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Use of QuEChERS as a manual and automated high-throughput protocol for investigating environmental matrices

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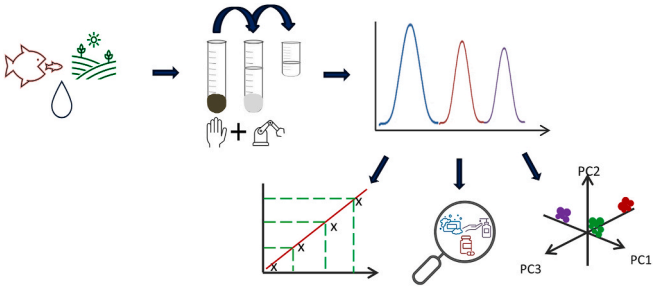
HIGHLIGHTS

- Chemometric differentiation of biota, effluent and seasonal sludge using QuEChERS.
- Identified detergents, polymers, pharmaceuticals and biocides in sludge.
- QAC biocides observed above EQSD levels in sludge.
- Rapid, automated analysis of soil and biota with good precision and recovery.
- Good accuracy and precision for pharmaceuticals and biocides when applied to clay.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental pollution has strong links to adverse human health outcomes with risks of pollution through production, use, ineffective wastewater (WW) remediation, and/or leachate from landfill. 'Fit-for-purpose' monitoring approaches are critical for better pollution control and mitigation of harm, with current sample preparation methods for complex environmental matrices typically time-consuming and labour intensive, unsuitable for high-throughput screening.

This study has shown that a modified 'Quick Easy Cheap Effective Rugged and Safe' (QuEChERS) sample preparation is a viable alternative for selected environmental matrices required for pollution monitoring (*e.g.* WW effluent, treated sludge cake and homogenised biota tissue). As a manual approach, reduced extraction times (hours to ~20 min/sample) with largely reproducible (albeit lower) recoveries of a range of pharmaceuticals and biocidal surfactants have been reported. Its application has shown clear differentiation of matrices via chemometrics, and the measurement of pollutants of interest to the UK WW industry at concentrations significantly

Abbreviations: BAC-C12, Benzyldimethyldodecylammonium chloride; BAC-C14, Benzyldimethyltetradecylammonium chloride; BAC-C14-d_7, Benzyldimethyltetradecylammonium chloride-d_7; dSPE, Dispersive solid-phase extraction; DDMA, Didecyldimethylammonium bromide; HDTMA, Hexadecyltrimethylammonium chloride; $MgSO_4$, Magnesium sulfate; NaOAc, Sodium acetate; PSA, Primary secondary amine; QAC, Quaternary ammonium compound; QuEChERS, Quick Easy Cheap Effective Rugged and Safe; BAC-C18, Stearalkonium Chloride.

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above suggested instrument detection limits (IDL) for sludge, indicating insufficient removal and/or bioaccumulation during WW treatment. Furthermore, new pollutant candidates of emerging concern were identified – these included detergents, polymers and pharmaceuticals, with quaternary ammonium compound (QAC) biocides observed at 2.3–70.4 mg/kg, and above levels associated with priority substances for environmental quality regulation (EQSD). Finally, the QuEChERS protocol was adapted to function as a fully automated workflow, further reducing the resource to complete both the preparation and analysis to <40 min. This operated with improved recovery for soil and biota (>62%), and when applied to a largely un-investigated clay matrix, acceptable recovery (88.0–131.1%) and precision (≤10.3% RSD) for the tested pharmaceuticals and biocides was maintained. Therefore, this preliminary study has shown the successful application of a high-throughput QuEChERS protocol across a range of environmental solids for potential deployment in a regulated laboratory.

1. Introduction

Environmental pollution has been strongly linked to adverse human health outcomes leading to growing research in this area. Given the breadth of chemicals used in everyday life and their resistance to environmental breakdown, the risk of pollution through release via production, use, ineffective wastewater (WW) remediation, and/or leachate from landfill is considerable. 'Fit-for-purpose' monitoring approaches are essential to understanding these relationships by determining the pollutant(s), degree of exposure, risk, and efficacy of management processes to better control release and mitigate harm. Monitoring approaches typically require individual sample collection onsite, with sample preparation and analysis using complex instrumentation in a laboratory. These result in the delayed deployment of mitigation measures and has driven research for analytical solutions that can be undertaken in a laboratory, and/or onsite, to provide accurate real-time data for an appropriate response that reduces environmental and public health risks. A recent paper that seeks to address this demand involves testing aqueous filtered WW or treated WW effluent using highly complex instrumentation within a mobile trailer (Stravs et al., 2021). However, even with this recent advance, there remains a need for more simplified analytical solutions that are applicable to the range of environmental matrices required for total ecosystem pollution monitoring.

QuEChERS is a two-step sample preparation method, originally developed for the extraction of acidic and basic pesticides from foodstuffs (Anastassiades and Lehotay, 2003), that has shown significant promise for the screening of environmental samples (Godfrey et al., 2014; Townsend et al., 2020; Xian et al., 2016; Slimani et al., 2017; Mishima-Kimura et al., 2018; Bergé et al., 2016). It uses a solvent extraction (typically using acetonitrile) and dispersive solid-phase extraction (dSPE) to target the removal of abundant interferences (*e.g.* humic acids, lipids etc.) (Anastassiades and Lehotay, 2003) rather than the selective isolation of the molecule to be measured. This less biased approach provides the distinct advantage of method flexibility for screening environmental samples, and is evidenced by recent work covering the breadth of analyte classes (Townsend et al., 2020; Godfrey et al., 2020) of emerging pollutants (*e.g.* pharmaceuticals (Godfrey et al., 2014; Cerqueira et al., 2014; Berlioz-Barbier et al., 2015; Chuang et al., 2015; Dulaurent et al., 2016; Amorim Alves et al., 2017; Kachhawaha et al., 2017; Salvia et al., 2012) and surfactants (Xian et al., 2016; Slimani et al., 2017; Mishima-Kimura et al., 2018; Bergé et al., 2016)), and environmental matrices (including WW, biota and soil) (Godfrey et al., 2014; Bergé et al., 2016, 2017; Cerqueira et al., 2014; Berlioz-Barbier et al., 2015; Kachhawaha et al., 2017; Salvia et al., 2012; Stöckelhuber et al., 2017; Lee et al., 2017; Nannou et al., 2019; Luo et al., 2022). Furthermore, QuEChERS is a low resource technique capable of extracting these matrices without the need for sophisticated equipment and offers a flexible approach for high-throughput or mobile (onsite) laboratories. However, a barrier limiting the adoption of QuEChERS for high-throughput monitoring has been the personnel costs associated with the manual sample preparation. This has led to an increasing number of studies aimed at automating part, or all, of the extraction process, but these have not been developed for the breadth of environmental solids and/or do not concern medicinal pollutants (Hakme and Poulsen, 2021; Lehotay et al., 2018; Michlig et al., 2021; Monteiro et al., 2021; Ninga et al., 2020; Lehotay et al., 2020; Monteiro et al., 2022; Zhang et al., 2019; Khaled et al., 2019; Ting et al., 2019; Jia et al., 2017; Mastrianni et al., 2018; Stremel et al., 2017; Chang et al., 2021; Trent, 2013; Kaewsuya et al., 2013; Roberts, 2009; Morris and Schriner, 2015; Huebschmann et al., 2016; Pfannkoch et al., 2011; Lehotay et al., 2016; Teledyne Tekmar, 2014). Nevertheless, with applicability to soils and foodstuffs, and the possibility of automation, QuEChERS offers significant potential as a relatively low-cost sample preparation for remote monitoring or high-throughput laboratory work, applicable to the breadth of pollutants anticipated across environmental matrices.

