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# Targeted multi-analyte UHPLC-MS/MS methodology for emerging contaminants in septic tank wastewater, sludge and receiving surface water†

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Septic tanks treat wastewater of individual houses and small communities (up to 2000 people in Scotland) in rural and semi-urban areas and are understudied sources of surface water contamination. A multi-analyte methodology with solid phase extraction (SPE), ultra-sonic extraction, and direct injection sample preparation methods was developed to analyse a comprehensive range of emerging contaminants (ECs) including prescription and over-the-counter pharmaceuticals and related metabolites, natural and synthetic hormones, and other human wastewater marker compounds in septic tank influent and effluent, river water, suspended solids, and septic tank sludge by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The number of quantifiable compounds in each matrix varied from 68 in septic tank wastewater to 59 in sludge illustrating its applicability across a range of matrices. Method quantification limits were  $2.9 \times 10^{-5}$ – $1.2 \mu\text{g L}^{-1}$  in septic tank influent, effluent and river water, with  $\leq 0.01 \mu\text{g L}^{-1}$  achieved for 60% of ECs in all three water matrices, and  $0.080$ – $49 \mu\text{g kg}^{-1}$  in sludge. The developed method was applied to a septic tank (292 population equivalents) and the receiving river in the North-East of Scotland. Across all samples analysed, 43 of 68 ECs were detected in at least one matrix, demonstrating the method's sensitivity. The effluent concentrations suggest limited removal of ECs in septic tanks and a potential impact to river water quality for some ECs. However, further monitoring is required to better appreciate this. The developed methodology for a wide variety of ECs in a range of liquid and solid phases will allow, for the first time, a comprehensive assessment of ECs fate and removal in septic tanks, and their impact to surface water quality.

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## Introduction

Over the past years, a large variety of emerging contaminants (ECs), such as prescription or over-the-counter pharmaceuticals and related metabolites, natural and synthetic hormones, and other human wastewater marker compounds (*e.g.*, caffeine), have been reported in various water sources worldwide in the ng to  $\mu\text{g L}^{-1}$  range.<sup>1–5</sup> Due to their incomplete removal in conventional (biological) wastewater treatment, and ubiquitous presence in influent, treated wastewater discharges are considered the main entry source of ECs into the environment.<sup>6–8</sup>

So far, research has focused on centralised wastewater treatment works (WWTWs) and their receiving surface

waters.<sup>1,7,9–11</sup> However, it is conservatively estimated that 9% of the Scottish population, are served by a public or privately owned septic tank.<sup>12–14</sup> Septic tanks are typically located in rural and semi-urban areas and treat wastewater from individual houses and small communities (up to 2000 people in Scotland).<sup>4,14</sup> In a watertight underground tank, often designed as a series of rectangular chambers, heavy solids settle as sludge to the bottom, while oil, grease and lighter solids float to the top.<sup>12</sup> The sludge and scum need to be removed from the tank (typically every few months to every few years), and transported to a centralised WWTWs for further treatment.<sup>15</sup> The septic tank effluent might be further treated, for example through subsoil infiltration systems, before being released into the ground or a nearby water body.<sup>12,16</sup>

Septic tank effluents can contain ECs in higher concentrations than in centralised WWTWs.<sup>13,17</sup> For instance, Stanford and Weinberg<sup>17</sup> reported the active ingredient in hormonal contraceptives  $17\alpha$ -ethinylestradiol up to  $0.4 \mu\text{g L}^{-1}$  in a septic tank effluent serving a boarding school for girls, which is 4- to

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400-times higher than centralised WWTWs influents.<sup>18</sup> In a septic tank, ECs can be removed through the physical separation of the sludge and scum, when they are bound to particles or oil, and *via* anaerobic biodegradation.<sup>19</sup> However, there is little information on the performance of septic tanks for the removal of ECs, and the effect of septic tank discharges to water quality. To this date, most studies focused on a few compounds only (maximum = 22),<sup>13,20–24</sup> and there is a lack of multi-analyte methods for the analysis of ECs in septic tanks.

Most commonly, ECs are analysed by reversed-phase liquid chromatography coupled to tandem mass spectrometry as a highly sensitive and selective detector (LC-MS/MS).<sup>9–11</sup> It is a suitable approach to determine low concentrations of ECs in the presence of other organics at comparatively high concentrations in complex environmental matrices, such as wastewater.<sup>9,11,19,25</sup> Typically, solid phase extraction (SPE) is used to enrich, isolate and/or purify the target ECs, with reversed-phase hydrophilic-lipophilic balanced (HLB) polymeric sorbents being the most common.<sup>3,26,27</sup> Although a wide range of compounds can be analysed with HLB sorbents, recoveries are low for very polar compounds such as the antidiabetic drug metformin.<sup>28–30</sup> Hence, for very polar ECs, direct injection is proposed as a second sample preparation method.<sup>30</sup> In wastewater, different ECs are present in a wide concentration range from low ng L<sup>-1</sup> (e.g., ciprofloxacin) to high µg L<sup>-1</sup> (e.g., metformin).<sup>29,31,32</sup> As septic tanks are used by fewer people than centralised WWTWs, the variations in concentration and detection of ECs in effluents can be higher.<sup>13</sup> The wide concentration range, poses a challenge for 'SPE-only' methods, as it requires the dilution and re-analysis of samples following data processing, when concentrations are above the calibration range.<sup>13,33</sup> At the same time, method detection limits for ECs present at lower concentrations might not be reached by direct injection. Analysing each sample by direct injection and after SPE, allows the determination of a comprehensive range of ECs of different polarities over a wide concentration range without the need for further sample processing (e.g., dilution) and re-analysis.

Environmental samples are typically filtered prior to analysis to remove suspended solids. Due to the extra effort associated with analysing both matrices, most studies focus on the aqueous part of the sample only.<sup>7,34</sup> However, ECs can adsorb to solid particulate matter, and desorb again once in the environment.<sup>1,35</sup> Thus, analysing the aqueous part of the sample only leads to underestimation of the total concentration in the sample.<sup>11</sup> Furthermore, in wastewater treatment, ECs can also adsorb to sludge, and for instance enter the environment when sludge is applied in agriculture.<sup>36</sup> Most studies analysed ECs only in the liquid phase of septic tank effluent,<sup>20,21</sup> and the receiving water bodies.<sup>13,22–24,37</sup> Developing a multi-analyte method for the analysis of ECs in septic tank influent and effluent, including suspended solids, sludge, and the receiving surface water will allow a more accurate assessment of the performance of septic tanks for the removal of ECs and their effect to water quality. The most common methods for the extraction of ECs from solid environmental matrices, such as suspended solids or sludge, are microwave accelerated extraction (MAE), pressurised liquid extraction (PLE), and ultra-sonic extraction (USE).<sup>1,11,30,38</sup> There is

little difference found in the performance and extraction efficiency of the three methods.<sup>36,39,40</sup> MAE and PLE are easier to automatise than USE. However, USE offers advantages due to low costs and easy operation for effective extraction of ECs from solid environmental samples.<sup>1,36</sup>

Therefore, the aim of the study was to develop a comprehensive multi-analyte methodology with SPE, USE, and direct injection as sample preparation methods to analyse a broad range of ECs in septic tank influent and effluent, river water, suspended solids, and septic tank sludge by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The developed method was applied to a septic tank and the receiving surface water in a rural area in the North-East of Scotland.

## Materials and methods

### Materials

A total of 68 ECs (prescription or over-the-counter pharmaceuticals and related metabolites, natural and synthetic hormones, and other human wastewater marker compounds) were selected for method development (S1: Table S1†). The selection included those identified in prioritisation schemes by the European Union (EU) and the United Kingdom (UK),<sup>41–46</sup> and those which posed the greatest threat to Scotland based on environmental risk assessment calculations (S2). Chemical names and properties of selected ECs and where they were obtained from are detailed in Tables S1 and S2.† Water was produced at ultra-pure quality in the laboratory (resistivity = 18.2 MΩ cm at 25 °C, PurA-Q18.2, LabPro, European Instruments, Oxford, UK), and methanol (HPLC grade, ≥99.9%) was purchased from Fisher Scientific (Loughborough, UK). Formic acid (≥99.0%, Fisher Scientific), ammonium formate (≥99.0%, Sigma Aldrich, Gillingham, UK), ammonium fluoride (NH<sub>4</sub>F, ≥99.99%, Sigma Aldrich), and ammonium hydroxide (NH<sub>4</sub>OH, 35%, Fison Instruments Ltd, Glasgow, UK) were used as mobile phase buffers and in ultra-sonic extraction. Oasis HLB (60 mg, 3 mL; and 200 mg, 6 mL) SPE cartridges were purchased from Waters (Manchester, UK). Poly-tetrafluoroethylene (PTFE), cellulose acetate (CA), polyvinylidene fluoride hydrophilic (PVDF-HL), and polyvinylidene fluoride hydrophobic (PVDF) Q-Fil syringe filter (13 mm, 0.22 µm) from Greyhound (Birkenhead, UK) were received from Crawford Scientific Ltd (Strathaven, UK) and glass fibre filter (GF/F) discs (0.7 µm, 47 mm) were purchased from Fisher Scientific.

Liquid samples (1 L septic tank influent, septic tank effluent, and river water), used during method development and validation were collected in the North-East of Scotland in polypropylene bottles in summer 2021. Samples were transported to the laboratory and frozen within 1 h after collection. The septic tank sludge (0.5 L) was collected in November 2021 with a custom-made polyvinylchloride sludge sampler (Fig. S1†), and frozen until processing.

