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HITLIST4

Non-targeted and suspect screening of sewage sludge

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Abstract

This project applied non-targeted and suspect screening to activated sludge from five Danish wastewater facilities. In addition, the concentrations of 61 elements were also determined in the sludge.

The sludge concentrations showed relatively high levels of mercury (0.4-1.4 mg/kg) and cadmium (0.4-1.4 mg/kg), compared to natural levels in arable soils. Also, a high copper (203-571 mg/kg) and zinc (502-1616 mg/kg) concentrations in sludge relative to arable soil show that the use of sludge is likely to increase the level of copper and zinc over time in sludge amended fields.

Five non-targeted screening platforms were applied to analyse the sewage sludge. After data analysis, thousands of substances were discovered in the non-targeted dataset. Searching the data against in-house and international databases 41 substances were determined at the highest annotation level (1), 1,751 molecules were identified at the second highest level (2), 7,091 compounds at level 3, and a total of 15,471 combined at level four and five. Examples of confirmed substances are 1H-benzotriazole, 2,6-dichlorophenol, bisphenol S, methylparaben, terbutryn and prosulfocarb.

By applying novel semi-quantitative concepts, it was possible to predict concentrations of 513 substances, e.g. PFOS was detected at all five sites with concentrations ranging from 2.4-47.4 µg/kg sludge, while 6PPD-quinone was only found in three sites (14-74 µg/kg). Endogenous or natural metabolites were found, e.g. bile acids as deoxycholate (58-3247 µg/kg) and peptides as Gly-Leu-Lys (53-938 µg/kg).

Acronyms

dw	Dry weight
EI	Electron impact ionization (GC)
ESI	(Heated) electrospray ionisation (LC)
GC	Gas chromatography
HLB	Hydrophilic-lipophilic balanced polymer
HRMS	High-resolution mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
LC	Liquid chromatography
LOD	Limit of detection
MFS	Miljøfarlige stoffer (substances of emerging concern)
MS	Mass spectrometry
nLC	nano-liquid chromatography
NTS	Non-targeted screening analysis
PFAS	Per- and polyfluoroalkyl substance
PFCs	Perfluorochemicals
ppb	Parts-per-billion (ng/mL, µg/L)
ppm	Parts-per-million (µg/mL, mg/L)
QC	Quality control
SPE	Solid phase extraction

1. Introduction

Sewage sludge is rich in nutrients and can be applied to agricultural soils (BEK nr 1001 af 27/06/2018). However, sludge may also contain elevated levels of contaminants such as heavy metals that can have adverse effects on the environment and pose a health risk for human consumption of the agricultural produce. Organic micropollutants, or substances of environmental concern (termed MFS in Danish) is another category of contaminants that may enter the environment via agricultural sludge amendment to exert unwanted public and environmental health side-effects.

Recently, it was demonstrated that, holistic non-targeted screening analysis (NTS) provides the means to perform mass suspect screening and go beyond to discover previously unknown molecular entities in environmental samples. NTS is revolutionary and fundamentally different from targeted monitoring strategies and has a large potential for effective evaluation of water quality regulations. NTS is based on high-resolution mass spectrometry (HRMS), that rapidly profile thousands of (unknown) substances in complex environmental samples [1]–[3]. The NTS strategy is used when former unknown compounds are detected in a sample and data is investigated without any presumptions or knowledge of the sample [4]. Suspect screening is another strategy used for searching HRMS data for known chemicals, i.e. by using a reference list of pesticides and biocides which are expected to be present in the sample [4]. As such, NTS is used to describe this entire field of research.

The NTS concept was recently developed and applied in a research project (HITLIST1) under the Danish Environmental Protection Agency's Pesticide Research Program [1]. The work was followed by a recent Danish EPA research project (HITLIST2) that mapped the detectable chemical space and further validated the NTS methodology, so it can be used as a reliable monitoring method for the NOVANA water quality program. Finally, a third research report is currently under review (HITLIST3) on applying the NTS concept on Danish surface water samples.

The aim of the present project 'HITLIST4' was to apply NTS and ICP-MS methodologies to determine inorganic and organic micropollutants in sewage sludge. A secondary objective was to compile a list of substances identified at each location, make concentration estimations based on novel semi-quantitative approaches and use multivariate statistics for in-depth data exploration.

2. Methodology

2.1 Sewage sludge samples

A total of five facilities provided activated sludge samples in November and December 2021 (Table 1). In all cases sludge were stored in anaerobic digesters following by dewatering. All sludges are approved for use on agricultural soils (grade A).

Table 1. Overview of studied wastewater sludge samples and facility details. PE, actual person equivalents burden.

