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Solid-phase extraction as sample preparation of water samples for cell-based and other *in vitro* bioassays†

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In vitro bioassays are increasingly used for water quality monitoring. Surface water samples often need to be enriched to observe an effect and solid-phase extraction (SPE) is commonly applied for this purpose. The applied methods are typically optimised for the recovery of target chemicals and not for effect recovery for bioassays. A review of the few studies that have evaluated SPE recovery for bioassays showed a lack of experimentally determined recoveries. Therefore, we systematically measured effect recovery of a mixture of 579 organic chemicals covering a wide range of physicochemical properties that were spiked into a pristine water sample and extracted using large volume solid-phase extraction (LVSPE). Assays indicative of activation of xenobiotic metabolism, hormone receptor-mediated effects and adaptive stress responses were applied, with non-specific effects determined through cytotoxicity measurements. Overall, effect recovery was found to be similar to chemical recovery for the majority of bioassays and LVSPE blanks had no effect. Multi-layer SPE exhibited greater recovery of spiked chemicals compared to LVSPE, but the blanks triggered cytotoxicity at high enrichment. Chemical recovery data together with single chemical effect data were used to retrospectively estimate with reverse recovery modelling that there was typically less than 30% effect loss expected due to reduced SPE recovery in published surface water monitoring studies. The combination of targeted experiments and mixture modelling clearly shows the utility of SPE as a sample preparation method for surface water samples, but also emphasizes the need for adequate controls when extraction methods are adapted from chemical analysis workflows.

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Environmental significance

Solid-phase extraction (SPE) is commonly applied for sample enrichment prior to bioanalysis. While many studies have assessed recovery of targeted chemicals, much less is known about effect recovery using common SPE methods. Using a complex mixture of chemicals spiked into a pristine surface water sample, the current study shows acceptable effect recovery for a range of bioassays after enrichment using large-volume SPE. Reverse recovery modelling was applied to predict effect loss by SPE in previously published water quality monitoring studies, with no substantial loss of effect by SPE found in most cases. With effect recovery similar to chemical recovery, the current study provides support for the application of bioassays for water quality monitoring.

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1. Introduction

There is increasing interest in applying bioanalytical tools complementary to chemical analysis for water quality monitoring.^{1,2} While targeted chemical analysis provides information about the presence of known chemicals in a sample, bioanalysis yields information about the mixture effects of the known and unknown bioactive chemicals in the sample. This complementary approach has been applied to a range of water samples including wastewater, surface water and drinking water,^{3–5} with studies showing that many more chemicals than those quantified contribute to the biological effects for many endpoints. As the concentration of chemicals in environmental waters is

typically in the nanogram per litre to microgram per litre range, sample preparation prior to bioanalysis is required, with solid-phase extraction (SPE) commonly applied to enrich water samples.^{6–10} As bioassays are increasingly applied to cleaner matrices, such as surface water and drinking water, samples often need to be enriched up to 100 times to detect an effect.¹¹ For practical purposes, the extracts are diluted in the bioassays, hence the initial enrichment of the water sample by SPE is typically 1000 to 2000 fold. Many studies have evaluated the recovery or the fraction of individual chemicals retained by SPE based on chemical analysis in a range of water matrices,^{12–15} with recovery dependent on the physicochemical properties of the target chemical, the matrix, the SPE material and the extraction conditions. To capture a broad range of chemicals, including very polar chemicals, combinations of SPE materials, such as reverse-phase materials with ion-exchange materials, are used.^{12,16,17} However, there is considerably less work on understanding the recovery of biological effects by typically applied enrichment techniques, but this is essential for the application of bioassays for water quality monitoring and for regulatory acceptance of these tools.

The aim of the current study was to review the different approaches applied to evaluate effect recovery by SPE from the literature and to propose a new approach to experimentally determine effect recovery for bioassays. This approach will be applied to assess the recovery of a complex mixture of 579 chemicals spiked into surface water prior to large volume solid-phase extraction (LVSPE) using a combination of chemical analysis and bioassays. For water quality monitoring, bioassays covering different stages of the cellular toxicity pathway, as well as apical effects, are recommended.¹⁸ Therefore, we applied nine cell-based bioassays indicative of xenobiotic metabolism, hormone receptor-mediated effects and adaptive stress responses, as well as the fish embryo toxicity test with *Danio rerio* as a representative for an *in vitro* assay covering apical effects in whole organisms. A single bioassay will not be able to detect all potential effects, but by using a test battery with assays that target specific modes of action, as well as assays that detect more integrative effects, such as adaptive stress responses and apical effects in whole organisms, we are able to detect the effects of a wide range of chemicals.

Another potential issue associated with the application of SPE extracts to bioassays is effects caused by impurities captured during the extraction process. The SPE material and solvents used for high enrichment of many different chemicals with diverse physicochemical properties might lead to unwanted blank effects. Therefore, in addition to high recovery of individual chemicals and effects, a low blank effect is a prerequisite for sample preparation with SPE for bioanalytical assessment. Potential blank effects from two different SPE methods recently used for water quality monitoring, LVSPE and multi-layer SPE, were evaluated in the current study using the bioassay test battery described above.

This study represents the most comprehensive experimental evaluation of effect recovery by SPE to date. In addition, we also applied chemical recovery data from two SPE methods and reverse recovery modelling to estimate how much the measured

effects underestimate predicted effects by back-calculating measured effects to expected effects for 100% chemical recovery using water quality monitoring case studies from the literature. Three of the case studies focused on samples collected from European rivers^{19–21} extracted with a LVSPE method using the same neutral HR-X sorbent applied in the current study. The fourth case study on Swiss effluent-impacted streams used a multi-layer SPE method with multiple layers of solid phases, namely Oasis HLB, a mixture of Strata-X-CW, Strata-X-AW and Isolute Env+, and Supelclean EnviCarb, for maximum chemical recovery.²²

2. Current state of knowledge on effect recovery

While studies on recovery of individual chemicals with SPE in the preparation of chemical analysis are abundant, very little systematic work has been performed on effect recovery by SPE. Effect recovery for bioassays in the literature is typically assessed by spiking a cocktail of chemicals into a water matrix before enrichment by SPE. Since it is most often not possible to measure the water sample prior to SPE directly in the bioassays, the effect of the extract expressed as a bioanalytical equivalent concentration from bioanalysis ($BEQ_{bio,extract}$) is often compared to the predicted mixture effect using the BEQ approach, which assumes that the spiked chemicals are acting in a concentration additive manner. BEQ for chemical analysis can be calculated based on either the concentration of individual chemicals detected in the extract ($BEQ_{chem,extract}$) or the nominal concentration of spiked chemicals ($BEQ_{chem,nominal}$), along with the potency of the individual chemicals in the assay.^{23,24} This type of mixture modelling and comparison between BEQ_{bio} and BEQ_{chem} has been applied extensively to quantify the effect triggered by unknown chemicals in environmental samples^{5,19,21,22} but can also be used to quantify effect recovery in SPE, provided that the effect is dominated by the spiked chemicals. The ratio of $BEQ_{bio,extract}/BEQ_{chem,nominal}$ or $BEQ_{bio,extract}/BEQ_{chem,extract}$ is a measure of the spiked chemical SPE recovery expressed as effect and assumes that the water sample receiving the spiked chemicals does not contribute to the effect and that the spiked chemicals act concentration-additive in mixtures (Fig. 1A).

