Application of effect-based methods (EBM) to water quality monitoring: Answering frequently asked questions by water quality managers, regulators and policy makers

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Abstract

Effect-based methods (EBM) have great potential for water quality monitoring as they can detect the mixture effects of all active known and unknown chemicals in a sample, which cannot be addressed by chemical analysis alone. To date, EBM have primarily been applied in a research context, with a lower uptake by the water sector and regulators. This is partly due to concerns regarding the reliability and interpretation of EBM. Using evidence from the peer-reviewed literature, this article aims to answer frequently asked questions about EBM. The questions were identified through consultation with the water industry and regulators and cover topics related to the basis for using EBM, practical considerations regarding reliability, sampling for EBM and quality control and what to do with the information provided by EBM. The information provided in this article aims to give confidence to regulators and the water sector to stimulate the application of EBM for water quality monitoring.

Keywords: chemical water quality; effect-based trigger values; *in vitro* bioassays; mixtures; well plate-based *in vivo* assays

Synopsis: Addressing commonly asked questions about effect-based methods (EBM) aims to help facilitate the uptake and application of EBM for routine water quality monitoring

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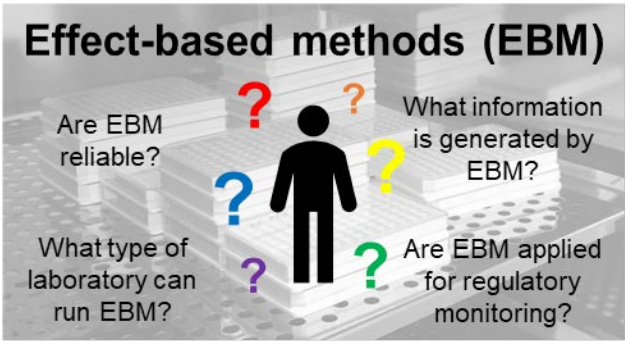

1. Introduction

With an estimated 350,000 chemicals and mixtures registered for commercial production and use,[1] it is no wonder that water bodies globally contain a varied and extensive mixture of chemical contaminants used as or included in pesticides, pharmaceuticals and personal care products, flame retardants, surfactants, industrial chemicals and their many environmental and disinfection transformation products.[2] Targeted chemical analysis of priority substances is typically used for water quality monitoring; however, only a small fraction of chemicals potentially present in the water will be detected by targeted analysis. Further, some chemicals may be present below the analytical limit of detection but may still contribute to a biological effect resulting from exposure to complex low-level mixtures of chemicals via different exposure routes.[3]

To overcome the limitations of applying only targeted chemical analyses, effect-based methods (EBM) using high-throughput *in vitro* bioassays (primarily mammalian cell models) and well plate-based *in vivo* assays (small organisms) are now recommended for water quality assessment.[4] EBM, also referred to as bioanalytical tools or effect-based monitoring, can be used complementary to chemical analysis as they can detect all chemicals in a sample that are active in the applied bioassay, including known and unknown chemicals. Water samples can contain many chemicals, often present at low concentrations, but they can act together to elicit significant mixture effects, even when individual chemicals are present at or below no observable effect concentrations.[5, 6] EBM can account for the mixture effects of the many chemicals potentially present in a water sample. Further, EBM are risk-scaled, with more potent chemicals eliciting a greater response than less potent chemicals when present at similar concentrations. Key terms and definitions related to EBM in the context of water quality monitoring are provided in Table 1.

EBM have been applied to a range of water types, including drinking water, surface water, recycled water, wastewater and stormwater.[7-10] The application of EBM for water quality monitoring is

described in detail in Escher et al.[11] *In vitro* bioassays used for water quality monitoring often apply mammalian cell lines and are run in 96-well or 384-well plate format, with bioassays indicative of different stages of cellular toxicity pathways, including induction of xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and cytotoxicity, available. Certain *in vivo* assays, such as the zebrafish embryo toxicity test or the algal toxicity test can be run in 24- and 96-well plate format. They are typically indicative of apical effects, observable outcomes such as growth, immobilization, and mortality in whole organisms, though behavioural and morphological endpoints can also be observed in fish embryos, as well as specific effects such as photosynthesis inhibition in algae.

To facilitate the uptake of EBM by regulators and the water industry, this perspective article aims to address common questions about EBM using evidence from the peer-reviewed literature. The questions, identified through consultation with industry, regulators and academic colleagues, cover the basis for using EBM, practical considerations regarding reliability and sampling, quality control considerations and what to do with the information provided by EBM.