### Sample preparation of liquid samples

In a preliminary study, four different syringe filters were tested to minimize loss of ECs during the filtration step. Wastewater



samples spiked with 60 ECs (available at the time of the experiment) were filtered through PVDF-HL, PTFE, CA, and PVDF syringes to determine any losses.

The SPE method (Fig. 1) was developed based on a previous method for the analysis of septic tank effluent and river water.<sup>13</sup> Initially, the samples were filtered under vacuum with a GF/F filter. Oasis HLB cartridges (3 mL, 60 mg) were conditioned under gravity with 2 mL methanol and 2 mL water for equilibration at a flow rate of 1 mL min<sup>-1</sup>. 50 mL wastewater, and 100 mL river water, were spiked with a 50 µL isotopic labelled surrogate working mix ( $c = 100 \mu\text{g L}^{-1}$ ), mixed and loaded onto the cartridges using vacuum at a flow rate of 5 mL min<sup>-1</sup> and then dried for 20 min. The samples were eluted under gravity with 4 mL methanol at a flow rate of 1 mL min<sup>-1</sup>, and the solvent was evaporated at 40 °C under nitrogen.<sup>13</sup> The dried residue was then redissolved in 500 µL water/methanol (95/5, v/v), and filtered through a PVDF-HL syringe filter prior to UHPLC-MS/MS injection. For direct injection, environmental samples were filtered through a PVDF-HL syringe filter, before 450 µL of the sample was spiked with 50 µL isotopic labelled surrogates ( $c = 100 \mu\text{g L}^{-1}$ ).

### Extraction of solid matrices by ultra-sonic extraction

The sludge was frozen and freeze dried using a Heto Drywinner freeze dryer by Copley. The selected ECs were extracted from solid matrices with a Clifton Range ultra-sonic water bath (280 W, 50/60 Hz) using three extraction cycles similar to that described by Al-Khazrajy and Boxall.<sup>25</sup> Briefly, 0.1 g of freeze-dried sludge (dry weight) was weighed into a 10 mL polypropylene centrifuge tube, spiked with 50 µL isotopically labelled surrogates ( $c = 100 \mu\text{g L}^{-1}$ ) and left overnight. In the first cycle, 2 mL of 2% NH<sub>4</sub>OH in methanol was added. The suspension was vortexed, ultra-sonicated for 15 min at 50 °C, and centrifuged at 2260 g for 15 min. The supernatant was collected in a 50 mL Duran® glass bottle. The extraction was repeated using 2 mL of 2% formic acid in methanol and then 2 mL of methanol. The combined supernatants were filtered through a wet GF/F disc and diluted with water to 100 mL

(methanol < 5%). The extracts were cleaned up by Oasis HLB SPE cartridges (6 mL, 200 mg) following the same procedure as described for the extraction of liquid samples. For sludge samples, the reconstituted extract was centrifuged for 10 min at 17 000g prior to filtration through a PVDF-HL syringe filter.

### Liquid chromatography tandem mass spectrometry

Samples were analysed with UHPLC-MS/MS using an ACQUITY UPLC system from Waters (Waters Corporation, Milford, MA) with a Xevo TQ-XS Triple Quadrupole Mass Spectrometer. Electrospray ionisation (ESI) was performed in both positive and negative modes with a capillary voltage of 2.6 kV, 3.00 low-mass (LM) resolutions, and 15.00 high-mass (HM) resolutions. The nebulising and desolvation gas was nitrogen, and the collision gas was argon. The gas temperature was 400 °C with a desolvation gas flow of 550 L min<sup>-1</sup>, and a nebulising pressure of 7.0 bar. The cone gas flow was 150 L h<sup>-1</sup>. The optimised ion energies were ion energy 1 = 0.1 V and ion energy 2 = 1.0 V in positive ionisation mode, and ion energy 1 = 1.0 V and ion energy 2 = 2.0 V in negative ionisation mode, respectively.

Two different mobile phases were used for the analysis of basic and acidic compounds in positive and negative ionisation, respectively.<sup>30</sup> Different additives to the mobile phase were tested. If not otherwise stated, the parameters were identical in both methods. Chromatographic separation was performed using reversed-phase ACQUITY UPLC Ethylene Bridged Hybrid (BEH) C18 columns (1.7 µm, 2.1 × 100 mm, Waters). The column temperature was kept constant at 50 °C. The injection volume was 2 µL and the flow rate was 350 µL min<sup>-1</sup>. A methanol-water-gradient along with additives was used as the mobile phase (S4: Table S3†). Additives were 5 mM ammonium formate and 0.1% formic acid in the positive ionisation method, and 0.5 mM NH<sub>4</sub>F in the negative ionisation method.

### Instrumental performance

The instrumental performance was validated in terms of detection and quantification limits, linearity, intra- and inter-day precision, and accuracy. All samples were spiked with isotopically labelled analytes as surrogate to correct for matrix effects and analyte loss during sample preparation ( $c = 10 \mu\text{g L}^{-1}$  at injection).<sup>13</sup>

The instrument detection (IDL) and quantification limits (IQL) for each analyte were determined by the lowest concentration with a signal-to-noise ratio (S/N) ≥ 3 or ≥ 10, respectively. Linearity was established through the injection of a range of standards between 0.05 and 100 µg L<sup>-1</sup> (S8: eqn S3†).

Intra-day precision and accuracy were determined by injecting standards at concentrations of 1, 10, and 50 µg L<sup>-1</sup> in triplicate within 24 h (S8: eqn S4 and S5†). This was repeated every 24 h over 3 days to establish inter-day precision and accuracy.

### Method performance

The method performance was assessed for septic tank influent and effluent wastewater, river water, and sludge, for detection and quantification limits, matrix effects, absolute and relative

Liquid Samples		Solid Samples	
<b>Sampling</b> <ul style="list-style-type: none"> <li>1 L grab sample in polypropylene bottle</li> <li>Stored at 4°C</li> <li>Filtration through GF/F (0.7 µm, 47 mm) within 24 h</li> </ul> <b>Direct injection</b> <ul style="list-style-type: none"> <li>PVDF-HL syringe filter (0.22 µm, 13 mm)</li> <li>450 µL waste-/river water + 5 ng surrogates</li> </ul> <b>SPE</b> <ul style="list-style-type: none"> <li>50 mL wastewater / 100 mL river water + 5 ng surrogates</li> <li>Oasis HLB (3 mL, 60 mg)</li> <li>Conditioning: 2 mL methanol, 2 mL water, flow rate 1 mL min<sup>-1</sup></li> <li>Sample loading: flow rate 5 mL min<sup>-1</sup>, dried for 20 min</li> <li>Elution: 4 mL methanol, flow rate 1 mL min<sup>-1</sup></li> <li>Evaporation (40°C, N<sub>2</sub>)</li> <li>Reconstitute in 500 µL water/methanol (95/5)</li> <li>PVDF-HL syringe filter (0.22 µm, 13 mm)</li> </ul>	<b>Sludge</b> <ul style="list-style-type: none"> <li>0.5 g grab sample, transported at 4°C and frozen at -20°C within 10 h</li> </ul> <b>Ultra-sonic extraction</b> <ul style="list-style-type: none"> <li>100 mg freeze-dried solid sample + 5 ng surrogates</li> <li>3x ultrasonic extraction (50°C, 15 min), centrifuged for 15 min at 2260 g</li> <li>(1) 2 mL 2% NH<sub>4</sub>OH in methanol; (2) 2 mL 2% formic acid in methanol; (3) 2 mL methanol</li> <li>Combine supernatants in 100 mL water</li> <li>Filtration through wet GF/F (0.7 µm)</li> </ul>	<b>Suspended solids</b> <ul style="list-style-type: none"> <li>Filter paper frozen at -20°C after filtration</li> </ul> <b>Ultra-sonic extraction</b> <ul style="list-style-type: none"> <li>Filter paper (7 mg suspended solids) + 5 ng surrogates</li> </ul>	<b>SPE</b> <ul style="list-style-type: none"> <li>Oasis HLB (6 mL, 200 mg)</li> <li>Reconstituted in 500 µL water/methanol (95/5)</li> <li>Centrifuged (10 min, 17000 g)</li> <li>PVDF-HL syringe filter (0.22 µm, 13 mm)</li> </ul>
Analysis by UPLC-MS/MS			
<b>UPLC</b> <ul style="list-style-type: none"> <li>BEH C18 columns (1.7 µm, 2.1 × 100 mm)</li> </ul>		<ul style="list-style-type: none"> <li>Column temperature = 50°C</li> <li>Injection volume = 2 µL</li> <li>Flow rate = 350 µL min<sup>-1</sup></li> </ul>	<ul style="list-style-type: none"> <li>Methanol – Water – Gradient with additives</li> <li>ESI+ : 0.1% HCOOH + 5 mM NH<sub>4</sub>FO<sub>3</sub></li> <li>ESI- : 0.5 mM NH<sub>4</sub>F</li> </ul>
<b>MS/MS</b> <ul style="list-style-type: none"> <li>Low-mass (LM) resolution = 3.00</li> <li>High-mass (HM) resolutions = 15.00</li> <li>Capillary voltage 2.6 kV</li> </ul>		<ul style="list-style-type: none"> <li>Nebulising gas = N<sub>2</sub></li> <li>Desolvation gas = N<sub>2</sub></li> <li>Collision gas = Ar</li> <li>Nebulising pressure = 7.0 bar</li> </ul>	<ul style="list-style-type: none"> <li>Desolvation gas flow = 550 L min<sup>-1</sup></li> <li>Cone gas flow = 150 L h<sup>-1</sup></li> <li>ESI+ : ion energy 1 = 0.1 V, ion energy 2 = 1.0 V</li> <li>ESI- : ion energy 1 = 1.0 V, ion energy 2 = 2.0 V</li> </ul>

BEH = Ethylene Bridged Hybrid, C18 = Octadecyl carbon chain bonded to silica, ESI+/- = positive/negative electrospray ionisation, GF/F = glass fibre filter, HLB = Hydrophilic-lipophilic balance, PVDF-HL = Polyvinylidene fluoride hydrophobic.