The comparison of $BEQ_{bio,extract}$ with $BEQ_{chem,extract}$ is mathematically similar to iceberg modelling (Fig. 1B), which is often applied in water quality monitoring to quantify the fraction of unknown bioactive chemicals in a water sample by calculating $BEQ_{chem,extract}/BEQ_{bio,extract}$.¹⁹ The difference between iceberg modelling used for water quality monitoring and the current approach using the $BEQ_{bio,extract}/BEQ_{chem,extract}$ ratio is that for chemical SPE recovery expressed as effect we assume that we know all chemicals in the sample. In this application of mixture modelling, it is assumed that the spiked chemicals dominate the effect in the water sample and hence the evaluation of the $BEQ_{bio,extract}/BEQ_{chem,extract}$ ratio can be a proxy for effect recovery. However, because this approach compares chemical analysis and bioanalysis after extraction



Fig. 1 Overview of approaches commonly used in the literature to evaluate chemical SPE recovery of spiked chemicals expressed as effect (A) and iceberg modelling, which is a comparison of the effect observed in a water sample to the effects predicted for the quantified chemicals (B), with the complementary approach of true effect recovery by SPE applied in the current study (C).

only, it is, strictly speaking, a measure of quality/applicability of mixture toxicity models based on concentration addition rather than an effect recovery.

Studies that have determined the $\text{BEQ}_{\text{bio,extract}}/\text{BEQ}_{\text{chem,extract}}$ ratio are summarised in Table S1 of the ESI.† Leusch *et al.*²³ spiked eight estrogenic compounds to various water types and reported $\text{BEQ}_{\text{bio,extract}}/\text{BEQ}_{\text{chem,extract}}$ of 0.3 to 1.64 for five different estrogen receptor (ER) assays. Kolkman *et al.*²⁵ spiked a surface water sample with a mixture of 39 chemicals including natural and synthetic hormones, pesticides and pharmaceuticals and determined $\text{BEQ}_{\text{bio,extract}}$ and $\text{BEQ}_{\text{chem,extract}}$ for a suite of assays indicative of different hormone receptor-mediated effects. The $\text{BEQ}_{\text{bio,extract}}/\text{BEQ}_{\text{chem,extract}}$ ratio ranged from 0.02 and 1.06, with the low $\text{BEQ}_{\text{bio,extract}}/\text{BEQ}_{\text{chem,extract}}$ ratio in the assays indicative of activation of the androgen receptor (AR) and activation of the progesterone receptor (PR) attributed to the spiked mixture containing both agonists and antagonists. Using the $\text{BEQ}_{\text{bio,extract}}/\text{BEQ}_{\text{chem,extract}}$ ratio one can relate the extracted chemicals to the observed effects against a background water matrix, but this is not a recovery of the biological

effect in the true sense. Rather these studies compare the predicted effects in the extracts based on known bioactive chemicals with the measured effects of the extracts, similar to iceberg modelling.

In contrast, the $\text{BEQ}_{\text{bio,extract}}/\text{BEQ}_{\text{chem,nominal}}$ ratio is more useful for determining effect recovery by SPE as the effect in the bioassay is related to the predicted effect based on the nominal concentration, rather than the concentration measured in the extract. Studies that have applied this approach are summarised in Table S2.† For example, Neale and Escher²⁶ found a $\text{BEQ}_{\text{bio,extract}}/\text{BEQ}_{\text{chem,nominal}}$ ratio of 0.91 for six spiked herbicides in treated wastewater in the combined algae assay. Further, Kunz *et al.*²⁴ found a $\text{BEQ}_{\text{bio,extract}}/\text{BEQ}_{\text{chem,nominal}}$ ratio of 0.27 to 1.38 for spiked estrogenic compounds in assays indicative of estrogenic activity and a similar study using four estrogenic chemicals spiked into wastewater reported a ratio of 1.13 to 1.24 for the yeast estrogen screen (YES).²⁷

One issue with comparing $\text{BEQ}_{\text{bio,extract}}$ with $\text{BEQ}_{\text{chem,extract}}$ or $\text{BEQ}_{\text{chem,nominal}}$ based on the spiked chemicals alone is that the spiked water matrix may have an effect itself in the bioassay.

This is especially likely for complex matrices, such as wastewater. Therefore, it is important to consider the effect of the matrix itself when assessing effect recovery for bioassays. By adding a chemical cocktail to a urine sample, which was selected as a representative for a matrix-rich water, and testing both urine alone and urine spiked with the cocktail, Escher *et al.*²⁸ were able to confirm good effect recovery by SPE, with between 75 and 148% recovery for YES and the bioluminescence inhibition test.

To truly assess effect recovery by SPE one would need to spike water prior to SPE and compare the effects before and after SPE, which is technically challenging. As a proxy, Escher *et al.*²⁹ previously extracted spiked and unspiked wastewater with Lichrolut Env/C18 SPE cartridges and SDC Empore Disks and compared the resulting effects. Full bioassay recovery was achieved for spiked estradiol in YES, spiked parathion in the acetylcholinesterase inhibition assay and spiked diuron in the combined algae assay, confirming high extraction efficacy as well as concentration-additive mixture effects of the wastewater matrix and spiked chemicals.²⁹ A limitation of this study was that concentrations in the samples were not chemically verified.

