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Basic micro-pollutants in sludge from municipal wastewater treatment plants in the Northwest Spain: Occurrence and risk assessment of sludge disposal

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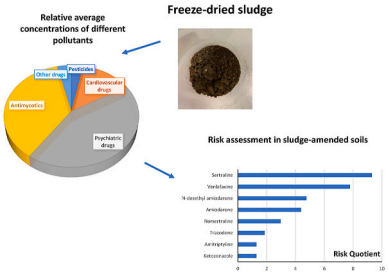
HIGHLIGHTS

- Single-step, effective extraction of basic micro-pollutants from sludge.
- Accurate recoveries using solvent-based calibration standards.
- Detection frequencies above 85% for 33 compounds in sludge from 45 STPs.
- Eight species highlighted as hazardous based on their RQs for sludge-amended soil.

GRAPHICAL ABSTRACT



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ABSTRACT

Sludge is one of the most problematic residues generated during wastewater treatment. Herein, we validate a single-step, sensitive procedure for the determination of a selection of 46 basic micro-pollutants, either used as pharmaceuticals or pesticides, in sludge from municipal sewage treatment plants (STPs), using liquid chromatography tandem mass spectrometry as determination technique. The proposed method permitted to achieve accurate recoveries (values from 70% to 120%, for samples spiked at different concentration levels) using solvent-based calibration standards. This feature, combined with limits of quantification lower than 5 ng g^{-1} (dry weight), allowed the rapid and sensitive quantification of target compounds in freeze-dried sludge samples. Out of 46 investigated pollutants, 33 species showed detection frequencies above 85% in a group of 48 sludge samples, obtained from 45 STPs located in the Northwest of Spain. The assessment of eco-toxicological risks associated to sludge disposal as fertilizer in agriculture and/or forestry, considering average concentrations found in sludge samples, highlighted eight pollutants (sertraline, venlafaxine, N-desethyl amiodarone, amiodarone, norsertraline, trazodone, amitriptyline and ketoconazole) representing an environmental hazard based on ratios between predicted soil levels and non-effect concentrations estimated using the equilibrium partition method.

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Victoria Fernández: Performing the experiments, Formal analysis, Writing-reviewing and editing. María Ramil: Formal analysis, Supervision, Writing-original draft. Isaac Rodríguez: Fund acquisition, Formal analysis, Writing-original draft, Supervision, Conceptualization.

1. Introduction

The occurrence of micro-pollutants of emerging concern in different environmental matrices has been systematically highlighted since the end of the previous century (Hirsch et al., 1998; Ternes, 1998). In most cases, these compounds enter the aquatic environment through an incomplete elimination at sewage treatment plants (STPs), and once they are introduced in the water cycle, they can trigger different harmful effects to the wildlife (Länge et al., 2001; Oaks et al., 2004). The non-quantitative removal of organic micro-contaminants in STPs may be due to limited biodegradability, transformation into other compounds, and/or adsorption on solid matrices such as sludge (Martín et al., 2012). Despite the concentrations of emerging pollutants in the water phase of STPs have been largely investigated in the literature (Tran et al., 2018; Wiest et al., 2021), limited information is available regarding their potential accumulation in sludge (Mailler et al., 2017; Peysson and Vulliet, 2013; Steele et al., 2022).

Assessing the residues of emerging pollutants in sludge is required to understand their behaviour and mass balances at STPs, and to implement additional facilities in the treatment of sludge, attending to found compounds and concentration levels (Patureau et al., 2021). Moreover, the identification and quantification of those contaminants existing in sludge is required to estimate the risk of re-introduction in the terrestrial environment through direct application of sludge as fertilizer (Beausse, 2004; Chen et al., 2013), or indirectly, using sludge for the production of compost. Depending on the sludge treatments applied at STPs, and the physico-chemical properties of wastewater discharged micro-pollutants, their residues in sludge might range from non-detected up to the μg per g level (expressed as dry matter) (Aydın et al., 2022; Castro et al., 2021; Peysson and Vulliet, 2013).

Analytical procedures applied to the determination of emerging contaminants in sludge are based on liquid chromatography (LC) combined with tandem mass spectrometry (MS/MS) (Martín-Pozo et al., 2019; Pérez-Lemus et al., 2019), after a previous extraction and sample preparation step. QuEChERS method (Campo et al., 2013; Peysson and Vulliet, 2013), ultrasound-assisted extraction (Aydın et al., 2022; Ivanová et al., 2018), pressurized liquid extraction (PLE) (Pang et al., 2017; Radjenović et al., 2009), and matrix solid-phase dispersion (Castro et al., 2021; Li et al., 2016) have been proposed for compounds extraction followed, either by direct analysis of primary extracts (Castro et al., 2021), or by combination with concentration approaches previously optimized for wastewater samples, i.e., solid-phase using reversed-phase materials (Aydın et al., 2022; Chen et al., 2013; Pang et al., 2017). Some of these methodologies show limited selectivity, ending in relatively complex extracts due to the high organic load of the sludge matrix and the lack of clean-up, or fractionation protocols, to separate emerging pollutants from other organic species present in sludge (i.e. natural origin organic compounds). Quite often, low selective extractions are associated to signal suppression effects during compounds ionization in the electrospray source (ESI) of LC-MS/MS systems (Radjenović et al., 2009). Obviously, signal suppression affects negatively the accuracy of the obtained results, and increase the LOQs of the procedure.

In a previous study (Castro et al., 2021), in which a non-target screening methodology was used, many compounds of basic nature were identified in sludge extracts obtained from a limited number of STPs; however, the performance of the sample preparation method, and thus the accuracy of found concentrations, were not fully validated, as the methodology was developed just for screening purposes. The prevalence of basic micro-pollutants in sludge can be explained considering that many pharmaceuticals, and/or their excretion metabolites, are poorly biodegradable compounds. Moreover, their interaction with sludge might be understood due to sorption of lipophilic compounds, and electrostatic interactions with the negatively charged surface of sludge particles.

The aims of this research were to validate a direct procedure for the determination of a selection of basic compounds in sludge using LC-MS/MS detection, to determine their presence in a representative number of STPs, from Galicia (Northwest Spain), and to assess the environmental risk associated to potential uses of the evaluated sludge samples as agriculture fertilizers. The suite of 46 compounds comprise psychiatric, cardiovascular and antimycotic drugs, in addition of certain pesticides. Some of these substances such as venlafaxine, O-desmethyl venlafaxine and several azolic compounds such as miconazole, penconazole and tebuconazole have been included in the 4th Watch list for Union-wide monitoring in the field of water policy (European Commission, 2022). Despite this fact, and the biocide properties of some of them, in the current regional legislation, there are not maximum threshold limits, neither referred directly to sludge from STPs, nor for compost (elaborated combining municipal sludge with vegetable stuff), intended to be used as fertilizers, either in agriculture, or in forestry.