90 **Table 1**: Key terms and definitions related to effect-based methods in the context of water quality
91 monitoring

Key term	**Definition**
Activity	Response induced by a chemical, group of chemicals, water sample or extract in a bioassay
Adverse outcome pathway	Schematic description of biological interactions and toxicity mechanisms at different levels of biological complexity (molecular initiating events and key events) that describe how exposure to a substance might cause illness or dysfunction (adverse outcomes). Adverse outcome pathways encompass cellular toxicity pathways
Bioassay	A bioanalytical method used to determine the concentration or potency of chemicals, chemical mixtures and water samples on whole organisms (*in vivo)* or cell lines, isolated tissue or enzyme extracts (*in vitro*)
Bioanalytical equivalent concentration (BEQ)	The concentration of a reference compound that elicits the same effect as the chemical mixture in a sample
Cellular toxicity pathway	A cellular response pathway that contributes to an adverse health effect when sufficiently perturbed
Concentration-response curve (CRC)	A plot of the response of a chemical, group of chemicals or water sample observed in a bioassay, commonly expressed as a percentage of the maximal response, against increasing exposure concentration. Also referred to as a concentration-effect curve
Effect-based methods (EBM)	High-throughput *in vitro* bioassays and well plate-based *in vivo* assays used to detect the effect of active chemicals in a sample.

Effect-based trigger value (EBT)	Acceptable effect level in a particular water type. EBTs reflect maximum allowed levels, derived in the context of human health risks or environmental health risks. Also referred to as monitoring trigger level
Effect concentration (EC_y)	The concentration of a chemical, group of chemicals or water sample causing a certain percent effect (*e.g.*, EC_{50} is the concentration causing 50% of the maximum effect). The abbreviation EC_x is also often used in the literature
Effect unit (EU)	The inverse of the effect concentration of the water sample (*e.g.*, $1/EC_y$)
Endpoint	An observed or measured biological event that serves as an indicator of an effect or toxicity
In vitro assay	Tests conducted with cell lines, tissues or enzymes
In vivo assay	Tests performed with whole organisms
Mode of (toxic) action	A shared set of physiological and behavioural indicators that describe a type of biological response
Relative enrichment factor	A measure of sample concentration that accounts for enrichment during sample extraction and sample dilution in the bioassay
Relative potency	The potency of a chemical in comparison to the potency of a reference chemical (often the most potent chemical in a chemical group)
Response	Any kind of biological response induced by exposure to chemicals including toxicity (*e.g.*, lethality, inhibition of cell viability) and effects (*e.g.*, binding to nuclear receptors, adaptive stress responses). Required for CRC modelling.
Test battery	A panel of bioassays. Test batteries may be designed to capture the response of as many active chemicals in a water sample as possible

	(chemical goal-motivated) or to target endpoints relevant for human or ecosystem health (protection goal-motivated)
Toxic unit (TU)	The inverse of the toxic concentration, expressed as either lethal concentration (LC_y) or inhibitory concentration (IC_y), of the water sample (*e.g.*, $1/LC_y$ or $1/IC_y$)

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2. Basis for using effect-based methods

2.1. Effect-based methods compared with other analytical methods

How do effect-based methods complement the targeted and non-targeted chemical analytical methods that are used for water quality assessment?

EBM provide different and complementary information compared to chemical analyses. EBM can detect the effect of mixtures of known and unknown active chemicals in a sample, although they cannot alone identify the individual chemicals that are contributing to the effect. Targeted chemical analysis can quantify the concentration of known, targeted chemicals, but cannot provide any information about their biological effect or potential mixture effects. Non-targeted analysis using high-resolution mass spectrometry can help to detect unknown chemicals present in a water sample but does not accurately quantify chemical concentrations or account for the biological effect.[11] The combination of EBM and chemical analysis overcomes many of their individual limitations.

What type of information is generated by effect-based methods?

