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RESEARCH PAPER

Solid-phase extraction of estrogens and herbicides from environmental waters for bioassay analysis—effects of sample volume on recoveries

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Abstract

Ecotoxicological screening of surface waters can involve multiple analyses using multiple bioassay and chemical analytical methods that require enriched samples to reach low concentrations. Such broad screening of the same sample necessitates sufficient sample volume—typically several liters—to produce a sufficient amount of enriched sample. Often, this is achieved by performing parallel solid-phase extractions (SPE) where extracts are combined into a pool—this is a laborious process. In this study, we first validated our existing SPE method for the chemical recovery of an extended set of compounds. We spiked four estrogenic compounds and 11 herbicides to samples from independent rivers (1 L) and wastewater treatment plant effluents (0.5 L). Then, we investigated the effect of increased sample loading of the SPE cartridges on both chemical and biological recoveries by comparing the validated volumes with four times larger sample volumes (i.e., 4 L river water and 2 L effluent). Samples were analyzed by LC-MS/MS and three bioassays: an estrogen receptor transactivation assay (ERα-CALUX), the combined algae test, and a bacterial bioluminescence inhibition assay. Our existing SPE method was found to be suitable for enriching the extended set of estrogens and herbicides in river water and effluents with near to perfect chemical recoveries (~ 100%), except for the herbicide metribuzin (46 ± 19%). In the large volume river and effluent samples, the biological activities and concentrations of the spiked compounds were between 87 and 104% of those measured with the lower sample loading, which is adequate. In addition, the ratio between the large and original volume SPE method for the non-target endpoint (bacterial bioluminescence inhibition) was acceptable (on average 82 ± 9%). Results indicate that our current water extraction method can be applied to up to four times larger sample volumes, resulting in four times more extract volumes, without significant reductions in recoveries for the tested estrogens and herbicides.

Keywords Photosynthesis inhibition · *Raphidocelis subcapitata* · Bacterial bioluminescence inhibition · ERα-CALUX · LC-MS/MS · Solid-phase extraction · LiChrolut EN/RP-18

Introduction

Effect-based techniques (i.e., bioassays) play an increasing role in ecotoxicological assessments of surface waters and monitoring of micropollutant removal efficiency of wastewater treatment plants (WWTPs) [1–7]. Often, bioassays are used together with chemical analysis, to complement conventional chemical monitoring (e.g., [8]). Numerous studies showed the beneficial use of such combined approaches (e.g., [9–13]). For example, bioassays can be deployed as pre-screening tools to distinguish between clean and contaminated sites. In addition, in combination with chemical analytics, bioassays can reveal the identity of unknown bioactive micropollutants in water samples, as often the target-analyzed chemicals are only partly contributing to the observed effects.

Prior to such joint biological and chemical analyses, environmental water samples are often enriched—typically with solid-phase extraction (SPE) or sometimes liquid-liquid extraction—enabling the measurement of low (effect) concentrations, often in picogram per liter ranges (e.g., [14, 15]). Enrichment is also needed to compensate for sample dilutions

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during bioassay-specific exposure designs [16]. Proper sample preparation is crucial for the outcome of further analysis [5, 17–19]. In spite of current advances in high-resolution chromatographic instrumental techniques, which can detect analytes at femtogram levels (e.g., [20]) and highly sensitive bioassays with limits of detection at picomolar levels (e.g., [19]), the analysis can be spoiled by unsuitable sample preparation.

SPE is the most widely used technique for concentrating water samples due to its numerous advantages, such as high efficiency, low organic solvent consumption, and broad spectrum of commercially available sorbent material and formats [17, 21, 22]. A disadvantage of SPE is that the sample loadability of an SPE cartridge is limited and depends on the type and composition of the sample extracted. Wastewater effluents or wastewater receiving surface waters, in particular, can contain surfactants, salts, and large organic/humic compounds and vary in pH levels. It also has to be kept in mind that sorption materials in SPE cartridges are more or less selective and do not sorb all organic compounds with the same efficiency.

Besides the selection of the applied sample enrichment method, the choice of the bioassay battery greatly depends on the aim of the study and the chemical groups of interest that share a common mode of action (i.e., exerting similar effects) [3, 16, 23, 24]. Here, we focus on estrogenic and herbicidal activities, which are relevant endpoints for ecotoxicological water quality testing and investigated regularly in Swiss rivers [1, 2, 23, 25–28].

Concerning estrogenic effects, steroidal estrogens, bisphenol A, alkylphenols, and phthalates are considered to be the main contributing compounds (e.g., [29, 30]). Three steroidal estrogens (i.e., 17α-ethinylestradiol [EE2], 17β-estradiol [E2], and estrone [E1]) are currently included in the so-called watch list mechanism of the EU Water Framework Directive (WFD) [31]. This means that more monitoring data on these compounds in European water bodies must be generated to assess their ecological risk. In vitro bioassays with various cell lines (e.g., yeast, fish, human) carrying estrogen receptor (ER) alpha constructs (in most of the cases, the human ER) are commonly used to monitor estrogenicity [32]. These assays have been used worldwide [9, 30, 33–37], were shown to be robust [32, 38, 39], and have recently been adopted as ISO standards [40].

Concerning herbicidal effects, compounds such as terbuthylazine, terbutryn, and diuron are important drivers of photosystem II (PSII) inhibition in surface waters. These compounds predominantly originate from agricultural activities and their use as biocides [27, 41, 42]. Currently, six PSII inhibitors can be found among the WFD priority substances (atrazine, irgarol, diuron, isoproturon, simazine, and terbutryn) [43]. The combined green algae test robustly measures PSII inhibition and effects on algal growth rates exerted by a broader spectrum of herbicidal compounds as well as baseline toxicants (i.e., non-specifically acting chemicals exerting non-specific toxicity) [44–46].

The objective of the present study was twofold. First, we validated the LiChrolut EN/RP-18 SPE method for a broad range of herbicides and estrogenic compounds that are routinely monitored surface water contaminants in Switzerland. This extraction method was developed earlier [47] for the analysis of estrogenic compounds in municipal wastewaters and used and further assessed for measuring unspecific toxicity and estrogenic and PSII-inhibiting effects by in vitro bioassays [1, 2, 18, 39]. However, a comprehensive validation, especially for a broad range of herbicides often found in Swiss water bodies (i.e., atrazine, chlortoluron, diuron, irgarol, isoproturon, lenacil, linuron, metribuzin, simazine, terbutryn, terbuthylazine), has not yet been performed.