In this study QuEChERS will be used within a typical laboratory-based environmental monitoring workflow (*e.g.* extraction, liquid chromatography-mass spectrometry (LC-MS) analysis, chemometric and molecular qualitative data processing, and target molecule quantitation) for the characterisation and reliable discrimination of the breadth of environmental samples (WW effluent, WW sludge, and biota) required by relevant legislation (European Union, 2000, European Union, 2013; European Union, 2008). The presence of medicinal chemical pollutants will be explored via their accurate mass, isotope and fragmentation data, and quantified within these samples. Furthermore, QuEChERS will be used as a fully automated protocol for high-throughput monitoring with improved method performance (*e.g.* recovery, precision and accuracy etc).

2. Materials and methods

2.1. Chemicals and reagents

Based on initial screening data and the needs of UK WW research initiatives (*e.g.* CIP), a selection of compounds anticipated from pharmaceutical and personal care products were purchased as pollutant targets (see Supplementary Information Table S1). These were quantified using 10,11-dihydrocarbamazepine (10,11-DHC), pronethalol hydrochloride, talopram hydrochloride (sourced from Tocris (Abingdon, England), and benzyldimethyltetradecylammonium chloride-d_7 or BAC-C14-d_7 (Toronto Research Chemicals (Ontario, Canada)) as internal standards (ISs). Sample extraction materials for a modified QuEChERS protocol (Townsend et al., 2020) were obtained from Biotage (Uppsala, Sweden) with the automated QuEChERS SPE sorbent from ITSP Solutions Inc (Georgia, USA). Finally, formic acid, and acetonitrile (ACN) and water (HPLC grade) solvents were sourced from Fisher Scientific (Loughborough, UK).

2.2. Instrumentation

Initial qualitative investigations were carried out using a Dionex Ultimate 3000 (Hemel Hempstead, UK) liquid chromatography (LC) system, with a reversed phase Waters (Elstree, UK) XSelect HSS T3 LC column (1 × 100 mm, 3.5 μm) and a Phenomenex KrudKatcher Ultra online filter (Macclesfield, UK). The LC system was operated using Chromeleon 6.8 software (Thermo Scientific) with detection performed by an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) using an ESI source in positive ion mode operated with

Xcalibur 3.0 software. For the quantitative method, a dual low resolution mass spectrometry method developed in-house (Townsend et al., 2020) was used due to differing analyte sensitivity, selectivity and scan speeds required by the sample types, and operated with a Thermo Scientific (Hemel Hempstead, UK) Micro AS autosampler and MSPump Plus using the column described above. To automate both stages of the QuEChERS protocol, a GERSTEL MultiPurpose Sampler Robotic/Robotic^{Pro} 2m was used to perform the extraction, with extracts analysed using a 1260 LC system and 6470 mass spectrometer (Agilent, Cheadle, UK). Again, the separation was carried out using the pre-column filter and analytical column described above, and instrumentation controlled using Agilent MassHunter software.

2.3. Stock and working solutions

To minimise degradation during storage, each standard was prepared as 1 mg/mL stock solutions in water, while loratadine, carbamazepine, 10,11-dihydrocarbamazepine and surfactants were prepared in neat ACN for solubility. Working solutions, calibration standards and quality control (QC) samples were prepared as a pharmaceutical and quaternary ammonium compound (QAC) mixture in 50:50 acetonitrile/water with a relevant IS at concentrations previously published (Townsend et al., 2020). These were randomised within each quantitative batch, and a "double" blank (S_B) containing just solvent and an IS blank (S_0) were also used judiciously within each batch of samples to detect carryover and confirm method selectivity.

2.4. Sample collection

To characterise and show application of a manual QuEChERS method for monitoring the breadth of environmental samples, garden soil (from an undisclosed location in West Wales), WW sludge cake and a complimentary treated effluent sample (both from a local municipal WW plant), and biota (from undisclosed locations in Southwest Wales and Southwest England) were collected as grab samples. Of these, the sludge was sampled seasonally (August and December to represent summer and winter, respectively) to assess the method applicability in detecting differences in drug usage, and a test portion of mixed biota samples (molluscs) were collected during routine monitoring, with both sample types homogenised by grinding prior to use. Given biota is commonly lyophilised for preparation (US EPA; Radiation Protection Division), aliquots were extracted as wet (from homogenisation) and lyophilised (FD) materials to determine whether the removal of water affected the sample composition. For the automation of the QuEChERS protocol, fresh biota (molluscs and Dab fish) from the same location was collected to compare with the original sample and analysed using the protocol described in section 2.5.2.

2.5. Liquid chromatography-mass spectrometry (LC-MS) analysis

2.5.1. Manual method

Each mass spectrometer was tuned and calibrated for the operating mass range and chromatographic conditions to ensure validity of performance. Following preparation, samples were held at 4 °C on the autosampler prior to injecting 5 μL on-column. The methods used were optimised and evaluated as described in Table S2-S4 and past work (Godfrey et al., 2014; Townsend et al., 2020). Targeted and untargeted elemental formula assignments were made for even electron ions using the search criteria $C_{40}H_{70}N_{10}O_{15}Cl_2F_3$ with a mass tolerance of 3 ppm for precursor species and 10 ppm for fragment ions using Xcalibur 3.0 software. For low mass resolution (quantitative) acquisitions, a combination of full mass scan and selected reaction/ion monitoring modes were used to facilitate target identification for quantitative analyses (see Table S4). Integrated peak areas from the selected reaction/ion monitoring chromatograms were used to generate relative response factors for the calibration graphs over selected concentration ranges (Townsend et al., 2020) (estimated from in-house sample screening), with simple statistics calculated using Microsoft Excel 2010.