EBM provide a sum measure of the active chemicals present in a water sample that act by a particular mode of action. To express the results of EBM in quantitative terms, water extracts are tested in a dilution series, similar to testing for individual chemicals. This allows a concentration-response curve to be generated and the effect concentration (EC) causing a certain percent effect, such as 10% effect (EC_{10}) or 50% effect (EC_{50}), to be determined. The inhibitory concentration (IC) or lethal concentration (LC) in *in vivo* bioassays is also determined in the same way. EC, IC and LC values for water extracts cannot be expressed in mass or molar units but are instead expressed as a relative enrichment factor (REF) in units of $L_{water}/L_{bioassay}$. REF takes into consideration enrichment of the water sample via, for example, solid-phase extraction (SPE), (units of $L_{water}/L_{extract}$) and dilution of the sample extract in the medium volume of a bioassay (units of $L_{extract}/L_{bioassay}$). At a REF of 1 the sample concentration in the bioassay is equivalent to the original concentration in the water sample, assuming completely recovery, while a REF of 10 indicates a water sample was enriched 10 times. Often, enrichment is needed for a response to be observed. The lower the EC, IC or LC value, the

greater the response of the water sample as less enrichment is required to induce a response in the bioassay. The EC value can be converted to a bioanalytical equivalent concentration (BEQ), which is calculated by dividing the EC value of the reference compound by the EC value of the sample.[11] BEQ expresses the response of a chemical mixture in a sample as the concentration of a reference compound that would elicit the same effect, which makes it easier to compare bioanalytical results with chemical results. For example, estrogenic activity can be expressed as an estradiol equivalent concentration (EEQ). The higher the BEQ, the greater the response in the water sample.

What are the advantages and disadvantages of *in vitro* bioassays compared to conventional vertebrate (*in vivo*) assays?

In vitro bioassays are generally less variable, faster and have a lower financial and ethical cost than whole animal *in vivo* assays. As *in vitro* bioassays can be run in 96-well or 384-well plate format, they require smaller sample extract volumes and have greater potential for automation and high-throughput screening, which makes them more practical for water quality monitoring.[12] *In vitro* bioassays can also provide information about specific modes of action, such as estrogenicity or genotoxicity, while *in vivo* assays integrate the effects from multiple toxicity pathways and provide information about apical (adverse) effects. Further, many *in vitro* bioassays utilize human cells, allowing the response of water extracts to be tested on test systems derived from human physiology, rather than from animal models. *In vivo* assays with animal models can, however, better capture absorption, distribution, metabolism and excretion (ADME) processes. Typically, one would require a battery of *in* vitro bioassays to cover one or more relevant *in vivo* outcomes because *in vitro* assays typically only yield information on one molecular initiating event or key event but do not relate directly to an adverse outcome. It can be difficult to link a response in an *in vitro* bioassay to adverse outcomes at the organism level, though effects *in vitro* can be extrapolated through quantitative *in vitro* to *in vivo* extrapolation (QIVIVE).[13] *In vivo* assays are more resistant to external challenges ~~and more suitable for whole-effluent toxicity (WET) testing, which provides information about the~~

~~mixture effects of micropollutants, metals and salts~~. *In vitro* bioassays, on the other hand, provide information about specific effects of organic micropollutants in a water extract, though a limited number of studies have also tested whole water samples in *in vitro* bioassays.[14, 15]

How do effect-based methods in water quality monitoring differ from whole-effluent toxicity testing?

Whole-effluent toxicity testing has been used for decades for a site-specific assessment of water quality in all its aspects from inorganics (salts), metals to organics (Chapter 3 in ref. 11). Traditionally WET relied on *in vivo* assays, e.g., direct testing with algae, daphnia or fish (embryos) (ref) but also *in vitro* bioassays have been applied for WET (e.g., bacterial toxicity, genotoxicity). Such direct testing of diluted water was mainly done for industrial and municipal effluents and contaminated sites. Today, how we typically use EBM is much broader in terms of water types, encompassing not only contaminated waters but also surface, recycled and drinking water. This necessitates enrichment of water and together with a focus on organic micropollutant this development has also led to mostly exclusive use of extracts of the organic micropollutants leaving behind salts and methods in typical EBM applications.

2.2. In the context of water regulation

Which endpoints can be used as bioassays for water quality assessment?

There are many different bioassays available, including multiple bioassays responsive to the same endpoint, as well as bioassays measuring multiple endpoints (multiplexed bioassays). This raises questions about which bioassays and how many should be applied for water quality assessment. The answers to these questions depend heavily on the specific scope of the study, plus available funding and resources. As complex low-level mixtures of chemicals are commonly present in environmental water extracts, a single bioassay cannot capture all of the responses that may be induced by these complex mixtures.[12] As a starting point, a practical test battery of at least three or four bioassays responsive to effects commonly detected in water samples and aligned with relevant steps of adverse

outcome pathways is recommended. *In vitro* bioassays responsive to activation of the aryl hydrocarbon receptor (AhR), activation of the estrogen receptor (ER) and the oxidative stress response are recommended for wastewater and water reuse for non-potable use.[16] These three endpoints can detect effects in a range of water types, as demonstrated by the use of both individual and multiplexed bioassays.[7, 17] In the context of drinking water or water reuse for potable use, a bioassay responsive to either genotoxicity or mutagenicity is recommended in addition to activation of AhR, activation of ER and the oxidative stress response due to the potential formation of disinfection by-products.