2. Materials and methods

2.1. Solvents, sorbents and standards

Methanol (MeOH), formic acid (FA), both LC-MS grade, and ammonia (NH_3, 7 M solution in MeOH) were purchased from Merck (Darmstadt, Germany). Ultrapure deionized water (18.2 MΩ cm^{-1}) was obtained from a Genie U system (Rephile, Shanghai, China). Bondesil-C_{18} (40 μm particle size) and ethylenediamine-N-propyl-bonded silica (PSA), both as bulk sorbents, were purchased from Agilent (Santa Clara, CA, USA) and Merck, respectively. Cationic exchange SCX-type 500 mg cartridges, containing sulfonic functionalities bonded to silica particles, were obtained from Agilent. All sorbents used in the sample preparation process (C18, PSA and SCX) were employed as received, without any previous clean-up or conditioning step. Polypropylene syringes (10 mL), and 20 μm pore-size polyethylene frits, employed for matrix solid-phase dispersion extraction (MSPD), were provided by BD Biosciences (Franklin Lakes, New Jersey, USA) and Phenomenex (Torrance, CA, USA) respectively.

Individual standards of target pollutants were purchased from Sigma-Aldrich (St, Louis, MO, USA). Most of them (33 compounds) are human use drugs, and the other 13 species are employed as pesticides. Many of the selected pesticides have been previously found either in agriculture soils (Pérez-Mayán et al., 2020), or in groundwater samples (Herrero-Hernández et al., 2016), from regions impacted by agriculture activities in Spain. As regards pharmaceuticals, the choice was done attending to prescription rates and previous reports of occurrence in liquid and solid matrices, from municipal wastewater (Tran et al., 2018) to agricultural soils amended with STP sludge (Chen et al., 2013). Table 1 summarizes the list of substances considered in this research, together with the surrogate standard (SSs, either deuterated or ^{13}C species) associated to each species for quantification purposes. SSs were provided either by Merck, or by Toronto Research Chemicals (North York, Ontario, Canada). In addition to the basic substances included in Table 1, a selection of four additional pollutants: chlorpyrifos, clofentezine, diclofenac and candesartan were considered as model compounds to assess the behavior of neutral and zwitterionic species during the on-line extraction and clean-up methodology applied in this research.

Stock solutions of each compound were prepared in MeOH. Further dilutions and mixtures were made in the same solvent. Individual solutions and concentrated mixtures of native compounds and SSs were kept at −18 °C, and used throughout this study (c.a. 6 months). Solvent-

Table 1
Summary of target compounds, including retention times, ESI-MS/MS determination conditions, SSs, instrumental LOQs, and linearity evaluation (0.1–150 μg L^{-1}, n = 10 levels).

Compound	Retention time (min)	Precursor ion	Q1 (CE)	Q2 (CE)	Ratio (Q2/Q1)	IS	LOQ (μg L^{-1})	Linearity (R^2, 0.1–150 μg L^{-1})
PESTICIDES								
Ametoctradin	12.81	276.2	176.1 (48)	70.0 (32)	0.15	Myclobutanil-d4	0.2	0.9997
Carbendazim	3.27	192.1	160.1 (16)	132.1 (32)	0.2	Carbendazim-d3	0.1	0.9987
Difenoconazole	13.11	406.0	251.1 (25)	111.1 (60)	0.25	Myclobutanil-d4	0.1	0.9998
Imazalil	8.80	297.1	69 (16)	255.0 (12)	0.27	Imazalil-d5	0.5	0.9975
Metconazole	12.78	320.1	70 (28)	124.9 (52)	0.09	Tebuconazole-d9	0.2	0.9987
Myclobutanil	11.71	289.1	70.1 (16)	125.1 (32)	0.25	Myclobutanil-d4	0.3	0.9964
Penconazole	12.42	284.1	70.1 (15)	159.0 (30)	0.45	Tebuconazole-d9	0.2	0.9996
Propiconazole	12.66	342.1	159 (32)	69.1 (16)	0.8	Myclobutanil-d4	0.5	0.9963
Tebuconazole	12.53	308.1	70.0 (40)	124.9 (47)	0.08	Tebuconazole-d9	0.2	0.9991
Terbutryn	9.84	242.0	185.9 (20)	68.0 (60)	0.32	Imazalil-d5	0.5	0.9980
Tetraconazole	12.08	372.0	70.0 (24)	158.8 (32)	1	Myclobutanil-d4	0.3	0.9995
Thiabendazole	4.94	202.0	175.0 (28)	131.1 (40)	0.73	Tramadol 13C d3	0.1	0.9990
Triadimenol	11.81	296.1	70.0 (8)	99.1 (8)	0.04	Myclobutanil-d4	0.5	0.9989
PHARMACEUTICALS								
Cardiovascular drugs								
Amiodarone	12.09	646.0	100.1 (36)	201.1 (40)	0.66	Miconazole-d5	0.5	0.9975
Atenolol	1.69	267.0	190.0 (16)	144.9 (25)	1.33	Tramadol 13C d3	0.1	0.9990
Carvedilol	8.86	407.2	100.1 (32)	224.1 (24)	0.52	Flecainide-d4	0.2	0.9981
Flecainide	8.14	415.1	398.0 (24)	301.0 (40)	0.61	Flecainide-d4	0.1	0.9980
Irbesartan	10.99	429	207 (24)	195.0 (24)	0.18	Irbesartan-d4	0.1	0.9983
N-desethyl amiodarone[a]	11.89	618	72.1(28)	546.9 (24)	0.53	Miconazole-d5	0.1	0.9973
Propranolol	7.59	260.16	116.1(20)	183.1 (20)	0.6	Propanolol-d7	0.5	0.9963
Psychiatric drugs								
Amisulpride	5.49	370.2	242.0 (20)	196.0 (40)	0.75	Flecainide-d4	0.1	0.9984
Amitriptyline	9.21	278.2	233.1 (16)	91.1 (36)	0.96	Imazalil-d5	0.1	0.9987
Citalopram	8.02	325.2	109.0 (28)	262.1 (16)	0.32	N-desmethyl citalopram-d3	0.1	0.9987
Clomipramine	9.85	315.2	86.1 (20)	58.1(56)	0.75	Clotrimazole-d5	0.1	0.9986
Clozapine	7.25	327.1	270.1 (24)	191.9 (52)	0.66	Imazalil-d5	0.5	0.9982
Haloperidol	8.68	376	123.0 (44)	165.1 (24)	0.92	Flecainide-d4	0.2	0.9986
Lamotrigine	5.57	256.0	43.1(40)	210.8 (32)	0.22	Lamotrigine-13C3	0.2	0.9987
Mirtazapine	5.51	266.2	194.9 (28)	72.0 (20)	0.73	Lamotrigine-13C3	0.2	0.9970
N-desmethyl citalopram[a]	8.06	311.2	108.9 (28)	262.0 (16)	0.49	N-desmethyl citalopram-d3	0.1	0.9983
Norsertraline[a]	9.91	275.0	158.8 (20)	129.1 (30)	0.1	Myclobutanil-d4	0.5	0.9941
O-desmethyl venlafaxine[a]	5.32	264.0	58.0 (17)			Venlafaxine-d6	0.1	0.9982
Sertraline	9.78	306.1	158.9(36)	275.0 (12)	0.83	Myclobutanil-d4	0.2	0.9979
Trazodone	7.17	372.2	176.1 (24)	147.9 (40)	0.7	Tramadol 13C d3	0.1	0.9991
Venlafaxine	7.24	278.0	58.0 (25)	260.0 (9)	0.27	Venlafaxine-d6	0.2	0.9992
Antimycotic drugs								
Climbazole	9.20	293.1	197.1 (16)	141.0 (24)	0.89	Climbazole-d4	0.3	0.9964
Clotrimazole	9.93	277.1	164.9 (28)	239.0 (60)	0.45	Clotrimazole-d5	0.1	0.9989
Fenticonazole	12.02	454.9/456.9	198.9 (36)	198.9 (36)	0.64	Miconazole-d5	0.1	0.9980
Fluconazole	7.40	307.1	219.9 (20)	70.0 (44)	0.5	Tramadol 13C d3	0.5	0.9987