2.5.2. Automated method

The MultiPurpose Sampler was operated with the 6470 LC-MS system using ESI in positive ion mode, with source temperature of 250 °C, at a gas flow of 12 L/min, a sheath gas flow of 11 L/min at 325 °C, and a capillary voltage of 3.5 kV. Data was acquired via multiple reaction monitoring (MRM) at collision energies optimised for each ion (see Table S5). Sample volumes of 10 μL were injected on the Waters HSS T3 column with separation conditions described above but at a flow rate of 0.1 mL/min due to higher allowable back pressure within the system. As a measure of signal intensity, the peak areas from the MRM chromatograms were integrated using Agilent MassHunter Quant B.09 and used to calculate the recovery by quantifying the analytes spiked within the samples (versus the amount recovered) using a calibration graph prepared over an appropriate dynamic range (see Table S6).

2.6. Sample preparation

2.6.1. Manual method

Each matrix (3.5 mL of effluent or 2.5 g of homogenised solid (*e.g.* biota or sludge)) was prepared in triplicate using the optimised QuEChERS method to ensure consistent conditions. Of these, one replicate was spiked with IS (at 100 ng/mL and 10 ng/mL for the pharmaceuticals and QACs, respectively) post-extraction for quantifying suspected medicinal pollutants. For the solid samples, 3.5 mL of water was added pre-extraction to provide fluidity to the mixture and these samples, along with the effluent, were extracted by adding 10 mL of ACN with 4 g magnesium sulphate ($MgSO_4$) and 1.5 g of sodium acetate (NaOAc). The mixtures were shaken for 1 min, centrifuged for 5 min at 4000 rpm, and the supernatant added to a mixture of 150 mg PSA and 900 mg $MgSO_4$(Townsend et al., 2020) for further extraction. The samples were vortexed for 1 min and centrifuged for 5 min, with the post-extraction supernatant transferred to a clean vial and evaporated to dryness under nitrogen at room temperature. For analysis, dried extracts were reconstituted in a total volume of 500 μL, containing an IS spike at the concentration noted above and/or an appropriate volume of mobile phase (50:50 water/acetonitrile). Sample matrices were analysed with standards and QCs for quantitation, along with blanks (matrix and solvent) to detect carryover and confirm method selectivity, with in-sample analyte concentrations determined using the recoveries (and matrix effects) published in allied work (Townsend et al., 2020).

2.6.2. Automated method

The complete automation of the QuEChERS extraction used the multipurpose sampler and ITSP SPE cartridges deemed equivalent to the sorbent used in the manual method (*e.g.* 9:1 $MgSO_4$/PSA) with a bed mass of 45 mg. These were washed with 300 μL of ACN prior to clean-up and used as part of an adjusted protocol (reduced in scale by a factor of 3.2) to accommodate the lower sample volumes and vial size (see section S7). In summary, the soil and clay matrices were fortified as triplicate aliquots with in-matrix concentrations of 512.5 ng/g for the pharmaceuticals and 102.5 ng/g for the QACs, with IS concentrations of 1025 ng/g and 205 ng/g, respectively. Similarly, triplicate aliquots of biota were prepared with in-matrix concentrations of 400 ng/mL and 80 ng/mL for pharmaceuticals and QACs, respectively, with IS concentrations of 800 ng/mL for the pharmaceuticals and 160 ng/mL for the QACs. The samples were then extracted by adding 4 mL of ACN that was shaken for 1 min at 2000 rpm, followed by the addition of 1.72 g of the extraction salts. The mixture was shaken again for 8 s at 2000 rpm, centrifuged at 2000 g for 5 min and 0.3 mL of supernatant taken for automated SPE clean-up. Following SPE, the total recovered volume of supernatant was diluted by 50% with water prior to analysis to facilitate chromatographic retention.

2.7. Chemometrics

For a 'global' view of sample differentiation a Principal Component Analysis (PCA) was undertaken using MzMine2 (Pluskal et al., 2010) and Aabel NG2 (Gigawiz Ltd. Co., OK, USA) to establish data correlations between sample types and as proof of use across open access platforms. To identify the sample components and confirm the selectivity of the PCA, blank samples (mobile phase only) were used to perform a background subtraction on the raw data of the matrices prepared in Section 2.6.1 and an unextracted QC (100 ng/mL pharmaceuticals, 30 ng/mL QACs with relevant ISs (100 ng/mL and 10 ng/mL for the pharmaceuticals and QACs, respectively)). Once subtracted, the data was converted into mzML format using MSConvert via ProteoWizard (Chambers et al., 2012) and processed using MzMine2 using the parameters included in section S8. Given MzMine2 does not generate loading scores and an Eigen value, the Aabel NG2 software was used (see section S9) to establish the *m/z* that contributed most to the principal components (PCs) identified via the PCA (*e.g.* a high score indicating a strong contribution, and the sign representing a positive or negative correlation). However, this software has a data point capacity of 27,000 and thus, the peak areas were summed across all samples to accommodate the data set, with the data ordered according to the summed values, and the PCA generated from a more manageable sample size (3000) of ~two orders of magnitude in spread. However, to ensure this data reduction did not adversely affect the PCA groupings, the PCA plots were compared with that from MzMine2 to confirm similar clustering could be achieved. Further chemometric analysis used logarithmic-ratio plots to directly compare sample compositions and provide an understanding of the degree of change between the sample types.

3. Results and discussion

With many environmental qualitative workflows involving generic extraction methods, LC-HRMS, chemometric processing and structural elucidation, an established manual QuEChERS protocol (Townsend et al., 2020) was tested within this process. Specifically, this modified QuEChERS approach was used to prepare a range of environmental matrices (effluent, sludge and biota) over different scenarios encountered during screening (*e.g.* sampling over calendar seasons for sludge), and the use of alternative biota preparations (*e.g.* lyophilised *vs.* wet) as proof of application.