Second, we investigated increased loading of the LiChrolut EN/RP-18 SPE cartridges to enhance efficiency and reduce sample preparation procedures. To provide for a robust evaluation and to have a representative matrix diversity, we sampled 12 independent WWTP effluents and associated receiving rivers. Samples were spiked, enriched, tested in bioassays, and subjected to chemical analysis. Recoveries were compared between original sample volumes (i.e., 0.5-L effluent and 1-L river water) and four times larger volumes (i.e., 2-L effluent and 4-L river water), after extraction on single cartridges. The maximal factor (four)—by which the sample load was increased—was chosen based on practical reasons. An optimal, maximal volume was selected that provides large enough extract volume for multiple chemical and biological analyses, while it is still practically workable (i.e., can be performed on the same single cartridge per sample without any clogging and is still doable within a day).

Samples were spiked with a mixture of estrogenic compounds and PSII-inhibiting herbicides at quantifiable levels (i.e., well above the bioassay quantification limit and within the calibration range of the chemical analysis). To explore the extraction efficiency of cartridges for a wider range of unknown and unspiked chemicals, the extracts were additionally tested using the bacterial luminescence inhibition assay [48, 49].

Methods and materials

Sampling of effluents and river water

Samples were collected from 12 independent locations in three Swiss cantons: six locations in the canton of Zürich, three in Aargau, and three in Thurgau, to obtain a representative sample matrix diversity. Sampling was spread over four sampling campaigns, in each campaign, three locations were visited. At each location, WWTP effluent and river water

downstream from the outlet of the WWTP were sampled. Samples were collected in aluminum bottles (MENKE Industrieverpackungen GmbH & Co. KG, Seevetal, Germany) and transported at 4 °C to the lab to minimize any bio-, chemical-, or photodegradation. As field blank, ultrapure water was transported along during sampling.

An overview of samples within a sampling campaign and their subsequent testing is depicted in the Electronic Supplementary Material (ESM, Figs. S1 and S2).

Sample preparation and extraction

Water samples were filtered over glass fiber filters (Milian, Wohlen, Switzerland) and acidified to pH 3 ± 0.1 with HCl on the day of sampling. Then, 0.5 and 2 L of effluent and 1 and 4 L of river water were spiked to evaluate the extraction method performance and monitor the recovery of the SPE with different volumes. The spiking mixtures consisted of four estrogenic compounds (estrogen mixture) and 11 PSII-inhibiting herbicides (herbicide mixture) (Table 1, Table S2 in ESM). Individual compounds were dissolved in ethanol and then mixed together at the desired spiking concentration (Table 1). The spiking mixtures only contained compounds, of which relative potency (REP) towards the reference substance in the pertinent bioassay was determined earlier in our lab (data not shown). We used three criteria to set the desired spiking concentration: (1) roughly equipotent contributions from each individual mixture component; (2) a total mixture concentration well above the quantification limit (LOQ) of the different bioassays; (3) suitable for the chemical analytical calibration range. One batch of each mixture was prepared for spiking all samples in each sampling campaign. The spiking volume for each mixture was 100 μL/L water.

Estrogen and herbicide spiking mixtures were analyzed before the experiment started to verify (1) concentrations of the individual spiked compounds and (2) effect concentrations in the bioassays. In addition, the spiking mixtures were also tested for their actual concentrations and activity in each sampling campaign ($n = 4$) to ensure and control the correctness of the spiking. Results of these control measurements are shown in Tables S3 and S4 in the ESM.

Spiked samples were stored overnight at 4 °C. The next day, samples were extracted using a solid-phase extraction (SPE) 20-port vacuum manifold system (Biotage, Uppsala, Sweden). SPE cartridges (6 mL, filled with 100 mg LiChrolut EN and 200 mg LiChrolut RP-C18 [VWR, Dietikon, Switzerland]) were conditioned using two times 1 mL of hexane, two times 1 mL of acetone, three times 2 mL of methanol, and three times 2 mL of acidified water (pH 3.0). After application of samples (at maximal 3–4 drops

Table 1 Overview of the estrogens and photosystem II-inhibiting herbicides used for spiking samples and field blanks. Bioassay reference compounds are highlighted in italic font

Compound	CAS registry no.	Purity*	Nominal conc. in the spiked water samples (ng/L)**
Estrogen mixture (nominal activity, 35 ± 2 ng 17β-estradiol-equivalence [EEQ]/L)***			
Estrone	53-16-7	≥ 99%	56
17β-Estradiol hemihydrate	50-28-2	97.5%	14
17α-Ethinylestradiol	57-63-6	≥ 98%	13
Bisphenol A	80-05-7	≥ 99%	56
Herbicide mixture (nominal activity, 404 ± 26 ng diuron-equivalence [DEQ]/L)***			
Atrazine	1912-24-9	99.1%	200
Chlortoluron	15545-48-9	99.5%	100
Diuron	330-54-1	99.0%	100
Irgarol	28159-98-0	98.4%	20
Isoproturon	34123-59-6	99.9%	100
Lenacil	01.08.2164	99.9%	40
Linuron	330-55-2	99.7%	100
Metribuzin	21087-64-9	99.7%	50
Simazine	122-34-9	99.9%	200
Terbutryn	886-50-0	99.1%	20
Terbuthylazine	5915-41-3	99.4%	100

*Some of the product purities might be corrected by the supplier when manufactured

**Nominal chemical concentration indicates the theoretical concentration of the individual substances added to the spiking mixture. These concentrations were measured and verified by LC-MS/MS and listed in Table S3

***Nominal activity indicates the activity measured in the respective assays for these spiking mixtures (more details in Table S4, ESM)

per second), cartridges were washed with 10 mL acidified water (pH 3.0), dried under a nitrogen stream for about 1 h, and then eluted sequentially four times with 1 mL acetone and once with 1 mL methanol. The volume of the extracts was reduced under a gentle nitrogen stream to ca. 500 μL; "original volume" samples were filled up with ethanol to a final volume of 1 mL, "large volume" samples to 4 mL. As a result, effluents were nominally enriched by 500 times and river water samples by 1000 times.