Facility	Type	PE	Digestion
Roskilde	Biodenipho	125.000	3 weeks anaerobic digestion
Måløv	Biodenipho	70.000	3-4 weeks anaerobic digestion
Ejby Mølle	Biodenipho	225.000	4 weeks anaerobic digestion
Egå	Biodenipho (modified)	90.000	3 weeks anaerobic digestion
Herning	Biodenipho	150.000	Thermophilic digestion (2 weeks) followed by mesophilic digestion (2 weeks)

2.2 Analytical platforms

Five high-resolution mass spectrometry NTS analytical platforms were used to analyse the samples for organic micropollutants. In addition, an accredited inductively coupled plasma mass spectrometry (ICP-MS) methodology was used to determine concentrations of 61 inorganic elements in the sludge. The five NTS platforms were reverse-phase nano-liquid chromatography electrospray ionisation high-resolution tandem mass spectrometry (nLC-ESI-HRMS/MS in positive and negative ionisation modes), cap-flow cLC-ESI-HRMS/MS methods (in positive and negative ionization mode) directed towards detecting e.g. perfluorochemicals and gas chromatography electron impact ionisation high-resolution mass spectrometry (GC-EI-HRMS) and are further detailed in Appendix 2.

- 1) nLC-ESI(+)-HRMS/MS
- 2) nLC-ESI(-)-HRMS/MS
- 3) cLC-ESI(+)-HRMS/MS
- 4) cLC-ESI(-)-HRMS/MS
- 5) GC-EI(+)-HRMS
- 6) ICP-MS

2.3 Sample preparation

Samples were collected in Rilsan bags and immediately stored in cooler box (5 °C) and transported to the analytical lab and stored at -20 °C. Sample aliquots of 0.20 grams for inorganic element analysis were acid extracted using microwave extraction and analysed by ICP-MS according to EPA method 3051A. Sample aliquots of 0.20 grams for organic micropollutant analysis were spiked with isotope-enriched internal standards and extracted using pressurized liquid extraction in a two-way workflow (one for LC and another for GC). Sample extracts for GC-HRMS workflows were analysed directly, while extracts for LC-HRMS workflows were further purified using solid-phase extraction (for details see Appendix 2).

2.4 Data analysis

The LC and GC-HRMS raw data were processed using Compound Discoverer 3.3 (Thermo Fisher) for peak detection, retention time alignment and peak picking for non-target screening.

An overview of the Compound Discoverer and non-target data processing workflow can be found in (Appendix 2.6). The output of this is a feature list, i.e. a table with m/z and retention time pairs (features) and their peak area, which were further processed for the identification and structural elucidation of contaminants. The detected peaks were prioritized based on the criteria such as peak intensity threshold, blank subtraction, reasonable peak symmetry (sharp peak apex), molecular formula predicted from the exact mass and the isotopic pattern as well as structural similarity match with the analytical reference standard. The data analysis filtration and decision tree(s) are available in detail in Appendix 2.7. The features/compounds were confirmed to different identification levels (level 1 to 5) as suggested by Schymanski et al. [5]. In short, a level 1 substance has been confirmed by a reference standard on the same NTS platform (MS, MS/MS and retention time matching). A level 2 substance is a highly probable structure matched to literature and/or public MS/MS spectral libraries, and may further be supported by retention time predictions. A level 3 substance is tentative candidate structure where no MS/MS libraries exist, however the experimental evidence is supported by in silico MS/MS fragmentation predictions. A level 4 substances is when an unequivocal chemical formula can be assigned from MS spectral data (e.g. adduct, isotopic pattern). A level 5 substance is when only the exact mass (m/z) can be assigned. Concentrations of level 1 and 2 identified substances were performed by novel semi-quantitative approaches [6], and are described in detail in Appendix 2.8. Statistical analyses were performed using GraphPad Prism 9.3 and MetaboAnalyst 5.0.

3. Results and discussion

3.1 Inorganic elements

The ICP-MS analyses included screening of 61 elements of the five sludge samples (Figure 1). Specific element concentrations across the five sites were relatively similar, e.g. arsenic (As) varied from 2.99 in Roskilde to 8.26 mg/kg dw in Ejby Mølle and mercury (Hg) from 0.41 in Ejby Mølle to 1.41 mg/kg dw in Måløv. Iron (Fe) was observed in the highest concentration (46.2 g/kg dw in Måløv), while rhenium (Re) was found at the lowest levels (0.002 mg/kg dw in Ejby Mølle and Måløv). A complete and detailed dataset of all 61 elements is available in Appendix 4.

