbon receptor
AOP	Adverse Outcome Pathway
BAM	Bromoacetamide
CAA	Chloroacetic acid
DB-ALM	Database on alternative methods, produced by the European Union Reference Laboratory on Alternatives to Animal Testing (EURL ECVAM)
DMSO	Dimethyl sulfoxide
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
E2	17β-Estradiol
FCS	Fetal calf serum
HLB SPE	Hydrophilic-lipophilic balanced copolymer solid phase extraction
HMs	Halomethanes
HANs	Halogenated acetonitriles
IAM	Iodoacetamide
LEC	Lowest effect concentration
Nrf2	Nuclear factor erythroid 2–related factor 2
OECD	Organisation for Economic Co-operation and Development
PFOA	Perfluorooctanoic acid
PXR	Pregnane X receptor
SOP	Standard Operation Procedure
TBT	Tributyltin
TCDD	2,3,7,8-tetra-dibenzo-dioxine
THM	Trihalomethanes
TR	Thyroid hormone receptor
TTR	Thyroid transport protein transthyretin

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Introduction

An integrated testing strategy to evaluate toxicological safety issues of disinfection by products (DBPs) and their complex mixtures in water samples is needed for monitoring of an European strategy for toxic-free and zero pollution. Here we apply for the first time a strategy consisting of a human CALUX® cell-based assay panel that can rapidly evaluate major types of toxicity relevant for regulatory risk assessment.

One of the aims of the SafeCREW project is to develop and apply in vitro toxicity profiling for DBPs in various water samples. Optimised protocols for various relevant general toxicological endpoints (cytotoxicity, genotoxicity p53 DNA repair, oxidative stress Nrf2, early warning activities PXR, PAH-like activities via Ah receptor ligand binding), as well as specific endocrine disrupting activities (estrogenicity ER, inhibition androgenicity anti-AR, thyroid hormone transport inhibition TTR) were first applied on several DBPs.

Based on the first results, a set of CALUX bioassays was then selected for monitoring DBPs in disinfected water to apply in the first two partner locations. The obtained in vitro toxicity results of the selected CALUX panel in the water have been then evaluated by comparison to some published effect-based trigger values (see in Table 1; NORMAN, 2019; Alygizakis et al, 2023; Been et al., 2021; Escher et al., 2018; Oost van der et al 2017; Phan et al., 2021; Behnisch et al 2021, 2023):

Table 1 – Overview about published effect-based trigger values (EBTs) for the eight selected CALUX assays. [na means not available].

EBT range	Cytotox [µg TBT-eq/L]	p53 [µg ACT-eq/L]	Nrf2 [µg Cur-eq/L]	PXR [µg Nic-eq/L]	ERα [ng E2-eq/L]	anti-AR [ng Flut-eq/L]	PAH [ng BaP-eq/L]	TTR TR [µg PFOA-eq/L]
EBT range			**10 to 21**	**3 to 54**	**0.008-0.28**	**14 to 270**	**6.2 to 150**	**0.56 to 22**
NORMAN 2021: surface water	na	na	na	na	0.10 to 0.28	14	6.2	na
Alygizakis et al, 2023: surface water	na	na	10	3	0.10	14	6.2	na
Escher at al. 2018: surface water	na	na	21	54	0.10	14	6.2	na
Oost van der et al, 2017: Passive sampling of surface water	na	na	10	3	0.50	25	150	na
Phan et al 2021: wastewater	na	na	10	3	0.10	14	6.2	na
Been et al 2021: Drinking water	na	na	na	na	0.008	270	19	na
Behnisch et al 2021: drinking water	na	na	na	na	na	na	na	2.9 to 4.6
Behnisch et al 2023: surface water (P5% to P80%)	na	na	na	na	na	na	na	0.56 to 4.3
Behnisch et al 2023: waste water (P5% to P80%)	na	na	na	na	na	na	na	0.71 to 22

The CALUX reporter gene assay panel

Signal transduction controls cellular programs in development and responses to chemical stressors. (In)direct modulation of cell signalling responses by chemicals can easily be evaluated using reporter gene assays. The CALUX reporter gene assay panel, where each of the tests specifically measures activation of one key event, is ideally suited for this task.