Quality control

In each sampling campaign, three field blanks (nanopure water in an aluminum bottle; Fig. S3 in ESM) were carried along to the field, extracted in the lab, and subjected to bioassay and LC-MS/MS analyses. Two field blanks were spiked with estrogen and herbicide mixtures prior to the extraction (SPE). One of those was additionally spiked with an internal standard (IS) mixture. The third field blank was not spiked (Fig. S3 in ESM).

To control each SPE run, 4 L of ultrapure water was extracted along with the samples and analyzed (SPE blank). The volume of the field and SPE blanks was set to 4 L, as this was the highest extracted sample volume in this study.

The organic solvent used to add extracts to the bioassays was tested bioassays in each run (solvent blank).

LC-MS/MS analysis of estrogens and herbicides

Chemical analysis of estrogens and herbicides was performed on an Agilent G6495A Triple Quadrupole (QQQ) mass spectrometer coupled to an UHPLC system for chromatographic separation (more measurement parameters are described in the ESM). The external standard calibration with matching deuterated analytes (i.e., adding the IS mixture to the extracts directly before LC-MS/MS analysis) was performed to determine the concentrations (ng/L) of the individual estrogens and herbicides by Agilent MassHunter quantitative analyses. Calibration and IS solutions are described in Table S2 (see ESM).

Identification of the selected analytes was performed based on two multiple reaction monitoring (MRM) transitions between the precursor ion and the two most abundant product ions. The first transition was used for quantification purposes ("quantifier"), whereas the second ("qualifier") was used to confirm the presence of the target compound in the sample. Quantified analytes were identified by comparing the retention time (RT) of the corresponding standard and the ratio between two ion transitions recorded (± 30%) in the standard and water samples. Limit of detection (LOD) for the chemical analysis was determined as a signal-to-noise (S/N) ratio of 3:1, and limit of quantification (LOQ) as an S/N ratio of 10:1. However, as all samples were spiked, determining and reporting concentrations around LOD or LOQ was never an issue in this study.

Bioassays and derivation of effect concentrations

Quality control samples (field, SPE, and solvent blanks), extracts of spiked water samples, and the estrogen and herbicide spiking mixtures were tested for estrogenicity using the ERα-CALUX assay, for herbicidal effects using the combined algae test, and for unspecific toxicity, using the bacterial bioluminescence inhibition assay. Brief method descriptions are provided in Table 2 and more details provided in ESM.

Biological activities of sample extracts were expressed as biological equivalence concentrations (ng or mg BEQ/L water). To derive BEQ, concentration-response relationships of reference compounds and a dilution series of sample extracts, blanks, and spiking mixtures were fitted using a four-parameter non-linear regression with normalized data as described earlier in similar studies [6, 24, 39, 49]. The fit was computed with variable slope in the ERα-CALUX. For the PSII inhibition endpoint in the combined algae test, the slope of the sample curve was adapted to the slope of the reference, diuron. A fixed slope of 1 was applied for the inhibition of bacterial bioluminescence and algal growth in the combined algae test. Normalization was performed as follows. The minimum was fixed based on the bioassay induction measured for the negative control (0% effect level) in all three assays. The maximum (100% effect level) was fixed (i) based on the highest fitted induction of the reference substance in the ERα-CALUX, (ii) the maximal bioluminescence inhibition, and the complete growth and photosynthesis inhibition in the algae test. The response of the sample dilution causing 10% (ERα-CALUX) or 50% effect (combined algae test and bacterial bioluminescence inhibition assay) was interpolated from the reference curve to determine the positive control concentration needed to induce 10 or 50% effect (PC10 and PC50). This positive control concentration was then divided by the corresponding sample concentration to obtain the BEQ of the sample. The sample concentration incorporates the enrichment by SPE and the dilution of the extract in the bioassay.

Fitting and normalization differences are due to the different nature of the assays applied. The bacterial bioluminescence inhibition assay and combined algae tests are tests with known maximum response (e.g., no cell growth or inhibition of photosynthetic yield), where responses can be converted to a maximum effect (100%). The ERα-CALUX is a reporter gene assay capturing the transactivation of the receptor, where the maximum response is defined by the reference compound saturating the receptor without causing cytotoxicity [24].

Table 2 Testing conditions of the original volume and large-volume SPE extracts, spiking mixtures, field, SPE, and organic solvent blanks in three in vitro bioassays. Extracts were tested in twofold dilution series and each test concentration in duplicate for the algae test and bacterial bioluminescence assay and in threefold dilution series in triplicate for ERα-CALUX. These exposure designs aimed to provide full concentration-response relationships and suitable plate layouts

	Test system*	Highest sample concentration** tested in the assay		Reference compound	Measured endpoint
		Effluent	River water		
ERα-CALUX	Stably transfected U2OS human osteosarcoma cell line with hERα construct	0.5	1	17β-Estradiol	Luminometric measurement of the luciferase activity after 24 h
Combined algae test	Unicellular green algae (*Raphidocelis subcapitata*)	67	133	Diuron for PSII inhibition and virtual toxicant for the growth inhibition	Fluorometric measurement of the effective quantum yield of energy conversion at PSII reaction after 2 and 24 h and photometric measurement of the cell growth after 24 h
Bacterial bioluminescence inhibition assay	Bioluminescent marine bacteria (*Aliivibrio fischeri)*	125	333	Virtual toxicant	Photometric measurement of the bioluminescence after 30 min

*Detailed references to these protocols are in the ESM

**Sample concentration includes all sample manipulation steps and equals the enrichment factor$_{extraction}$ × dilution factor$_{bioassay}$

Validation of the original SPE method

One field blank, one effluent, and one river water sample in each of the four sampling campaigns were collected (in total, eight samples and four field blanks) and extracted in duplicate. One set of duplicates was spiked with estrogenic and herbicidal chemical mixtures as well as the IS mixture before extraction ("IS-controlled SPE extract"). The experimental design is described in Figs. S1-S3, (see ESM). These extracts were analyzed solely by LC-MS/MS but not by bioassays. The other set of samples was spiked with estrogenic and herbicidal chemical mixtures prior to extraction; the IS mixture was then added to the extract only prior to LC-MS/MS analysis and not prior to SPE ("IS-non-controlled SPE extract"). This way, the extraction efficiency (i.e., the recovery) for individual spiked compounds (i) was controlled and determined using Eq. 1:

$$\text{Recovery}_i\ (\%) = \frac{\text{Concentration}_i\ (\text{IS}-\text{non}-\text{controlled SPE extract})}{\text{Concentration}_i\ (\text{IS}-\text{controlled SPE extract})}\ \text{x}100 \quad (1)$$

An extraction efficiency (chemical recovery) above 80% for individual compounds and a measurement error of 15% (i.e., relative standard deviation, RSD%) were considered acceptable.