3.1. Chemometric analysis of effluent, sludge, biota screening

Regardless of chemometric software, each replicate clustered closely, indicating that the PCA conditions were suitable in presenting the datasets. Importantly for classification, the data showed that the less biased QuEChERS sample preparation could reproducibly provide datasets that differentiate (and attribute) each environmental matrix (see plots S10–S18), even with a relatively low number of replicates. Furthermore, this could be extended to delineate each sub-class of matrix (*e.g.* seasons and biota preparation) and, with the sample matrix positioning, enable additional information to be derived. For example, WW effluent showed a similar PCA position to the solvent-based standard (and a clear difference to that of the sludge), indicating that this may contain a lower proportion of pollutants than the remaining WW fraction. This was supported by the log-ratio analysis for the standard and effluent matrix, with a significant number of data points exhibiting little difference between these samples (*e.g.* values near zero (see plot S11)). This lower sample complexity for effluent was not unexpected given lipophilic substances (with higher Log P values) are known to predominantly adsorb to sludge (Bhal, 2007), suggesting that this latter matrix may provide greater pollutant information for this analyte type.

It was anticipated that seasonal changes in drug usage may result in PCA differences for the summer and winter months and, from the log-ratio plot, a change in abundance of polar and semi-polar low mass species (~up to 20 min) was observed, with higher levels associated with the summer sample (see Fig. 1). However, upon closer inspection, this variation appeared to be described by a number of *m/z* differences, spanning the hydrophobicity of the chromatogram, and polymer-type substances (presenting as a series of repeating mass units (Thurman et al., 2014) at 13–20 min), emphasising the complexity of the samples and application breadth of QuEChERS. Interestingly, these suspected polymer ions were not apparent in the biota plots, suggesting that they may be either strongly retained within the sludge or sufficiently biodegradable, reducing the likelihood of bioaccumulation within these organisms. This alternative sample composition was also reflected in the PCA data, and further differentiation of sample composition was noted with the biota extraction conditions (*e.g.* wet *vs.* lyophilised). From examining the log-ratio plot, lyophilisation appears to result in a change in sample composition with a higher proportion of non-polar, larger mass ions observed in wet biota, potentially indicating a degradation or transformation of these analytes with lyophilisation (see plot S12). This finding is critical in properly interpreting monitoring data given this change in sample composition with preparation method may significantly mask the true level of pollution within these samples.

To understand what may result in the spatial differences of the sludge and biota matrices, the top 5 *m/z* for the highest loading scores for the PC with the highest variance, were examined and searched within the chromatograms as matrix 'markers' (see Supplementary Excel for PCA loading scores via Aabel). Based on the positioning within the Aabel PCA, these markers would be primarily associated with the sludge and therefore, present at a higher abundance than the remaining samples. Of these ions, the most notable was *m/z* 276.2687, which provided a highly abundant peak at ~25 min and was not observed as a distinct signal within the biota samples. This ion correlated with a formula indicative of a quaternary ammonium biocide ($C_{19}H_{34}N$) and, given a less abundant marker for PC1 also matched this compound class (*m/z* 553.2119 at ~25 min), the data indicates the potential of ammonium biocides as sludge markers and pollutants that warrant further investigation (see section 3.2.1). Furthermore, and more generally, the data evidences that the less targeted QuEChERS sample extraction with averaged chemometric data can generate potential candidates for subsequent monitoring programmes.

3.2. Investigating WW sludge cake

3.2.1. Qualitative analysis

The samples were searched to determine the presence of pollutants of interest to the UK WW sector and based on a pilot in-house screen. Identifications were made via the criteria as per section 2.5.1 within the raw, unadjusted HR-MS data. Four pharmaceuticals and five biocides were assigned within the sludge samples (see Table S19). For the pharmaceuticals, these were consistent with a Log P > 2.5 and, as noted above, this was not unexpected given their propensity to adsorb to this matrix (Berthod et al., 2014). Other targets were suspected (*e.g.* diphenhydramine and BAC-C14) however, despite comparative RT, precursor *m/z* and low-resolution MS/MS data, these identifications were less certain due to a lack of quality HR-MS/MS spectra due to spectral interference or the dynamic exclusion criteria of the HR-MS method. Of those detected, propranolol, carbamazepine, citalopram and loratadine appeared common to summer and winter samples, suggesting a persistent use and loading of these pharmaceutical pollutants throughout the year, and is consistent with other sludge monitoring work (Aydın et al., 2022). For the QACs, the applicability of the lipophilicity criterion was less clear, with some of those observed in sludge with a lower Log P value than 2.5. Again, this was not unexpected given the biphasic nature of QACs (Ruan et al., 2014), and only highlights the difficulty in designing a WW management procedure and monitoring workflow that can segregate (and isolate) these amphiphilic chemicals.

Following these assignments, the base peak chromatogram was explored to identify abundant non-targeted species. Interestingly, the