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The CALUX® battery of tests contains a range of specific assays that can be used in assessing chemical safety. It measures chemical interactions with key adverse outcome pathways (AOPs, (Leist et al. 2017)). Because the CALUX assay results are supported by a mechanistic base, and because several assays have already undergone ECVAM/OECD validation, the chance of regulatory acceptance is greatly increased. Secondly, this mechanistic base aims to provide input for decisions on further test requirements and risk assessment (Figure 1).

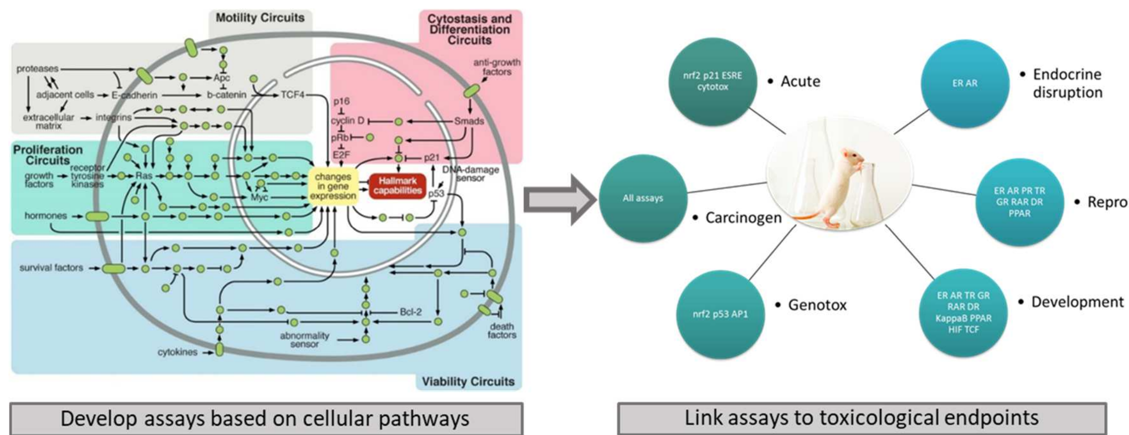

Figure 1 – The general principle of risk assessment using the CALUX assay panel

Approach

This panel is based on the human U2-OS cell line, which is modified to express reporter genes for separate pathways (van der Burg et al. 2013). The high selectivity is achieved through the use of multimerized, highly selective response elements driving the expression of the reporter gene luciferase, which provides a straightforward readout (Figure 2). The panel of selective assays was designed to create very low levels of false-positives. The panel contains a single reporter gene assay per pathway, and appropriate controls for non-specific effects.

In general, CALUX assays focus on molecular initiating and early key events that are the primary target of toxicants (Becker et al. 2015; van der Burg et al. 2013). These events are involved in cellular events key in cell growth and organismal development, and therefore deregulation can cause developmental disorders and diseases, such as cancer (Hanahan and Weinberg 2011).

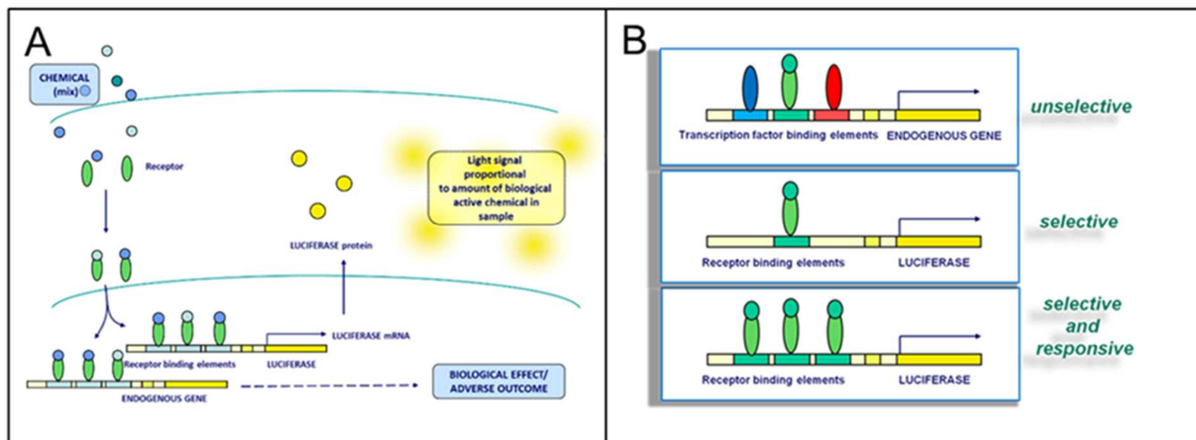

Figure 2 – A. the CALUX assay principle. B. the CALUX reporter gene assays are highly selective and responsive due to multimerization of specific responsive elements.