In addition to chemical recoveries, matrix effects on extraction efficiency of spiked compounds were determined using Eq. 2. Here, we followed methods described in previous studies with slight modifications [14, 18].

$$\text{Matrix effect}_i = \frac{\text{Extraction efficiency}_i\ (\text{river water or effluent})}{\text{Extraction efficiency}_i\ (\text{field blank})} \quad (2)$$

For ideal performance, matrix effects are close to one. Matrix effects higher than one suggest that there is a background concentration of the respective spiked compound. Matrix effects lower than one indicate the interference of the matrix during extraction and thus a reduced extraction efficiency for the spiked compound. There could also be a combined effect, i.e., a value close to one could also be caused by a combination of background concentration and matrix interference.

Evaluation of the large volume SPE performance

To assess the performance of the SPE with a large sample volume, we compared results of analytical and bioassay measurements between the two methods. Results are expressed as percentage average recovery and the standard deviation of the average recovery of the

respective samples (% ± SD). A maximum deviation of 20% was considered acceptable.

Quality control of the methodological aspects

Performance of the chemical analysis and bioassays were comprehensively assessed by negative and positive control measurements. All validity criteria (listed in the ESM) were met, e.g., no biological activities or concentrations above LOQ of the target analytes (spiked compounds) were detected in the solvent-, extraction-, and field blanks.

An aliquot of the estrogen- and herbicide-spiking mixtures in each sampling campaign ($n = 4$) was always separated when the samples were spiked. These aliquots were then tested along the sample extracts by both bioassays and LC-MS/MS and considered as 100% recovery of the (effect)concentrations.

The measured individual concentrations of the compounds (by LC-MS/MS) in the neat mixture and their effect concentrations tested in the bioassays matched perfectly with the theoretical concentrations (on average 108 ± 7%; Tables S3 and S4 in ESM). High precision (RSD% ≤ 6%) was found for the four measurements in both bioassay and chemical analyses (Tables S3 and S4 in ESM). The neat spiking mixtures showed pure estrogenic and herbicidal activities (i.e., the estrogenic mixture was inactive in the combined algae test and the herbicide mixture in the ERα-CALUX; data not shown).

Results and discussion

Robust SPE method with original sample volumes—with one exception

The original SPE protocol was developed for a selection of estrogenic compounds and PSII inhibitors which are routinely monitored in Swiss surface waters. Both estrogenicity and PSII inhibition are ecologically relevant endpoints [1, 2, 18, 39]. We intended to validate this SPE method for additional PSII inhibitors and at the same time investigate the impact of extracting larger sample volumes with various sample matrix compositions (i.e., 12 river waters and 12 effluents). The extended, large volume LiChrolut SPE EN/RP-C18 extraction method was found to meet essential requirements for high accuracy (i.e., acceptable chemical recovery of the spiked compounds; > 80%) and high precision (i.e., low relative standard deviation of the parameters measured in a large number of samples; < 15%). Extraction efficiency in the field blanks was nearly perfect (99 ± 4%, average) for 14 spiked compounds and somewhat lower for BPA (in average 76 ± 12%) (Fig. 1).

For all but one compound (i.e., metribuzin), recoveries remained high in the presence of sample matrix: 101 ± 6% for the four river water samples (1 L) and 99 ± 8% for the three effluents (0.5 L) (Fig. 2; ESM Table S7). One of the four effluent (i.e., from Fehraltorf) samples was excluded. Reasons are described in detail later (in the section "Larger sample volumes hardly impact SPE recoveries of estrogen and

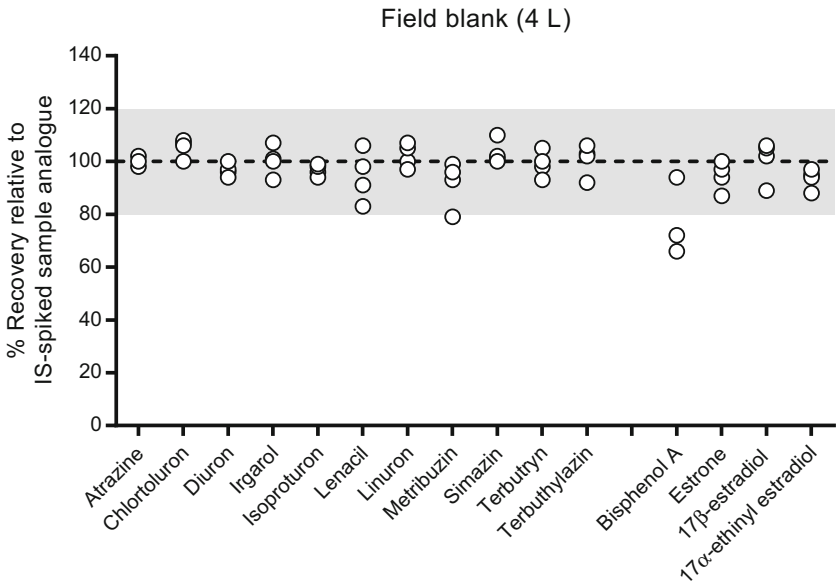

Fig. 1 SPE recoveries (%) of the spiked compounds determined in the four field blank samples by LC-MS/MS. Concentrations measured in the internal standard (IS)-spiked sample pairs (analogue) were taken as nominal concentrations (i.e., 100% recovery as indicated by the dashed line). Dots represent single-recovery ratios of the pertinent spiked compound in a single sample. An acceptable recovery range of ± 20% is indicated by the shaded area