Fig. 1 Overview of analytical workflow from sample preparation to analysis, for liquid and solid samples by ESI+ and ESI- methods.



recoveries, precision, and accuracy. Samples were prepared at three concentrations in triplicate. Spike concentrations were 1, 10, and 50  $\mu\text{g L}^{-1}$  for direct injection of influent, effluent, and river water; 0.01, 0.1, and 0.5  $\mu\text{g L}^{-1}$  for SPE of influent and effluent; 0.005, 0.05, and 0.25  $\mu\text{g L}^{-1}$  for SPE of river water, and 50, 250, 500  $\mu\text{g kg}^{-1}$  for sludge (S5: Table S4†). Prior to spiking with ECs, samples were spiked with isotopically labelled ECs only and analysed to determine the analyte concentrations in the environmental samples. Water samples were analysed by direct injection and SPE (S5: Table S4†).

Absolute ( $\text{REC}_{\text{abs}}$ ) and relative recoveries (REC) were calculated following eqn (1) and (2) from peak areas ( $A$ ) and area ratios (ar) of spiked and unspiked (US) samples and standards (std), respectively.

$$\text{REC}_{\text{abs}} = \frac{(A_{\text{spiked}} - A_{\text{US}})}{A_{\text{std}}} \times 100\% \quad (1)$$

$$\text{REC} = \frac{(\text{ar}_{\text{spiked}} - \text{ar}_{\text{US}})}{\text{ar}_{\text{std}}} \times 100\% \quad (2)$$

The method detection (MDL) and quantification limits (MQL) were calculated for each analyte from the IDL and IQL, respectively, the recovery and concentration factor  $c_F$  using eqn (3) and (4).

$$\text{MDL} = \frac{(\text{IDL} \times 100)}{\text{REC} \times c_F} \quad (3)$$

$$\text{MQL} = \frac{(\text{IQL} \times 100)}{\text{REC} \times c_F} \quad (4)$$

REC and  $c_F$  were specific for each matrix and sample preparation method.  $c_F$  was 0.9 for direct injection, 100 for septic tank influent and effluent in the SPE method, and 200 for river water in the SPE method. For solid matrices,  $c_F$  is replaced with a conversion factor of 0.2  $\text{g mL}^{-1}$ , based on the extraction of 0.1 g sludge.

The relative standard deviation of the replicates was calculated for method precision. Accuracies were determined from the percentage deviation of the concentrations added to the samples from the calculated concentrations.

To ensure instrumental and method performance, blanks and quality control standards with concentrations of 1, 10, and 50  $\mu\text{g L}^{-1}$  were injected before and after every batch of samples.

### Application to a septic tank and receiving river

A septic tank and the receiving surface water in a rural area in the North-East of Scotland was investigated. The septic tank serves 292 population equivalents, with no tourist impact and around 8% non-household contribution.<sup>14</sup> The nominal dilution of the septic tank discharge into the river was calculated (S6: eqn S1 and S2†). The receiving river mainly flows through agricultural land, with single houses and smaller villages along side. In the catchment area, 1% of land use is classified as urban.<sup>47</sup> The largest settlement in the catchment area with a population of 3140 (mid-2020 estimate)<sup>48</sup> is located roughly 7

km upstream of the studied septic tank. It is served by a secondary biological WWTW that discharges into the river.

Sampling was conducted on the 10th of November 2021. Grab samples (1 L) were collected in polypropylene bottles at the influent and effluent point of the septic tank, in the river upstream and downstream of the septic tank discharge point at a minimum distance of five river widths, and from the sludge. Samples were transported to the laboratory at 4 °C. Liquid samples were filtered through 0.7  $\mu\text{m}$  GF/F membrane filters within 24 h, processed as described previously, and analysed within 48 h. The filter papers were frozen at  $-20$  °C until processing. The solids were extracted by ultra-sonic extraction following the previous description. All samples were prepared in duplicate.

## Results and discussion

### Liquid chromatography tandem mass spectrometry

All ECs were analysed using multiple reaction monitoring (MRM) transitions. The protonated ( $[\text{M} + \text{H}]^+$ ) or deprotonated molecular ion ( $[\text{M} - \text{H}]^-$ ) was monitored in ESI- and ESI+ mode, respectively. Following EU guidelines,<sup>49</sup> two MRM transitions were monitored for most ECs (one in the case of isotopic labelled surrogates), using the fragment with the highest response for quantification and the fragment with the second highest response for confirmation. Ion ratios were monitored. In accordance with the literature, only one stable fragment was found for ibuprofen, gemfibrozil and lidocaine,<sup>3,30,50</sup> which is considered semi-quantitative (optimised MS/MS parameters in S7: Table S5†).

Following optimisation of MS/MS parameters for all compounds the chromatography methods were developed using a methanol–water–gradient with additives as the mobile phase and a reversed-phase BEH C18 column. Two different mobile phases were used, since basic and neutral compounds are best analysed in positive ionisation mode from acidic solutions, whereas acidic compounds are more efficiently analysed in negative ionisation mode from basic solutions.<sup>51</sup> Different additives were tested to optimise separation, peak shape, and sensitivity. In the positive ionisation mode for the analysis of basic ECs, the use of 5 mM ammonium acetate with 0.1% formic acid was compared to using 5 mM ammonium formate and 0.1% formic acid. While the choice of ammonium salt generally had little effect on the chromatography, the peak shape improved substantially with ammonium formate in the mobile phase for metformin, guanlylurea, and paracetamol. The highly polar drug metformin and its aerobic bacterial metabolite guanlylurea are more suited to analysis by hydrophilic interaction chromatography (HILIC) columns,<sup>2,29</sup> but satisfactory chromatography could be achieved under reversed phased conditions with ammonium formate as an additive.

In the negative ionisation mode, ammonium hydroxide (0.1%) and different concentrations of  $\text{NH}_4\text{F}$  (0.1, 0.5 and 1 mM) in a methanol–water–gradient were considered to enable the analysis of estrogens together with acidic drugs.<sup>30,52</sup> Overall,  $\text{NH}_4\text{F}$  resulted in greater peak areas and sharper peaks than ammonium hydroxide. Improved sensitivity with  $\text{NH}_4\text{F}$  might



be due to the strong basicity of the fluoride anion, and hence increased deprotonation of ECs in the gas phase.<sup>53</sup> Lower  $\text{NH}_4\text{F}$  concentration increased the sensitivity for estrogens, with optimum concentrations being 0.1 mM. However, decreased sensitivity for ibuprofen was noted. Since estrogens are expected to be found in significantly lower concentrations in wastewater and river water compared to ibuprofen,<sup>6</sup> 0.1 mM  $\text{NH}_4\text{F}$  was considered for further method development. However, in wastewater a contamination was present in the  $17\alpha$ -ethinylestradiol MS/MS spectrum at the same retention time. This was resolved from the  $17\alpha$ -ethinylestradiol peak by increasing the  $\text{NH}_4\text{F}$  concentration to 0.5 mM. With the reversed-phase BEH C18 column, good separation, sensitivity, and peak shape was achieved for all compounds using a methanol–water–gradient along with 5 mM ammonium formate and 0.1% formic acid in the ESI+ method, and 0.5 mM  $\text{NH}_4\text{F}$  in the ESI– method (Fig. 2).

### Instrument performance

The IDL and IQL were determined as the lowest concentration with a  $\text{S/N} \geq 3$  and  $\geq 10$  and ranged from  $0.002$  to  $1 \mu\text{g L}^{-1}$ , and from  $0.005$  to  $5 \mu\text{g L}^{-1}$ , respectively (S8: Table S6†). For the majority of compounds,  $\text{IQL} \leq 0.5 \mu\text{g L}^{-1}$  was achieved. A wide range of IQLs is commonly observed in multi-analyte methods for compounds with a variety of physicochemical properties, and similar to what has been reported before.<sup>3,27,28,30</sup>

Linearity was established through the injection of standards at concentrations between  $0.05$  and  $100 \mu\text{g L}^{-1}$  ( $500 \mu\text{g L}^{-1}$  for paracetamol, ibuprofen, and metformin due to their higher concentrations in wastewater). A linear regression model was fitted (S8: eqn S3†), and the  $R^2$  was calculated. For the compounds without the isotopically labelled EC, a different deuterated surrogate was assigned (S8: Table S6†). The choice was based on retention time, structural similarity, and eventually linearity. The linear dependency was in range of  $0.938 \leq R^2 \leq 1.000$  (S8: Table S6†). Approximately two thirds of the ECs, 52 compounds in the positive method and four compounds in the negative method, have  $R^2$  values  $\geq 0.997$ . Atorvastatin and

miconazole were calibrated externally using peak area as there was no suitable deuterated surrogate. Calibrations with  $R^2 \geq 0.991$  were sufficient for accurate quantification, as indicated by the other instrumental performance criteria. Published studies for multi-analyte analysis of pharmaceuticals in wastewater accept  $R^2 \leq 0.990$ .<sup>11</sup> Miconazole, clotrimazole, and climbazole, have  $R^2 < 0.980$ , most likely due to the absence of suitable deuterated surrogate, and were analysed semi-quantitatively. Most compounds were linear over the whole concentration range from 0 to  $100 \mu\text{g L}^{-1}$ .