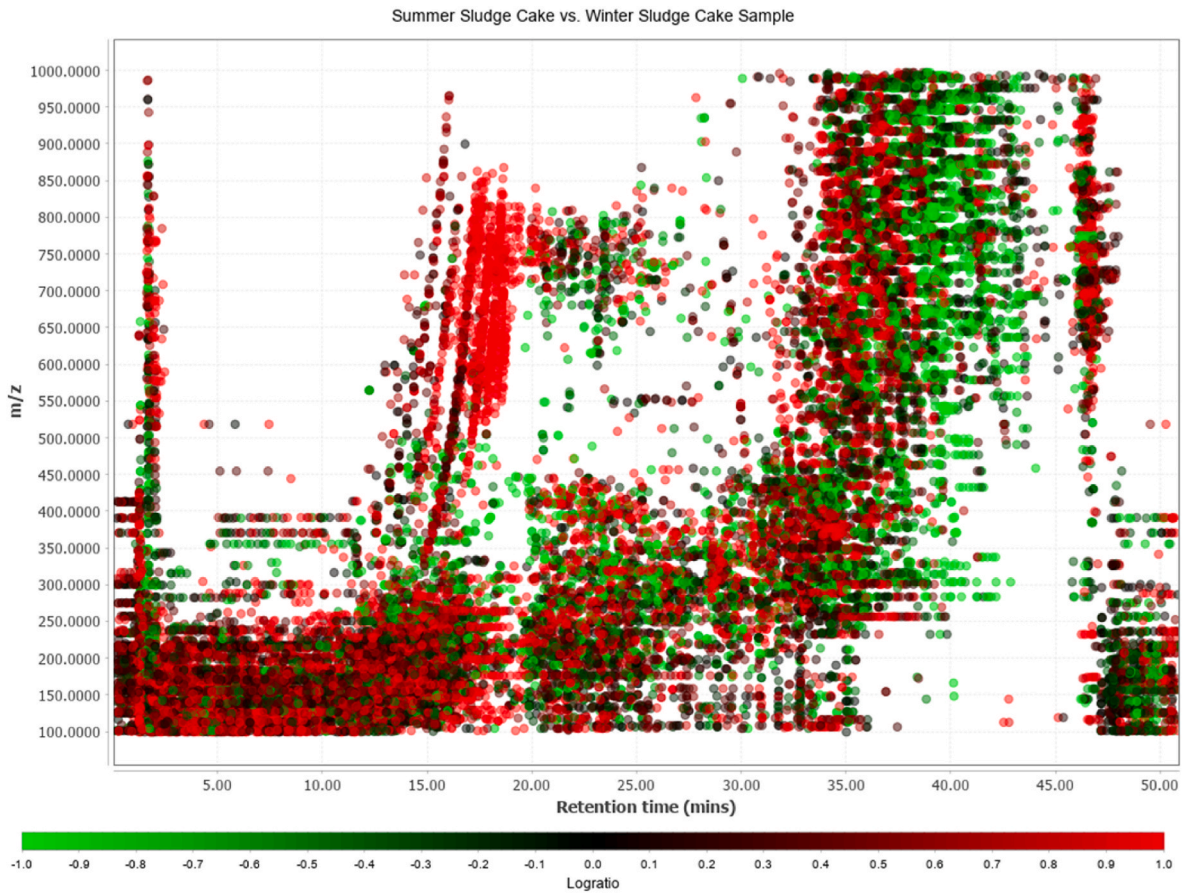

Fig. 1. Log-ratio analysis comparing the summer vs. winter sludge cake samples, showing the degree of change observed for the components detected within these samples (*e.g.* red and green indicate a higher and lower abundance, respectively, of the relevant *m/z* within the summer sample). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

differences between the sludge samples were not particularly obvious from the most abundant species; for example, ions of *m/z* 455.29044 and 267.1859 indicative of verapamil and cyclizine respectively, were not consistently observed across sludge samples (see Table S20). Furthermore, given these *m/z* did not correlate highly with the PCs, it suggests that less abundant ions may be responsible for the seasonal differences in sludge composition and further data mining or sample concentration would be required to identify these candidates. However, data that offered the most interest in terms of novelty were common to both the summer and winter sludge samples, and spanned a *m/z* 200–700. Those of lower *m/z* (200–370) eluted under highly hydrophobic conditions (22–33 min), with *m/z* 200.2371, 228.2688, 270.3157, 276.2687, 312.3621, 332.3312, 360.3630, 368.4256 indicative of $C_{13}H_{30}N$, $C_{15}H_{34}N$, $C_{18}H_{39}N$, $C_{19}H_{34}N$, $C_{21}H_{46}N$, $C_{23}H_{42}N$, $C_{25}H_{46}N$, $C_{25}H_{54}N$, respectively (see Table S21). With a highly similar RT, isotope pattern and elemental formula to QACs, these species are indicative of ammonium compounds, with *m/z* 276.2687, 312.3621 and 368.4256 correlating with BAC-C10, octadecyltrimethylammonium and trioctylmethylammonium cations, respectively. Both aliphatic and aromatic QACs have been observed in sludge and discussed in the literature (Ruan et al., 2014; Li and Brownawell, 2009; Martínez-Carballo et al., 2007; Zhang et al., 2015) however, these studies were largely targeted to specific QACs for samples collected outside of the UK, underlining the limited scope of work and disparity in UK-based monitoring (Li and Brownawell, 2009; Martínez-Carballo et al., 2007; Clara et al., 2007; Breen et al., 1996). Furthermore, with the majority of these ions not correlating with known pollutants (*e.g.* the NORMAN list), this emphasises the need to explore these analytes more broadly as candidate emerging pollutants within monitoring programmes. In addition to these targets, further similarities between these samples included the tentatively assigned sertraline (matched to published works), along with a selection of ions that did not match the NORMAN database (*e.g. m/z* 211.0866 and 258.2794 (see Table S20). These latter unidentified ions indicate the presence of oxygen functionalities with a product ion loss consistent with water (18 Da) however, without further information assignments were not possible.

For the higher *m/z* species, these were indicative of several polymers (see Fig. 2 and sections S22 and 23). The first was observed as a series of peaks at ~14 min that spanned *m/z* 415.2540–789.4464, and were consistent with the findings of the log-ratio plots noted earlier as a polymer containing a subunit of mass 44 Da. These ions showed an isotope pattern (^{13}C), relevant adduct *m/z* of the precursor molecule (protonated, ammonium and sodium species at +1, +17 and + 22 mass units, respectively), and product ion data (loss of 17 or 18 from NH_3 or H_2O, respectively), consistent with polyethylene glycol (PEG) 400 (Thurman et al., 2014; Vijaya Bhaskar et al., 2013). Given PEG 400 is common pharmaceutical excipient (Basit et al., 2001), this was not unexpected however, despite its perceived low toxicity, there is increasing concern of the environmental fate and persistence of these water-soluble polymers, and the toxicity of related species (*e.g.* transformation products) (Huppertsberg et al., 2020). These data, therefore, underline the need and use of QuEChERS as a possible solution in achieving a reliable and sensitive monitoring method for these pollutants.

In addition to PEG 400, the data showed a peak series corresponding to Triton QS-15 and the less abundant structural analogues of the detergent (Hu and Cole, 2019). Whilst the detection of detergents is also not unexpected (Bergé et al., 2016, 2018; Olkowska et al., 2011), this