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Assay selection

To select a panel of relevant CALUX assays for the SafeCREW project, a literature review was performed on known DBPs. DBPs are most widely feared for their cytotoxic, genotoxic, mutagenic, and tumorigenic effects (Kim et al., 2020; Stalter et al., 2020). Therefore, the Cytotoxicity CALUX and the p53 GENTOX CALUX (DNA damage) were included in the SafeCREW panel (van der Burg et al. 2013; van der Linden et al. 2014). Since genotoxicity is often mediated through oxidative stress, the Nrf2 CALUX was also included (van der Linden et al. 2014).

Several DBPs have been shown to exhibit effects on the endocrine system, in particular via interaction with the estrogen- and androgen hormone receptors (Kim et al., 2020). The CALUX panel has a particularly strong coverage of assays for nuclear hormone receptors relevant for endocrine disruption and reproductive toxicity; this includes assays to measure interaction with the estrogen and androgen receptors (Era and AR assays) (Lewin et al. 2015; Piersma et al. 2013; Sonneveld et al. 2005; Sonneveld et al. 2011; Sonneveld et al. 2006; van der Burg, Pieterse, et al. 2015; van der Burg et al. 2013).

In addition to interaction with the endocrine and androgen axis of the hormonal system, interaction with the thyroid axis has also been shown, in the form of human transthyretin (TTR) binding by polar phenolic DBPs (Yang et al., 2019). Therefore, the TTR-TRβ CALUX was added to the SafeCREW panel as well (Collet et al. 2020).

In addition, two assays were included that serve as xenobiotics sensors, and are as such an early warning system of the cell: the pregnane X receptor (PXR) CALUX and the aryl hydrocarbon receptor (AhR) CALUX.

With this concise panel of CALUX assays we can identify the most relevant toxicological endpoints for DBPs (Table 2).

Table 2 – the eight selected CALUX assays, the associated toxicological endpoints, and examples of DBPs able to activate these endpoints according to literature studies. TBT tributyl-tin; TCDD 2,3,7,8-Tetra-dibenzo-dioxine; E2 17β-estradiol; IAM iodoacetamide; BAM Bromo acetamide; THM trihalomethanes; CAA chloroacetic acid

CALUX assay	Endpoint	Reference	DBP
Cytotox CALUX	Cytotoxicity	TBT	IAM, BAM
p53 GENTOX CALUX	Genotoxicity, DNA damage	Actinomycin D	Halogenated
Nrf2 CALUX	Oxidative stress	Dichlorvos	THM
PXR CALUX	Early warning, xenobiotic sensor	Nicardipine	
AhR CALUX	Ah receptor ligands	TCDD	
ERα CALUX	Estrogen agonism	E2	CAA, BAM
AR CALUX	Androgen antagonism	Flutamide	CAA, BAM
TTR-TRβ CALUX	TTR binding inhibition	PFOA	Phenolics

All CALUX assays have been automated in a high throughput format. Data storage and analysis has been set up and more than 600 compounds have been screened on the CALUX panel. Finally, although the CALUX panel endogenously has little metabolic activity, it can be performed in the presence of a fraction of metabolic enzymes (rat or human liver S9), allowing assessment of involvement of metabolism in chemical effects (van der Linden et al. 2014; van Vugt-Lussenburg et al. 2018).

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Validation and performance

Already in 2005, we developed a panel of mechanism-based CALUX assays to assess hormonal activity of compounds (Sonneveld et al. 2005), a panel which has shown to be highly predictive for such activities in experimental animals (Sonneveld et al. 2011; Sonneveld et al. 2006). Several of these assays have now been successfully engaged in extensive validation exercises as alternatives to animal experiments via EURL-ECVAM, OECD, and others (OECD 2012, 2016; van der Burg, Winter, Man, et al. 2010; van der Burg, Winter, Weimer, et al. 2010). Further formal validations have been initiated in the area of thyroid disruption, metabolic activation of endocrine assays, and skin sensitization, while SOPs, including one on the assay panel automation, have been submitted to the EURL-ECVAM database of alternative methods DB-ALM (DB-ALM 197).