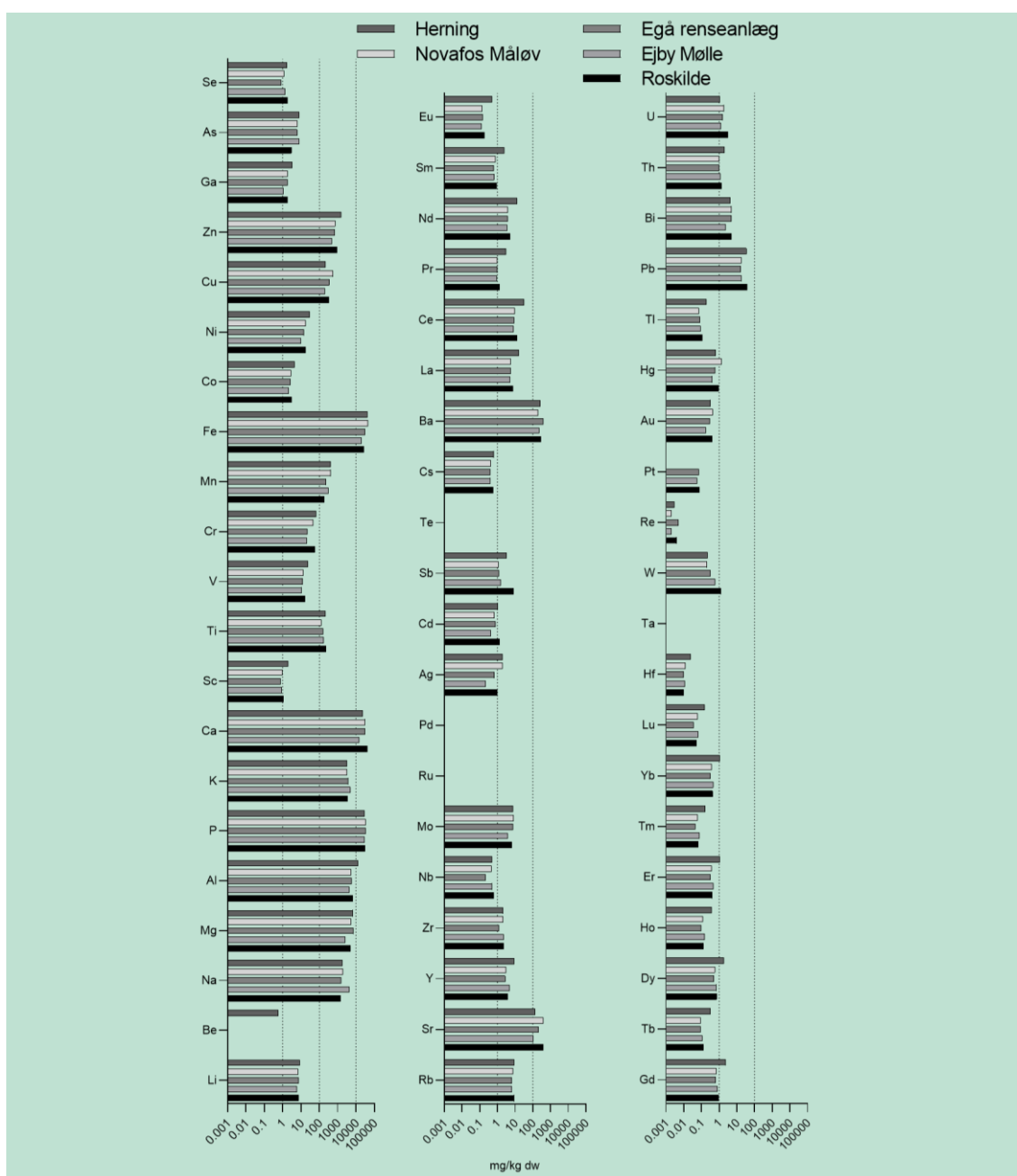


Figure 1. Concentrations of 61 inorganic elements in wastewater activated sludge (mg/kg dry weight) determined with ICP-MS.

A comparison with limit values in BEK nr 1001 of 27/06/2018 for inorganic elements in waste applied for agricultural or private gardens use are compiled in Table 2. As shown, all five sludge samples comply with the safety limit values. The limit values expressed on a dry weight basis were exceeded for cadmium and/or mercury (and just exceeded for nickel) in three of the samples but were below the limit value expressed per amount phosphorus. According to BEK nr. 1001, the waste must comply with the limit values expressed on either dry weight basis or phosphorus basis, so all samples comply with this criterion. However, the results indicate that the metals of particular concern in the waste are cadmium and mercury, which should be monitored closely especially if large amounts of waste are spread or if a significant variation in composition is expected.

Comparison with average levels from 430 samples of arable soil [7] in 1992 and 1993 indicate levels in the sludge is within a factor of two for arsenic, between 1.4 and 7 times higher for cadmium, chromium, nickel and lead, whereas mercury is a factor of 8-28 times higher in sludge than in arable soil. Copper and zinc are used in many household products and leaks from plumbing, so levels are 17-73 times higher in sludge than arable soils. It has been argued, that the sludge amendment leads to increasing levels of copper and zinc in arable land topsoil (0-25 cm) of respectively 0.16 and 0.96 mg/kg/y, or 1.7 and 3% increase per year from 1998 to 2014, and slightly less below the ploughing zone (25-50 cm) at 1.6 and 2.8% increase per year [8].