In summarising the available literature, there is a lack of experimentally determined effect recoveries for bioassays using commonly applied SPE techniques. To fill this knowledge gap, the current study evaluated the effect recovery of a mixture of micropollutants by SPE using a combination of bioanalysis and chemical analysis (Fig. 1C). Spiked and unspiked water samples were enriched using LVSPE, and chemical analysis was performed on the spiked and unspiked SPE extracts. Effect recovery was calculated by applying mixture modelling based on the assumption that the chemical mixture and the unspiked water extract would act in a concentration additive manner. Effect recovery was hence defined as the ratio of the difference in BEQ_{bio} between the spiked and unspiked extract to the BEQ_{bio} of the spiked chemical mixture. Thus all parameters of the recovery calculations are derived from experimentally quantified effects. In addition, reverse recovery modelling was applied to determine how much greater the predicted effect would be if all chemicals had been completely recovered by SPE (Fig. 1C). This was termed $BEQ_{chem,modelled\ 100\% \text{ recovery}}$ and was also calculated for existing iceberg modelling studies from the literature and compared with the reported $BEQ_{chem,extract}$ values.

3. Materials and methods

3.1 Chemical mixture

The spiked chemical mixture (sample “mix”) contained 579 chemicals in methanolic solution. The spiked mixture contained chemical classes commonly detected in environmental waters and wastewater³⁰ including pharmaceuticals, pesticides, industrial compounds and natural and synthetic hormones. This set of chemicals covers a wide range of physicochemical properties, including acids and bases as well as multiprotic chemicals to explore the applicability domain of SPE. The test set of 579 chemicals includes and expands our previous study of the chemical recovery of 251 organic chemicals.¹² The

concentrations of 532 compounds in the mix stock solution were 800 ng mL^{-1} , though the concentrations of the 47 steroidal hormones were 20 ng mL^{-1} to account for their high bioactivity. A list of the spiked chemicals is provided in Table S3† along with selected chemical properties, such as octanol–water partition constant ($\log K_{ow}$) and the ionisation-corrected octanol–water distribution ratio ($\log D_{ow}$).

3.2 Sample collection and extraction

Surface water from Wormsgraben, a pristine creek in the Harz Mountains, Germany, was used as the water matrix for the effect recovery experiments. Ninety litres of the water were collected using a submersible rotary pump (Comet, Pfaffschwende, Germany) equipped with polytetrafluoroethylene tubing and stored in three solvent-cleaned stainless steel drums. The flow rate of the pump was 20 L min^{-1} . Therefore, it can be assumed that the water condition of the creek was not altered during the short sampling period (approximately 5 min) and thus the water composition was similar in all drums. The samples were stored at 4°C in a cooling chamber for three weeks until performance of the spiking experiments. The mix stock solution was diluted with methanol by a factor of five prior to spiking, with 10 mL of the diluted mix stock solution spiked into 20 L Wormsgraben water to give final concentrations of 80 ng L^{-1} for the majority of compounds and 2 ng L^{-1} for steroidal hormones. The spiked water sample was enriched using LVSPE with the neutral HR-X sorbent (sample “water + mix”), with further information about the LVSPE method available in Schulze *et al.*¹² A modified elution procedure with neutral, acidic and basic elution steps was used as detailed in Vältälto *et al.*³¹ The final extract had a volume of 20 mL, giving an enrichment factor of 1000 based on the water volume. Twenty litres of unspiked Wormsgraben water were also extracted by the same LVSPE method (sample “water”). Five litres of ultrapure water (LCMS grade water) were extracted using LVSPE by circulating the water four times to obtain a process blank containing possible impurities from the extraction process (*e.g.*, leachates from machine materials or residues from SPE sorbent) as described in Schulze *et al.*¹² Both the unspiked Wormsgraben water and the process blank had a final enrichment factor of 1000. In addition to the process blank, a methanol solvent blank was also included.

Recovery of a suite of chemicals spiked in surface water from the Rhine River was also evaluated using multi-layer SPE cartridges. These multi-layer SPE cartridges have been previously applied to extract wastewater and surface water samples for bioanalysis²² and the recovery data were used for reverse recovery modelling in the current study. Briefly, Rhine water was filtered with a glass microfiber filter (GF/F, 47 mm, Whatman) and adjusted to pH 6.5. Three different sample types were prepared, a background sample with no chemicals spiked and recovery samples where 193 chemicals were spiked before SPE and after elution, respectively. Internal standards were also spiked into samples to account for possible analyte loss.

One litre of water was enriched using the multi-layer SPE cartridge, which was composed of 200 mg of Oasis HLB (Waters, U.S), 350 mg of a mixture of Strata-X-CW, Strata-X-AW

(Phenomenex, USA) and Isolute Env+ (1 : 1 : 1.5) (Separtis, Germany) and 200 mg of Supelclean EnviCarb (Sigma-Aldrich, Germany). The cartridges were conditioned with 5 mL methanol and 10 mL nanopure water, then the samples were loaded onto the cartridges and dried completely by pumping air through the cartridge. Elution occurred in back flush mode with ethyl acetate/methanol (1 : 1, 6 mL) containing ammonium (0.5%), followed by ethyl acetate/methanol (1 : 1, 3 mL) containing formic acid (1.7%) and then pure methanol (2 mL), which resulted in a final neutral elution volume of 11 mL. The samples were then concentrated to a volume of 100 µL under a gentle nitrogen flow, diluted with nanopure water (100 µL) and filtered (4 mm Cronus Filter, regenerated cellulose, 0.45 µm, Infocroma, Switzerland). The vial and filter were rinsed with nanopure water (800 µL), giving a final volume of 1 mL and thus an enrichment factor of 1000.

3.3 Chemical analysis

Analysis of all spiked compounds was performed using liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) or high-resolution tandem mass spectrometry (HRMS/MS). For the LVSPE recovery experiment, 561 compounds were analysed by a LC-HRMS/MS target screening method in positive and negative electrospray ionization (ESI⁺/ESI[−]) using a QExactive Plus instrument (Thermo). An additional 18 compounds (phenols and steroids) were analysed by LC-MS/MS in ESI-mode on a QTrap 6500 instrument (ABSciex), as the sensitivity of the LC-HRMS screening method was not sufficient. Details on the analytical method used can be found in Section S1.† Analysis of 193 chemicals in the multi-layer SPE extracts was also conducted using LC coupled to a QExactive HRMS. Further information is provided in Section S2.†

3.4 Bioanalysis

Ten bioassays covering 9 different endpoints were selected in the current study (Table 1). The assays were indicative of

activation of the aryl hydrocarbon receptor (AhR, AhR CALUX), activation of the pregnane X receptor (PXR, HG5LN-hPXR), binding to peroxisome proliferator-activated receptor gamma (PPARγ, PPARγ GeneBLAzer), activation of ER (ER GeneBLAzer, MELN), activation of AR (AR GeneBLAzer), activation of the glucocorticoid receptor (GR, GR GeneBLAzer), activation of PR (PR GeneBLAzer), oxidative stress response (AREC32) and fish embryo toxicity (FET). Cell viability was assessed in parallel for all assays indicative of non-apical effects. Detailed information about the studied assays can be found in König *et al.*,²¹ Neale *et al.*¹⁸ and Nivala *et al.*³²

The mix stock solution and the five times diluted mix stock solution were also analysed in the bioassays in their original methanolic form and were equivalent to an enrichment factor of 10 000 and 2000, respectively, of a water sample that had 100% recovery. As both the mix stock solution and the diluted mix stock solution gave consistent concentration–effect curves in all bioassays they were evaluated together as sample “mix”.