Interestingly, these mechanistic assays also performed very well as part of a battery of tests and stand-alone for developmental and reproductive toxicity used in the EU Framework program (FP) ReProTect and ChemScreen projects (Schenk et al. 2010; van der Burg et al. 2013). This was surprising because of the complexity of the process of mammalian reproduction. This led to the belief that only a very complex battery of tests would suffice to give reliable predictions in this area, while animal experiments would remain indispensable. Nevertheless, results from the ReProTect project very clearly showed that an in vitro test battery covering only part of the reproductive cycle processes can provide very promising results with respect to reproductive toxicity testing.

Another area where the CALUX assay panel has been used successfully is for early toxicity testing for safe design. Using comparative analyses, it is possible to rank (groups of) compounds by activity or potency, rather than to give a definite measure or prediction of toxicity. Trends in activity with increasing chain length, or the effect of the introduction or removal of a specific functional group can be identified. It is also possible to compare candidates with their parent platform molecules and/or the substances they aim to replace to determine if they have a more favorable toxicity profile. Examples are green solvents in BBI EU project ReSolve, and furan-based plasticizers (Byrne et al. 2020; Sherwood et al. 2020; van Vugt-Lussenburg et al. 2020; Liu et al. 2022).

Existing methods: adaptation for purpose

For the SafeCREW project, the CALUX reporter gene assay screening was performed using an automated protocol (DB-ALM 197). According to this protocol, CALUX cells are seeded in 384 wells plates, and exposed to a 14-point dilution curve of the test compound (typically 1E-10M to 1E-3M in 0.5 Log increments) by a Hamilton STARlet liquid handling robot. After 24h exposure, the cells are analysed using a luminometer coupled to a robotic stacker.

Due to the nature of the compounds to be analysed in the SafeCREW project, however, several adaptations to the published protocol had to be made.

Compound volatility

Several of the DBPs are known or expected to be volatile. In previous projects, BDS has successfully analysed volatile compounds by applying a breathing plate seal that is compatible with the cell culture (Tolosa et al., 2022). For the SafeCREW project, all samples are analysed in combination with such a breathing plate seal.

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Creating a point of departure: reference compounds

BDS' role in the SafeCREW project is to analyse (water) samples containing DBPs. To ensure that these compounds are effectively detected by the selected CALUX panel (Table 2), and to get an idea of the detection limit of these compounds, a selection of reference DBPs was first analysed.

Compound selection

Sixteen reference DBPs were selected from literature (Kim et al., 2020; Stalter et al., 2020; Yang et al., 2019) (Table 3). Most of the selected compounds are under regulation (highlighted in green).

Table 3 – the 16 selected reference DBPs that were analyzed on the CALUX panel to create a point-of-departure. DBPs highlighted in green are under regulation.

HMs	HANs	HAAs
chloroform	dichloroacetonitrile	chloroacetic acid
bromodichloromethane		bromoacetic acid
chlorodibromomethane		dichloroacetic acid
bromoform		dibromoacetic acid
iodoform		trichloroacetic acid

Nitrosamines	Oxyhalides	Phenolics
n-nitrosodiethylamine	potassium bromate	2,4,6-trichlorophenol
	sodium chlorite	2,4,6-tribromophenol

Analysis of reference compounds for DBPs

The sixteen DBPs from Table 3 were analysed on the CALUX panel. Results are presented in Table 4 as lowest effect concentrations (LECs) in LOG(M). The table shows that almost all compounds are able to activate at least one CALUX assay: only four of the 16 compounds are not 'picked up' by any of the assays. This suggests that the selection of assays is appropriate for the detection of these compounds.

The assays most frequently activated are related to acute and general toxicity, like cytotoxicity, oxidative stress (Nrf2 CALUX), or DNA damage (p53 GENTOX CALUX). Active compounds in these bioassays are typically tributyl-tin (cytotoxic), bromoacetic acid (oxidative stress) or N-DBPs with aminooxy- or cyano- structure (in vitro genotoxicity; see Liu et al., 2021).