Intra- and inter-day accuracy and precision (S8: Table S7†) were determined by injecting three standards ( $c = 1 \mu\text{g L}^{-1}$ ,  $10 \mu\text{g L}^{-1}$ , and  $50 \mu\text{g L}^{-1}$ ) three times within 24 h, and repeatedly every 24 h over three days (S8: eqn S4 and S5†). In multi-analyte methods, accuracies are generally expected to be within an ideal range of 90–110%, or within the accepted range 80–120%.<sup>10,28,54</sup> A total of 63 compounds were accurate within the range of 90–110% in most samples above the IQL, with little or no difference between the intra- and inter-day accuracy ( $p > 0.05$ , S8: Table S7†). The remaining five compounds also have intra-day accuracies from 90% to 110% in most samples, but inter-day accuracies were 80% to 120% in most samples ( $0.004 \geq p \leq 0.046$ ). As repeating the calibration every day is time-consuming, few ECs with inaccuracies are accepted in multi-analyte methods.<sup>28</sup> QC standards were therefore injected with every batch to ensure accuracies stay within the accepted range. Calibrations were repeated after the mass spectrometer was turned off for an extended period of time, at least once a year, or if the QC data fell out with the performance data.

In general, relative standard deviations  $\leq 10\%$  are expected in the instrumental performance. However, higher standard deviations  $\geq 20\%$  are accepted for few ECs in multi-analyte methods, as long as other validation parameters are suitable.<sup>11,27</sup> In the developed instrumental method, 50 ECs were very precise over all concentrations studied above the IQL with a relative standard deviation  $\leq 10\%$  except the occasional one concentration in the intra- and inter-day analysis. Of the remaining compounds, 15 had a relative standard deviation  $\leq 20\%$  over all three concentrations above the IQL. The remaining three ECs had relative standard deviation  $\leq 10\%$  in most samples. Overall, the method was very precise with relative standard deviations  $\leq 10\%$  for the majority of compounds.

The intra- and inter-day instrumental performance was high across the majority of ECs. In total, 94% of the compounds were precise and accurate with a suitable linear calibration using the area ratio. Atorvastatin was linear, precise and accurate using the peak area, and miconazole, clotrimazole and climbazole could be analysed on a semi-quantitative basis as they showed satisfactory accuracy and precision data.

### Method performance

The most common syringe filter membrane used for ECs prior to UHPLC-MS/MS is PTFE.<sup>11,30,31,54</sup> However, low recoveries have been observed for some ECs including erythromycin and gemfibrozil.<sup>55</sup> Therefore, a range of syringe filters including PVDF-

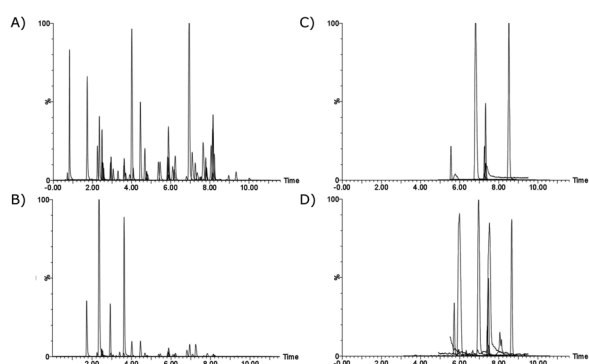


Fig. 2 Chromatograms (quantification MRM) of septic tank effluent spiked at  $c = 62.5 \mu\text{g L}^{-1}$  and analysed by direct injection (A and C), and at  $c = 0.5 \mu\text{g L}^{-1}$  and analysed by SPE (B and D) (details in S5: Table S4†), analysed with the ESI+ (A and B) and ESI– (C and D) method.



HL, PTFE, CA, and PVDF were investigated to minimize loss of ECs during the filtration step.

Absolute recoveries were >75% for all four syringe filters for 49 ECs (S9: Table S8†). Similarly, Darwano *et al.*<sup>1</sup> reported high recoveries for most analytes with little variation between different syringe filters. However, for clarithromycin, erythromycin, chlorpheniramine, cetirizine and citalopram poorer recoveries were found with PVDF, which is in line with what has been reported before for antibiotics including clarithromycin.<sup>56</sup> For all five compounds recoveries were at least 20% higher in the other filters, with CA and PVDF-HL being more effective than PTFE. However, CA gave lower recoveries for amoxicillin, estrone, and 17 $\beta$ -estradiol than what was achieved with PTFE, PVDF, and PVDF-HL syringe filters (>80%). PVDF-HL syringe filters were the best compromise for the studied EC, giving recoveries >70% for the majority of ECs. The effective use of PVDF-HL syringe filters has, for example, also been reported by Wang *et al.*<sup>57</sup> Low recoveries of approximately 10% were only found for fluoxetine, miconazole, and clotrimazole, and this was observed for all four syringe filters. All samples were filtered

through PVDF-HL syringe filters prior to UHPLC-MS/MS injection.

To determine method performance, septic tank influent and effluent, river water, and sludge samples were spiked at three concentrations (S5: Table S4†). Water samples were analysed by direct injection and SPE. Calculations were not practical for 29 ECs in at least one sample, when the environmental concentration exceeded the spike concentration, most common at lowest spike concentrations in effluent SPE samples.

In direct injection samples, absolute recoveries were 23–209% in septic tank influent, 19–192% in septic tank effluent, and 19–186% in river water (S9: Table S9†). Most ECs have absolute recoveries from 25 to 125% (Fig. 3). Recoveries over 100% were due to signal enhancement. This highlights the requirement of the use of deuterated surrogates to correct for matrix effects and variations in the instrumental and method performance.

For 41 ECs (63%), relative recoveries by direct injection were in the range of 90% to 110% in all three matrices, and in the range of 75% to 125% for a further 11 ECs (Fig. 4). The remaining 14 ECs have relative recoveries from 22 to 197%, most likely due to the absence of a suitable deuterated surrogate to account for matrix effects and analyte loss. Similar results have been reported by Oliveira *et al.*<sup>58</sup> who found relative recovery from 20 to 230%, with the majority recoveries being in the range of 70–150% in the analysis of ECs in wastewater influent and effluent by direct injection LC-MS/MS. The direct injection MDLs were  $3.3 \times 10^{-3}$ – $3.0 \mu\text{g L}^{-1}$  in influent, were  $4.1 \times 10^{-3}$ – $3.7 \mu\text{g L}^{-1}$  in effluent, and  $3.6 \times 10^{-3}$ – $3.4 \mu\text{g L}^{-1}$  in river water. MQLs were  $6.7 \times 10^{-3}$ – $8.8 \mu\text{g L}^{-1}$  in influent,  $8.1 \times 10^{-3}$ – $14 \mu\text{g L}^{-1}$  in effluent, and  $7.2 \times 10^{-3}$ – $8.3 \mu\text{g L}^{-1}$  in river water (S9: Table S10†). While these MQLs were sufficient for the determination of high use compounds, such as metformin or paracetamol,<sup>29,31,32</sup> hormones and antibiotics have predicted no-effect concentrations (PNEC) <  $1 \mu\text{g L}^{-1}$  and are reported in freshwater at  $\text{ng L}^{-1}$ . Hence, the use of a SPE method was

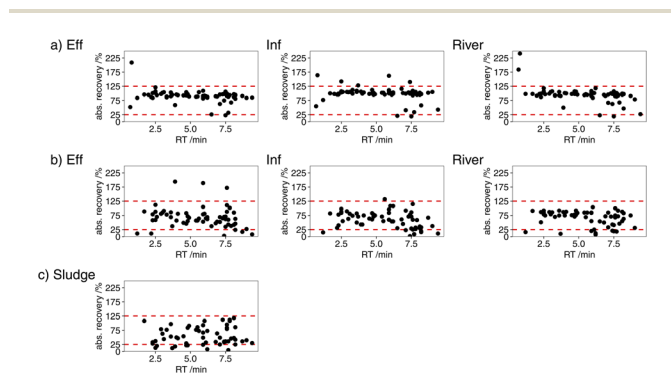


Fig. 3 Absolute recoveries (%) in influent, effluent and river water analysed by (a) direct injection and (b) SPE, and in (c) sludge.