SPE process blank samples from LVSPE and multi-layer SPE were also tested in all assays, with the exception of HG5LN-hPXR and MELN in the case of multi-layer SPE. In addition, blank samples from different materials used in multi-layer SPE (*e.g.*, Oasis HLB, Oasis HLB + Strata-X-AW, Strata-X-CW and Isolute ENV+) were tested, as well as different conditioning solvents.

3.5 Data evaluation

The concentration causing 10% effect (EC₁₀) was derived from linear concentration–effect curves for the assays indicative of xenobiotic metabolism and hormone receptor-mediated effects, while the effect concentration causing an induction ratio of 1.5 (EC_{IR1.5}) was derived from linear concentration–effect curves for the AREC32 assay. Log-sigmoidal concentration–effect curves were applied to the FET assay to determine the concentration causing 50% effect (EC₅₀). Further information about the applied data evaluation methods can be found in Escher *et al.*³³ and Neale *et al.*¹⁸ The EC values for all samples were expressed

Table 1 Summary of applied bioassays

Bioassay	Endpoint	Method reference	Positive reference compound	EC value	Positive reference compound EC value (M)
AhR CALUX	Activation of aryl hydrocarbon receptor (AhR)	Brennan <i>et al.</i> ³⁸	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin (TCDD)	EC ₁₀	$(5.68 \pm 0.17) \times 10^{-13}$
HG5LN-hPXR	Activation of pregnane X receptor (PXR)	Lemaire <i>et al.</i> ³⁹	SR 12813	EC ₁₀	$(1.41 \pm 0.15) \times 10^{-8}$
PPARγ GeneBLAzer	Binding to the peroxisome proliferator-activated receptor gamma (PPARγ)	Neale <i>et al.</i> ¹⁸	Rosiglitazone	EC ₁₀	$(9.87 \pm 0.14) \times 10^{-10}$
MELN	Activation of estrogen receptor (ER)	Balaguer <i>et al.</i> ⁴⁰	17β-Estradiol	EC ₁₀	$(2.42 \pm 0.06) \times 10^{-12}$
ER GeneBLAzer	Activation of ER	König <i>et al.</i> ²¹	17β-Estradiol	EC ₁₀	$(2.50 \pm 0.08) \times 10^{-11}$
AR GeneBLAzer	Activation of androgen receptor (AR)	König <i>et al.</i> ²¹	Metribolone (R1881)	EC ₁₀	$(2.37 \pm 0.07) \times 10^{-10}$
GR GeneBLAzer	Activation of glucocorticoid receptor (GR)	König <i>et al.</i> ²¹	Dexamethasone	EC ₁₀	$(8.49 \pm 0.36) \times 10^{-10}$
PR GeneBLAzer	Activation of progesterone receptor (PR)	König <i>et al.</i> ²¹	Promegestone	EC ₁₀	$(1.52 \pm 0.06) \times 10^{-10}$
AREC32	Oxidative stress response	Wang <i>et al.</i> ⁴¹ , Escher <i>et al.</i> ⁴²	<i>tert</i> -Butylhydroquinone (tBHQ)	EC _{IR1.5}	$(1.93 \pm 0.04) \times 10^{-6}$
Fish embryo toxicity (FET)	Mortality	OECD ⁴³	3,4-Dichloroaniline	—	—

in units of relative enrichment factor (REF), which was calculated based on the SPE enrichment factor, or equivalent enrichment factor in the case of sample “mix”, and the dilution factor in the bioassay.⁶ The EC values for the assay positive reference compounds were expressed in molar units.

To relate the effect of the sample in a bioassay in units of REF to the concentration of a reference compound (ref) in molar units that would elicit the same effect the EC values were converted to BEQ_{bio} using eqn (1).

$$\text{BEQ}_{\text{bio}} = \frac{\text{EC}_{10}(\text{ref})}{\text{EC}_{10}(\text{sample})} \text{ or } \frac{\text{EC}_{\text{IR1.5}}(\text{ref})}{\text{EC}_{\text{IR1.5}}(\text{sample})} \quad (1)$$

Effect recovery for the bioassays was calculated for each assay using eqn (2) with the BEQ_{bio} value of the spiked Wormsgraben water extract (BEQ_{bio,extract} (water + mix)), the BEQ_{bio} value of the unspiked Wormsgraben water extract (BEQ_{bio,extract} (water)) and the BEQ_{bio} of the mix stock solution (BEQ_{bio} (mix)).

Effect recovery by SPE

$$= \frac{\text{BEQ}_{\text{bio,extract}}(\text{water} + \text{mix}) - \text{BEQ}_{\text{bio,extract}}(\text{water})}{\text{BEQ}_{\text{bio}}(\text{mix})} \quad (2)$$

The effect based on spiked chemicals was modelled using BEQ_{chem,extract} based on the concentration of the individual chemical in the extract (C_i) and its relative effect potency (REP_{*i*}) in the studied bioassay (eqn (3)). REP_{*i*} was calculated using eqn (4), with effect concentrations of the individual chemicals collected from the peer reviewed literature or the US EPA ToxCast database.³⁴ As the data in the ToxCast database were expressed as 50% activity concentrations (AC₅₀), EC_{10,absolute} was calculated using the reported AC₅₀ value and the maximum of the concentration–effect curve based on the approach described in Neale *et al.*²²

$$\text{BEQ}_{\text{chem,extract}} = \sum_{i=1}^n [C_i \times \text{REP}_i] \quad (3)$$

$$\text{REP}_i = \frac{\text{EC}_{10}(\text{ref})}{\text{EC}_{10}(i)} \text{ or } \frac{\text{EC}_{\text{IR1.5}}(\text{ref})}{\text{EC}_{\text{IR1.5}}(i)} \quad (4)$$