Compared to the levels in the Earth's Crust particularly bismuth and gold are concentrated in the sludge samples (270-610 resp. 45-110 times above Earth Crust levels), but also antimony, selenium, phosphorous, silver, zinc, mercury and platinum are present in up to 10-40 times the level in the earth crust (in declining order). Bismuth present at very low levels in the Earth Crust (around 0.009 mg/kg), but is used both for medicine, in personal care products, as a catalyst for rubber and fibers, in alloys and have been used in shot and bullets as replacement for lead. The use of gold in electronics, medical treatments and nano-gold are potential sources for the sludge. Antimony is also used in electronics, as alloys (mainly with lead), and antimony compounds are used for flame-retardant materials, paints, glass and pottery. Selenium is used as additive to glass and in photocells, solar cells and photocopiers, it is toxic and used in anti-dandruff shampoos, but it is also an essential element needed in small amounts, with both carcinogenic and teratogenic effects at raised levels. Silver is used in jewelry and utensils, in mirrors and solder, electrical contacts and batteries. It was previously used in photography, and now as antibacterial nano-particles in e.g. clothes, foot ware and touchscreen-enabled gloves. Platinum is also used for jewelry, but today mainly as catalytic converters for cars, trucks and busses. Also, other industrial catalytically uses, in electronics including optical fibers and LCDs, and in chemotherapy drugs. All uses are cited from the Royal Society of Chemistry [9]. The high ratio metals can mostly be attributed to industrial, therapeutic or household product use of rare metals or metalloids, whereas the more ordinary elements like phosphor and zinc are used in large quantities by households, farmers and industry to give the overrepresentation in sludge. Mercury is supposed to be more or less banned, and the high levels in sludge are mainly attributed to legacy usage. In Europe it is probably mainly used in fluorescence lighting, the majority is from long range transport from artisan goldmining in Africa, together with pollution from coal burning in powerplants in Eastern Europe and Asia.

Table 2. Limit values of inorganic elements in waste for agricultural use and private gardens in BEK no. 1001 of 27/06/2018 in comparison to measured values in the five sludge samples. The sludge must comply with either the limit value for an element in mg kg⁻¹ dry weight or the limit value in mg kg⁻¹ total phosphorus (P). Limit values for inorganic elements in soil in BEK no 1001 and the average concentrations in Danish soils are listed in the bottom of the table for comparison. A detailed table is available in Appendix 4.

	Concentrations in mg kg ⁻¹ dry weight								Concentrations in mg kg ⁻¹ total P			
	Cr	Ni	Cu	Zn	As	Cd	Hg	Pb	Ni	Cd	Hg	Pb
<i>Safety limit values for waste used for agriculture (BEK no. 1001, 27/06/2018)</i>	100	30	1000	4000	-	0.8	0.8	120	2500	100	200	10000
<i>Safety limit values for waste used for private gardens (BEK no. 1001, 27/06/2018)</i>	100	30	1000	4000	25	0.8	0.8	60	2500	100	200	5000
N001 Roskilde (n=2)	57	19	335	926	3	1.4	1.0	38	605	44	31	1235
N004 Ejby Mølle	20	10	203	502	8	0.4	0.4	18	363	15	14	640
N007 Egå	23	14	348	693	6	0.8	0.6	17	410	23	18	491
N010 Måløv	47	19	571	736	6	0.7	1.4	18	556	20	42	550
N013 Herning (n=2)	68	31	214	1616	8	1.1	0.6	35	1061	37	22	1228
<i>Safety limit values for soil (BEK no. 1001, 27/06/2018)</i>	30	15	40	100		0.5	0.5	30				
<i>Average soil level 1992/3 N=430 (Larsen et al, 1996)</i>	12	6	8	30	4	0.2	0.05	12				

3.2 NTS dataset

After data analysis pipelines were applied (Appendix 2.6) to the NTS HRMS dataset (LC and GC), nearly half a million features were observed, and they could be grouped into thousands of substances. After lab procedural blank background filtration, the GC EI dataset was assembled into c. 2,300 substances whereas 1,056 substances were annotated at level 2, 189 compounds at level 3 and 1027 compounds at level 5. The combined LC datasets contained over 20,000 features across both positive and negative ionization modes. Nearly 15,000 of these could only be annotated as either *m/z* or as a calculated chemical formula (levels 5 and 4 respectively). 6,902 compounds (not taking duplicates into account) were annotated at level 3 across both platforms, i.e. with proposed chemical structure based on MS2 fragments. A total of 725 unique compounds were identified at level 2 across both platforms, with 546 detected in positive mode, 215 in negative mode, and 36 compounds detected in both platforms. 29 compounds were detected at level 1 using positive ionization and 15 using negative, with a combined total of 41 unique level 1 compounds across the two platforms (LC negative and LC positive).