(*continued on next page*)

Table 1 (*continued*)

Compound	Retention time (min)	Precursor ion	Q1 (CE)	Q2 (CE)	Ratio (Q2/Q1)	IS	LOQ (μg L^{-1})	Linearity (R^2, 0.1–150 μg L^{-1})
Ketoconazole	9.72	531.2	82.0 (52)	489.1 (36)	0.4	Tebuconazole-d9	0.5	0.9988
Miconazole	11.25	417.0	158.8 (36)	160.8 (40)	0.98	Miconazole-d5	0.5	0.9966
Sertaconazole	11.31	437/439	180.9 (40/36)		0.63	Miconazole-d5	0.1	0.9971
Tioconazole	10.42	387.0	130.9 (32)	68.9 (24)	0.06	Miconazole d5	0.1	0.9968
Other drugs								
Cinnarizine	10.34	369.2	166.9 (16)	151.9 (60)	0.32	Climbazole-d4	0.1	0.9974
Cloperastine	9.53	330.2	201 (16)	166.1 (40)	0.58	Clotrimazole-d5	0.1	0.9989
Tramadol	5.83	264.2	58.1(15)			Tramadol 13C d3	0.1	0.9996
Trimethoprim	4.99	291.4	230.2 (25)	122.8 (25)	0.59	Lamotrigine-13C3	0.1	0.9986

[a] Drug metabolite.

based calibration mixtures, from 0.1 to 150 μg L^{-1}, were prepared in MeOH: NH_3 (98:2) and maintained at 4 °C. These solutions were renewed every 2 months. SSs were included in the set of calibration standards at a constant level of 10 μg L^{-1}

2.2. Samples and sample preparation

Sludge samples were obtained from 45 municipal STPs located in the Northwest of Spain (all but one in the region of Galicia) during 2021 and 2022. Globally, they represent around 30% of the municipal STPs with a capacity above 2000 habitant equivalents (h.e.) in this region. Location of STPs is shown in Fig. S1. The depuration capacity of each plant (as h.e.), together with existing water and sludge treatment facilities are given as supplementary information, Table S1. Most of them lack of a physico-chemical (flocculation-coagulation) unit, and consider the use of activated sludge as biological treatment. Regarding the sludge line, the use of thickening tanks followed by centrifugation, or belt filters, for physical dewatering of sludge is the most common approach. Only some of the largest STPs consider anaerobic digestion of sludge, or thermal stabilization combined with lime addition, Table S1. Sludge samples, representing the final solid residue generated at each of the considered facilities, were supplied by staff responsible for STPs management.

After reception, samples were lyophilized, within the next 48 h, and stored in polyethylene vessels at 4 °C. Sample preparation conditions were adapted from previous studies, using MSPD, as extraction technique, on-line combined with a clean-up cationic exchange sorbent (Casado et al., 2015). In brief, a fraction of 0.5 g of lyophilized sludge, spiked with of the mixture of SSs at a concentration of 100 ng g^{-1} (referred to the dry sludge matrix), was dispersed using 2 g of C_{18} in a glass mortar, with a pestle, for 5 min. The blend was packed into a polypropylene syringe, already containing 1 g of PSA and one polyethylene frit at the bottom. Another frit was used to compact the sorbents at the top of the MSPD syringe. Then, the packed MSPD syringe was connected to a SCX cartridge and both were eluted together using 10 mL of MeOH. This step allows the extraction of basic compounds from the dispersed sludge sample followed by their retention in the SCX sorbent. Acidic species are expected to remain in the layer of PSA, at the bottom of the MSPD syringe, and neutrals are removed with MeOH. After discarding the MSPD syringe, compounds were recovered from the SCX sorbent using MeOH: NH_3 (5 mL of a 98:2 solution).Fig. S2 summarizes a scheme of the sample preparation procedure. This extract was filtered, using a 0.2 μm pore size PTFE filter, and injected in the UPLC-MS/MS system without any additional treatment.

Spiked pooled sludge samples, employed to evaluate the efficiency of the MSPD extraction, were prepared by addition of methanolic mixtures (500 μL) of selected compounds to freeze-dried sludge at three different concentration levels (40, 100 and 250 ng g^{-1}). Then spiked samples were allowed to stand overnight before extraction. Procedural blanks (without any sludge in the MSPD packed syringe) were prepared every 8 samples in order to investigate possible contamination problems during the extraction protocol.

2.3. LC-MS/MS determination conditions

Compounds were determined using an ultra-performance liquid chromatography (UPLC) triple quadrupole-type MS system provided by Agilent. The UPLC was a 1290 Infinity II connected, through a jet-stream ESI source, to an i-funnel Agilent 6495 QqQ instrument. Compounds were separated in a Kinetex PS C_{18} column (50 × 2.1 mm; 2.6 μm) purchased from Phenomenex connected to a Supelco® Column Saver 0.5 μm Filter from Sigma-Aldrich. Utrapure water (0.1% FA) (phase A), and MeOH (0.1% FA) (phase B) were combined as follows: 5% B (0–2 min), 100% B (15–16 min), 5% B (16.1–19 min). The flowrate of mobile phase was 0.3 mL min^{-1}. The ESI source was operated in the positive mode, using needle and fragmentor voltages of 1500 V and 166 V, respectively. The MRM parameters for each compound, including the ratio between qualification (Q2) and quantification (Q1) transitions, are compiled in Table 1. In case of O-desmethyl venlafaxine and tramadol, just one transition was available. MRM parameters for compounds employed as SSs are given as supplementary information, Table S2.

2.4. MSPD recoveries, matrix effects, method accuracy and samples quantification

The efficiency of the extraction on-line clean-up sample preparation process, independent of potential variations in the efficiency of ESI ionization, was calculated as the ratio between the responses (peak area for Q1 transition without SSs correction) measured for spiked sludge samples and MeOH:NH_3 (98:2) extracts, from non-spiked fractions of the same sludge matrix, fortified at identical concentration levels. Matrix effects (MEs, %) were evaluated comparing the difference of responses for spiked (50 ng mL^{-1}) and non-spiked extracts, obtained from a pooled sludge matrix, with those observed for a solvent-based standard of the same concentration. Values close to 100% indicate the absence of signal suppression or enhancement for sample extracts versus solvent-based standards (Matuszewski et al., 1998).

The accuracy and precision of the analytical procedure was investigated with sludge samples spiked at several concentrations. The difference in responses for spiked and non-spiked fractions of each sludge were corrected with those measured for the associated SS. Thereafter, they were converted to concentrations (using solvent-based standard calibration curves, 0.1 μg L^{-1} to 150 μg L^{-1} range) and compared with fortified values.