To evaluate how much effect would be overlooked due to loss of chemicals during SPE, we predicted the biological effect if the recovery of all chemicals by SPE were 100%, BEQ_{chem,modelled 100% recovery}, using eqn (5), where $f_{\text{recovery},i}$ is the fraction of each chemical *i* recovered by SPE. $f_{\text{recovery},i}$ was calculated using eqn (6), where $C_{\text{extract}}(\text{water} + \text{mix})$ is the measured chemical concentration in the spiked water extract (ng L⁻¹), $C_{\text{extract}}(\text{water})$ is the measured chemical concentration in the unspiked water extract (ng L⁻¹) and $C_{i,\text{nominal}}$ is the nominal chemical concentration spiked into the water (ng L⁻¹).

$$\text{BEQ}_{\text{chem,modelled 100\% recovery}} = \sum_{i=1}^n \left[\frac{C_i}{f_{\text{recovery},i}} \times \text{REP}_i \right] \quad (5)$$

$$f_{\text{recovery},i} = \frac{C_{i,\text{extract}}(\text{water} + \text{mix}) - C_{\text{water},i,\text{extract}}(\text{water})}{C_{i,\text{nominal}}} \quad (6)$$

Reverse recovery modelling was also applied to existing iceberg modelling studies from the literature.^{19–22} The predicted loss of effect by SPE was calculated using eqn (7).

Predicted loss of effect after SPE

$$= 1 - \frac{\text{BEQ}_{\text{chem,extract}}}{\text{BEQ}_{\text{chem,modelled 100\% recovery}}} \quad (7)$$

4. Results and discussion

4.1 Recovery of individual chemicals

The concentration of each chemical measured in the spiked water extract, along with the calculated $f_{\text{recovery},i}$ values, is provided in Table S4.† Of the 579 chemicals spiked, 29 were not detected at all after LVSPE, while a further 88 were not measurable as no calibration was obtained, either due to lack of ionization or high background noise. The majority of the 29 chemicals that were not detected after LVSPE were hydrophilic or charged compounds, with predicted log D_{ow} values less than 0.5. Another three compounds, 4-*n*-octylphenol, benzyldimethyldodecylammonium and lauramidopropylbetaine, were detected in the unspiked water extract at similar concentrations as in the spiked water extract due to background contamination, resulting in negative $f_{\text{recovery},i}$ values. Of the remaining 459 compounds, $f_{\text{recovery},i}$ ranged from 0.01 for the insecticide ethion to 3.08 for the pharmaceutical metabolite canrenone, with an average $f_{\text{recovery},i}$ of 0.70. As can be seen from Fig. 2, $f_{\text{recovery},i}$ for the majority of chemicals was between 0.75 and 1.25. The low recovery of ethion fits with previous studies, with Schulze *et al.*¹² finding no recovery of hydrophobic ethion by LVSPE using HR-X, the weak anion exchanger HR-XAW and weak cation exchanger HR-XCW. Thirty six chemicals had a $f_{\text{recovery},i}$ greater than 1. The higher recovery is likely to be due to the presence of an isobaric compound or a mismatch of the internal standard used for quantification, as only 40 isotope-labelled compounds were available, with the one with the closest retention time used for quantification. $f_{\text{recovery},i}$ was set to 1 for reverse recovery modelling for these chemicals.

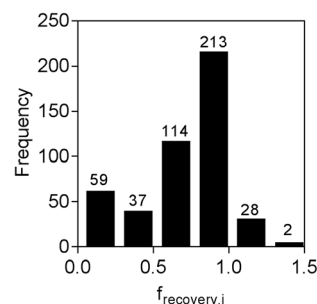


Fig. 2 Distribution of $f_{\text{recovery},i}$ for spiked chemicals ($n = 459$) in LVSPE. Six chemicals had a $f_{\text{recovery},i}$ greater than 1.5 (not shown).

The ratio of $f_{\text{recovery},i}$ for LVSPE from the current study to $f_{\text{recovery},i}$ from Schulze *et al.*¹² for HR-X only was calculated to compare recovery between studies (Fig. S1†). For 79% of common chemicals (165 out of 208 chemicals), the ratio was within a factor of 2, indicating that the results are generally reproducible. While the same sorbent, HR-X, was used, neutral, acidic and basic elution steps were undertaken in the current study, while only a neutral elution step was applied in Schulze *et al.*¹² As a result, greater recovery of some chemicals, such as positively charged quaternary ammonium compounds, was achieved in the current study.

To compare chemical recovery in the current study with a mixed sorbent SPE cartridge designed to capture a wide range of neutral and charged chemicals, the ratio of $f_{\text{recovery},i}$ for LVSPE from the current study to $f_{\text{recovery},i}$ for multi-layer SPE was determined (Fig. S1†). The calculated $f_{\text{recovery},i}$ values for multi-layer SPE are provided in Table S5.† Despite using different sorbents, $f_{\text{recovery},i}$ for 87% of common chemicals (153 out of 175 chemicals) was within a factor of 2 of the LVSPE method. Many of the compounds with greater than two times higher recovery in multi-layer SPE were either charged hydrophilic compounds with $\log D_{\text{ow}}$ less than 0 or hydrophobic compounds with a $\log D_{\text{ow}}$ greater than 4.

4.2 SPE process blank effects in bioassays

Bioassays cannot differentiate between the effects of micro-pollutants in a water sample and the effects associated with impurities from the extraction process, but their benefit is that they can provide information about the mixture effects of all bioactive chemicals. Solvent traces in bioassays, non-volatile residues from solvents, leachates from the LVSPE device, residues from SPE material and dirty glassware can potentially cause blank effects. Consequently, it is important to include procedural blanks as part of bioassay quality control. In the current study with LVSPE two procedural blanks were included, namely a process blank, where 5 L of ultrapure water was extracted by circulating through LVSPE four times, and a methanol blank to act as a solvent control. The volume of the ultrapure water in the process blank was restricted to 5 L to prevent potential effects from any trace level contamination in the water itself, rather than from the LVSPE material, glassware or solvents used.¹² Neither of the process nor solvent blanks induced a response in the bioassays, though cytotoxicity was observed at high sample enrichment (REF 306) in the AhR CALUX assay. For the other assays, no induction or cytotoxicity was observed up to the maximum tested REF, which ranged from 50 (MELN, HG5LN-hPXR) to 150 (GeneBLAzer assays) (Table S6, Fig. S2 to S11†).

In contrast, the multi-layer SPE produced higher blank effects, with the cytotoxicity 10% inhibitory concentration (IC_{10}) around REF 20 for several of the assays (Table S7 and Fig. S12†). We also tested the SPE blanks with the EnviCarb layer removed and with Oasis HLB only, as well as the effect of using methanol only or ethyl acetate and methanol for conditioning. The different sorbents and conditioning solvents did not have a significant influence on cytotoxicity (Table S7 and Fig. S12†).