Are effect-based methods currently applied for regulatory monitoring?

In vitro bioassays responsive to activation of ER and activation of AhR are used to monitor recycled water quality intended for both groundwater recharge and reservoir water augmentation in California.[18] Health-based monitoring trigger levels of 3.5 ng/L EEQ and 0.5 ng/L 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) equivalent concentration (TCDDEQ) have been set to interpret observed activation of ER and activation of AhR activity, respectively. Different response actions are to be taken if the BEQ (observed response) to trigger level ratio exceeds certain thresholds. For example, operators should consult with regional and state water boards if the BEQ to trigger level ratio is between 10 to 1,000, with possible actions including targeted chemical analysis and increased bioassay monitoring.[18] It should be noted that these assays are currently used for monitoring and not compliance. Except for this example, EBM have not yet been implemented in any other legislation to date.

How can we use effect-based methods in water safety planning?

Water Safety Plans (WSP) aim to ensure the safety of drinking water and assess risks associated with microbial, chemical, physical and radiological hazards in source waters.[19] Together with chemical analysis, EBM can be applied in WSPs to assess chemical hazards. While EBM have not been used

formally in WSPs to date, it is clear that EBM can be integrated in WSPs. Specifically, EBM have the potential to be applied in several of the WSP modules, including those that describe the water supply system (Module 2), identify hazards and assess risks (Module 3) and determine and validate the control measures, reassess and prioritize the risks (Module 4). Requirements to support the uptake of EBM into WSPs are discussed further in Neale et al.[20]

3. Practical considerations

3.1. Reliability and logistics

What type of laboratory is needed to run effect-based methods?

In vitro bioassays are generally run in cell culture laboratories. Certified facilities with appropriate biosafety measures are often required as many cell lines are genetically modified. The minimum equipment required includes incubators to grow and expose cells, a biosafety cabinet to ensure a sterile environment for cell culture and a plate reader to measure the bioassay output. Specific bioassays may require more advanced equipment. Access to chemical laboratory facilities is also required for sample processing and extraction prior to bioanalysis. If (advanced) cell culture laboratories are not available, simple bacterial toxicity assays, such as Microtox or BLT-Screen, can be used in locations that only have access to microbiological laboratory facilities. These assays provide information about the non-specific toxicity of a water sample, with no information about specific endpoints, but they require much less bioassay operator training compared to cell-based bioassays. Further, other bacterial assays, such as the Ames assay for mutagenicity, may also be suitable in locations without cell culture facilities.

Are effect-based methods cheaper than chemical analysis?

The argument related to cost-effectiveness for the inclusion of EBM is not one based on costs, but rather based on effectiveness. EBM provide complementary information about mixture effects and links water quality with risk assessment as BEQ are potency-scaled sum concentrations. That said,

applying a carefully selected bioassay test battery, such as discussed in Section 2.2, along with targeted chemical analysis of relevant chemicals, can reduce the need to monitor large numbers of chemicals, and thus keep analytical costs within reason. The price of EBM can vary depending on the type of bioassay(s) run. For example, simple bacterial toxicity assays are much cheaper than cell-based reporter gene *in vitro* bioassays, due to differences in consumable costs and operator time requirements. Broadly, the per-sample cost of analysing water extracts in a high-throughput cell-based bioassay is in the same order of magnitude as trace chemical analysis screening methods.

Are effect-based methods reliable?

A commonly voiced criticism of EBM is that the results are not reliable or repeatable, but these statements are unfounded. Many *in vitro* bioassays and well plate-based *in vivo* assays used for water quality assessment are validated to ensure that they are accurate, precise, robust and sensitive.[21-24] The variability associated with many *in vitro* bioassays is similar to targeted chemical analysis methods.[25, 26] Once a bioassay is validated, standard operating procedures (SOP) that cover consumable and equipment requirements, detailed bioassay procedures and data analysis are developed for routine application. Quality assurance and quality control (QA/QC) procedures are included in every bioassay run to ensure consistent bioassay performance over time. QA/QC is discussed further in Section 3.3.