2.5. Environmental risk assessment

Predicted environmental concentrations in amended soil (PEC_{soil}), estimated 1 year after one sludge-dose application, were calculated using the following equation (eq. (1)) (European Commission, 2008; Martín et al., 2012).

$$PECsoil = \frac{(Csludge\ x\ APPLsludge)}{(DEPTHsoil\ x\ RHOsoil)} \quad (1)$$

C_{sludge} is the concentration measured in sludge expressed as μg Kg^{-1} dry mass, $APPL_{sludge}$ is the application rate of dry sludge onto soils (0.5 kg m^{-2} $year^{-1}$ for agricultural soils), and RHO_{soil} is the average bulk density of wet soil (1700 kg m^{-3}). The $DEPTH_{soil}$ was fixed at 0.20 m, considering a homogeneous mixture of sludge and soil in this upper layer of agriculture fields.

In the absence of acute ecotoxicological data for soil organisms, and considering the selected compounds as non-volatile, their Predicted Non-Effect Concentration in soil ($PNEC_{soil}$) were estimated from available data for acute water toxicity ($PNEC_{water}$), using the equilibrium partitioning method (EPM) (Aydın et al., 2022; Martín et al., 2012). Thus, the $PNEC_{soil}$ was calculated using the following simplified equation (eq. (2)), obtained considering standard values for the density of wet soil, the solid phase of soil (1700 kg m^{-3} and 2500 kg m^{-3}, respectively), and a 2% of organic carbon in the solid fraction of soil (European Commission, 2008). Koc is the predicted value for the organic carbon-water partition coefficient of each compound at pH 7. It was obtained from SciFinder database (Search | CAS SciFinder^n). $PNEC_{water}$ was taken from Norman Ecotoxicity Database (NORMAN Ecotoxicology Database (norman-network.com)).

$$PNEC_{soil} = (0.1176 + 0.01764 * K_{oc}) * PNEC_{water} \quad (2)$$

Finally, environmental risks to terrestrial ecosystems derived from sludge application in agriculture soils were estimated from risk quotients (RQs), calculated as the ratio between PEC_{soil} and $PNEC_{soil}$ for each compound (eq. (3)).

$$RQ = \frac{PEC_{soil}}{PNEC_{soil}} \quad (3)$$

3. Results and discussion

3.1. Performance of the analytical methodology

LC analysis of strong basic compounds using conventional C_{18} columns represents a challenging issue. Secondary interactions between positively charged species and residual silanol groups, in silica particles, led to broad, non-symmetric peaks (Golpe et al., 2022). The PS C_{18} column used in this study contains, in addition to C_{18} chains, positively charged sites surrounding silica particles; thus, basic drugs with the same charge are repelled not being able to establish secondary electrostatic interactions with silanol groups responsible for peak broadening. Using the gradient of mobile phases reported in section 2.3, compounds showed retention times between 1.7 min (atenolol) and 13.1 (difenoconazole), Table 1, with LC separations lasting 19 min. The chromatographic behavior of the PS C18 during analysis of sludge extracts was compound dependent. The weakly basic triazolic compounds showed symmetric peaks, with stable retention times throughout the study (figure not shown). On the other hand, during optimization of extraction conditions (particularly considering direct extraction of sludge dispersed with C_{18} without employing any clean-up sorbent), we observed a trend towards increased retention times and non-symmetric peaks for strong bases, as it is the case of haloperidol, mirtazapine and ketoconazole. This trend was associated with saturation of positively charged sites with compounds of the opposite charge. The original peak shape and retention time could be recovered after rinsing the column with a 50 mM solution of ammonium bicarbonate, prepared in ACN:H_2O (1:3), for 20 min at 0.2 mL min^{-1}. Fig. 1 illustrates the above behavior using haloperidol as model compound. The column conditioning procedure was repeated at the end of each series of samples, even under optimized sample preparation conditions, considering just the MeOH: NH_3 (98:2) extract for analysis.

When developing multianalyte LC-MS/MS methods, the possibility of having compounds with common, even interfering, transitions is not negligible. The pair tramadol- O-desmethyl venlafaxine, showing a single intense common MRM transition, was baseline separated, Table 1. Another situation where interferences can occur is during determination of parent drugs and their de-alkylated metabolites. In-source fragmentation of the $[M+H]^+$ ion of the parent pharmaceutical might led to a fragment with an identical *m/z* value to that corresponding to the protonated form of its dealkylated metabolite. In this case, if the spectra of both compounds contain common product ions, the concentrations of metabolites might be overestimated. Under employed chromatographic conditions, amiodarone and venlafaxine were well separated from their de-alkylated forms. Citalopram and norcitalopram showed the same retention time; however, the product ion spectra of the former did not contain the ($[M+H]^+$) ion of the N-desmethyl metabolite. Finally, sertraline and norsertraline could not be baseline separated, and the MRM transitions of the metabolite are partially interfered by those of sertraline. When present at similar concentrations, both compounds could be discriminated (Fig. S3); however, quantification of norsetraline is more challenging in those extracts containing lower levels of the metabolite vs the parent drug.

The linearity of the LC-MS/MS method was evaluated using solvent-based standards, in the range of concentrations from 0.1 μg L^{-1} to 150 μg L^{-1}. The peak area for the Q1 transitions of each compound was corrected with that measured for the associated SS (10 ng mL^{-1}) and plotted versus its concentration. Determination coefficients (R^2) higher than 0.994 were observed for all compounds, Table 1. Instrumental limits of quantification (LOQs), corresponding to a signal to noise ratio (S/N) of 10 for the Q1 transition, were estimated from ratios obtained for the lowest level calibration solutions. LOQs varied between 0.1 μg L^{-1} to 0.5 μg L^{-1}, Table 1. Intra-day (n = 3) and inter-day repeatabilities (n = 12 injections during 4 days) were investigated at the 10 ng mL^{-1} level. Relative standard deviations (RSDs,%) of peak areas, without SS correction, varied between 2% (ametoctradin, cinnarizine) and 15% (clotrimazole), and between 3% (myclobutanil) till 18% (carvedilol, miconazole), under intra- and inter-day assessment conditions.

The efficiency and selectivity of the MSPD sample preparation procedure was evaluated with extraction recoveries and MEs. Recoveries higher than 80% were obtained for most of the compounds involved in the study, Table 2. The efficiency of the procedure to remove neutrals was evaluated using fractions of the same pooled sludge, previously spiked with the pesticides chlorpyrifos and clofentizine, and as well as monitoring the responses for non-charged pollutants, ubiquitous in sludge (i.e. the UV filter octocrylene (Tran et al., 2018)). As expected, the three species were noticed only in the methanolic extract flowing through the MSPD syringe and the clean-up SCX sorbent, but not in the MeOH: NH_3 (98:2) fraction collected from the latter. Zwitterionic pollutants, such as diclofenac and candesartan (both contain a carboxylic group in their molecules in addition to basic moieties) were not recovered in any of the two fractions. The most probable reason was the strong interaction of the carboxylic moiety with the amine groups of PSA. This sorbent was included in the MSPD extraction cartridge to remove, among others, fatty acids from the sludge matrix (Peysson and Vulliet, 2013). This assumption was confirmed with additional extractions, comparing the responses for both compounds in extracts obtained from MSPD packed syringes with, and without, PSA as clean-up co-sorbent. In summary, the extraction-fractionation protocol permitted to isolate compounds within a large range of Log D values at pH 7 (from −2.1 to 5.5), and slight to moderate basic character (pKa values from 2.1 to 9.6 units, for their protonated forms), covering the extraction of amine, azol (imidazole and triazol), pyrimidine and