The current state-of-the-art semi-quantitative methods can predict concentrations of LC (ESI) data, however not GC (EI) data and more research efforts are urgently needed in this area.

3.3 Identified organic substances

The identity of 41 substances were confirmed (level 1) across the dataset (Table 2). See Appendix 2.8 for a detailed description of data curation for level 1 annotation. The complete dataset with level 1-5 annotations is available through Appendix 5.

Table 3. Substances identified at the highest level (1) with observed minimum and maximum sludge concentrations (across all identified platforms) and detection frequencies (D_f). n.c. denoted concentration not calculated by the semi-quantitation algorithm.

Name	C_{min} ($\mu\text{g}/\text{kg}$)	C_{max} ($\mu\text{g}/\text{kg}$)	D_f (%)
<i>Industrial substances</i>			
1H-Benzotriazole	57	3283	100
4-Methyl-1H-benzotriazole	204	617	100
5-Methyl-1H-benzotriazole	31	1577	100
Bisphenol S	25	116	60
Dimethyl phthalate	189	964	80
Ethylparaben	287	952	100
Methylparaben	287	952	100
Tributyl phosphate	93	343	100
Triisobutyl phosphate	n.c.	n.c.	100
Tris(2-butoxyethyl) phosphate	127	750	40
Vanillin	639	17431	100
<i>Pesticides and biocides</i>			
2,6-Dichlorophenol	n.c.	n.c.	40
Clomazone	n.c.	n.c.	40
Prosulfocarb	81	305	60
Terbutryn	73	654	60
<i>Pharmaceuticals</i>			
Aspartame	90	685	60
Azithromycin	11	2807	40
Caffeine	103	9075	40
Carbamazepine	n.c.	n.c.	100

Cetirizine	16	52	80
Citalopram	114	1120	80
Cotinine	n.c.	n.c.	60
Fexofenadine	17	189	100
Furosemide	31	85	100
Lamotrigine	n.c.	n.c.	80
Losartan	69	496	100
Metoprolol	82	1020	80
Miconazole	n.c.	n.c.	60
Nicotine	104	809	60
Propranolol	19	449	80
Salicylic acid	99	4048	100
Sertraline	9	362	100
Venlafaxine	4	599	100
Diclofenac	4	70	100
Ibuprofen	15	90	80
<i>Natural products</i>			
Daidzein	19	2962	80
Genistein	42	70	20
<i>PFAS substances</i>			
Perfluorodecanoic acid (PFDA)	2	11	100
Perfluorooctanesulfonic acid (PFOS)	3	53	100
Perfluorononanoic acid (PFNA)	1	2	60
Perfluorooctanesulfonamide (PFOSA)	1	3	40

3.4 Suspects

A list of suspects was used for screening throughout the dataset. The combined dataset also revealed the tyre residues 6PPD and 6PPD-quinone were present in sludge samples. The two substances were detected using two platforms, i.e. demonstrating the importance of combining NTS platforms to obtain a more complete chemical fingerprint.

It is expected the applied NTS platforms will be able to detect all listed suspects of interest (Table 3), however substances could be present below detection limits or hampered by matrix interference. Some substances are identified to level 2 as no in-house reference standard was available. Other substances as level 3 (tentative candidates), as the obtained MS/MS-fragmentation spectral score was low.

Table 4. Suspects extracted from the NTS dataset.

Compound name	Found	Annotation level
<i>Industrial substances</i>		
6PPD	Yes	2
6PPD-Quinone	Yes	2
1,2,4-Triazole	No	
<i>Pesticides and biocides</i>		
Trimethoprim	Yes	3
Pyrethrin	Yes	2
Cypermethrin	No	