Are the results from effect-based methods comparable between different bioassays, different sites and different studies?

While results were often reported as simply "positive (+)" or "negative (-)" in the early days of applying EBM, the science of bioassay data analysis has greatly advanced since.[27] The results of EBM are now routinely expressed as EC values, BEQ values, toxic units (TU) or effect units (EU). From experience with interlaboratory comparison studies, the same bioassay tested in different laboratories gives reasonably similar results.[24, 28, 29] However, bioassays responsive to the same

endpoint using different cell lines or different testing conditions may exhibit larger differences due to biological variation in the ligands/receptors that may lead to different relative effect potencies of the same chemicals in the different bioassays.[26] Expressing the results as BEQ can reduce some of the variability, which facilitates the comparison of results between different bioassays, different sites and different studies.

3.2. Sampling

Which sampling locations should be included for water quality assessment?

As is the case with conventional chemical analysis, the sampling locations selected will depend on the purpose of the sampling campaign.[30] For example, if the purpose of the sampling campaign is to assess treatment process efficacy in a drinking water treatment plant, then source water and product water should be collected. If the purpose of the sampling campaign is to understand critical control points in a wastewater treatment plant, then it would be necessary to collect samples from the influent, after the critical control point(s), and from the effluent. For routine monitoring, product water from a drinking water treatment plant or effluent from a wastewater treatment plant can be collected to verify the quality of the final water.

What type of sampling (*e.g.*, grab or composite) should be used?

As it is for chemical analysis, the type of sampling depends on the water type. Composite samples are recommended for wastewater influent and effluent to correct for the diurnal variation observed for some micropollutants,[31, 32] with many studies collecting 24 hour composite influent and effluent samples.[33-35] Grab sampling is suitable when little difference in quality over time is demonstrated, which is common for drinking water or recycled water. Other sampling options that are compatible with EBM include large volume SPE, where up to 1000 L of water can be sampled at once,[36] and passive samplers, which are devices that collect micropollutants from the water environment over a longer period of time to enable time-integrated sampling.[37]

How many samples should be collected at each sampling location?

Like chemical analysis, the number of samples collected will depend on the sampling campaign. Truly independent replicate samples, collected at a predefined interval, should be collected in duplicate or triplicate and analysed in appropriately designed monitoring programmes. A careful distinction needs to be made in the reporting of EBM between sample replicates and replicate analysis in a bioassay (see Section 3.3).

Do water samples need to be prepared prior to testing with *in vitro* bioassays?

Water samples commonly undergo pre-treatment and extraction prior to testing with *in vitro* bioassays, with concentrated extracts typically dosed and diluted in the bioassay. Common pre-treatment steps include adjusting the pH, quenching the disinfectant residual, and filtration, with further information provided in Escher et al.[11] Water samples are commonly extracted using SPE, though passive sampling and liquid-liquid extraction are also used to extract water samples prior to bioanalysis. SPE has several advantages including good recovery of a wide range of contaminants, low solvent requirements and the ability to be automated. SPE cartridges can clog when extracting water samples with a high particulate content,[38] so filtration prior to SPE is recommended for turbid water samples.[11] While filtration will remove the particulate matter, previous studies have shown that particulate matter can have considerable biological activity.[39, 40] Therefore, to understand the biological effect of the whole-water sample it may be necessary to retain the filtered particulate matter and extract with solvents.[38]

Is the composition of the water sample changed after sample preparation?

Extraction methods commonly used for EBM, namely SPE, liquid-liquid extraction and passive sampling, target organic micropollutants and exclude salts, metals and other inorganics. Common SPE sorbents such as Oasis HLB, Chromabond HR-X and StrataX, can capture a range of

hydrophobic and hydrophilic micropollutants but are known to recover a lower fraction of charged chemicals compared to neutral chemicals.[41, 42] Further, parameters such as temperature and flow velocity can affect the uptake of chemicals into passive samplers.[43]

Unlike in chemical analysis, assessing effect recovery in bioassays is challenging as internal standards cannot be used as they may induce an effect in the bioassay that cannot be distinguished from that of the micropollutants in the sample. Effect recovery by SPE has been evaluated in the literature by considering the effect of a spiked mixture of micropollutants alone, the effect of the extracted spiked sample and the effect of the unspiked water alone.[41] Effect recovery ranged from 35% to 236% for assays indicative of activation of AhR, activation of ER and the oxidative stress response. Effect recovery was within a factor of two of the optimal 100% recovery for most bioassays, which suggests that SPE captures the majority of active chemicals. In the same study, the recovery of 459 spiked micropollutants for conventional chemical analysis ranged from 0.8 to 308%, with an average recovery of 70%.[41]

Do I need to extract samples prior to bioanalysis?