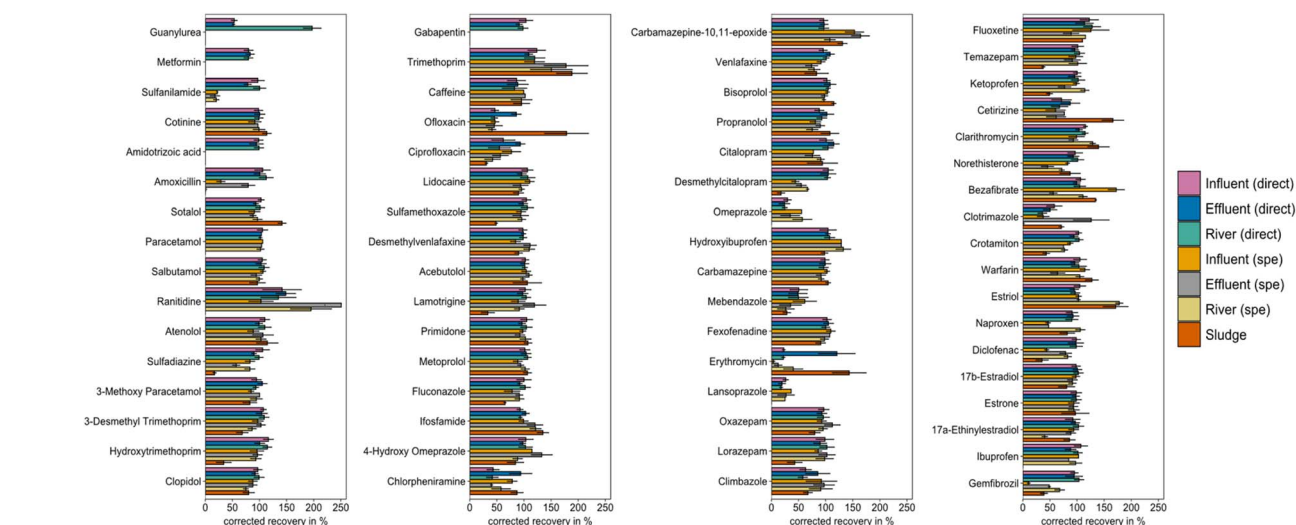


Fig. 4 Relative recoveries (%) in influent, effluent and river water analysed by direct injection and SPE, and in sludge, for the 66 ECs with assigned deuterated surrogate.



necessary to determine all ECs at the relevant concentrations. In direct injection, 29 ECs were very precise over all three concentrations with a relative standard deviation  $\leq 10\%$  in influent, effluent and river water (S9: Table S11†). Of the remaining compounds, 29 ECs were precise with a relative standard deviation  $\leq 20\%$  over all three concentrations in all matrices. The remaining ECs were precise for most spiked concentrations and matrices. Accuracies within the range of 75–125% were observed for the majority of 54 ECs. Most remaining ECs were accurate for most concentrations and matrices. This is similar to the results reported by Rapp-Wright *et al.*<sup>31</sup> for direct injection LC-MS/MS, and considering the complexity of matrices and the number of analytical steps involved, precision and accuracy were considered acceptable.

Absolute recoveries following SPE were 0–194% in septic tank influent, 1–200% in septic tank effluent, and 0–122% in river water (Fig. 3). The measured absolute recoveries were in the range of what has been previously reported using LC-MS/MS to determine multiple ECs in wastewater.<sup>10,30,59</sup> While the lack of selectivity of HLB allows the extractions of a wide range of analytes, matrix can be co-extracted and cause significant signal interference.<sup>10</sup> Signal interference is typically reported to be high in multi-residue LC-MS/MS methods using ESI as ionisation method and HLB columns in SPE due to lack of selectivity.<sup>11,30,54</sup> Lowest and no absolute recoveries from SPE were observed for the very polar compounds guanylurea, metformin, gabapentin, sulfanilamide, and amidotrizoic acid, and amoxicillin from river water (S9: Table S9†). HLB sorbents are known for their low recovery of very polar compounds,<sup>27,28,30</sup> *e.g.*, Klančar *et al.*<sup>28</sup> reported recoveries of 0.3% for metformin and 2.6% for gabapentin from river water. Due to the low absolute recoveries, guanylurea, metformin, gabapentin and amidotrizoic acid were determined by direct injection only. Relative recoveries for the remaining ECs analysed by SPE were 90–110% in all three matrices for 16 ECs and 75–125% for 17 ECs in all three matrices (Fig. 4). The remaining ECs had relative recoveries  $<75\%$  or  $>125\%$  in at least one water matrix. Similar relative recoveries have been reported by Anumol and Snyder in wastewater,<sup>37</sup> and the results used in the determination of concentrations to account for differences in the behaviour of the deuterated surrogate and analyte. The MDLs for SPE were  $5.4 \times 10^{-5}$ – $0.073 \mu\text{g L}^{-1}$  in influent,  $5.3 \times 10^{-5}$ – $0.033 \mu\text{g L}^{-1}$  in effluent, and  $2.9 \times 10^{-5}$ – $0.40 \mu\text{g L}^{-1}$  in river water. MQLs were  $1.5 \times 10^{-4}$ – $0.096 \mu\text{g L}^{-1}$  in influent,  $1.6 \times 10^{-4}$ – $0.22 \mu\text{g L}^{-1}$  in effluent, and  $6.6 \times 10^{-5}$ – $0.50 \mu\text{g L}^{-1}$  in river water (S9: Table S10†). Including SPE in the method preparation allows the determination of ECs at the relevant concentrations. The precision of 58 ECs was high over all three concentrations in influent, effluent and river water with relative standard deviations  $\leq 20\%$ . The remaining ten ECs were precise over most concentrations and matrices (S9: Table S12†). Similar precision were obtained by Ofrydopoulou *et al.*<sup>27</sup> The majority of ECs analysed by SPE had accuracies within the range of 75–125% for all concentrations above the MQL in influent, effluent and river water. Comparatively lower accuracies were found when the EC was present in the sample, *e.g.*, sulfanilamide in the effluent, trimethoprim at the smallest spike concentration in river water,

and citalopram in influent. Lower accuracies were also found for amoxicillin in river water, with a low absolute recovery, and for warfarin at  $1 \mu\text{g L}^{-1}$  close to the MQL (S9: Table S12†).

The USE method for the extraction of sediments described by Al-Khazrajy and Boxall<sup>25</sup> was modified to optimise extraction of the selected 68 ECs from sludge. To accommodate the higher concentrations of ECs in sludge compared to sediments,<sup>60</sup> a smaller mass of 0.1 g was used. Furthermore, the clean-up step was adjusted to keep it as similar as possible to the SPE of liquid samples. However, a larger SPE cartridge (200 mL for sludge) was chosen to avoid blocking of the cartridge during sample loading. Furthermore, an additional centrifuge step prior to filtration through a PVDF-HL syringe filter was necessary. The method was successfully applied for the extraction of 59 out of 68 ECs from sludge (S9: Table S9†). Due to the complexity of the environmental matrices, a different number of analytes is often reported for different matrices in multi-analyte methods.<sup>10,30</sup> For example, the USE method is not suitable for very polar compounds, such as metformin, sulfanilamide and gabapentin with low absolute recoveries from SPE. Due to their high polarity they are more likely to stay in the water phase and less likely to be found in the sludge.<sup>61</sup> For the remaining compounds absolute recoveries from sludge were 12–112% (Fig. 3). The majority of ECs had relative recoveries of 75–125% from sludge (Fig. 4). Low relative recoveries below 50% (*e.g.*, diclofenac and sulfadiazine) and high relative recoveries over 150% (*e.g.*, trimethoprim and estriol) were found for ECs when the deuterated surrogate behaved differently than the analyte. MDLs and MQLs were  $0.025$ – $7.4 \mu\text{g kg}^{-1}$  and  $0.080$ – $49 \mu\text{g kg}^{-1}$ , respectively. However, only five ECs have MQLs  $> 10 \mu\text{g kg}^{-1}$  and only mebendazole has an MQL  $> 15 \mu\text{g kg}^{-1}$  (S9: Table S10†). Most ECs have accuracies within the range of 75–125% for all spike concentrations (S9: Table S12†). Lower accuracies were found for few ECs at one spike concentration, *e.g.*, for sulfadiazine at  $50 \mu\text{g kg}^{-1}$  and for hydroxyibuprofen at  $500 \mu\text{g kg}^{-1}$ . The precision of 53 ECs was high over all three spike concentrations with relative standard deviations  $\leq 20\%$ ; the remaining six compounds have higher relative standard deviations at one concentration only.

The number of quantifiable compounds in each matrix varied from 68 in effluent to 59 in sludge, demonstrating the method's wide applicability.

### Application to environmental matrices

The developed method was applied to samples collected from a septic tank in the North-East of Scotland at the influent and effluent point, from the sludge, and from the receiving river upstream and downstream of the septic tank's discharge point. Additionally, the suspended solids from the influent and effluent were analysed. At sampling time, the dilution factor of effluent into the river was 756.<sup>62</sup>

Across all samples analysed, 43 ECs were detected at least once (Table 1). Fifteen ECs from six different groups (analgesics, antibiotics, anticonvulsants, antihistamines,  $\beta$ -blockers, wastewater discharge marker) were found in all matrices.



Table 1 Concentrations detected in influent (Inf), effluent (Eff), river water upstream (Up) and downstream (Down) of the effluent discharge point, suspended solids (SuS), and sludge<sup>a</sup>