In addition to cytotoxicity, some of the SPE blanks induced a response in the xenobiotic metabolism assays and the oxidative stress response assay. None of the SPE blanks had an effect at 24 or 48 h in the FET assay, though all blanks induced mortality after 120 h, with EC_{50} values ranging from REF 29 to 59. While the multi-layer SPE has the highest chemical recovery, this comparison also demonstrates that when optimising a SPE method not only maximum chemical recovery but also effect recovery and bioassay blank effects must be considered.

We recommend that samples are tested up to enrichments where no blank effects occur. In exceptional situations and at very high enrichments, when the process blank has an effect, the blank effect concentration in units of REF cannot be simply subtracted, but the BEQ of the blank can be subtracted by applying eqn (8). For this equation to be valid, the process blank should be prepared using the same SPE extraction and elution conditions as the sample.

$$\text{BEQ}_{\text{bio}}(\text{blank-corrected sample}) = \text{BEQ}_{\text{bio}}(\text{sample}) - \text{BEQ}_{\text{bio}}(\text{blank}) \quad (8)$$

4.3 Pristine water effects in bioassays

A pristine surface water sample was used as the matrix to assess effect recovery by SPE in the current study. Chemical analysis revealed that 43 chemicals were present at low concentrations in the unspiked water extract. Consequently, the effect of the water alone, $\text{BEQ}_{\text{bio,extract}}(\text{water})$, was included in eqn (2). In any case, it is still important to consider the effect of the water matrix alone even if no chemicals are detected as chemicals may still be present at concentrations below the analytical limit of detection. The $\text{BEQ}_{\text{bio,extract}}(\text{water})$ correction was zero for ER GeneBLAzer, AR GeneBLAzer, GR GeneBLAzer and PR GeneBLAzer as no effect was observed in the unspiked water extract in these assays, though it had a minor effect in the other assays (Table 2) and this effect was subtracted using eqn (2). Some of the chemicals detected in the unspiked water extract, including bisphenol A, estriol and propylparaben, are active in the studied bioassays (Tables S8 and S9†) and may have contributed to the effect observed in the unspiked water. It should be noted that the 43 chemicals found in the unspiked water extract were also detected at similar concentrations in the process blank (Table S4†), suggesting that they may have originated from the LVSPE material or solvent residues, rather than from Wormsgraben. Interestingly, the process blank did not induce a response in any of the assays, suggesting that other undetected chemicals may be contributing to the effect observed in the unspiked water extract.

4.4 Effect recovery by SPE

BEQ_{bio} values for the unspiked water extract ($\text{BEQ}_{\text{bio,extract}}(\text{water})$), mix stock solution ($\text{BEQ}_{\text{bio}}(\text{mix})$) and spiked water extract ($\text{BEQ}_{\text{bio,extract}}(\text{water} + \text{mix})$) are provided in Table 2, along with effect recovery (eqn (2)). All EC values are provided in Table S6,† along with the full concentration–effect curves in Fig. S2 to S11.† Effect recovery could not be calculated for FET as

Table 2 BEQ_{bio} values (M) for unspiked Wormsgraben water extract (water), mix stock solution (mix) and spiked Wormsgraben water extract (water + mix), with calculated bioassay recovery (%). Standard errors were calculated using error propagation

Assay	BEQ _{bio,extract} (water) (M) ± standard error	BEQ _{bio} (mix) (M) ± standard error	BEQ _{bio,extract} (water + mix) (M) ± standard error	Effect recovery (%) ± standard error
AhR CALUX	$(2.39 \pm 0.11) \times 10^{-14}$	$(1.56 \pm 0.08) \times 10^{-13}$	$(1.20 \pm 0.07) \times 10^{-13}$	61.2 ± 5.6
HG5LN-hPXR	$(3.90 \pm 0.69) \times 10^{-10}$	$(2.59 \pm 0.51) \times 10^{-10}$	$(3.76 \pm 0.86) \times 10^{-9}$	1300 ± 420
PPAR γ GeneBLAzer	$(2.95 \pm 0.27) \times 10^{-11}$	$(1.83 \pm 0.09) \times 10^{-10}$	$(3.10 \pm 0.29) \times 10^{-10}$	153 ± 18
MELN	$(6.68 \pm 0.96) \times 10^{-14}$	$(2.38 \pm 0.31) \times 10^{-11}$	$(2.94 \pm 0.34) \times 10^{-11}$	124 ± 21
ER GeneBLAzer	$<8.33 \times 10^{-13}$	$(4.27 \pm 0.17) \times 10^{-11}$	$(1.49 \pm 0.07) \times 10^{-11}$	34.9 ± 2.1
AR GeneBLAzer	$<2.63 \times 10^{-12}$	$(3.46 \pm 0.12) \times 10^{-11}$	$(4.33 \pm 0.16) \times 10^{-11}$	125 ± 6.4
GR GeneBLAzer	$<2.84 \times 10^{-11}$	$(1.76 \pm 0.08) \times 10^{-10}$	$(1.24 \pm 0.07) \times 10^{-10}$	70.5 ± 5.4
PR GeneBLAzer	$<5.07 \times 10^{-12}$	$(4.47 \pm 0.18) \times 10^{-11}$	$(2.96 \pm 0.15) \times 10^{-11}$	66.2 ± 4.3
AREc32	$(8.43 \pm 0.27) \times 10^{-8}$	$(1.49 \pm 0.05) \times 10^{-8}$	$(1.19 \pm 0.04) \times 10^{-7}$	236 ± 29

the unspiked water extract, mix stock solution and spiked water extract all resulted in similar concentration–effect curves (Fig. S11†).

If all bioactive chemicals spiked in the water extract were 100% recovered by SPE, BEQ_{bio} (mix) and BEQ_{bio,extract} (water + mix) (after subtraction of BEQ_{bio,extract} (water)) should be the same. Effect recovery for the studied bioassays was calculated using eqn (2) and ranged from 35% for ER GeneBLAzer to 236% for AREc32, with one extreme value of 1300% in HG5LN-hPXR. The effect recovery in HG5LN-hPXR is not likely to be representative, but is instead related to the small and rather variable effect of the mix stock solution in the assay, which is in the denominator of eqn (2) and therefore is strongly driving the effect recovery. Similarly, the variable response in the mix stock solution in ER GeneBLAzer may also explain the low recovery reported.

Effect recovery was within a factor of two of the optimal 100% effect recovery for AhR CALUX, PPAR γ GeneBLAzer, MELN, AR GeneBLAzer, GR GeneBLAzer and PR GeneBLAzer, suggesting that LVSPE is suitable to capture bioactive chemicals for the majority of applied assays.