Also frequently activated is the TTR-TRβ CALUX, which measures interference with TTR binding and is related to thyroid hormone disruption. Active compounds are typically many different PFAS (Behnisch et al. 2021), chlorinated paraffins (Sprengel et al.) or 4-nonylphenol (Collet et al., 2020).

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Table 4 – CALUX results for sixteen reference DBPs. Results are lowest effect concentrations in LOG(M). Grey cell: not active up to highest concentration tested.

compound	Cytotox20%	ERa	AR-anti	PXR	AhR	Nrf2	p53 GENTOX	TTR-TRb
Assay-specific reference	-6.6	-12.2	-7.7	-7.1	-12.3	-5.4	-9.0	-9.0
chloroform	>-3	>-4	>-4	>-4	>-3	>-3	>-3	>-2.5
chlorodibromomethane	>-3	>-4	>-4	>-4	>-3	>-3	>-3	>-2.5
bromoform	>-3	-4.2	>-4	>-4	>-3	-3.4	>-3	-2.5
iodoform	-3.4	>-4	>-4	>-4	>-3	>-3	>-3	-4.0
dichloroacetonitrile	-3.5	>-4	>-4	>-4	>-3	-3.5	-3.6	>-2.5
chloroacetic acid	>-3	>-4	>-4	>-4	>-3	-4.0	-3.0	>-2.5
bromoacetic acid	-5.0	>-4	>-4	-5.5	>-3	-7.0	-5.6	>-2.5
dichloroacetic acid	>-3	>-4	>-4	>-4	>-3	>-3	>-3	>-2.5
dibromoacetic acid	>-3	>-4	>-4	>-4	-4.0	-4.0	-3.0	>-2.5
trichloroacetic acid	>-3	>-4	>-4	>-4	>-3	-3.1	>-3	-2.5
bromodichloromethane	>-3	>-4	>-4	>-4	>-3	>-3	>-3	-2.5
n-nitrosodiethylamine	>-3	>-4	>-4	>-4	>-3	>-3	>-3	>-2.5
Sodium chlorite	>-3	>-4	>-4	>-4	>-3	-3.1	>-3	>-2.5
2,4,6-trichlorophenol	-4.1	>-4	>-4	-4.2	>-3	>-3	-3.5	>-2.5
2,4,6-tribromophenol	-4.6	>-4	>-4	-4.2	-3.0	>-3	-4.0	-2.5
potassium bromate	>-3	>-4	>-4	>-4	>-3	-3.5	-3.3	-2.9

Extraction

Since some DBPs are known or expected to be volatile, special attention was paid to testing and evaluation of extraction procedures of DBPs from water samples. The testing and evaluation of extraction procedures were performed using water samples obtained from two project partner locations.

Two extraction procedures were tested and compared:

1. Liquid/liquid extraction using ethyl acetate as solvent (3x 200 mL, 3x 30 minutes shaking)

2. Solid-phase-extraction using Oasis HLB SPE cartridges

For both extraction methods approximately 1 Liter of water sample was used. For quality control purposes, a positive control sample (enriched ELGA water (150 µl BRM44(30); 150 µl BRM31; 150 µl PFOA) and a procedure blank (1L distilled water) have been added to each of the shipments as additional external samples.

Following extraction and evaporation to dryness, the final sample extracts were reconstituted in 150 µL DMSO and gently vortexed to make a homogenous solution. The homogenous solutions were then diluted with DMSO into a 1x, 3x, 10x, 30x and 100x dilution (concentration factors ranging from a) in stock: 67 to 6700 and b) in exposure medium: for example for cytotox CALUX (in 1% DMSO): 0.67 to 67).

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CALUX® bioassays

All extracts were tested on a panel of human cell based CALUX® bioassays as indicated in Table 3. For determination of the CALUX® activities, CALUX® cells were seeded in 96 wells plates in assay medium. To test the effectiveness of using breathing plate seals (see above), all sample extracts tested for bioactivity in the presence and absence of the breathing seals.

Prior to evaluation of the bioactivity of the sample extracts on the various bioassays, they were tested for cytotoxicity using the U2OS based CALUX® cytotox bioassay, by exposing cytotox CALUX® cells to the prepared serial sample dilutions (1% DMSO). The cytotox CALUX® cells constitutively express luciferase. Exposure of the cytotox CALUX® cells to compound causing cytotoxicity will result in a reduction of luminescence. Concentrations of final extract causing 20% reduction of luminescence are considered cytotoxic.