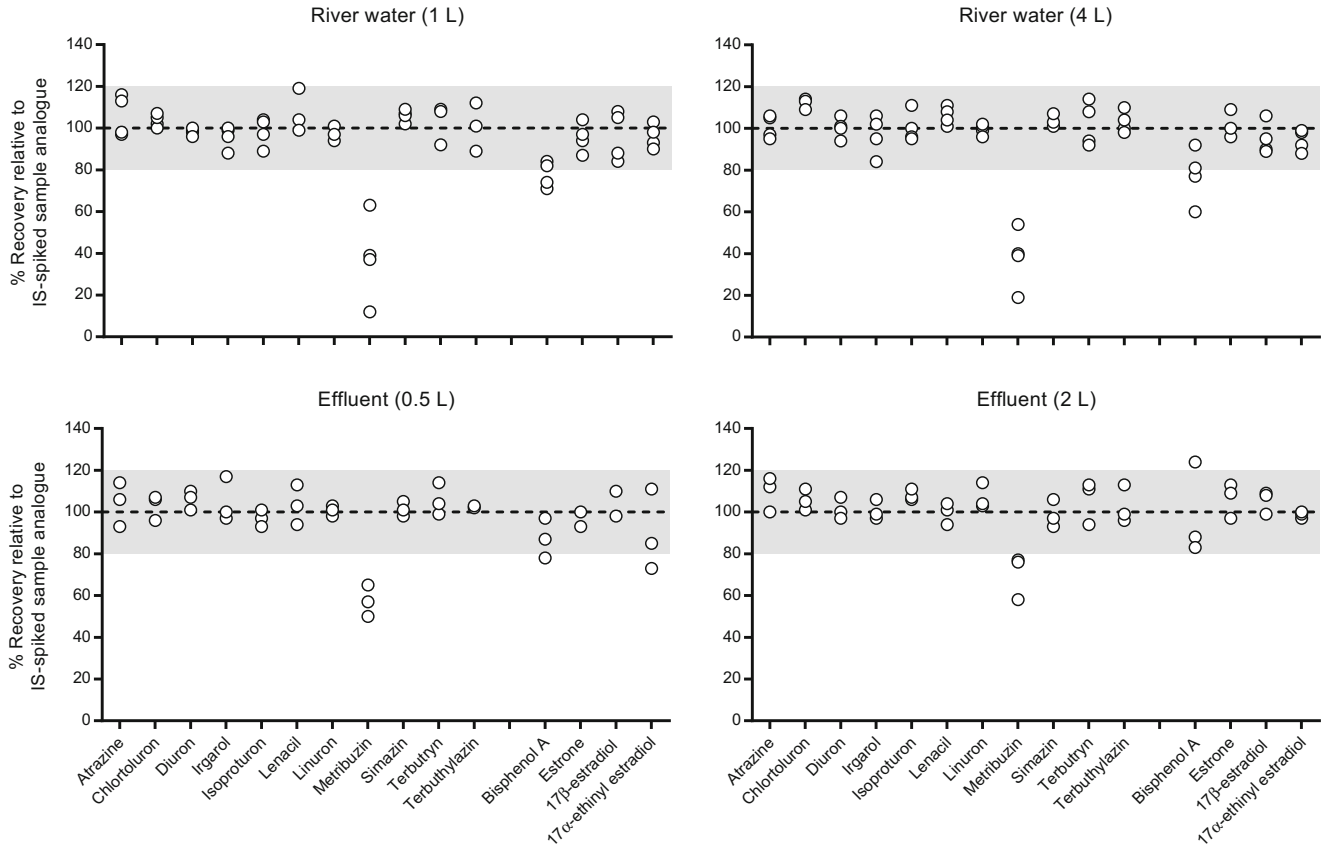

Fig. 2 SPE recoveries (%) of the spiked compounds determined in both original and large-volume river water samples ($n = 4$) and effluent samples ($n = 3$) by LC-MS/MS. Concentrations measured in the internal standard (IS)-spiked sample pair (analogue) were taken as nominal concentrations (i.e., 100% recovery as indicated by the dashed lines). An acceptable recovery range of ± 20% is indicated by the shaded area

herbicide mixtures"). There was no matrix effect on extraction efficiency for any spiked compound (i.e., no interference of the matrix with the extraction efficiency; data not shown) except for metribuzin (matrix effect of 0.49 ± 0.24).

Metribuzin challenges

Metribuzin (a triazine herbicide, such as atrazine and simazine) was the only compound where the extraction efficiency was affected by sample matrix. Its IS-controlled recovery ratio in the spiked field blanks, without any sample matrix, was high (92 ± 7%), with the exception of one replicate field blank, where recovery ratio was slightly below 80% (Fig. 1) most likely being an outlier. Whereas, in effluent samples, 57 ± 7% of the spiked metribuzin was recovered, and in river water, it was only 38 ± 7%.

Metribuzin is known to show high (often irreversible) binding affinity towards humic acids and organic matter in waters leading to poor recoveries [50, 51]. Such interference of organic material with SPE recoveries of other triazine herbicides (atrazine and simazine) was described earlier. Johnson et al. [52] suggested the formation of humic acid-herbicide complexes which are then retained by the C18-bonded silica cartridges as well as the saturation of sorptive sites of the cartridge material resulting in lower recoveries of the desired analytes. Similarly, in the study of Hela et al. [53], recoveries of different classes of pesticides decreased with increasing organic matter content in water samples. In addition, the interaction of metribuzin with dissolved organic matter in water seems to be highly pH dependent, explaining our low SPE recoveries for metribuzin at pH = 3. Acceptable recovery ratios were found for metribuzin at pH = 7, especially when salts were added to increase the ionic strength of the water samples [54]. Sabik et al. [55] recommended the elimination of humic acid interferences by using polymer-based cartridges at pH = 7 instead of C18-based cartridges at pH = 3 when extracting pesticides from water.

Besides a poor recovery, the quantification of metribuzin by LC-MS/MS was also challenging. Signal-to-noise ratios were often low (< 10) which hampered a proper quantification of metribuzin (Table S5 in ESM).

The dilemma of robust SPE recovery controls for bioassays

When SPE recovery is poor, LC-MS/MS results are not necessarily negatively affected. Suitable IS controls can easily be implemented to overcome extraction and quantification challenges and to correct for losses during extraction and ionization matrix effects (i.e., suppression or enhancement of the analyte signal). However, for bioassays, a poor SPE recovery will directly lead to an underestimation of ecotoxicological effects in the extracted sample and the exploration of possible sample matrix interferences is challenging. The application of internal standards in bioassays is not recommended, since they may contribute to the observed bioassay responses and lead to overestimation of the total biological activities [19] and because the relationship between concentrations of mixtures and bioassay response is not necessarily linear. Bioassays measure combined effects, and they cannot differentiate between the effect of the bioactive chemicals present in the sample and the effect caused by the co-extracted sample matrix impurities or internal standards. Extensive QA/QC data collection and interpretation, particularly of replicate samples, are necessary to validate the performance of an extraction method and asses matrix effects on recoveries of the selected compounds. An independent recovery analysis on a per sample basis is not possible. To obtain a minimum level of robustness, each SPE run should not only include a parallel blank SPE control and field blank, but also a recovery control with spiked substances. In addition, several real matrix recovery tests should be included to safeguard a minimum assurance of a satisfactory recovery across a sampling campaign. As an alternative to a spiking mixture, the inclusion of a well-characterized and representative internal reference material as recovery control into each SPE can be considered. Even so, checking such positive control SPEs will not provide an absolute guarantee that all other SPEs run in the same sampling campaign were also adequate. Finally, even the best recovery controls can only control for effects of target compounds and not "unknown compounds," particularly those compounds causing non-specific toxicity.