Class	EC	Inf ( $\mu\text{g L}^{-1}$ )	Eff ( $\mu\text{g L}^{-1}$ )	Up ( $\mu\text{g L}^{-1}$ )	Down ( $\mu\text{g L}^{-1}$ )	SuS Inf ( $\mu\text{g kg}^{-1}$ )	SuS Eff ( $\mu\text{g kg}^{-1}$ )	Sludge ( $\mu\text{g kg}^{-1}$ )
Anaesthetics Analgesics	Lidocaine	0.025 ± 0.0056	0.043 ± 0.0020	nd	nd	(2.6 ± 0.14) × 10 <sup>3</sup>	(1.1 ± 0.084) × 10 <sup>2</sup>	nd
	3-Methoxyparacetamol	8.2 ± 0.44	13 ± 0.85	(2.3 ± 0.10) × 10 <sup>-3</sup>	0.026 ± 0.0013	(1.1 ± 0.064) × 10 <sup>2</sup>	(1.6 ± 0.40) × 10 <sup>2</sup>	68 ± 8.3
	Diclofenac	2.4 ± 0.10	0.58 ± 0.0047	0.023 ± 0.0024	0.016 ± 0.00097	78 ± 2.6	47 ± 3.2	(6.2 ± 1.3) × 10 <sup>2</sup>
	Hydroxybupropfen	2.7 ± 0.25	17 ± 0.56	(2.9 ± 0.41) × 10 <sup>-3</sup>	0.019 ± 0.0017	nd	nd	nd
	Ibuprofen	34 ± 1.8	26 ± 0.71	0.47 ± 0.083	0.63 ± 0.070	(2.1 ± 0.067) × 10 <sup>4</sup>	(6.1 ± 0.55) × 10 <sup>3</sup>	(1.6 ± 0.26) × 10 <sup>3</sup>
Antibiotics	Ketoprofen	nd	nd	nd	nd	nd	nd	nd
	Naproxen	3.7 ± 0.079	26 ± 0.26	(3.2 ± 0.12) × 10 <sup>-3</sup>	0.032 ± 0.0017	(5.1 ± 0.62) × 10 <sup>2</sup>	(7.0 ± 0.88) × 10 <sup>2</sup>	(3.0 ± 0.41) × 10 <sup>2</sup>
	Paracetamol	(2.0 ± 0.12) × 10 <sup>2</sup>	(2.9 ± 0.076) × 10 <sup>2</sup>	0.039 ± 0.0043	0.59 ± 0.053	(1.4 ± 0.45) × 10 <sup>3</sup>	(1.3 ± 0.18) × 10 <sup>4</sup>	(3.6 ± 0.045) × 10 <sup>3</sup>
	3-Desmethyl-trimethoprim	0.013 ± 0.0053	0.16 ± 0.014	(1.8 ± 0.23) × 10 <sup>-3</sup>	(2.5 ± 0.30) × 10 <sup>-3</sup>	78 ± 5.4	84 ± 10	4.8 ± 0.63
	Amoxicillin	nd	nd	nd	nd	—	—	—
	Ciprofloxacin	10 ± 0.61	2.4 ± 0.43	nd	nd	(3.8 ± 0.93) × 10 <sup>3</sup>	(4.9 ± 0.59) × 10 <sup>2</sup>	nd
	Clarithromycin	nd	nd	nd	nd	(3.1 ± 0.027) × 10 <sup>3</sup>	(1.3 ± 0.13) × 10 <sup>3</sup>	13 ± 2.0
	Erythromycin	0.077 ± 0.020	0.14 ± 0.016	nd	nd	nd	nd	—
	Ofloxacin	nd	nd	nd	nd	(1.2 ± 0.16) × 10 <sup>2</sup>	69 ± 15	—
	Sulfadiazine	nd	nd	nd	nd	nd	nd	nd
Sulfamethoxazole	0.029 ± 0.0032	0.59 ± 0.029	(3.1 ± 0.55) × 10 <sup>-3</sup>	(3.1 ± 0.56) × 10 <sup>-3</sup>	nd	43 ± 3.7	nd	
Anticoagulants Anticonvulsants	Sulfamilamide	0.33 ± 0.031	0.13 ± 0.018	nd	nd	—	—	—
	Trimethoprim	0.014 ± 0.0047	0.25 ± 0.018	(8.8 ± 1.8) × 10 <sup>-4</sup>	(1.1 ± 0.14) × 10 <sup>-3</sup>	44 ± 3.0	63 ± 1.8	7.5 ± 0.67
	$\alpha$ -Hydroxytrimethoprim	nd	(7.0 ± 2.8) × 10 <sup>-3</sup>	nd	nd	nd	nd	nd
	Warfarin	nd	nd	nd	nd	nd	nd	nd
	Carbamazepine	nd	(7.2 ± 0.43) × 10 <sup>-4</sup>	(2.0 ± 0.14) × 10 <sup>-4</sup>	(1.4 ± 0.097) × 10 <sup>-4</sup>	nd	nd	nd
Antidepressants	Carbamazepine-10,11-epoxide	nd	nd	nd	nd	nd	nd	nd
	Gabapentin	1.9 ± 0.30	7.4 ± 0.56	nd	nd	—	—	—
	Lamotrigine	0.97 ± 0.17	1.2 ± 0.055	(3.0 ± 0.26) × 10 <sup>-3</sup>	(4.5 ± 0.27) × 10 <sup>-3</sup>	(4.3 ± 0.68) × 10 <sup>2</sup>	(1.3 ± 0.15) × 10 <sup>3</sup>	70 ± 11
	Primidone	nd	nd	nd	nd	nd	nd	nd
	Citalopram	0.19 ± 0.014	0.14 ± 0.0091	nd	nd	(7.2 ± 1.5) × 10 <sup>2</sup>	(1.3 ± 0.26) × 10 <sup>2</sup>	(1.4 ± 0.088) × 10 <sup>2</sup>
Anti-diabetics	Desmethylcitalopram	0.14 ± 0.0084	0.079 ± 0.0030	nd	nd	(1.0 ± 0.22) × 10 <sup>3</sup>	(1.2 ± 0.26) × 10 <sup>2</sup>	(1.5 ± 0.067) × 10 <sup>2</sup>
	Desmethylvenlafaxine	0.028 ± 0.0015	0.21 ± 0.011	nd	(6.3 ± 1.7) × 10 <sup>-4</sup>	44 ± 7.2	47 ± 7.2	15 ± 3.3
	Fluoxetine	0.013 ± 0.0026	0.016 ± 0.0025	nd	nd	(1.4 ± 0.54) × 10 <sup>2</sup>	68 ± 12	nd
	Venlafaxine	0.081 ± 0.0025	0.14 ± 0.0084	nd	nd	(2.0 ± 0.12) × 10 <sup>2</sup>	66 ± 3.6	55 ± 10
	Guanylfurea	nd	nd	nd	nd	—	—	—
Anti-fungals	Metformin	(2.2 ± 0.082) × 10 <sup>2</sup>	(1.6 ± 0.067) × 10 <sup>2</sup>	0.85 ± 0.031	1.1 ± 0.038	—	—	—
	Climbazole	nd	nd	nd	nd	nd	nd	nd
	Clotrimazole	nd	nd	nd	nd	(5.1 ± 0.64) × 10 <sup>2</sup>	(4.8 ± 0.89) × 10 <sup>2</sup>	(1.7 ± 0.11) × 10 <sup>2</sup>
	Fluconazole	nd	nd	nd	nd	nd	nd	nd
	Miconazole	nd	nd	nd	nd	nd	nd	78 ± 25





Table 1 (Contd.)

Class	EC	Inf ( $\mu\text{g L}^{-1}$ )	Eff ( $\mu\text{g L}^{-1}$ )	Up ( $\mu\text{g L}^{-1}$ )	Down ( $\mu\text{g L}^{-1}$ )	SuS Inf ( $\mu\text{g kg}^{-1}$ )	SuS Eff ( $\mu\text{g kg}^{-1}$ )	Sludge ( $\mu\text{g kg}^{-1}$ )
Anti-helminthics	Mebendazole	nd	nd	nd	nd	nd	nd	nd
	Cetirizine	$1.2 \pm 0.050$	$1.2 \pm 0.046$	$(3.4 \pm 0.32) \times 10^{-3}$	$(5.6 \pm 0.36) \times 10^{-3}$	$(1.0 \pm 0.18) \times 10^2$	$(1.2 \pm 0.14) \times 10^2$	$(1.1 \pm 0.060) \times 10^2$
Antihistamines	Chlorpheniramine	nd	$(1.8 \pm 0.034) \times 10^{-3}$	nd	nd	$93 \pm 4.0$	$39 \pm 3.0$	$22 \pm 6.0$
	Fexofenadine	$0.16 \pm 0.0097$	$0.81 \pm 0.018$	$0.013 \pm 0.0014$	$0.012 \pm 0.0016$	$(2.6 \pm 0.049) \times 10^2$	$(1.1 \pm 0.088) \times 10^2$	$(3.4 \pm 0.67) \times 10^3$
	Crotamiton	$0.74 \pm 0.061$	$1.5 \pm 0.13$	nd	nd	$(2.2 \pm 0.14) \times 10^2$	$(2.3 \pm 0.51) \times 10^2$	$89 \pm 6.6$
	Ranitidine	nd	nd	nd	nd	nd	nd	nd
	4-Hydroxymeprazole	$0.33 \pm 0.023$	$0.43 \pm 0.035$	nd	nd	$(3.6 \pm 0.19) \times 10^2$	$45 \pm 4.5$	$(1.1 \pm 0.027) \times 10^2$
Benzodiazepines	Lansoprazole	nd	nd	nd	nd	—	—	—
	Omeprazole	nd	nd	nd	nd	—	—	—
	Lorazepam	nd	nd	nd	nd	nd	nd	nd
	Oxazepam	nd	nd	nd	nd	nd	nd	nd
	Temazepam	nd	$0.019 \pm 0.0026$	nd	nd	nd	nd	nd
Betablockers	Acebutolol	nd	nd	nd	nd	nd	nd	nd
	Atenolol	$(7.5 \pm 0.60) \times 10^{-3}$	$0.088 \pm 0.0067$	$(3.8 \pm 0.27) \times 10^{-4}$	$(6.3 \pm 0.16) \times 10^{-4}$	$59 \pm 4.1$	$50 \pm 12$	$53 \pm 4.6$
	Bisoprolol	$0.23 \pm 0.020$	$0.086 \pm 0.0095$	$(4.0 \pm 2.5) \times 10^{-4}$	$(3.6 \pm 0.42) \times 10^{-4}$	$54 \pm 10$	$41 \pm 3.6$	$3.8 \pm 0.36$
	Metoprolol	nd	nd	nd	nd	nd	nd	nd
	Propranolol	$0.43 \pm 0.035$	$0.24 \pm 0.029$	nd	nd	$(6.2 \pm 0.98) \times 10^2$	$(3.7 \pm 0.77) \times 10^2$	$46 \pm 6.5$
	Salbutamol	$(3.1 \pm 0.27) \times 10^{-3}$	$0.011 \pm 0.00030$	nd	nd	$11 \pm 1.1$	$9.5 \pm 1.0$	$5.7 \pm 1.8$
Chemotherapeutic Coccidiostat Hormones	Sotalol	nd	nd	nd	nd	nd	nd	nd
	Ifosfamide	nd	nd	nd	nd	nd	nd	nd
	Clopidol	nd	nd	nd	nd	nd	nd	nd
	17 $\beta$ -Estradiol	nd	nd	nd	nd	nd	nd	nd
	17 $\alpha$ -Ethinylestradiol	nd	nd	nd	nd	nd	nd	nd
	Estrilol	$0.093 \pm 0.0086$	$0.70 \pm 0.013$	nd	nd	nd	nd	$16 \pm 3.3$
	Estrone	$0.15 \pm 0.0030$	$0.054 \pm 0.0035$	nd	nd	$(1.2 \pm 0.14) \times 10^2$	nd	$15 \pm 1.4$
Lipid regulators	Norethisterone	nd	nd	nd	nd	nd	nd	nd
	Atorvastatin	$2.1 \pm 0.19$	$1.9 \pm 0.090$	nd	nd	nd	nd	$(3.9 \pm 2.3) \times 10^2$
	Bezafibrate	nd	nd	nd	nd	nd	nd	nd
	Gemfibrozil	nd	nd	nd	nd	nd	nd	nd
Wastewater discharge marker	Caffeine	$(1.4 \pm 0.12) \times 10^2$	$41 \pm 4.1$	$0.079 \pm 0.025$	$0.19 \pm 0.017$	$(1.0 \pm 0.17) \times 10^4$	$(9.2 \pm 1.2) \times 10^3$	$(2.7 \pm 0.35) \times 10^3$
	Cotinine	$1.1 \pm 0.10$	$1.2 \pm 0.086$	$(1.1 \pm 0.055) \times 10^{-3}$	$(2.8 \pm 0.14) \times 10^{-3}$	$85 \pm 22$	$(1.1 \pm 0.12) \times 10^2$	$31 \pm 4.3$
X-ray contrast	Amidotrizoic acid	nd	nd	nd	nd	—	—	—
		nd	nd	nd	nd	—	—	—