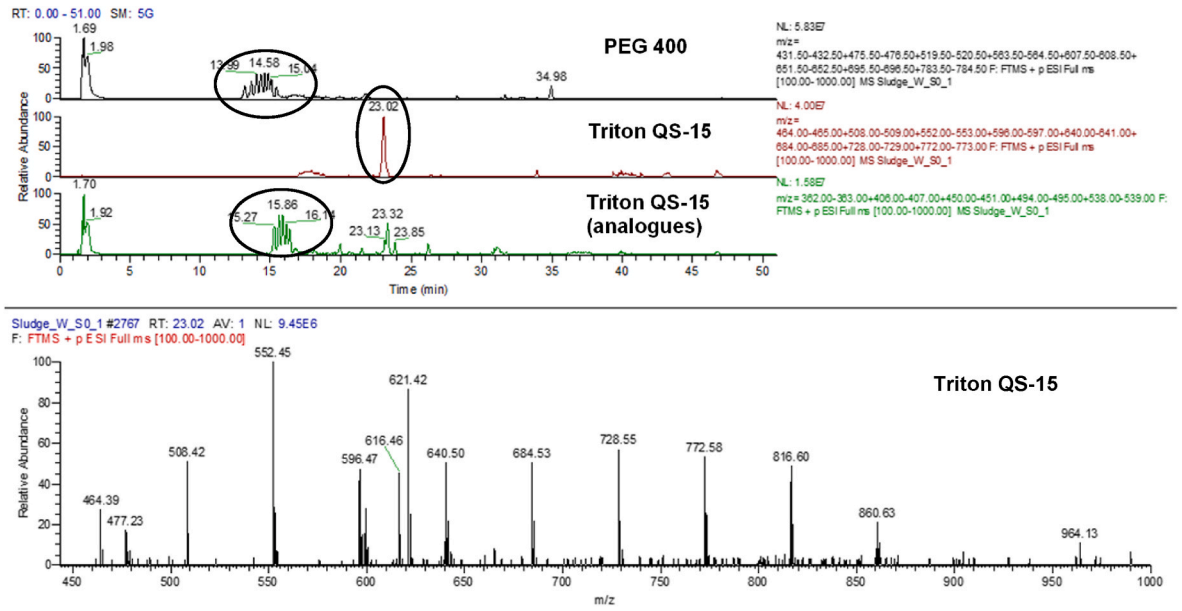

Fig. 2. The extracted ion chromatograms of suspected PEG 400 polymer, Triton QS-15 surfactant, and the Triton QS-15 analogue (top), and the mass spectrum of the peak at 23 min, with an aggregate pattern indicative of Triton QS-15, suggesting that this aggregation may be from the ESI process. Interestingly, the Triton QS-15 analogue shows a chromatographic pattern indicative of a polymeric structure, suggesting that this species is not a simple surfactant moiety.

substance has, to the best of the authors' knowledge, yet to be recognised within literature as an emerging pollutant. Therefore, given the limited number of studies that have specifically monitored these polymer and surfactant substances in sludge (Petrovic and Barcelo, 2000), this new data again underlines the need to explore the environmental impact of these substances and related products, and the value of QuEChERS in extracting them from sludge.

3.2.2. *Quantitative analysis*

To establish if the differences observed for the summer and winter sludge in the chemometric data were supported by changes in drug usage, these samples were extracted with and without the addition of IS to ensure selectivity for accurate quantitation. However, this required the use of pronethalol to quantify citalopram in the winter sample, and talopram for propranolol within the summer sample due to a high background response causing limited selectivity of the original structural analogue ISs. Despite this, acceptable quantitative performance was achieved for these compounds, with good linearity ($R^2 \geq 0.99$), precision and accuracy (<20% RSD and deviation, respectively) at concentrations across the dynamic range (see Tables S24a). When applied to winter and summer sludge samples, clear differences were observed for the pharmaceuticals, with four measured at or above the IDL of a S/N $\geq$ 3 (Cheeseman et al., 1989) (see Table S26). However, of these pharmaceuticals, only citalopram was sufficiently abundant to quantify within the winter sludge (*e.g.* S/N > 10), and this was present at levels (995.3 μg/kg) consistent with previous studies concerning this matrix (Magnér et al., 2016; Evans et al., 2015).

Unlike the pharmaceuticals, peaks indicative of the biocides were observed with a much higher signal intensity, and as such, a 1:400 dilution of the sludge extracts was required for accurate quantitation within the method dynamic range. When applied, BAC-C12, BAC-C14, BAC-C18, HDTMA and DDMA were measured at milligram amounts (2.3–70.4 mg/kg, see Table S26), exceeding previous reported values (Martínez-Carballo et al., 2007) and emerging compound limits for sludge (0.1 μg/kg) (European Union, 2013). These concentrations also nearly doubled for 3 out of 6 QACs between the winter and summer samples, highlighting potential differences in seasonal usage (*e.g.* as an algaecide for swimming pools during the summer (Baracuda Pool Algaecide) and the changing demands of WW treatment. Unfortunately, a direct comparison with more substantial UK-based monitoring programmes that detect QACs throughout the year was not possible given published work has involved alternative locations (Martínez-Carballo et al., 2007) or the measurement of QAC sub-classes (Fernández et al., 1996) rather than individual analytes. This lack of monitoring, with known links to antimicrobial resistance (AMR) (Mulder et al., 2018; Gerba, 2015) at the levels observed in this current work, and additional unidentified ammonium compounds indicated by the screen, further underlines the need for QACs to be included within UK (and global) monitoring programmes as emerging substances. Importantly, this work shows the usability of QuEChERS in detecting these substances in sludge across seasons, to ensure levels are accurately understood for risk assessment and mitigation, particularly if sludge is re-used or disposed of on land/in landfill.

3.3. *Investigating wastewater: effluent (winter season)*

3.3.1. *Qualitative analysis*

The QuEChERS method was applied to a treated effluent sample that was complimentary to the sludge cake and processed according to the methodologies described in sections 2.5-2.7. The chemometric data showed significant differences between effluent and sludge/biota samples, and a sample complexity similar to the pharmaceutical standard mixture according to the cluster analysis. This, with the log-ratio plots (see plots S16 and 17), suggested that the effluent extract is less populated than sludge (regardless of season), with the latter potentially offering a richer source of pollutants when using this QuEChERS sample preparation. This was evident for the target pharmaceuticals (and additional compounds observed in the sludge (see S19-23)), where precursor ions indicative of propranolol, citalopram and carbamazepine were observed but at very low levels in the effluent sample. This was not unexpected given these compounds have significant lipophilicity (log P values 2–3 (Silverman and Holladay, 2015)) and are capable of partitioning between effluent and sludge (Berthod et al., 2014). Therefore, given the poor signal intensity (S/N ~3) leading to an inconclusive identification, no further analysis was undertaken. However, unlike the pharmaceuticals and other components identified within the sludge, ions indicative of four of the biocides were detected within effluent, with that for BAC-C12 observed in the greatest abundance (see Table S19).

Given the amphiphilic nature of these analytes, these were anticipated to be present within sludge and effluent, and this data further supports reports of QACs within aqueous samples highlighting the challenge for WW treatment in segregating these chemicals to a specific WW pathway.