Permethrin	No	
Deltamethrin	No	
Lamda-cyhalothrin	No	
Spinosad A	No	
<i>Pharmaceuticals</i>		
beta-Estradiol	Yes	2
(+)-Estrone	Yes	3
Azithromycin	Yes	1
Citalopram	Yes	1
Clarithromycin	Yes	2
Diclofenac	Yes	1
(-)-Erythromycin	Yes	2
Naproxen	No	
Ibuprofen	Yes	2
2-hydroxyibuprofen	Yes	1
Propranolol	Yes	1
Tramadol	Yes	3
Carbamazepine	Yes	1
Telmisartan	Yes	2
Metformin	No	
Metoprolol	Yes	1
Sertraline	Yes	1
Ciprofloxacin	No	
Venlafaxine	Yes	1
<i>PFAS substances</i>		
Trifluoroacetic acid	No	
Heptafluorobutyric acid (PFBA)	No	
Perfluoropentanoic acid (PFPeA)	No	
Perfluorohexanoic acid (PFHxA)	No	
Perfluoroheptanoic acid (PFHpA)	No	
Perfluorooctanoic acid (PFOA)	No	
Perfluorononanoic acid (PFNA)	Yes	1
Perfluorodecanoic acid (PFDA)	Yes	1
Perfluorododecanoic acid (PFDoA)	Yes	3
Perfluorotridecanoic acid (PFTrDA)	No	
Perfluorobutanesulfonic acid (PFBS)	No	
Perfluorohexanesulfonic acid (PFHxS)	No	
Perfluoroheptanesulfonic acid (PFHpS)	No	
Perfluorooctanesulfonic acid (PFOS)	Yes	1
Perfluorodecanesulfonic acid (PFDS)	No	
Perfluorooctanesulfonamide (PFOSA)	Yes	1
6:2 FTSA (1h,1h,2h,2h-perfluorooctanesulfonic acid)	No	

3.5 Multivariate data analysis and data exploration

The analysed NTS data revealed very different molecular fingerprints between wastewater treatment plants (Figure 2). Clearly, the sewage sludge samples fall into three groups having very similar molecular signatures; Zealand (Roskilde, N01-02 and Måløv, N10-12); Fuenen (Ejby Mølle, N04-06) and Jutland (Egå, N07-09 and Herning, N13-15).

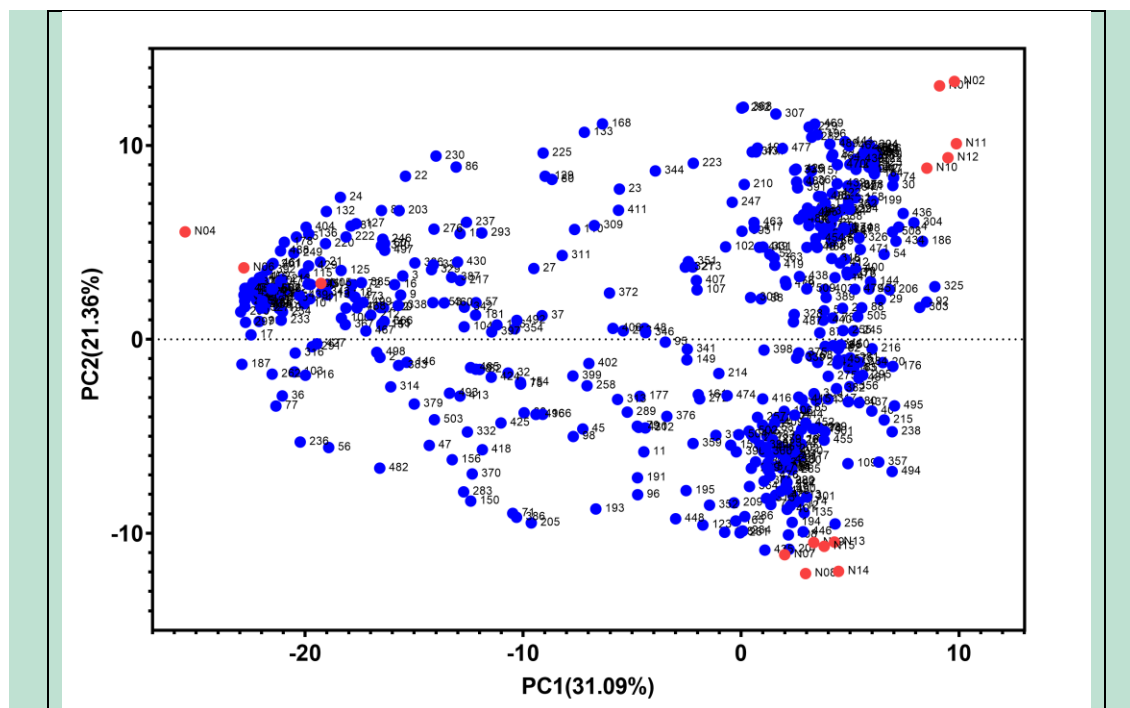


Figure 2: Scores (red) and loadings (blue) in biplot using multivariate principal component analysis is used for data exploration of the samples (red) and concentrations of 513 substances (blue). Samples clustered in proximity to each other has very similar chemical profiles, whereas samples far from each other are very dissimilar. Substances in proximity with specific samples are highly associated with its occurrence at this site. For simplicity substances has been numbered from 1-513 and table with substance names are found in Appendix 5.