Few studies have run unenriched or native water samples in *in vitro* bioassays.[14, 15, 44] This is equivalent to WET testing and would incorporate the effect from different components in water including salts, metalsand other inorganics, as well as organic micropollutants. This means it is difficult to isolate the effect of the micropollutants alone. Testing unextracted water samples prevents the risk of losing compounds during sample extraction, but confounding factors, such as microbial activity or pH changes, can result in potential false-positive and false-negative responses for unextracted samples.[45] Moreover, the volume that can be dosed in an *in vitro* bioassay is limited (microliter range), so cells will be exposed to much lower concentrations than when extracts are diluted in the bioassay medium. Therefore, this approach is unlikely to be suitable for water samples

with a lower micropollutant burden, such as surface water, drinking water and recycled water, where the samples need to be enriched to detect a response.

3.3. Quality control in running bioassays

Which quality control samples should be included during routine bioanalysis?

QA/QC are critical when running bioassays, to ensure that the results are reliable and reproducible. Quality control samples, including a positive reference compound, negative control, solvent control and blank samples, should be included as part of routine bioanalysis. The positive reference compound is a potent chemical in the bioassay and ideally an environmentally relevant chemical (*e.g.*, the herbicide diuron in the photosynthesis inhibition assay). The positive reference compound should be tested at different dilutions to generate a concentration-response curve in every bioassay run. This allows an EC value (*e.g.*, EC_{50}) to be determined, which can be compared between runs and over time using a control chart (*e.g.*, Shewart chart). The negative control (*i.e.*, test medium alone) is used to determine the minimum response of the test system, while the solvent control (*i.e.*, same volume of solvent as the sample extract added to test medium) is used to confirm that the solvent itself does not induce a response in the bioassay. Blank samples, including field blanks (*i.e.*, ultrapure water taken into the field and processed the same way as the actual water samples) and laboratory blanks (*i.e.*, ultrapure water processed the same way as the actual water samples), should be tested to ensure that the sampling and sample processing steps do not introduce any contamination (*e.g.*, impurities in solvents used for sample extraction that could induce or mask bioactivity). Measuring cytotoxicity concurrently in each bioassay is critically important to ensure that the response reported is indeed specific to the effect, rather than due to interference caused by cytotoxicity. For example, cytotoxicity can look like antagonism in reporter gene bioassays, and induction of cell growth may induce additional mutations in a mutagenicity bioassay. Measuring cytotoxicity and excluding any results at cytotoxic concentrations will prevent cytotoxicity from being incorrectly reported as a specific effect

induced by micropollutants. Further information about bioassay QA/QC is available in Escher et al.[11] and Denison et al.[46]

Why do samples need to be analysed in replicate?

Experimental replication is critical for any type of analysis, including EBM, with replicates being an important part of EBM quality control. Firstly, sample extracts should be run in duplicate or triplicate on the same well plate (*i.e.*, intra-plate replication) to determine if there is any variability between the wells due to variations in handling (*e.g.*, operator error) or external factors (*e.g.*, humidity). Secondly, the same sample extract should be run on different plates or different parts of the same plate to determine if there is any variability between bioassays, *e.g.*, due to temporal drift from environmental factors or instrument issues during the bioassay run (*i.e.*, intra-assay or inter-plate replication). Finally, each sample extract should be run at least twice in independent bioassay runs on different days to ensure there is no bias introduced over time (*i.e.*, inter-assay replication). Further information is available in Escher et al.[11]

4. What to do with information provided by effect-based methods

4.1. Understanding the significance of bioassay results

Does a response in a bioassay mean the chemical water quality is not acceptable?