Spiking experiments—such as we describe in this study—can also help to better explore the recovery of biological effects. Here, three elements have to be considered [15, 56]: testing a neat spiking mixture in the bioassay representing the maximal predictive effect (1). Then, testing the procedure blank (e.g., HPLC-grade water for water analysis) previously spiked with the spiking mixture representing the extraction efficiency without the sample matrix (2). Finally, if possible, extracting one or more environmental water sample(s) directly (native sample) and after the addition of the spiking mixture (matrix spike) (3). Ideally, the unspiked sample analogues gather information on background activities present. In the current study design, testing of the native water samples was, however, not included. Our ultimate goal was to study the impact of larger sample load on the extraction performance relative to our original method and irrespective to the individual chemical or effect recoveries. Ideally, the (un)spiked procedure blanks and water samples should be analyzed not only after, but prior to the enrichment as well, which is technically challenging. Neale et al. [15] also pointed out the lack and necessity of adequate controls for combined biological and chemical validation of extraction methods and designed an extensive study to investigate possible solutions to overcome these difficulties. Good correlations were observed between individual chemicals and total effect recovery of mixture components for both multilayer- and large volume SPE. However, currently, no golden rules exist that solve uncertainties associated with recovery, matrix effects, and compound-specific matrix interferences that are involved with SPE followed by bioassay analyses.

Larger sample volumes hardly impact SPE recoveries of estrogen and herbicide mixtures

Comparability of the original and large volume SPE was extensively investigated: we used a relatively large and representative sample set (independent samples from 12 rivers and 12 effluents), employed three bioassays and analyzed 15 compounds by LC-MS/MS (ESM Table S6). To evaluate results, we used recoveries obtained with our original sample volumes as a basis (i.e., 100%) and compared them to recoveries obtained with large sample volumes. One effluent sample pair (original and large volume SPE samples from "Fehraltorf") was excluded from the data set due to a technical failure (see ESM, text "Exclusion Fehraltorf sample pair" and ESM Table S1). LC-MS/MS data indicated that part of the sample—used for the original SPE method—most likely was lost during sample handling. Therefore, the number of tested effluents was reduced from 12 to 11.

Figure 3 summarizes the results of three bioassays and chemical analysis of two groups of chemicals (with effluent "Fehraltorf" excluded). Both LC-MS/MS and bioassay results indicate that the currently used water volumes extracted per cartridge can be increased fourfold without negative effects on recoveries of a wide range of compounds. The average biological activities and chemical concentrations in the large volume SPE (i.e., four times larger volume) effluent and river water extracts were between 79 and 104% of nominal values (i.e., those measured in the original extraction procedure) (Fig. 3; Table S5 in ESM).

Estrogens were extracted with high accuracy and precision (100 ± 2%; RSD% = 2%) from large sample volumes of both river water and effluent (Fig. 4; Table S5 in ESM). The LC-MS/MS results were in line with ERα-CALUX results (94 ± 10%; RSD% = 11%; Table S5 in ESM). Apart from metribuzin, excellent agreement was also obtained between