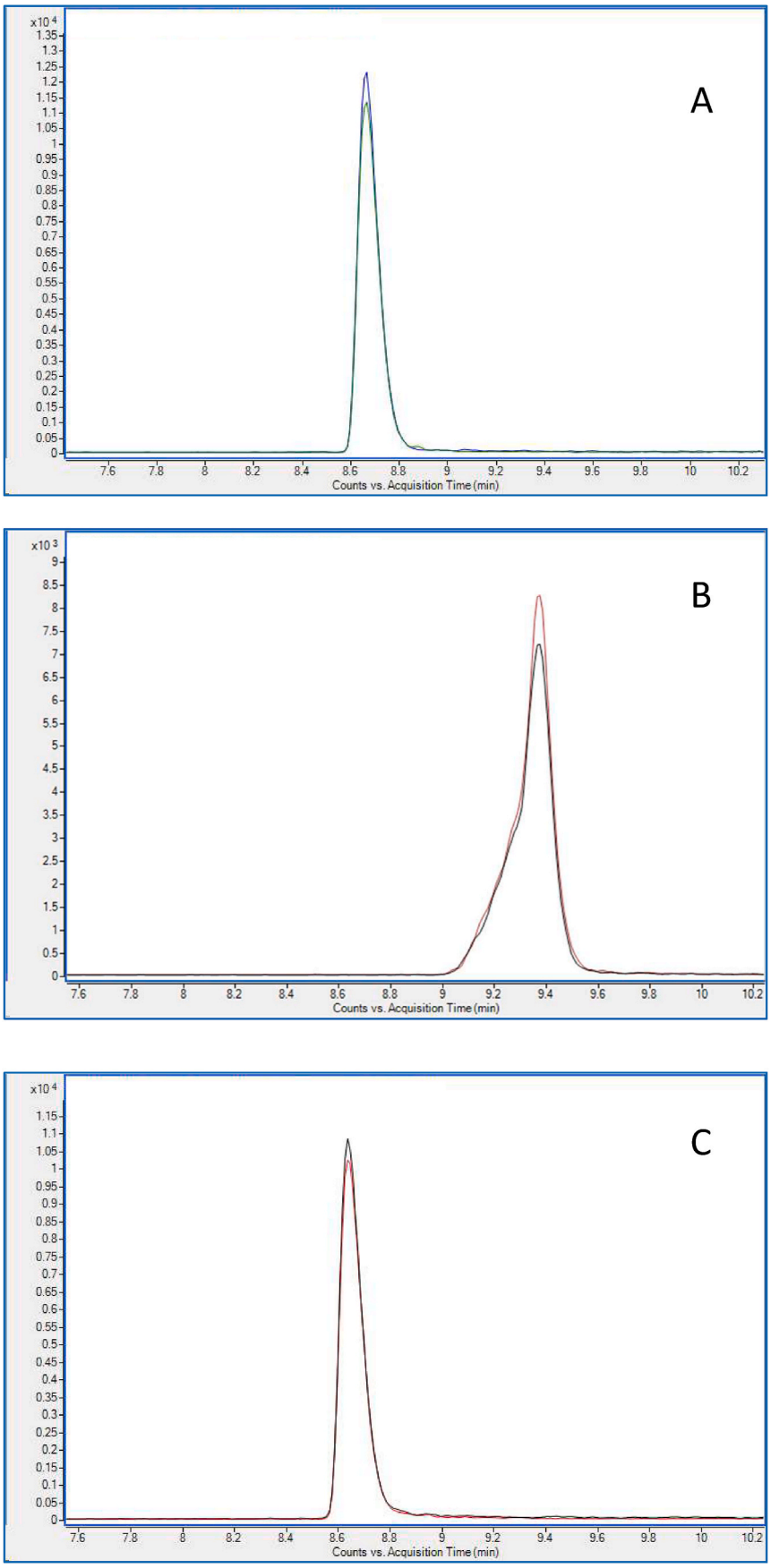

Fig. 1. Chromatograms for the Q1 and Q2 transitions of a standard of haloperidol (10 ng mL^{-1}). A, new column. B, after 100 injections of raw sludge extracts (without compounds fractionation). C, same column rinsed with a 50 mM solution of ammonium bicarbonate in ACN: water (1:3).

Table 2
Efficiency of MSPD extraction and fractionation, matrix effects (MEs), method accuracy for sludge samples spiked at 3 different concentration levels (n = 3 replicates per level) and LOQs (ng g^{-1} freeze-dried sludge).

Compound	Extraction efficiency (500 ng g^{-1}) (%)		MEs (%)		Accuracy (40 ng g^{-1})		Accuracy (100 ng g^{-1})		Accuracy (250 ng g^{-1})		Estimated LOQs
	Mean	SD	Mean	RSD	Mean	SD	Mean	SD	Mean	SD	ng g^{-1}
PESTICIDES											
Ametoctradin	94%	3%	92%	3%	83%	3%	84%	3%	84%	3%	2
Carbendazim	92%	1%	101%	3%	88%	4%	96%	2%	92%	3%	1
Difenoconazol	92%	2%	91%	2%	80%	2%	85%	2%	84%	4%	1
Imazalil	91%	3%	74%	3%	77%	9%	83%	5%	94%	4%	8
Metconazole	91%	2%	95%	3%	93%	5%	99%	6%	91%	2%	2
Myclobutanil	94%	2%	96%	4%	93%	3%	94%	3%	92%	3%	1
Penconazole	93%	3%	83%	2%	83%	8%	87%	6%	77%	1%	5
Propiconazole	92%	5%	96%	1%	102%	22%	99%	12%	91%	5%	5
Tebuconazole	91%	2%	97%	2%	98%	4%	95%	6%	88%	2%	2
Terbutryn	91%	1%	103%	4%	76%	10%	100%	12%	97%	4%	5
Tetraconazole	92%	3%	88%	2%	75%	2%	76%	5%	81%	5%	3
Thiabendazole	89%	1%	102%	4%	89%	3%	93%	3%	96%	1%	1
Triadimenol	88%	2%	106%	2%	95%	11%	99%	6%	97%	3%	5
PHARMACEUTICALS											
Cardiovascular drugs											
Amiodarone	81%	3%	92%	5%	n.e.		80%	12%	73%	7%	5
Atenolol	89%	1%	71%	1%	62%	4%	66%	3%	61%	3%	2
Carvedilol	86%	3%	102%	5%	76%	8%	94%	3%	76%	4%	2
Flecainide	93%	1%	105%	3%	86%	8%	96%	4%	91%	3%	1
Irbesartan	75%	2%	101%	4%	83%	6%	100%	4%	93%	2%	1
N-desethyl amiodarone[a]	90%	3%	95%	4%	n.e.		91%	14%	90%	8%	1
Propranolol	89%	1%	99%	3%	92%	3%	96%	3%	92%	3%	5
Psychiatric drugs											
Amisulpride	87%	3%	107%	3%	76%	9%	96%	5%	86%	3%	1
Amitriptyline	84%	3%	103%	3%	74%	12%	90%	2%	85%	5%	1
Citalopram	90%	1%	104%	5%	84%	7%	109%	5%	96%	3%	1
Clomipramine	83%	3%	105%	3%	88%	8%	96%	4%	82%	2%	1
Clozapine	88%	1%	96%	3%	82%	8%	95%	9%	91%	5%	5
Haloperidol	89%	2%	104%	4%	88%	5%	92%	2%	85%	3%	2
Lamotrogine	91%	1%	97%	4%	92%	5%	96%	3%	93%	2%	2
Mirtazapine	88%	1%	97%	4%	89%	7%	95%	5%	92%	3%	3
N-desmethyl citalopram[a]	88%	0%	104%	4%	86%	7%	101%	4%	92%	3%	5
Norsertraline[a]	95%	4%	104%	3%	80%	10%	111%	10%	74%	4%	1
O-desmethyl venlafaxine[a]	91%	1%	103%	4%	91%	4%	93%	2%	94%	1%	2
Sertraline	91%	2%	102%	6%	85%	10%	112%	8%	79%	2%	2
Trazodone	90%	1%	100%	3%	81%	5%	96%	4%	89%	2%	1
Venlafaxine	91%	2%	103%	4%	85%	4%	95%	4%	92%	1%	2
Antimycotic drugs											
Climbazole	94%	1%	99%	4%	82%	3%	89%	2%	86%	1%	3
Clotrimazole	92%	2%	107%	4%	119%	4%	94%	7%	93%	3%	1
Fenticonazole	91%	1%	90%	3%	80%	5%	82%	6%	78%	2%	1
Fluconazole	93%	2%	100%	3%	90%	8%	96%	5%	90%	2%	5
Ketoconazole	94%	3%	94%	4%	96%	6%	83%	7%	79%	4%	5
Miconazole	94%	2%	106%	3%	112%	8%	118%	3%	86%	4%	5
Sertaconazole	93%	7%	108%	6%	81%	8%	101%	17%	88%	3%	1
Tioconazole	90%	3%	104%	3%	86%	6%	107%	7%	92%	3%	1
Other drugs											
Cinnarizine	91%	2%	105%	4%	95%	7%	90%	4%	93%	1%	1
Cloperastine	91%	3%	109%	3%	89%	9%	113%	3%	89%	3%	1
Tramadol	91%	1%	103%	4%	86%	4%	96%	1%	93%	1%	1
Trimethoprim	84%	2%	100%	4%	95%	3%	95%	5%	91%	2%	1