Following exposure of the CALUX® cells to serial sample dilutions in triplicate, the induction of luciferase production is quantified by measuring luminescence (Berthold luminometer) following addition of the substrate luciferin. On each 96-well plate, a complete calibration curve for each respective bioassay is also analysed using the relevant reference compounds.

For testing of antagonistic activity, the respective CALUX® cells were incubated in the presence of a fixed concentration of reference compound (PC_{80} concentration) after which the cells were exposed to sample dilutions as described above.

For testing the potency of sample extracts to interfere with thyroid-hormone binding to the blood-transport protein transthyretin (TTR), serial dilutions of sample extracts were incubated in a Tris-buffer (pH 8.0) overnight at 4°C in the presence of TTR (0.058 µM) and a fixed concentration of T_4 (0.052 µM) (3.2% sample dilution in incubation mixture). After incubation, TTR-bound and free T_4 were separated on a Bio-Gel P-6DG column. The eluate was added to assay medium after which $TR_β$ CALUX® cells were exposed for 24 hours.

In Table 5 the exposure conditions for the various bioassays are given.

Table 5: *BDS CALUX® cell culture and exposure information*

Assay	(anti-)ERα, (anti-)AR, (anti-)PR, TTR-TRβ, PXR	Cytotox, Nrf2, P53(-S9),	(anti-)DR
Cell type	U2OS	U2OS	H4IIE
Species	Human	Human	Rat
%DMSO	0.1%	1%	0.8%
Fold dilution	1000	100	125
%CO2	5%	5%	5%
Exposure time	24 hrs	24 hrs	24 hrs
Confluence	10000 cells per well	10000 cells per well	10000 cells per well
Medium used	DMEM/F12	DMEM/F12	αMEM
Additions to medium	-Stripped FCS -Non essential amino acids	-Stripped FCS -Non essential amino acids	-FCS -Non essential amino acids

Note: for (anti-)TRβ CALUX and TTR- TRβ CALUX bioassays, the exposure medium does not contain FCS.

Data analysis

Analysis results of extracts of the water samples, expressed as induction relative to the standard reference compound, are interpolated in the calibration curves of each respective bioassay for quantitative determination of disruptive potential using the statistical software package GraphPad Prism V5.03. Only dilutions that do not show any signs of cytotoxicity (relative induction in the cytotox

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CALUX® bioassay > 80%) are used for final evaluation of analysis results. All analysis results are expressed as amount of reference compound equivalents per g processed sample.

Results summary:

The results of the different CALUX analyses of water samples obtained from two partner locations are presented in Tables 6 - 8. Two different sample processing methods were tested. The first sample preparation method is a solid-phase-extraction method using HLB SPE cartridges (from OASIS). The second sample preparation method that has been tested is a liquid/liquid extraction using ethyl acetate as solvent. There were also two different exposure methods tested for exposing the cells (with and without a breathing seal covering the plate). Samples from the first partner location were used to evaluate the effect of the extraction procedure and the exposure conditions whereas samples from the other location were only used for evaluation of using breathing seals (or not) after SPE-extraction.

1. **Location study 1 – with two different extraction methods: HLB-SPE (1st result) vs Liquid/Liquid with ethyl acetate (2nd result)**

In **Table 6,** the effect of using either a SPE extraction method or the liquid/liquid extraction method with ethyl acetate is presented (first partner location samples). Similar results have been obtained for all CALUX bioassays for both extraction methods (sealed vs non-sealed). Water samples from first partner location showed no activities in the cytotoxicity, genotoxicity/DNA repair (p53), estrogen (ER)- and anti-androgenic (anti-AR)-like activities. Only in case of oxidative stress (Nrf2), early warning (PXR) and thyroid hormone transport competition (TTR TR) slightly elevated levels above the published effect-based trigger values have been analysed here.