Many *in vitro* bioassays, particularly mammalian reporter gene bioassays, are highly sensitive by design and can detect effects in relatively clean waters, such as drinking water and recycled water, after sufficient enrichment.[8, 10, 47, 48] However, the detection of an effect does not necessarily mean that the chemical water quality is unacceptable, just like detecting a chemical in a water sample does not necessarily mean that the water is not fit for purpose – the chemical concentration needs to be compared to a guideline value to determine risk. Effect-based trigger values (EBT) have been introduced to help bioassay users differentiate between an acceptable and unacceptable response.[49-52] EBTs are commonly given as a BEQ value (EBT-BEQ), allowing the measured effect in a water

sample for a particular bioassay (expressed as BEQ) to be compared with the corresponding EBT-BEQ. This is similar to the comparison of a detected chemical concentration with the corresponding water quality standard or guideline value. An effect below the EBT indicates the chemical water quality is acceptable, while further action is required if the effect of a sample exceeds the EBT (see Section 4.2).

Does a lack of response in a bioassay indicate that chemical water quality is acceptable?

The answer to this question depends on the bioassay(s) used and the sample enrichment factor. Low sensitivity bioassays, such as most yeast reporter gene bioassays, may be suitable for monitoring wastewater quality, but will not be sensitive enough to detect effects in drinking water or recycled water where micropollutants are present at lower concentrations.[53] In these cases, highly sensitive mammalian reporter gene bioassays are recommended. However, even sensitive bioassays will not be able to detect an effect if the water sample is not sufficiently enriched. The final enrichment (e.g., REF) in a bioassay can be up to 20 for wastewater effluent, 100 for surface water and 200 for drinking water. If a suitably sensitive bioassay is used and the water sample is sufficiently enriched, then a lack of response indicates that the chemical water quality is acceptable with respect to the tested biological effect. It is also important to confirm that the method detection limit, inclusive of the assay sensitivity and sample enrichment, is below any relevant EBT. In addition, it is important to consider if the test battery applied sufficiently captured all relevant bioactivity before drawing more a general conclusion about the chemical water quality.

Which effect-based trigger value should I use?

A number of different EBTs are available in the literature for bioassays responsive to the same endpoint. For example, EBTs for estrogenic activity range from 0.2 to 12 ng/L EEQ for drinking water and recycled water[49, 52, 54], and from 0.1 to 2.2 ng/L EEQ for surface water[50, 51, 55]. This range results from differences in bioassay sensitivity and chemical potency, as well as differences in the

EBT deviation method. It should be noted that there is no widely accepted approach to derive EBTs, with approaches applied in the literature ranging from simple translation from chemical guideline values[56] to using multiple lines of evidence.[50] EBTs are usually bioassay-specific, and a specific EBT, rather than a generic EBT for an endpoint, should be used where available. Further, EBTs are developed for either the protection of human health or ecological health and should be applied in the correct context (*e.g.*, human health-relevant EBTs applied to drinking water and recycled water).

4.2. Operational response

What do I do if there is significant bioassay activity in a water sample?

If the measured BEQ value exceeds the EBT-BEQ in the corresponding bioassay, the bioassay quality control should be checked, and another sample collected and re-tested immediately (to determine if the response is still present). Further action is required if the second sample confirms that the BEQ value is greater than the EBT-BEQ. The action taken depends on the type of water sample, the type of bioassay and the magnitude of the exceedance. For bioassays where few known and potent chemicals contribute to the effect, such as bioassays responsive to receptor-mediated effects (*e.g.*, estrogenic activity), targeted analysis of known potent chemicals to compare with chemical guideline values is recommended. Identification of the chemicals that contribute to the bioassay response can also be pursued using an effect-directed analysis approach, in which bioanalysis and chemical analysis of fragmented extracts are coupled.[57] If the detected chemical concentration exceeds the guideline value, then the well-established chemical guideline exceedance response procedure outlined in the relevant guideline document (e.g., European Union (EU) Drinking Water Directive for EU Member States) should be followed. If the observed effect cannot be explained by detected chemicals there are several options depending on the magnitude of the exceedance and advice from the regulatory authority. These include optimising treatment processes and assessing the quality of surface water.

For bioassays where many low potency chemicals can contribute to the response, such as bioassays responsive to adaptive stress responses or apical effects in whole organisms, targeted chemical analysis cannot be used to determine the causative chemicals. Instead, it becomes important to determine if the cytotoxicity of the sample exceeds an acceptable level. If there is significant cytotoxicity, options include optimising treatment processes and assessing the quality of surface water. The response will depend on the magnitude of the exceedance and advice from the regulatory authority. In cases where there is no significant cytotoxicity, but the effect in the assay still exceeds the corresponding EBT (e.g., oxidative stress EBT for an oxidative stress assay), applying bioassays responsive to receptor-mediated effects is recommended to determine if they are also exceeding their respective EBTs. Further information and an interpretation framework for EBT exceedance can be found in Neale et al.[58]

5. Case studies

Are effect-based methods used for monitoring in the water sector?