n.e., not evaluated.
[a] Drug metabolite.

pyperazine compounds, Table S3.

The evaluation of MEs, through comparison of differences of responses for spiked and non-spiked sludge extracts versus solvent-based standards, shows that only two compounds (atenolol and imazalil) were affected by moderate signal suppression effects (26% and 29%, respectively) during analysis of sludge extracts, Table 2. For the rest of analytes, variations in the efficiency of ionization between spiked sludge extracts and solvent-based standards were in the range of ± 20%, confirming a low effect of the sample matrix in the performance of ESI ionization, Table 2. Compared to these values, the combination of PLE extraction followed by reversed-phase clean-up of primary sludge extracts led to signal suppression effects up to 70% for several pharmaceuticals (Radjenović et al., 2009). Accuracy was investigated evaluating the recoveries corresponding to the addition of three different concentrations of the compounds (40, 100 and 250 ng g^{-1}) to sludge. Taking into account the small variation of compounds ionization efficiency and the high extraction yield of the extraction process, recoveries were determined using solvent-based calibration standards. The obtained values, with associated standard deviations (n = 3 blanks, and 3 extractions injected in duplicate) are compiled in Table 2. The background level of amiodarone and its N-desethyl form prevented to calculate their recoveries at the lowest investigated addition level. Atenolol showed repeatable, but not quantitative recoveries, at the three spiked concentrations (from 61% to 66%, with standard deviations between 3% and 4%). These values are coherent with the observed signal suppression for this species, not corrected with the considered SS

(Tramadol-^{13}C-d$_3$). For the rest of compounds (45 species), recoveries ranged from 70% to 120%. The variability of these data, expressed as standard deviations, decreased with the added concentrations, varying between 1% to a maximum of 22%, Table 2. The LOQs of the analytical procedure were estimated from instrumental values (Table 1), considering the ratio between final extract and the mass of sludge, and corrected with signal suppression effects observed for atenolol and imazalil. LOQs stayed between 1 ng g^{-1} and 8 ng g^{-1}, Table 2. These values are lower (sometimes in one order of magnitude) than those attained using more generic extraction procedures, not involving the fractionation of basic drugs during the sample preparation process (Castro et al., 2021; Peysson and Vulliet, 2013).

3.2. Analysis of sludge samples

The developed protocol was applied to 48 sludge samples, obtained from 45 different STPs located in the Northwest of Spain, Fig. S1. Every sample was extracted and injected in duplicate. Quality control actions included the analysis of one procedural blank and one spiked sludge (100 ng g^{-1}) with each series of samples (at least every 8 samples). Positive identifications are based on retention times and Q2/Q1 ratios match with those observed for solvent-based standards, Table 1, within ranges of 0.1 min and ± 30%, respectively.

Out of 46 species considered in the procedure, only three compounds (clozapine, fluconazole and triadimenol) remained below the LOQs of the method in the set of processed samples. Levels measured for the rest of species are provided as supplementary information, Table S4. Their average, minimum and maximum concentrations, as well as the frequency of detection are summarized in Fig. 2. Data have been organized from highest to lowest detection frequencies, considering also their decreasing average concentrations, Fig. 2. The LOQs provided by the developed method permitted to quantify 33 compounds (most of them pharmaceuticals) in at least 85% of the processed samples. Substances with lower detection frequencies (10 compounds) corresponded in most cases to pesticides. Within this group, only carbendazim, imazalil, terbutryn and thiabendazole were quantified in more than 85% of the samples. The sum of concentrations for compounds above their LOQs in sludge samples varied between 2083.8 ng g^{-1}, for sample code S16, and 6937.0 ng g^{-1}, for sample S26, Table S4.

Sertraline was the pollutant showing the highest average residues (619 ng g^{-1}), whilst miconazole presented the top minimum and maximum values, with concentrations in sludge ranging between 104 ng g^{-1} and 2881 ng g^{-1}, respectively, Fig. 2. It is worth noting that 19 compounds were ubiquitous in sludge. This list includes not only highly lipophilic species, but also relatively polar drugs and/or their excretion metabolites, with Log D values lower than 1, as it is the case of the opioid tramadol, the cardiovascular drug flecainide, O-desmethyl venlafaxine and mirtazapine, Table S3. The four species exhibit a poor biodegradability and exist in their protonated form at neutral pH; thus, electrostatic interactions might contribute significantly to their presence in this environmental compartment. Given that some of the more polar compounds considered in this research (i.e. tramadol, flecainide, venlafaxine, propanolol) have been also reported in the water phase of STPs (Fernández-Fernández et al., 2022; Tran et al., 2018; Wiest et al., 2021) sludge residues need to be considered in further studies assessing their mass balances at these facilities.

Average concentrations measured for high detection frequency compounds are in agreement with data reported for sludge samples from different geographical areas. As example, levels of clotrimazole and miconazole in the range of 400 ng g^{-1} to 500 ng g^{-1} have been found in sludge from STPs in China (Chen et al., 2013). Another study, in the same geographical area in Asia, reported concentrations up to 1400 ng g^{-1} for both drugs (Huang et al., 2010). The survey of pharmaceuticals in sludge from 5 different STPs in Slovakia highlighted sertraline, citalopram and their N-desmethyl forms as ubiquitous in sludge, with similar average concentrations to those shown in Fig. 2 (Ivanová et al., 2018). The screening of micro-contaminants in sludge collected from more than 100 STPs in USA ranked sertraline in 2nd place with average and maximum concentrations of 458 ng g^{-1} and 636 ng g^{-1}, respectively (Chari and Halden, 2012).