Evidently, some chemicals are highly associated with sewage sludge from specific sites. E.g. the industrial chemical 1-Methyl-1H-benzotriazole (substance no. 74 in proximity to sample N10) is in high abundance in Roskilde and Måløv sludges (305-504 µg/kg), yet still occurring in the Herning sludge, albeit at lower concentrations (140-181 µg/kg, Appendix 5). Some chemicals are common across the samples and located at the centre region of the PCA biplot, e.g. Mono(2-ethylhexyl) phthalate (MEHP, substance no. 402 and is present in all samples at similar levels; 20-57 µg/kg, Appendix 5).

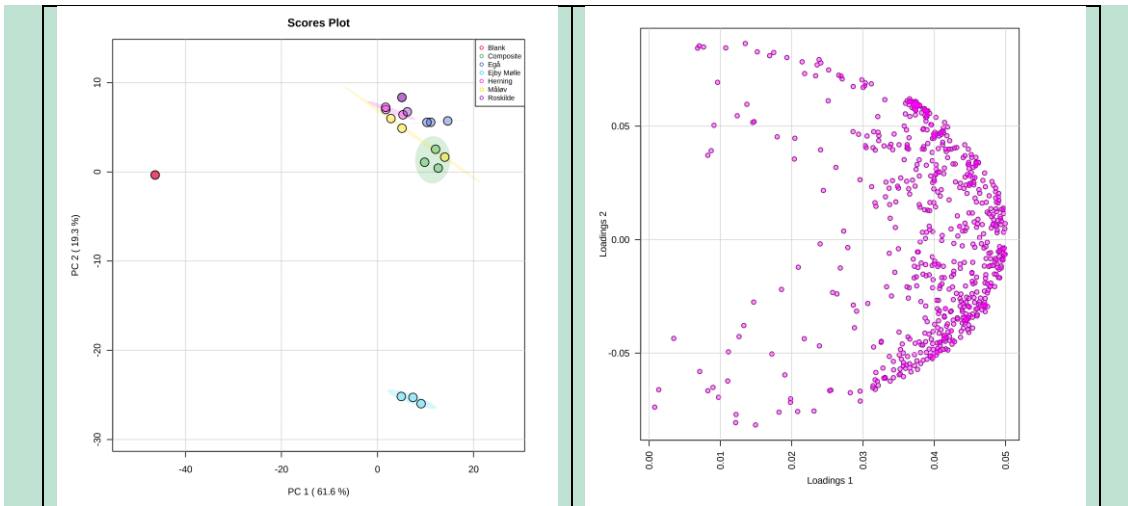


Figure 3: Scores and loadings plot in multivariate principal component analysis is used for data exploration of the samples analysed in GC EI dataset. Each sample is displayed in the scores plot (left). Samples clustered in proximity to each other has very similar chemical profiles, whereas samples far from each other are very dissimilar. The loadings plot (right) displays every detected substance present in the given dataset (1,056 substances annotated at level 2 for GC IE). Overlaying the two plots the association between substance and sample is visualised. One sample (Roskilde) were omitted as outlier.