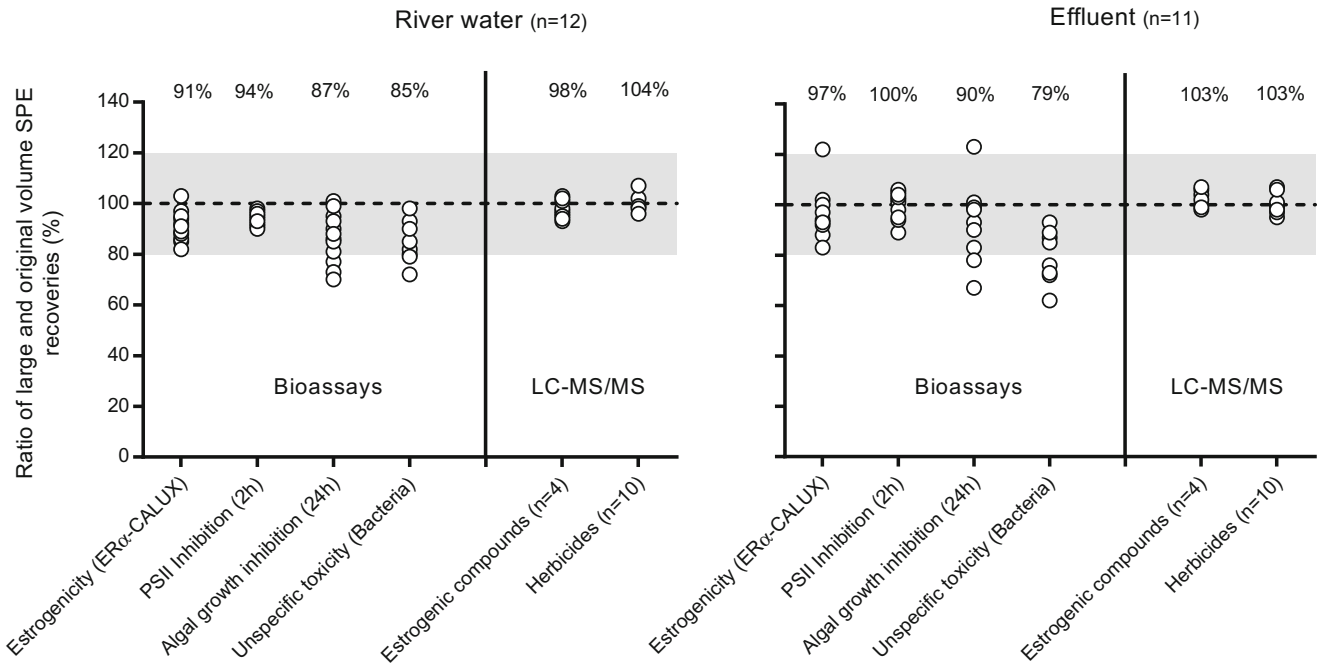

Fig. 3 Comparison of the large-volume SPE method to the original volume SPE method. The *y*-axis shows the ratio of recoveries of biological activities and chemical concentrations of the spiked compounds: Measurement $_{\text{large-volume SPE}}$/Measurement $_{\text{original volume SPE}}$. Dashed lines indicate 100% comparability between the large-volume and the original SPE method. Dots show recovery ratios of 11 independent effluents and 12 independent river water samples. For bioassays, each dot represents a single-effect concentration measured in the sample. For chemical analysis, each dot represents averaged concentrations of either four estrogens or ten herbicides in each sample (metribuzin measurements were excluded, due to the large distribution of the metribuzin data, see Fig. 5). Data labels show the average of the respective recovery ratios. An acceptable recovery range of ± 20% is indicated by the shaded area

spiked herbicides (100 ± 2%, RSD% = 2%; Fig. 5; Table S5 ESM) and results of the algae test (97 ± 7%, RSD% = 7% for the PSII inhibition endpoint and 88 ± 14%, RSD% = 16% for algal growth inhibition).

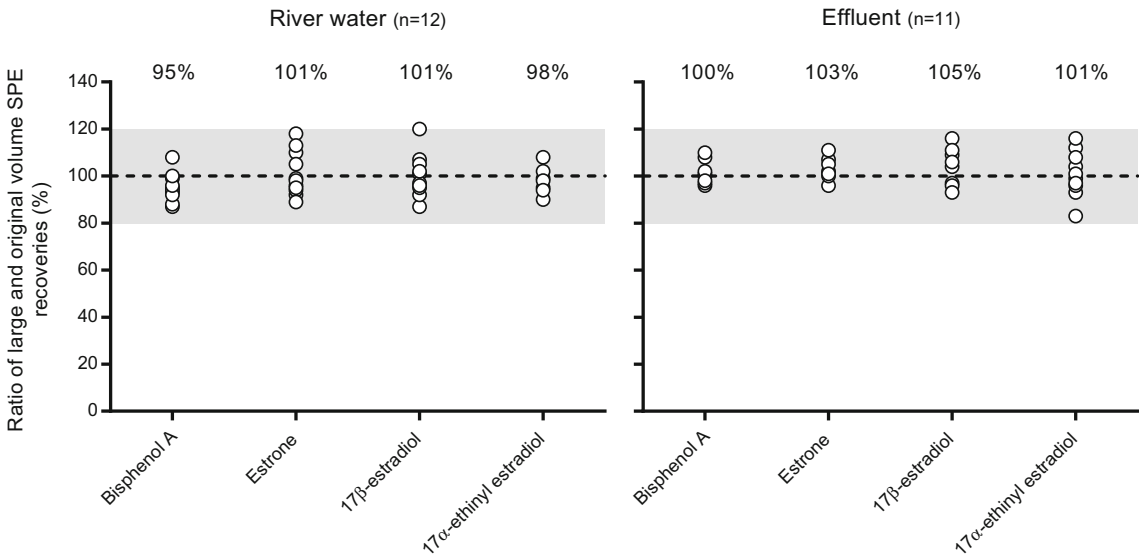

Fig. 4 Comparison of the large-volume SPE method to the original SPE method for the four estrogens in both river water and effluent samples. Dots show the recovery ratio = Measurement $_{\text{large-volume SPE}}$/Measurement $_{\text{original SPE}}$. Dashed lines indicate perfect comparability between the large-volume and the original SPE method for the estrogenic compounds. An acceptable recovery range of ± 20% is indicated by the shaded area