Fig. 3A presents the Box-Whisker plots for the sum of concentrations corresponding to four different groups of compounds: pesticides, cardiovascular and psychiatric drugs, and antimycotic compounds of medical use, together with the sum of all compounds. In agreement with detection frequencies and average concentrations summarized in Fig. 2, the presence of pesticide in sludge samples from the investigated area was residual compared with pharmaceuticals and particularly, with the groups of psychiatric and antimycotic drugs. In the particular case of imazalil, which is mainly employed as post-harvest fungicide, the maximum concentration measured in this study (94.2 ng g^{-1}) is significantly lower than values reported by J. Campo et al. (2013) for samples obtained from STPs in the South and Southwest of Spain, with maximum values up to 1000 ng g^{-1}. The most probable reason is the importance of food transforming and packing industry in the two former areas. The groups of psychiatric and antimycotic pharmaceuticals include relatively lipophilic, low biodegradable active ingredients, which explain their accumulation in sludge. Furthermore, the topic application of most antimycotics might contribute to their direct entrance in municipal sewers due to wash out from treated areas, with little, or null, metabolization.

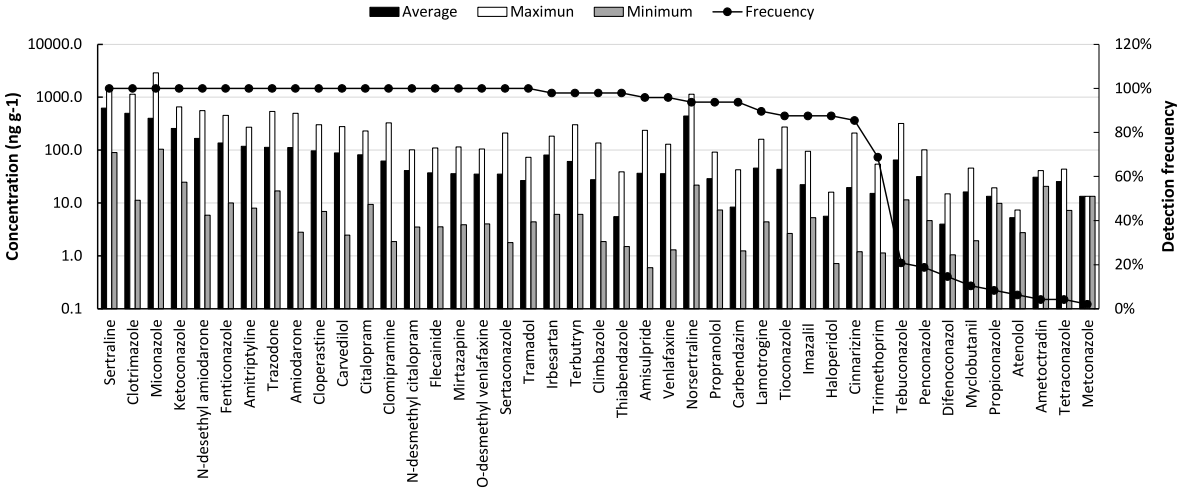

Fig. 2. Summary of compounds occurrence in sludge samples.

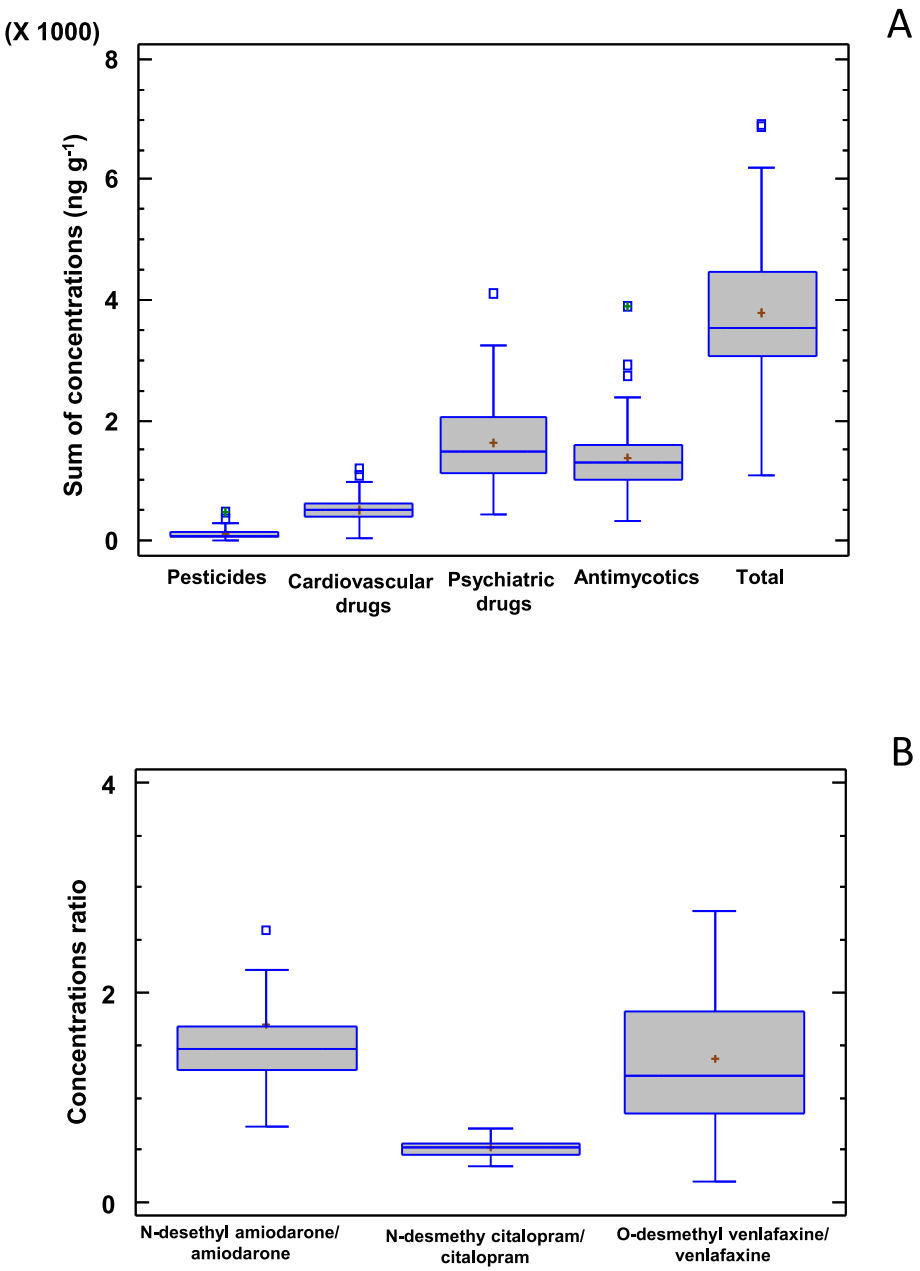

Fig. 3. A. Box-Whisker plot for the sum of concentrations corresponding to four families of compounds and total residues in sludge. B. Box-Whisker plots with the ratios of concentrations for metabolites and parent compounds of selected drugs.