<sup>a</sup> nd, not detected; —, method not suitable.

In the influent, 34 ECs were detected at concentrations from  $(7.5 \pm 0.60) \times 10^{-3} \mu\text{g L}^{-1}$  (atenolol) to  $(2.2 \pm 0.082) \times 10^2 \mu\text{g L}^{-1}$  (metformin). A wide concentration range is typically observed for different ECs in wastewater.<sup>29,31,32</sup>

The highest detection frequency was observed in the effluent, where 38 ECs could be quantified. ECs were found at concentrations lower (e.g., ciprofloxacin), similar to (e.g., venlafaxine) and higher (e.g., gabapentin) than in the influent. Some determined effluent concentrations were in the range of what is typically reported in the influent of centralised WWTWs; for instance, both influent and effluent concentrations of metformin, were found to be  $(2.2 \pm 0.082) \times 10^2 \mu\text{g L}^{-1}$  and  $(1.6 \pm 0.067) \times 10^2 \mu\text{g L}^{-1}$ , respectively.<sup>2</sup> This suggests that in contrast to the high removal efficiency in centralised WWTWs of over 90% from the liquid phase,<sup>2</sup> metformin is not degraded in the septic tank. Furthermore, effluent concentrations of some compounds exceeded concentrations typically reported from centralised WWTWs. For example, the antipruritic drug crota-miton was present at  $(1.5 \pm 0.13) \mu\text{g L}^{-1}$  in the effluent, higher than previously reported concentrations of 0.11–0.27  $\mu\text{g L}^{-1}$  by Nakada *et al.*<sup>26</sup> in the UK. On the other hand, effluent concentrations of ECs such as fexofenadine, cetirizine, ciprofloxacin and lidocaine were similar to what has been reported in centralised WWTWs.<sup>30,31</sup> Further research is necessary to better understand the removal of different ECs in septic tanks.

In the river, 18 ECs were detected upstream and 19 downstream of the septic tank discharge point. The EC found at the highest concentration in the river, both upstream and downstream, was the anti-diabetic metformin at  $(0.85 \pm 0.031) \mu\text{g L}^{-1}$  and  $(1.1 \pm 0.038) \mu\text{g L}^{-1}$ , respectively. Metabolites can potentially have a significant effect on the total concentration of ECs in the environment, e.g., both desmethylvenlafaxine and 3-desmethyltrimethoprim were detected at higher concentrations in the river than the parent compound. The contribution of the septic tank to the pharmaceutical concentrations in the river varied from no difference between upstream and downstream concentrations to a marked increase. The biggest contribution was found for paracetamol with an increase by a factor of 15 from  $(0.039 \pm 0.0043) \mu\text{g L}^{-1}$  to  $(0.59 \pm 0.053) \mu\text{g L}^{-1}$ . Other sources that contribute to ECs concentrations in the river are the secondary WWTWs and additional private septic tanks. Further work focussing on ECs in rural Scotland is needed to understand the impact of septic tank discharges on rivers.

With 30 detected ECs, detection frequencies in the suspended solids were similar to the wastewater. For most ECs, the liquid phase is the main contributor to the total concentrations in the septic tank discharge. However, clotrimazole, clarithromycin and ofloxacin that were not detected in the water, were found in the suspended solids at concentrations up to  $(1.3 \pm 0.13) \times 10^3 \mu\text{g kg}^{-1}$  for clarithromycin in the effluent. This stresses the importance of analysing the solids when assessing the impact of wastewater discharges to the environment. Most ECs had similar concentrations in the suspended solids of the influent and effluent, showing a potential for removal of ECs in the septic tank through sludge formation and consequent reduction of the total suspended solids in the effluent.

The 30 ECs that were determined in the sludge sample were found at concentrations from 4 (bisoprolol) to 3617  $\mu\text{g kg}^{-1}$  (paracetamol). A wide concentration range of ECs in digested sludge from centralised WWTWs was also reported by Aydın *et al.*<sup>63</sup> at mean concentrations from 0.73 (sulfamethazine) to 147  $\mu\text{g kg}^{-1}$  (clarithromycin), and a maximum concentration of 1496  $\mu\text{g kg}^{-1}$  (clarithromycin). Higher levels of some ECs such as fexofenadine and diclofenac in the sludge *versus* the suspended solids may reflect an accumulation over time, whereas lower levels of other ECs such as caffeine, paracetamol and clarithromycin could be due to degradation in the sludge.<sup>36</sup> Future research on the distribution of ECs between the liquid and solid phase could increase the understanding of the removal of different ECs through sorption or degradation.

The contribution of the septic tank to the pharmaceutical concentrations detected in the river varies from no difference between upstream and downstream concentrations to an increase by the factor 15. The observed effluent concentrations of some pharmaceuticals suggest less removal in septic tanks than in centralised WWTWs. Finally, the detection of 30 ECs in the suspended solids in the effluent stresses the importance of including solid analysis when analysing environmental samples to avoid underestimation of the total concentration in the sample.

## Conclusions

A new multi-analyte method was developed for the accurate determination of a broad range of ECs in liquid and solid environmental matrices of varying complexity. Analysing septic tank influent and effluent, including suspended solids, sludge, and the receiving surface water allows an accurate assessment of the performance of septic tanks for the removal of ECs and their effect on water quality. Including suspended solids in the analysis of environmental samples minimises underestimating the total concentration of ECs.

The reported effluent concentrations of some pharmaceuticals suggest less removal in septic tanks than in centralised WWTWs. Furthermore, the river sampling suggests that septic tanks have an impact on water quality for some ECs. Hence, a more robust sampling of septic tanks in Scotland is proposed to accurately determine their impact to the environment.

## Author contributions

Kai Wilschnack: conceptualisation, data curation, formal analysis, investigation, methodology, validation, visualisation, writing – original draft; Bess Homer: conceptualisation, resources, writing – review & editing; Elise Cartmell: conceptualisation, resources, writing – review & editing; Kyari Yates: conceptualisation, supervision, writing – review & editing; Bruce Petrie: conceptualisation, funding acquisition, methodology, project administration, supervision, writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.