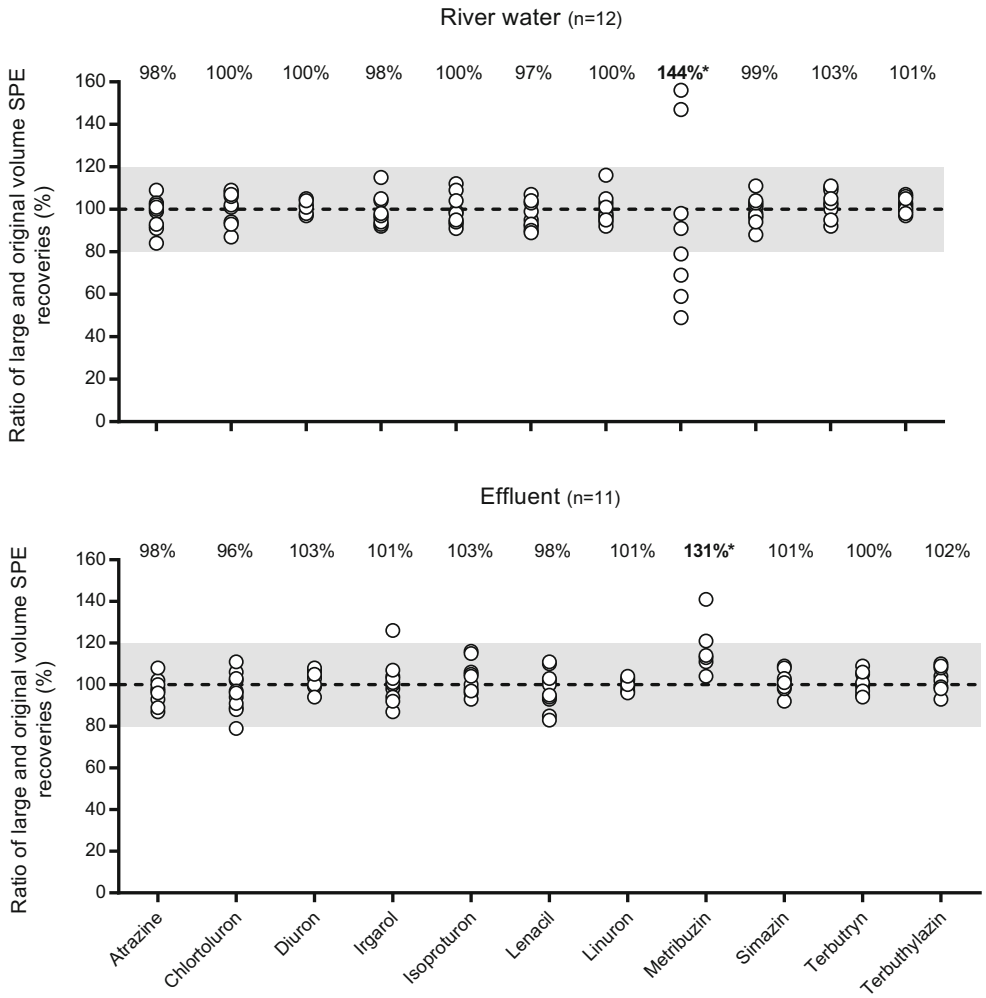

Fig. 5 Comparison of the large-volume SPE method with the original volume SPE method for the 11 herbicides in both river water and effluent samples. Recovery ratios (Measurement $_{\text{large-volume SPE}}$/ Measurement $_{\text{original volume SPE}}$) are shown as dots. Dashed lines indicate perfect comparability between the large-volume and the original SPE method. An acceptable recovery range of ± 20% is indicated by the shaded area. Asterisk symbol indicates recovery ratios above 160% were cut off and not presented in this figure (for these data, see Table S5 in the ESM)

In the majority of the samples (> 70%), metribuzin showed unacceptable recoveries (recovery ratios of 49–350% (Tables S5 and S7 in ESM). This was also observed with the original extraction procedure and was not a result of increased sample volume extraction.

Non-target endpoints show slightly reduced recoveries at higher sample volumes

One of the main aims of our study was to evaluate the extraction efficiency of our original "low volume" SPE method with a fourfold larger sample volume to support extended or multiple bioassay and LC-MS/MS analyses of a single sample. However, for bioassay analyses of environmental samples, one does not a priori know the involved chemicals and thus cannot control or assess their extraction efficiency. For LC-MS/MS analyses, we focused on spiked compounds (estrogens and herbicides). In addition, effects measured by ERα-CALUX were dominated by spiked compounds (estrogens). The combined algae test covers two endpoints: PSII inhibition and algal growth rate. The PSII endpoint is highly specific to and driven by PSII inhibitors—again, compounds that we spiked to all samples. For all these analyses, results from both SPE methods were very comparable and robust. It is, however, unclear how efficient both SPE methods are for non-specific effects. We explored this question in more detail for two non-specific endpoints: algal growth rate and bacterial bioluminescence.

Growth rate integrates effects of PSII inhibitors but also a much broader range on non-specifically acting compounds (i.e., baseline toxicants)—compounds that were likely present in our river water and effluent samples. Figure 3 shows that recovery of the growth endpoint in algae is slightly reduced compared with that of the PSII endpoint (there is a significant

difference between the means confirmed by a two-tailed paired t test, $p = 0.0011$). Though the recovery ratio for algal growth is still above the 80% target, this significant effect probably indicates a minor reduction in the recovery of unknown non-specifically acting compounds.

To assess the applicability of the large volume extraction method for another non-specific endpoint, we used the bacterial bioluminescence inhibition assay. Like algal growth, bacterial bioluminescence can be inhibited by non-specifically acting compounds. First, we tested the pure spiking mixtures in the bacterial luminescence inhibition assay. Neither the estrogen nor the herbicide mixture induced an effect (data not shown). Second, we tested all spiked sample extracts and blanks. Clear effects were observed in sample extracts (not in blanks), so non-specifically acting toxicity was present in our samples. Third, we compared the activities measured in the large volume SPE extracts with the measurements of the original method. Recovery ratios for effluent were 79 ± 9% ($n = 11$) and for river water 85 ± 7% ($n = 12$). These values are slightly lower when compared with the other (specific) effect-based methods (87–100%, Fig. 3; Table S5 in ESM). A recovery ratio of 79% is just below our target value of 80%.

As mentioned above, there is a dilemma that robust SPE recovery assessments are crucial for bioassays—particularly for unknown compounds—but at the same time, target analytical recovery verification or spiking of unknowns is not possible. Though this may seem an unsurpassable challenge, comparing SPEs with different sample volumes (focusing on unspecific toxic endpoints) could help to verify if recoveries are still within acceptable ranges (e.g., a recovery ratio > 80%).

Conclusions

We found that

i. LiChrolut EN/RP-C18 SPE is suitable to enrich the four tested estrogens (estrone, 17β-estradiol, 17α-ethinylestradiol, and bisphenol A) and a broad range of PSII-inhibiting herbicides (i.e., atrazine, chlortoluron, diuron, irgarol, isoproturon, lenacil, linuron, simazine, terbutryn, terbuthylazine) from river water and effluent. The extraction and chemical analysis of metribuzin was challenging, influenced by the sample matrix but independent from the extracted volume.
ii. Increased sample volume did not affect the extraction efficiency of mixtures of estrogens or herbicides. The extraction of 0.5-L effluent or 1-L river water gave near identical recoveries as 2-L effluent or 4-L river water. Therefore, up to four times larger sample volumes (4-L river water and 2-L effluent) can be extracted on single LiChrolut SPE EN/RP-C18 cartridges resulting in four times larger extract volumes.
iii. Our study targeted estrogens and herbicides. Although results were excellent for spiked compounds (except metribuzin) and the directly associated endpoints, caution is warranted concerning recovery of non-specifically acting compounds. Analysis of the samples with the bacterial bioluminescence inhibition assay (addressing unspecific toxicity) showed that the activities measured were just below our target recovery ratio of 80%.
iv. Robust recovery testing is crucial for SPE followed by bioassays, as no IS correction of results is possible as it is for LC-MS/MS analyses. We strongly advise to include various SPEs of samples spiked with relevant compounds—as employed in our study—to underpin results from bioassay analyses of SPE extracts. As an additional quality control, testing how recoveries are affected by sample volume could further support SPEs for bioassay analyses.

Author contributions Eszter Simon, Inge Werner, and Etiënne Vermeirssen conceived the study. Eszter Simon, Andrea Schifferli, Thomas Bucher, Daniel Olbrich, and Etiënne Vermeirssen planned the experiments. Andrea Schifferli, Thomas Bucher, and Daniel Olbrich carried out the sampling, the sample preparation and the bioassay and chemical analyses. Andrea Schifferli, Thomas Bucher, Daniel Olbrich, and Eszter Simon performed the calculations. Eszter Simon took the lead in writing the manuscript. All authors contributed to the revision of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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