Table 6: *In vitro toxicity profiling by various CALUX® for cytotoxicity, genotoxicity/DNA repair (p53), oxidative stress (Nrf2), early warning (PXR), estrogen (ER)-, anti-androgenic (anti-AR) and TTR TR-like activities for water samples from the first partner location treated by HLB-SPE (1st result) and liquid/liquid (2nd result)*

Sample	Cytotox [µg TBT-eq/L][1,2]	p53 [µg ACT-eq/L][1,2]	Nrf2 [µg Cur-eq/L][1,2]	PXR [µg Nic-eq/L][1,2]	ERα [ng E2-eq/L][1,2]	anti-AR [ng Flut-eq/L][1,2]	TTR TR [µg PFOA-eq/L][3]
EBT range [Literature 1, 2, 3)			10 to 21	3 to 54	0.10-0.28	14 to 25	0.56 to 22
WW1 input river water	LOQ vs LOQ	LOQ vs LOQ	69 vs LOQ (35)	9.3 vs 11	LOQ vs LOQ	20 vs 11	4.4 vs 3.2
WW1 after chlorination	LOQ vs LOQ	LOQ vs LOQ	54 vs 64	5.5 vs 5.3	LOQ vs LOQ	LOQ vs LOQ	4.9 vs 1.4
WW2 influent	LOQ vs LOQ	LOQ vs LOQ	93 vs 110	6.8 vs 4.7	LOQ vs LOQ	LOQ vs LOQ	3.4 vs 3.1
Blank	LOQ vs LOQ	LOQ vs LOQ	LOQ (35) vs 93	LOQ vs LOQ	LOQ vs LOQ	LOQ vs LOQ	LOQ vs LOQ

2. **Location study 1- with two different exposure procedure: non-sealed (1st result) vs sealed (2nd result) bioanalysis results of a panel of CALUX bioassays**

In **Table 7** (first partner location samples) and **Table 8** (second partner location samples), the effect on bioactivity of exposing cells in the presence or absence of breathing seals is presented.

Table 7: *In vitro toxicity profiling by various CALUX® for cytotoxicity, genotoxicity/DNA repair (p53), oxidative stress (Nrf2), early warning (PXR), estrogen (ER)-, anti-androgenic (anti-AR) and TTR TR-like activities for water samples from the first partner location treated by non-sealed (1st result) and sealed (2nd result) exposure methods*

Sample	Cytotox [µg TBT-eq/L][1,2]	p53 [µg ACT-eq/L][1,2]	Nrf2 [µg Cur-eq/L][1,2]	PXR [µg Nic-eq/L][1,2]	Erα [ng E2-eq/L][1,2]	anti-AR [ng Flut-eq/L][1,2]	TTR TR [µg PFOA-eq/L][3]
EBT range [Literature 1, 2, 3)			10 to 21	3 to 54	0.10-0.28	14 to 25	0.56 to 22
WW1 input river water	LOQ vs LOQ	LOQ vs LOQ	69 vs 38	9.3 vs LOQ (2)	LOQ vs LOQ	20 vs LOQ (8)	4.4 vs 4.2
WW1 after chlorination	LOQ vs LOQ	LOQ vs LOQ	54 vs 35	5.5 vs LOQ (2)	LOQ vs LOQ	LOQ vs LOQ	4.9 vs 7.7
WW2 influent	LOQ vs LOQ	LOQ vs LOQ	93 vs 61	6.8 vs 2.5	LOQ vs LOQ	LOQ vs LOQ	3.4 vs 4.6
Blank	LOQ vs LOQ	LOQ vs LOQ	LOQ (35) vs 93	LOQ vs LOQ	LOQ vs LOQ	LOQ vs LOQ	LOQ vs LOQ

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3. Location study 2 – with two different exposure procedure: non-sealed (1st result) vs sealed (2nd result)

Table 8: *In vitro toxicity profiling by various CALUX® for cytotoxicity, genotoxicity/DNA repair (p53), oxidative stress (Nrf2), early warning (PXR), estrogen (ER)-, anti-androgenic (anti-AR) and TTR TR-like activities for water samples from the 2nd partner location treated by non-sealed (1st result) and sealed (2nd result) exposure procedures*