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## References

- H. Darwano, S. V. Duy and S. Sauvé, *Arch. Environ. Contam. Toxicol.*, 2014, **66**, 582–593.
- M. Scheurer, A. Michel, H.-J. Brauch, W. Ruck and F. Sacher, *Water Res.*, 2012, **46**, 4790–4802.
- N. A. Alygizakis, P. Gago-Ferrero, V. L. Borova, A. Pavlidou, I. Hatzianestis and N. S. Thomaidis, *Sci. Total Environ.*, 2016, **541**, 1097–1105.
- Q. Gao, K. M. Blum, P. Gago-Ferrero, K. Wiberg, L. Ahrens and P. L. Andersson, *Sci. Total Environ.*, 2019, **651**, 1670–1679.
- S. Letsinger, P. Kay, S. Rodríguez-Mozaz, M. Villagrassa, D. Barceló and J. M. Rotchell, *Sci. Total Environ.*, 2019, **678**, 74–84.
- B. Petrie, R. Barden and B. Kasprzyk-Hordern, *Water Res.*, 2015, **72**, 3–27.
- S. Comber, M. Gardner, P. Sörme, D. Leverett and B. Ellor, *Sci. Total Environ.*, 2018, **613–614**, 538–547.
- D. White, D. J. Lapworth, W. Civil and P. Williams, *Environ. Pollut.*, 2019, **249**, 257–266.
- M. Gros, M. Petrović and D. Barceló, *Talanta*, 2006, **70**, 678–690.
- K. Proctor, B. Petrie, R. Barden, T. Arnot and B. Kasprzyk-Hordern, *Anal. Bioanal. Chem.*, 2019, **411**, 7061–7086.
- D. R. Baker and B. Kasprzyk-Hordern, *J. Chromatogr. A*, 2011, **1218**, 7901–7913.
- S. Richards, E. Paterson, P. J. A. Withers and M. Stutter, *Sci. Total Environ.*, 2016, **542**, 854–863.
- S. Ramage, D. Camacho-Muñoz and B. Petrie, *Chemosphere*, 2019, **219**, 191–201.
- Scottish Water, *List of Wastewater Treatment Works, Annual Return 2021*, Intern information, 2021.
- L. Gill, J. Mac Mahon, J. Knappe, S. Gharbia and F. Pilla, *Desludging Rates and Mechanisms for Domestic Wastewater Treatment System Sludges in Ireland (2016-W-DS-26)*, Environmental Protection Agency, Dublin, 2018.
- D. Dubber and L. Gill, *Sustainability*, 2014, **6**, 1623–1642.
- B. D. Stanford and H. S. Weinberg, *Environ. Sci. Technol.*, 2010, **44**, 2994–3001.
- Y. F. Ting and S. M. Praveena, *Environ. Monit. Assess.*, 2017, **189**, 178.
- L. A. Schaidler, K. M. Rodgers and R. A. Rudel, *Environ. Sci. Technol.*, 2017, **51**, 7304–7317.
- S. N. Garcia, R. L. Clubbs, J. K. Stanley, B. Scheffe, J. C. Yelderian and B. W. Brooks, *Chemosphere*, 2013, **92**, 38–44.
- C. A. James, J. P. Miller-Schulze, S. Ultican, A. D. Gipe and J. E. Baker, *Water Res.*, 2016, **101**, 241–251.
- J. A. Oppenheimer, M. Badruzzaman and J. G. Jacangelo, *Water Res.*, 2012, **46**, 5904–5916.
- B. Subedi, N. Codru, D. M. Dziewulski, L. R. Wilson, J. Xue, S. Yun, E. Braun-Howland, C. Minihane and K. Kannan, *Water Res.*, 2015, **72**, 28–39.
- E. Godfrey, W. W. Woessner and M. J. Benotti, *Groundwater*, 2007, **45**, 263–271.
- O. S. A. Al-Khazrajy and A. B. A. Boxall, *Anal. Methods*, 2017, **9**, 4190–4200.
- N. Nakada, S. Hanamoto, M. D. Jürgens, A. C. Johnson, M. J. Bowes and H. Tanaka, *Sci. Total Environ.*, 2017, **575**, 1336–1348.
- A. Ofrydopoulou, C. Nannou, E. Evgenidou and D. Lambropoulou, *J. Chromatogr. A*, 2021, **1652**, 462369.
- A. Klančar, J. Trontelj and R. Roškar, *Water, Air, Soil Pollut.*, 2018, **229**, 192.
- R. Oertel, J. Baldauf and J. Rossmann, *J. Chromatogr. A*, 2018, **1556**, 73–80.
- B. Petrie, J. Youdan, R. Barden and B. Kasprzyk-Hordern, *J. Chromatogr. A*, 2016, **1431**, 64–78.
- H. Rapp-Wright, F. Regan, B. White and L. P. Barron, *Sci. Total Environ.*, 2023, **860**, 160379.
- K. Styszko, K. Proctor, E. Castrignanò and B. Kasprzyk-Hordern, *Sci. Total Environ.*, 2021, **768**, 144360.
- R. López-Roldán, M. L. de Alda, M. Gros, M. Petrovic, J. Martín-Alonso and D. Barceló, *Chemosphere*, 2010, **80**, 1337–1344.
- L. Duan, Y. Zhang, B. Wang, G. Yu, J. Gao, G. Cagnetta, C. Huang and N. Zhai, *Water Res.*, 2022, **216**, 118321.
- I. L. Costa Junior, C. S. Machado, A. L. Pletsch and Y. R. Torres, *Int. J. Sediment Res.*, 2022, **37**, 346–354.
- J. L. Malvar, J. L. Santos, J. Martín, I. Aparicio and E. Alonso, *Microchem. J.*, 2020, **157**, 104987.
- T. Anumol and S. A. Snyder, *Talanta*, 2015, **132**, 77–86.
- A. Eaglesham, A. Scott and B. Petrie, *Environ. Chem. Lett.*, 2020, **18**, 2119–2126.
- P. N. Carvalho, A. Pirra, M. C. P. Basto and C. M. R. Almeida, *Anal. Methods*, 2013, **5**, 6503–6510.
- N. Dorival-García, A. Zafra-Gómez, F. J. Camino-Sánchez, A. Navalón and J. L. Vilchez, *Talanta*, 2013, **106**, 104–118.
- European Commission, *Commission Implementing Decision (EU) 2018/840 of 5 June 2018 Establishing a Watch List of Substances for Union-wide Monitoring in the Field of Water Policy Pursuant to Directive 2008/105/EC of the European Parliament and of the Council and Repealing Commission Implementing Decision (EU) 2015/495*, 2018, vol. 141.
- B. Ellor and M. J. Gardner, *The National Chemical Investigations Programme 2015–2020*, Monitoring Substances of Emerging Concern, UKWIR, vol. 2, 2018.
- European Commission, *Common Implementation Strategy for the Water Framework Directive and the Floods Directive: Voluntary Groundwater Watch List*, 2019.
- L. Gomez Cortes, D. Marinov, I. Sanseverino, A. Navarro Cuenca, M. Niegowska, E. Porcel Rodriguez and T. Lettieri, *Selection of Substances for the 3rd Watch List under the*



- Water Framework Directive. JRC121346*, Publications Office of the European Union, 2020.
- 45 B. Ellor, G. Castle and C. Yates, *The National Chemical Investigations Programme 2020–2022, Monitoring Substances of Emerging Concern*, UKWIR, vol. 5, 2023.
- 46 K. Helwig, A. Aderemi, D. Donnelly, S. Gibb, L. Gozdzielewska, J. Harrower, R. Helliwell, C. Hunter, L. Niemi, E. Pagaling, L. Price, J. Roberts and Z. Zhang, *CREW Research Summary: Pharmaceuticals in the Water Environment: Baseline Assessment and Recommendations*, 2021.
- 47 UK Centre for Ecology & Hydrology, *National River Flow Archive*, <https://nrfa.ceh.ac.uk/>.
- 48 National Records of Scotland, *Mid-2020 Population Estimates for Settlements and Localities in Scotland*, <https://www.nrscotland.gov.uk/statistics-and-data/statistics/statistics-by-theme/population/population-estimates/settlements-and-localities/mid-2020>, accessed 17 October 2022.
- 49 2002/657/EC: Commission Decision of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results (Text with EEA Relevance) (Notified under Document Number C(2002) 3044), 2002.
- 50 L. Boulard, G. Dierkes, M. P. Schlüsener, A. Wick, J. Koschorreck and T. A. Ternes, *Water Res.*, 2020, **171**, 115366.
- 51 M. Petrović, M. D. Hernando, M. S. Díaz-Cruz and D. Barceló, *J. Chromatogr. A*, 2005, **1067**, 1–14.
- 52 C. Ripollés, M. Ibáñez, J. V. Sancho, F. J. López and F. Hernández, *Anal. Methods*, 2014, **6**, 5028–5037.
- 53 O. Yanes, R. Tautenhahn, G. J. Patti and G. Siuzdak, *Anal. Chem.*, 2011, **83**, 2152–2161.
- 54 P. Paiga, L. H. M. L. M. Santos and C. Delerue-Matos, *J. Pharm. Biomed. Anal.*, 2017, **135**, 75–86.
- 55 K. B. Bodle, M. R. Pernat and C. M. Kirkland, *Water, Air, Soil Pollut.*, 2022, **233**, 505.
- 56 C. Miossec, T. Mille, L. Lancelleur and M. Monperrus, *Food Chem.*, 2020, **322**, 126765.
- 57 J. Wang, L. Qi, C. Hou, T. Zhang, M. Chen, H. Meng, M. Su, H. Xu, Z. Hua, Y. Wang and B. Di, *J. Pharm. Anal.*, 2021, **11**, 739–745.
- 58 T. S. Oliveira, M. Murphy, N. Mendola, V. Wong, D. Carlson and L. Waring, *Sci. Total Environ.*, 2015, **518–519**, 459–478.
- 59 Y. Li, M. A. Taggart, C. McKenzie, Z. Zhang, Y. Lu, S. Pap and S. W. Gibb, *J. Environ. Sci.*, 2021, **100**, 18–27.
- 60 A. Azzouz and E. Ballesteros, *Sci. Total Environ.*, 2012, **419**, 208–215.
- 61 Y. Luo, W. Guo, H. H. Ngo, L. D. Nghiem, F. I. Hai, J. Zhang, S. Liang and X. C. Wang, *Sci. Total Environ.*, 2014, **473–474**, 619–641.
- 62 SEPA, *SEPA Time series data service (API)*, <https://timeseriesdoc.sepa.org.uk/>.
- 63 S. Aydın, A. Ulvi, F. Bedük and M. E. Aydın, *Sci. Total Environ.*, 2022, **817**, 152864.