3.3.2. Quantitative analysis

Of the target analytes selected for monitoring, the QAC biocides were observed at sufficient abundance to allow detection however, only BAC-C12 was suitable for quantitation. This was not unexpected given this QAC showed the greatest abundance across sludge samples and its sorption to sludge was expected, reducing the resulting levels in effluent. However, despite this measured concentration being lower than the sludge data at 1.5 ng/mL (see Table S26), this was a comparative level to previous reports for effluent that had undergone a more laborious extraction process (0.081–4.1 μg/L) (Kreuzinger et al., 2007). This finding indicates that the QuEChERS protocol offers the potential to function across environmental sample types with an equivalent performance to established methods.

3.4. Investigating biota

3.4.1. Qualitative analysis

Locally sourced homogenised Mussel tissue was extracted using the manual QuEChERS method to determine whether the pharmaceuticals and biocides could collect within this matrix (as per the sludge due to a comparative lipid content (Zhu et al., 2017; Uno et al., 2001)). Like the sludge, the total ion chromatogram recorded for the wet and FD samples was considerably populated, with FD biota showing a marginally higher background signal, possibly due to sample concentration associated with lyophilisation (see section S27). These differences were also reflected in the PCA with clusters observed in alternative locations for the wet and FD samples. However, with the samples showing a similar profile for the target analytes, this data indicated that their presence alone was not contributing to segregation of samples in the PCA. Furthermore, with abundant non-targeted analytes also showing a similar profile across biota samples (*e.g.* m/z 430.2432, 150.0909, 228.2323, 254.2479, and 282.2795, see Table S20), this suggests that differences in analyte abundance, or un-investigated low-level analytes between sample types are resulting in the segregation observed within the PCA. This emphasises the value of chemometrics in classifying samples and providing additional information from large datasets. However, given m/z 430.2432 and 150.0909 were also observed in the effluent sample at similar retention times (~15 and 21 min, respectively) these were searched against a recent comprehensive list of biota pollutants (Miller et al., 2018) for potential identifications. With no correlating candidates, these were interpreted directly from the HR-MS data, and showed characteristics indicative of additional alkylated/aromatic amines to those observed in sludge; this again suggests the presence of an alternative biocide or ammonium transformation products following biota consumption or environmental exposure.

3.4.2. Quantitative analysis

To explore the levels of the targeted pollutants in each sample, these were analysed using the same protocol described for sludge. Interestingly, these samples appeared considerably populated with material, and this seemed to cause a shift in retention time and some signal suppression. As such many of the analytes were more difficult to observe, with only BAC-C12 and BAC-C18 measured, and at lower amounts than sludge with concentrations of 7.4 and 5.5 μg/kg, respectively within the wet biota (see Table S26). With a recent publication highlighting the need to explore the fate of these chemicals in biota following the COVID pandemic due to increased use of sanitisers (Hora et al., 2020), this further justifies the importance of monitoring BACs, particularly given biota may be exposed through indirect routes such as run-off from farmland, or via "clean" treated WW that is dispensed into the water course. BAC-C12 and BAC-C18 were also detected within FD samples however, these were at lower concentrations, with only BAC-C12 at a measurable concentration of 5.5 μg/kg. This effect may be due to "free" BAC binding to phospholipids within the biota sample (Maris, 1995) during water removal, potentially lowering their observed concentrations, however this would require further analysis for confirmation.

3.5. Feasibility testing of the modified QuEChERS method for high throughput analysis of environmental solids

The manual processing of samples is the main limitation of the standard QuEChERS method for routine analyses within a regulated environment. To explore the potential of automating the protocol, the method was transferred to a system with a robotic liquid sample handler, capable of performing the initial extraction and automated SPE. Admittedly, the latter SPE stage would operate differently with a stationary sorbent however, the aim of the mechanism remained (*e.g.* capturing targeted interferences and allowing the remaining sample to pass through the sorbent). To evaluate the extraction performance under real-world conditions, it was assessed in terms of recovery and precision for selected compounds that represented the diversity (acid, base, neutral, amphiphilic) of those needed for UK regulatory programmes and identified from screening. These were measured as spiked triplicate samples for example environmental matrices (soil, clay and biota), with the recovery determined using a solvent-based calibration graph due to the difficulty in determining analyte signal when spiked after extraction using this automated approach.

All compounds tested were recovered with a high degree of precision ($\leq$10.3% RSD) within soil and clay, and this was significantly improved (as expected) *vs.* the manual protocol (*e.g.* <18% RSD) (Townsend et al., 2020), showing the benefit of automation (see Table 1).

Furthermore, despite the over-recovery of citalopram (potentially due to slightly higher matrix effect as observed in past work for soil (Townsend et al., 2020)), the remaining test compounds showed improved analyte recovery (a minimum of 62.0%) with automation versus the manual method (Townsend et al., 2020).

Interestingly, the QACs showed slightly poorer precision within clay however, these remained within 10.3% RSD, offering a good degree of reliability within this challenging matrix and excellent recoveries. Unfortunately, there is little comparative work involving the extraction of these pharmaceuticals in clay (Acosta-Dacal et al., 2020) however, given the data presented in our current study ranges from 88.0 to 131.1% for analytes that extend a range of chemical types (including log P and pKa), this offers significant potential as a highly competitive protocol for these common environmental solids.

Finally, to investigate the potential of this automated approach for biota, an aliquot of the sample used in the original quantitation work above (stored at −20 °C between analyses), with a freshly homogenised Mussel (M) and Dab (D) fish tissue were prepared and analysed in triplicate. Interestingly, the precision data shown in Table 1 typically had greater variation than soil and clay for certain compounds (*e.g.* loratadine and QACs). These are compounds that can interact strongly with lipophilic environments given their partition coefficient (log P) or biphasic character (Comber et al., 2008), and as such, it is not unexpected that these would be more difficult to reliably extract from high fat matrices such as biota. Therefore, this data indicates that an additional step may be required to improve the extraction of these lipophilic species, such as an alternative homogenisation process (*e.g.* milling), given this can enhance the release of substances from particulate material (Sun et al., 2021). However, despite this limitation, the recoveries for these matrices remained high, indicating the excellent efficiency of this method in measuring loratadine at least, for biota contamination. Admittedly, the over-recovery of the QACs does indicate a degree of matrix effects that warrants further investigation however, when combined with the soil and clay data, the overall usability and benefits of automating QuEChERS for environmental analysis is clear. Specifically, by using this protocol it can reliably extract with a high degree of