It must be noted that the concentration axis in bioassays is typically on a logarithmic scale so a small variation on the concentration–effect curve might have quite dramatic effects on the calculated effect recovery. Most likely the samples where the BEQ_{bio,extract} (water) was below the detection limit are more robust than those where the BEQ_{bio,extract} (water) was subtracted from the BEQ_{bio,extract} (water + mix). Also, the BEQ addition and subtraction assumes that chemicals and samples act concentration additive, which is conceptually likely and recommended for mixture risk assessment of environmental mixtures^{35,36} but there might still be some variability due to the contribution of antagonistically or independently acting chemicals (*i.e.*, chemicals that act according to different modes of action).

This experimental case study demonstrates the difficulty in assessing recoveries directly with experimental data and the number of replicates would need to be increased to increase the power of the experiment. While a recovery range of 80–120% is desirable and within the range of uncertainty for chemical analysis, this range must likely be expanded for bioassays to a range of a factor of 2, *i.e.*, from 50% to 200%.

To get a better feeling of how much of the effect we would overlook by chemical losses incurred during SPE, we did reverse recovery modelling for the experimental data (Section 4.5) and also for case studies from the literature (Section 4.6).

4.5 Reverse recovery modelling of LVSPE extracts

The BEQ_{chem,extract} was calculated using the detected chemical concentration in the LVSPE extract and available REP_i values from the literature or the US EPA ToxCast database. REP_i values were available for between 4 and 45 chemicals in the different assays, with the EC and REP_i values provided in Tables S8 and S9.† Of the chemicals with REP_i values, five (4-*n*-octylphenol, acrylamide, amitraz, flufenoxuron and iopamidol) were not detected after LVSPE and could not be included in the BEQ_{chem,extract} calculations. No effect data could be found for the individual spiked chemicals in PR GeneBLAzer, so it was not possible to calculate BEQ_{chem,extract} for this assay, though some of the spiked chemicals, such as progesterone and canrenone, are active in other assays indicative of activation of PR.⁴

BEQ_{chem,extract} was compared to BEQ_{chem,modelled} 100% recovery predicted by reverse recovery modelling using $f_{\text{recovery},i}$ data from the current study to determine how much greater the effect would be if all chemicals were completely recovered by SPE. The predicted loss of effect after SPE ranged from 13% for the AR GeneBLAzer assay to 61% for the AREc32 assay (Table 3). For most assays, the predicted loss of BEQ by SPE was around 40%, which is less than a factor of two, *i.e.*, relatively small in relation to the variability of effect concentrations in bioassays. A ratio of two in effect concentrations such as EC₅₀ or EC₁₀ is a factor of ± 0.3 on a log scale and a standard deviation of ± 0.3 is more than typical for a log EC value derived from a log-sigmoidal concentration–effect curve.

4.6 Reverse recovery modelling of literature data

Recently, iceberg modelling using the BEQ concept (Fig. 1B) has been applied to determine the contribution of detected chemicals to the biological effect in surface water and wastewater.^{19,21,22} These studies all use the detected chemical concentrations after SPE to calculate BEQ_{chem,extract} but some chemicals may be poorly recovered by SPE, meaning BEQ_{chem,extract} may underestimate the true effect potential in

Table 3 $BEQ_{\text{bio,extract}}$, $BEQ_{\text{chem,extract}}$ and $BEQ_{\text{chem,modelled 100\% recovery}}$ for spiked Wormsgraben water

Assay	$BEQ_{\text{bio,extract}}$ (water + mix) (M)	$BEQ_{\text{chem,extract}}$ (M)	$BEQ_{\text{chem,modelled 100\% recovery}}$ (M)	% $BEQ_{\text{bio,extract}}$ (water + mix) explained by $BEQ_{\text{chem,extract}}$	% $BEQ_{\text{bio,extract}}$ (water + mix) explained by $BEQ_{\text{chem,modelled 100\% recovery}}$	% predicted loss by SPE
AhR CALUX	1.20×10^{-13}	4.31×10^{-17}	7.22×10^{-17}	0.04%	0.06%	40.3%
HG5LN-hPXR	3.76×10^{-9}	1.07×10^{-10}	2.06×10^{-10}	2.84%	5.48%	48.1%
PPAR γ	3.10×10^{-10}	1.31×10^{-12}	2.13×10^{-12}	0.42%	0.69%	38.6%
GeneBLAzer						
MELN	2.94×10^{-11}	1.90×10^{-11}	3.29×10^{-11}	64.7%	112%	42.1%
ER GeneBLAzer	1.49×10^{-11}	8.47×10^{-12}	1.29×10^{-11}	56.9%	86.8%	34.4%
AR GeneBLAzer	4.33×10^{-11}	8.31×10^{-11}	9.62×10^{-11}	192%	222%	13.6%
GR GeneBLAzer	1.24×10^{-10}	1.68×10^{-10}	2.91×10^{-10}	135%	234%	42.4%
AREc32	1.19×10^{-7}	1.28×10^{-10}	3.33×10^{-10}	0.11%	0.28%	61.6%

the water sample. Since concentration–effect curves are only linear at low effect levels we cannot just assume that an 80% average chemical recovery results in 80% effect recovery. In addition the composition might change in the extract and we do not expect any correlation between recovery and potency but this remains to be proven. Therefore, $BEQ_{\text{chem,modelled 100\% recovery}}$ was calculated for three studies that have previously applied LVSPE, Neale *et al.*,¹⁹ König *et al.*²¹ and Tousova *et al.*,²⁰ using $f_{\text{recovery},i}$ determined in the current study. Neale *et al.*¹⁹ used a LVSPE device with three sorbents in a row, HR-X, HR-XAW and HR-XCW, to extract surface water samples, but previous studies have shown that the majority of chemicals are primarily extracted using the neutral HR-X.¹² Despite the different elution steps used, results presented in Section 4.1 indicate that the majority of $f_{\text{recovery},i}$ values in the current study were similar to those obtained for HR-X by Schulze *et al.*,¹² therefore using $f_{\text{recovery},i}$ values from the current study for reverse recovery modelling for Neale *et al.*¹⁹ is still acceptable.