Sample	Cytotox [µg TBT-eq/L][1,2]	p53 [µg ACT-eq/L][1,2]	Nrf2 [µg Cur-eq/L][1,2]	PXR [µg Nic-eq/L][1,2]	Erα [ng E2-eq/L][1,2]	anti-AR [ng Flut-eq/L][1,2]	TTR TR [µg PFOA-eq/L][3]
EBT range [Literature 1, 2, 3)			**10 to 21**	**3 to 54**	**0.10-0.28**	**14 to 25**	**0.56 to 22**
SRNOM	LOQ vs LOQ	LOQ vs LOQ	37 vs LOQ (31)	3.1 vs LOQ (1.2)	LOQ vs LOQ	LOQ vs LOQ	13 vs 4.0
HOHLO	LOQ vs LOQ	LOQ vs LOQ	52 vs 61	4.3 vs 1.8	LOQ vs LOQ	LOQ vs LOQ	13 vs 2.9
SRNHO	LOQ vs LOQ	LOQ vs LOQ	81 vs 95	4.0 vs 4.4	LOQ vs LOQ	LOQ vs LOQ	13 vs 7.9
WW1	LOQ vs LOQ	LOQ vs LOQ	120 vs 160	18 vs 12	LOQ vs LOQ	LOQ vs LOQ	13 vs 3.6
Blank	LOQ vs LOQ	LOQ vs LOQ	LOQ vs LOQ	LOQ vs LOQ	LOQ vs LOQ	LOQ vs LOQ	1.2 vs LOQ (1.1)

There was no significant difference for the sealed and the non-sealed assay exposure procedures for any of the samples.

Water samples for both partner locations showed no activities in the cytotoxicity, genotoxicity/DNA repair (p53), estrogen (ER)- and anti-androgenic (anti-AR) activities. Only in case of oxidative stress (Nrf2), early warning (PXR) relevant and thyroid hormone transport competition (TTR TR) slightly elevated levels above the published effect-based trigger values have been measured here.

Similar bioassay results were found with both extraction procedures and both exposing conditions. Therefore, we propose to use for all future analysis the extraction method by HLB-SPE and the exposure method for volatile compounds with sealed 96 well plates.

It should be noted that the fact that there was no difference between the different extraction and exposure steps does not necessarily imply that all volatiles have been exposed to the human cells. It could also be due that volatiles were lost by some of the procedures (such as evaporation of solvent or binding to materials in the analysis procedure) to a similar extent. Therefore, further experiments are planned with a) spiked volatile compounds (in cooperation with UFZ) and b) with different kinds of levels of DBPs in water samples (in cooperation with EURECAT).

Conclusions

In the first part of the toxicity testing, sixteen DBPs were analysed on the CALUX panel. Almost all compounds were able to activate at least one CALUX assay: only four of the 16 compounds are not 'picked up' by any of the assays. This suggests that the selection of assays is appropriate for the detection of these compounds.

The assays most frequently activated are related to acute and general toxicity, like cytotoxicity, oxidative stress (Nrf2 CALUX), or DNA damage (p53 GENTOX CALUX). Also frequently activated is the TTR-TRβ CALUX, which measures interference with TTR binding and is related to thyroid hormone disruption.

In the second part of the toxicity testing, several samples from two partner locations (site 1 and 2) have been extracted by two different methods and by two different cell exposure methods. Similar results have been obtained for all CALUX bioassays for all four different extraction and cell exposure methods here validated.

As a result of these first water testing of these two partner locations, we can conclude that water samples from both case study showed no activities in the cytotoxicity, genotoxicity/DNA repair (p53), estrogen (ER)- and anti-androgenic (anti-AR)-like activities.

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Only in case of oxidative stress (Nrf2), early warning (PXR) and thyroid hormone transport competition (TTR TR) slightly elevated levels above the published effect-based trigger values have been measured in both partner locations.

In case of the thyroid hormone transport competition (TTR TR), so far, from the here tested DBPs only bromoform did show a low activity in this in vitro bioassay. Therefore, other active compounds such as PFAS (Behnisch et al. 2021), chlorinated paraffins (Sprengel et al. 2021) or 4-nonylphenol (Collet et al., 2020) are expected to be here with the current knowledge more relevant.

Comparing the four different analysis results from both partner locations indicates that similar results were obtained. Therefore, we propose to use for all future analysis the SPE extraction method with HLB sorbent and the exposure method for volatile compounds with sealed 96 well plates.

Further water sample bioanalysis of the different water treatments in the different partner locations is now planned in several other Tasks (T1.6; T2.6; T3.1 and T3.2) to find out if the here tested in vitro toxicity profiling with its related effect-based trigger values can be an added value for toxic-free and zero pollution water.

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