The ratios between the concentrations of metabolites and the parent pharmaceutical, for three compounds, in sludge samples is shown in Fig. 3B. Average and median values below the unit were observed for the pair N-desmethyl citalopram/citalopram. The desethyl form of amiodarone was more abundant than the parent cardiovascular drug. Finally, a large variability was noticed between the levels of O-desmethyl venlafaxine and venlafaxine. The question if ratios between dealkylated and parent compounds in sludge reflects those existing in wastewater is not evident. In the particular case of venlafaxine, the O-desmethyl form is normally predominant versus the commercial drug in wastewater (Fernández-Fernández et al., 2022). In this study, with data corresponding mainly to non-digested sludge, the average and median values for the ratio of concentrations (1.36 and 1.21, respectively) were also slightly higher than the unit; however, depending on the sludge sample different trends were noticed.

3.3. Environmental risk assessment

Once established the real concentrations of these target compounds in sludge of the considered STPs, their potential toxicity for the environment was evaluated, in this case, for the terrestrial environment, since the most frequent application of urban sludge is the use as fertilizer in agriculture, either directly, or preferably after a composting step (Ivanová et al., 2018).

Table 3 summarizes the calculated PNEC values for soil, PEC in soil and RQ values, calculated as defined in section 2.5, with compounds sorted from higher to lower RQs. The PEC and RQ values were obtained using average concentrations, measured for the set of sludge samples involved in this study, which represent a more conservative approach than the use of maximum residues. Under this assumption, a group of 20 compounds showed RQs above 0.1, pointing out to moderate (RQs from 0.1 to 1), or high (RQs above 1) eco-toxicological risks in the terrestrial

Table 3
Assessment of environmental risks on sludge amended soils for compounds determined in sludge samples above their LOQs.

Compound	[a]PEC in soil (ng g^{-1})	[b]Koc (L/Kg), pH 7	[c]PNEC in water (μg L^{-1})	PNEC in soil (ng g^{-1}), pH 7	RQ, pH 7
Sertraline	0.95	57.3	0.091	0.10	9.29
Venlafaxine	0.06	4.32	0.038	0.01	7.79
N-desethyl amiodarone	0.26	2410	0.0013	0.06	4.75
Amiodarone	0.18	2130	0.0011	0.04	4.39
Norsertraline	0.70	88.8	0.14	0.24	2.97
Trazodone	0.18	336	0.016	0.10	1.86
Amitriptyline	0.16	44	0.14	0.13	1.30
Ketoconazole	0.39	2140	0.0081	0.31	1.29
N-desmethyl citalopram	0.06	1	0.5	0.07	0.90
Clomipramine	0.10	53.2	0.11	0.12	0.88
Carvedilol	0.13	161	0.064	0.19	0.69
Propranolol	0.04	3.19	0.41	0.07	0.58
Flecainide	0.052	4.41	0.64	0.13	0.41
Ametoctradin	0.045	321	0.021	0.12	0.37
Fenticonazole	0.21	6030	0.0061	0.65	0.32
Amisulpride	0.054	1	1.43	0.19	0.28
Cloperastine	0.14	153	0.2	0.56	0.25
Mirtazapine	0.052	8.43	1	0.27	0.20
Miconazole	0.61	8390	0.025	3.70	0.17
Clotrimazole	0.76	10,000	0.03	5.30	0.14
Terbutryn	0.089	1640	0.065	1.89	0.05
Citalopram	0.12	6.47	16	3.71	0.03
Tramadol	0.037	1.84	9.65	1.45	0.03
Tioconazole	0.075	3470	0.054	3.31	0.02
Sertaconazole	0.055	22,300	0.012	4.72	0.01
Carbendazim	0.014	152	0.44	1.23	0.01
O-desmethyl venlafaxine	0.053	1.64	42	6.15	0.01
Cinnarizine	0.032	5960	0.042	4.42	0.01
Tebuconazole	0.068	2670	0.24	11.33	0.01
Lamotrogine	0.071	111	10	20.76	0.003
Haloperidol	0.008	173	0.76	2.41	0.003
Tetraconazole	0.037	2050	0.35	12.70	0.003
Climbazole	0.041	1709	0.52	15.74	0.003
Imazalil	0.032	1320	0.87	20.36	0.002
Metconazole	0.020	2450	0.29	12.57	0.002
Propiconazole	0.018	2300	1	40.69	0.00044
Trimethoprim	0.021	23.7	100	53.57	0.00039
Atenolol	0.008	1	150	20.29	0.00038
Thiabendazole	0.008	525	3.3	30.95	0.00025
Penconazole	0.052	7980	1.7	239.50	0.00022
Difenoconazol	0.003	4370	0.76	58.68	0.00005
Irbesartan	0.13	198	704	2541.67	0.00005
Myclobutanil	0.004	1120	0.021	79.50	0.00005

[a] Estimated with average of sludge concentrations.
[b] Values obtained from SciFinder database.
[c] Values from NORMAN Ecotoxicology Database.

environment. All of them were pharmaceuticals or their excretion metabolites, with the single exception of the fungicide ametoctradin, Table 3. Despite this compound showed an RQ of 0.37, it was noticed just in two samples.

Out of 8 pollutants with RQs above 1, five compounds are employed as drugs for the treatment of psychiatric problems, two are cardiovascular pharmaceuticals (amiodarone and its metabolite), and one is a topic application antimycotic (ketoconazole). Accordingly to recommendations provided by the EU, a more detailed assessment of the toxicity of these compounds, using model soil organisms, is required (European Commission, 2008). In any case, using the same calculation methodology, the RQs obtained in this study for several pharmaceuticals in sludge amended soils are higher than those reported for well-known endocrine disrupters, such as 17 β-estradiol (Martín et al., 2012). The highest RQs obtained from maximum, average and minimum concentrations measured for selected compounds are provided in Fig. S4. Considering maximum, instead of average concentrations, the number of compounds with RQs above the unit increased from 8 to 16 compounds, Fig. S4.

Another important variable affecting the calculated PNEC in sludge-amended soils using the equilibrium partition method described in eq. (2) is soil pH. For basic compounds, their Koc decrease with pH. Thus, in acidic soils, as those existing in the Northwest Spain (most soils show pH values between 5 and 6 units) (Fernández-Calviño et al., 2015), a significant reduction in the estimated PNEC values in soil, and thus higher RQs estimates, are predicted.

4. Conclusions

A sensitive and selective procedure has been validated for the determination of 46 pollutants of emerging concern, with a basic group in their structure, in sludge from municipal STPs. For most of them, the combination of quantitative extraction yields with low variations in the efficiency of ESI ionization between solvent-based standards and sludge extracts provided accurate concentration results using solvent-based calibration standards. Thus, a high sample analysis throughput was achieved. Attending to detection frequencies, average concentrations and estimated risk quotients, a group of 20 species with moderate to high environmental risks in sludge-amended soils was identified. Thus, additional treatments need to be implemented in the STPs from the investigated area before considering sludge as a safe source of nutrients for agriculture or forestry uses. Furthermore, tailored assays permitting a more accurate assessment of the toxicity of certain sludge pollutants towards soil organisms are required.

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

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.139094.

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