$BEQ_{\text{chem,extract}}$ and $BEQ_{\text{chem,modelled 100\% recovery}}$ from the current study and the literature^{19–21} were compared for assays indicative of activation of PXR, activation of ER and oxidative stress response in Fig. 3, with literature data shown for the FET

assay. Comparisons for assays indicative of binding to PPAR γ , activation of AR and p53 response are provided in Fig. S13.† Generally, $BEQ_{\text{chem,extract}}$ was within a factor of 2 of $BEQ_{\text{chem,modelled 100\% recovery}}$, indicating less than 50% loss of effect after LVSPE. The exceptions included one sample from Neale *et al.*¹⁹ (JDS 59) in the activation of PXR and FET assays, with 60% and 64% predicted loss of effect after LVSPE, respectively. Similarly, up to 92% loss by LVSPE was predicted for several samples in Tousova *et al.*²⁰ for the FET assay. In all examples this could be attributed to the poor recovery of triclosan, which had a $f_{\text{recovery},i}$ of 0.06. Similarly, low recovery of triclosan was also observed previously,^{12,37} and may be related to the hydrophobicity of triclosan, which has a log K_{ow} of 4.98 (Chemaxon, Table S3†). Therefore, strong sorption of triclosan to the HR-X sorbent and/or LVSPE materials is likely, with the solvents used for elution seemingly unable to completely desorb triclosan from the LVSPE device.

$BEQ_{\text{chem,modelled 100\% recovery}}$ was also calculated for Neale *et al.*,²² where a suite of bioassays were applied to surface water extracts collected from streams in Switzerland upstream and downstream of wastewater treatment discharges. As multi-layer SPE was used for sample enrichment prior to bioanalysis, multi-

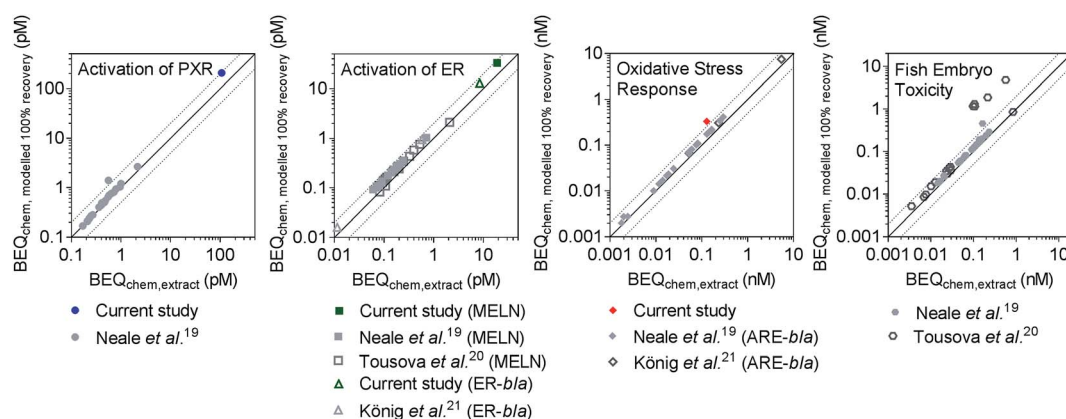


Fig. 3 Comparison of $BEQ_{\text{chem,extract}}$ and $BEQ_{\text{chem,modelled 100\% recovery}}$ for activation of PXR, activation of ER (both ER GeneBLAzer (ER-bla) and MELN), oxidative stress response and fish embryo toxicity for the current study, Neale *et al.*,¹⁹ König *et al.*²¹ and Tousova *et al.*²⁰ The dotted lines indicate a factor of 2 difference between $BEQ_{\text{chem,extract}}$ and $BEQ_{\text{chem,modelled 100\% recovery}}$. NB: two different oxidative stress response assays based on the same endpoint were included, with AREc32 applied in the current study and ARE GeneBLAzer (ARE-bla) applied in Neale *et al.*¹⁹ and König *et al.*²¹



Fig. 4 Comparison of $BEQ_{chem,extract}$ and $BEQ_{chem,modelled}$ 100% recovery for activation of AhR, activation of AR, oxidative stress response and the combined algae assay (open symbols indicate PSII inhibition and closed symbols indicate algal growth inhibition) from Neale *et al.*²² derived from the recovery data of the multi-layer SPE (Table S5†). The dotted lines indicate a factor of two difference between $BEQ_{chem,extract}$ and $BEQ_{chem,modelled}$ 100% recovery.

layer SPE $f_{recovery,i}$ values measured in the current study were applied for reverse recovery modelling. The studied assays were indicative of activation of AhR, activation of AR, oxidative stress response, photosystem II inhibition and algal growth inhibition. No information was available on the recovery of 4-nonylphenol, alifuzosin, bisphenol A, estrone, etodolac and ritonavir by multi-layer SPE. Therefore, $f_{recovery,i}$ data for LVSPE were used for 4-nonylphenol, bisphenol A and estrone, given similar recoveries in the multi-layer SPE and LVSPE (Fig. S1†), while a $f_{recovery,i}$ of 1 was assumed for the other chemicals. $BEQ_{chem,extract}$ was within a factor of 2 of $BEQ_{chem,modelled}$ 100% recovery for all assays (Fig. 4), indicating a good agreement and a minor loss of effect after SPE.

While the reverse recovery modelling approach has some limitations, such as the lack of recovery data for some of the detected chemicals and lack of effect data for some others, it suggests that there are no substantial losses of effect equivalents due to SPE. This indicates that the current method of iceberg modelling for environmental water samples is meaningful.

5. Conclusions

A complementary chemical analysis and bioanalysis approach was applied in the current study to assess the chemical and effect recovery of a complex mixture of chemicals by SPE. Overall, comparison with other studies and different extraction processes indicates that chemical recovery by LVSPE in the current study is within an acceptable range. The majority of chemicals were well recovered by LVSPE, with 79% and 87% of spiked chemicals having $f_{recovery,i}$ values within a factor of 2 of previously measured recovery values for LVSPE and for multi-layer SPE from the current study, respectively. Effect recovery was determined from experimentally quantified effects in the spiked water extract, the unspiked water extract and mix stock solution. For the majority of assays, experimental effect recovery was within a factor of 2 of the expected 100% recovery, though small variations in the concentration–effect curve may have implications for the calculated effect recovery. Reverse recovery modelling of existing published studies that applied LVSPE and multi-layer SPE for bioanalysis indicated that in most cases there was no substantial loss of effect by SPE. Further, the

theoretical correction for chemical losses using reverse recovery modelling is a useful approach to predict effect when chemical recovery is less than 100%. Overall, the current study found that available SPE methods for bioanalysis are appropriate, with effect recovery similar to recovery by chemical analysis and that we can confidently apply bioassays after SPE extraction without fear of substantial loss of effect due to incomplete SPE recovery. This provides support for the use of current SPE methods with low blank effects for bioanalytical assessment and the application of bioassays for water quality monitoring and for assessing treatment efficacy in natural and engineered treatment systems.

Conflicts of interest

There are no conflicts to declare.

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