As mentioned in Section 2.2, *in vitro* bioassays responsive to activation of ER and AhR are used to monitor recycled water quality in California.[18] In the Netherlands, EBM are not legally required but a nationwide monitoring framework was recently launched which recommends batteries of bioassays in tandem with extensive chemical analysis to provide water authorities and drinking water companies with additional information about the mixture effects of chemical hazards in their water systems and sources. Further information can be found at https://www.sleutelfactortoxiciteit.nl/.

Are there examples that demonstrate how the application of effect-based methods can improve water quality assessment?

The strengths of EBM, including detecting the effect of potent chemicals present at low concentrations and detecting the mixture effects of both known and unknown chemicals, are advantageous for water quality assessment. Some examples that highlight the utility of applying EBM

for water quality monitoring are listed below. These examples do not consider the application of other innovative monitoring approaches, such as non-targeted chemical screening approaches or effect-directed analyses, although these also have clear advantages for water quality assessment.

Steroidal hormones, estrone, 17β-estradiol and 17α-ethinylestradiol are included in the watch list of the EU Water Framework Directive, but the proposed environmental quality standards for 17β-estradiol and 17α-ethinylestradiol are below the limit of detection of many chemical analytical methods. Konemann et al.[59] applied both chemical analysis and EBM to wastewater and surface water extracts from Europe. While estrone could be quantified in most samples, 17β-estradiol and 17α-ethinylestradiol could only be quantified in a smaller number of samples, despite estrogenic activity being detected in all samples using *in vitro* bioassays responsive to estrogenic activity. Hence, *in vitro* bioassays enabled the detection of chemical hazards that would have been missed using chemical analysis alone. The limit of quantification for chemical analysis (0.04 to 1.5 ng/L for 17β-estradiol in surface water and 0.05 to 3 ng/L for 17β-estradiol in wastewater effluent) is higher than that for *in vitro* bioassays such as ERα CALUX (as low as 0.002 ng/L),[59] with robust EBTs available for estrogenic activity.[60]

In another example, Magdeburg et al.[61] assessed changes in chemical concentrations and biological effect in a pilot advanced wastewater treatment plant with conventional biological activated sludge, ozonation and sand filtration. Ozonation reduced the concentration of most analysed chemicals by more than 90%. Genotoxicity in the umuC bioassay was also reduced by more than 90% after ozonation, but mutagenicity increased using the Ames strain YG7108, revealing an otherwise overlooked chemical hazard. Enhanced mortality and genotoxicity in rainbow trout was also observed after ozonation, with the effect likely due to the formation of alkylating mutagenic oxidation by-products after ozonation. Sand filtration after ozonation reduced the observed effect, but not completely. Treatment with powdered activated carbon instead of ozonation was not as effective at

removing target chemicals and genotoxicity but did not result in significant mutagenicity or mortality. This example shows why targeted chemical analysis should be complemented with EBM when assessing new treatment processes. Many more examples are provided in Escher et al.[11]

6. Implications

The current state of knowledge and experience has opened the way for adoption of EBM to better assess water quality when it comes to complex mixtures of organic micropollutants, overcoming the limitations of the current chemical-by-chemical approach. EBM have many advantages including accounting for mixture effects and providing a sum parameter of all active chemicals with the same mode of action. However, they cannot identify the individual chemicals causing the effect. Consequently, EBM are complementary to existing chemical analysis methods, detecting otherwise overlooked effects, including unknown chemicals or potent chemicals present at concentrations below analytical detection limits. While the field has advanced greatly in the last decade, there are still some knowledge gaps that need to be addressed. This includes further work on assessing the validity of sample preparation methods, which is challenging for EBM as internal standards cannot be added to correct for any losses. Further, some relevant endpoints, such as reproduction and developmental toxicity, lack comprehensive *in vitro* models. Currently, most EBM are offline (*i.e.*, water samples are collected and then taken to a laboratory for processing and analysis), but there is also potential for the development of online EBM for surveillance monitoring. While there has so far only been tentative uptake of EBM by regulatory bodies, recent progress in establishing EBTs for a wide range of bioassays and developing frameworks to respond to EBT exceedances, as well as extensive experience with the systematic application of EBM to water quality monitoring, means that it is likely we will soon see greater acceptance of EBM in regulatory contexts to address the ever-increasing universe of potential chemical contaminants.

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