Table 1
Summary data of the extraction performance of the automated QuEChERS protocol for selected substances within soil, clay and biota, describing the mean amount recovered (Amnt), its standard deviation (sd), %RSD or precision (%P), and % recovery (%Rec). Values for triplicate samples have been determined by subtracting the relevant matrix blank to reduce matrix interference. The data for soils and clay were determined from triplicate quality controls prepared with in-matrix concentrations of 512.5 ng/g for the pharmaceuticals and 102.5 ng/g for the QACs, with an internal standard concentration of 1025 ng/g and 205 ng/g, respectively. Similarly, triplicate quality controls were prepared for biota samples with in-matrix concentrations of 400 ng/mL and 80 ng/mL for pharmaceuticals and QACs, respectively, with internal standard concentrations of 800 ng/mL for the pharmaceuticals and 160 ng/mL for the QACs. Biota matrix samples = 1 and 2) comparative biota (Mussel) sample to that used for the manual extraction, 6M and 7M) freshly homogenised biota (Mussel) sample, 7D and 8D) freshly homogenised fish (Dab) sample. Note: data for loratadine has been established from estimated values due to calibration issues that resulted in a reduced calibration range.

Compound		Matrix							
		Soil	Clay	Biota					
				1	2	6M	7M	7D	8D
Citalopram	Amnt (ng)	70.6	65.6	49.5	49.6	50.1	52.0	54.4	54.4
	sd	1.09	0.13	1.27	0.35	0.72	0.52	1.27	0.71
	%P	1.5	0.20	2.6	0.7	1.4	1.0	2.3	1.3
	%Rec	141.3	131.1	96.4	95.4	97.0	100.8	104.7	105.4
Carbamazepine	Amnt (ng)	54.3	54.7	51.3	51.4	52.2	52.2	51.1	51.8
	sd	1.76	1.44	0.74	0.19	0.71	0.71	0.14	0.11
	%P	3.2	2.6	1.4	0.4	1.4	1.4	0.3	0.2
	%Rec	108.6	109.4	101.5	100.6	102.7	102.6	99.6	101.9
Loratadine	Amnt (ng)	81.7	89.0	45.0	49.2	53.5	51.7	54.3	50.1
	sd	2.70	0.72	5.65	2.06	2.99	11.42	2.23	3.24
	%P	3.3	0.81	12.6	4.2	5.6	22.1	4.1	6.5
	%Rec	81.1	95.7	89.3	96.8	106.1	101.6	105.8	98.6
BAC-C12	Amnt (ng)	15.0	21.6	15.9	15.0	13.1	14.1	12.5	12.1
	sd	0.27	2.16	1.50	1.28	1.79	2.91	0.11	0.26
	%P	1.8	10.0	9.5	8.5	13.7	20.6	0.9	2.2
	%Rec	62.0	88.0	150.7	149.8	130.5	122.9	124.9	121.4
BAC-C14	Amnt (ng)	13.2	16.0	16.0	16.6	16.7	16.9	16.0	17.1
	sd	0.11	1.65	1.28	0.01	0.30	0.42	1.01	0.96
	%P	0.87	10.3	8.0	0.1	1.8	2.5	6.3	5.6
	%Rec	65.8	94.7	160.1	166.3	166.7	169.0	159.6	171.5

recovery amphiphilic species and those across the pKa range from soils and clay, with significant potential for application to biota.

4. Conclusion

Current sample preparation methods for WW products and biota are typically time-consuming and labour intensive, unsuitable for high throughput screening. We have shown that the QuEChERS method developed in this study is an alternative sample preparation for a range of environmental samples required for pollution monitoring, reducing the extraction time from hours to ~20 min/sample. The loss in extraction recovery observed for some compounds, does not outweigh the benefit of reduced preparation time, solvent usage (from ~200 mL to 15 mL), and need for complex vacuum or distillation apparatus seen in regulatory methods (*e.g.* EPA). The application of this simple, manual method has shown largely reproducible recovery of a range of pharmaceuticals and biocidal surfactants (Townsend et al., 2020), with clear, repeatable clustering of matrix types following PCA. Of these matrices, sludge and biota showed the highest level of pollutants and were observed at different regions of the PCA plot. Therefore, this data showed that despite its less biased approach, QuEChERS can extract sufficient sample information for the differentiation of each environmental matrix and sub-class, generating highly selective datasets for sample discrimination and attribution.

For the pollutants of interest to the UK WW industry, the calculated concentrations significantly exceeded suggested IDL values (at 0.01 μg/L) for sludge, indicating insufficient removal and/or bioaccumulation during WW treatment. Interestingly, some of these pharmaceuticals were common across seasons (suggesting all-year round release), with citalopram measured higher than previous studies in winter sludge. However, many abundant substances were beyond the scope of the initial investigation; QAC biocides, PEG, Triton QS-15 and associated analogues were detected, with BAC-C12, DDMA and HDTMA at higher levels than previous studies. Notably, the Triton QS-15 substances are yet to be recognised as emerging pollutants to the best of the authors' knowledge and, along with the detection of a range of ammonium species that do not correlate with existing pollutant databases, this highlights the need to better investigate detergent (and polymer) pollution within sludge, particularly for its re-use or deposition on land/in landfill. Furthermore, two of the QACs were also observed within effluent and both preparations of homogenised biota; given these were above the typical biota EQSD (European Union, 2008), and are linked to AMR, this data with the presence of the remaining detergents, underlines the need to monitor these substances across environmental samples, with QuEChERS proving to be a viable approach to achieve this.

Finally, we have also shown that the QuEChERS protocol can operate within a fully automated workflow, further reducing the resource and time for the total analysis to under 40 min. This method operates with improved precision for soil and biota and has been extended to the largely un-investigated clay matrix, with extractions showing a high degree of accuracy and precision for the tested pharmaceuticals and biocides. To the best of the authors' knowledge, this work is an advancement of QuEChERS for environmental monitoring and shows that this protocol can meet the needs of a high-throughput regulatory laboratory.

Author contribution

Ruth Godfrey – Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization, Supervision, Project administration. Jonathan Dunscombe - Methodology, Formal analysis, Investigation, Data curation, Visualization, Resources, Writing – review & editing. Anthony Gravell - Supervision, Writing – review & editing. Ann Hunter – Methodology, Resources, Writing – review & editing. Mark P. Barrow – Methodology, Resources, Formal analysis, Writing – review & editing, Visualization. Geertje van Keulen - Writing – review & editing, Supervision. Claire Desbrow - Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. Rachel Townsend - Conceptualization,

Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

doi for the data has been provided in the manuscript

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2022.136313.

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