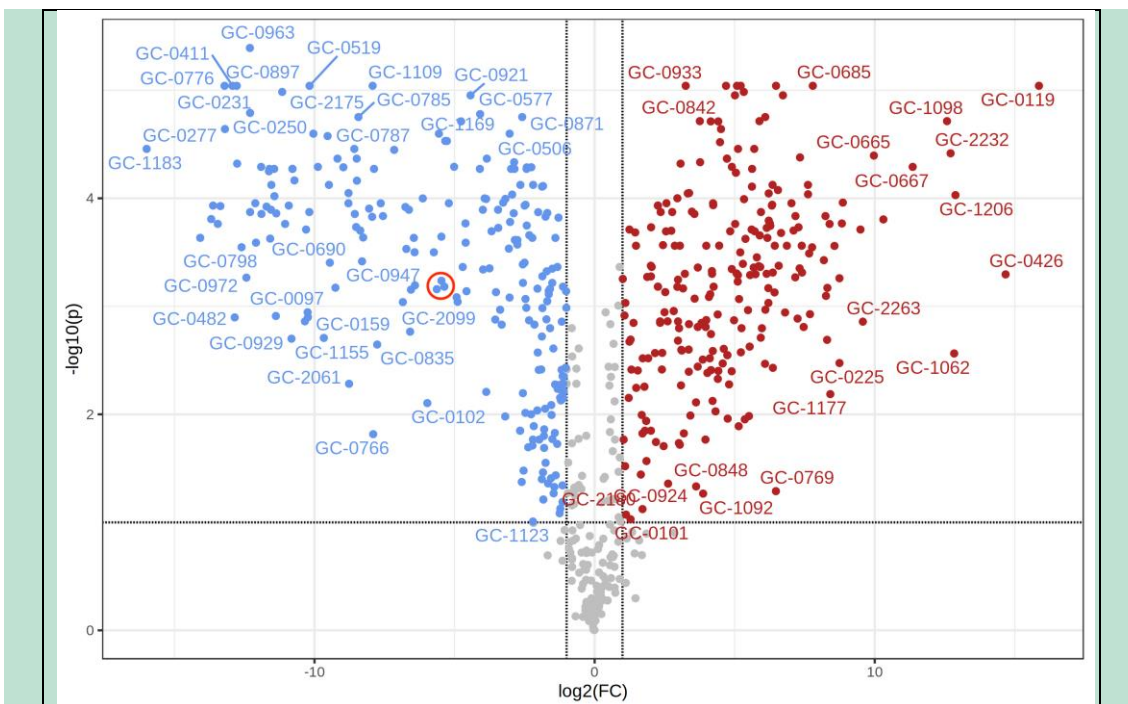


Figure 4: Differential analysis between Herring and Ejby Mølle for GC-EI dataset. Each dot is a substance and analysis are performed with $n=3$ for each group. Substances on left side (blue area) is in higher abundance in Herring when compared to Ejby Mølle. Substances on right side (red area) is in lower abundance in Herring when compared to Ejby Mølle. The higher (vertical y-axis) adjusted $-\log p$ value to more significant. 238 substances (blue) are at significantly higher abundance in Herring and another 227 substances (red) are significantly higher abundance in Ejby Mølle, while 168 substances (grey) are not significantly different in abundance between the two sites. One of the encircled substances (red ring) is sertraline (GC-2103, see Appendix 5 for substance names).

4. Conclusion

In terms of inorganic elements, all sludge samples complied with the criterion in the current BEK no. 1001 on waste applied for agriculture. The metals causing most concern in sludge are relatively high levels of mercury and cadmium, compared to natural levels in arable soils. But also interesting to see the high levels of particular rare metals as bismuth and antimony, and precious metals like gold, silver and platinum, all of which with many uses in both industry, as catalysts and alloys, as therapeutic products in hospitals and in house hold products like jewellery, electronics, nano-particles in clothes and cosmetic products. The high copper and zinc concentrations in sludge relative to arable soil show that the use of sludge is likely to increase the level of copper and zinc over time in sludge amended fields, both in the top soil and below the ploughing zone.

The NTS dataset revealed that a great number of micropollutants are present in waster sludge and the chemical fingerprint varies across wastewater sites. Combined with suspect screening and semi-quantitative concentration determinations it was possible to estimate sludge concentrations of more than 500 chemicals. To name a few substances observed at all sites, diclofenac with concentrations ranging 4-70 µg/kg, ethylparaben (287-953 µg/kg), perfluorodecanoic acid (PFDA, 2-11 µg/kg), azithromycin (11-2807 µg/kg) and tributyl phosphate (93-343 µg/kg). The chemical identity and occurrence were very different between sites, exemplified by the herbicide prosulfocarb observed at three out five sites at 61-305 µg/kg. Several parent chemicals and associated metabolites were also observed, such as venlafaxine and O-desmethyl-venlafaxine. The dataset also revealed five different per- and polyfluoroalkyl substances are present in sewage sludge. Especially, PFOS and PFDA were omnipresent and detected in all samples at 2-53 µg/kg.

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Appendix 1. Sample overview

Table 5. Overview of sludge samples and locations. The legends/names used in data analysis are underlined.

Site	Sample ID (n)	Sampling date
FORS <u>Roskilde</u> Spildevand A/S	N001, N002, N003	November 24, 2021
Vandcenter Syd, <u>Ejby Mølle</u>	N004, N005, N006	December 14, 2021
Århus vand, <u>Egå</u> renseanlæg	N007, N008, N009	December 14, 2021
Novafos <u>Måløv</u> Renseanlæg	N010, N011, N012	December 13, 2021
<u>Herning</u> Vand	N013, N014, N015	December 14, 2021

Appendix 2. Methods

Appendix 2.1 Liquid chromatography high-resolution mass spectrometry

This system was used for two NTS platforms (nLC-ESI(+)-HRMS/MS and nLC-ESI(-)-HRMS/MS). Nano-liquid chromatographic separation was performed on a Dionex Ultimate 3000 NCS-3500RS Nano Proflow system (Thermo Scientific). Ready samples were stored in glass 96-well plates in a Dionex WPS-3000 TPL RS autosampler at 8°C. Samples were loaded (1 µL) onto a nanoflow UHPLC column (PepMap RSLC, C18, 2 µm, 100 Å, 50 µm x 150 mm, Thermo Scientific) equipped with a titanium inline filter frit (0.5 µm). The flow rate of mobile phases was 300 nL/min. Chromatographic separation was achieved using a gradient beginning at 10 % mobile phase B (98 % acetonitrile, 2 % water, and 0.1 % formic acid) and 90 % mobile phase A (2 % acetonitrile, 98 % water, and 0.1 % formic acid) kept for 2 minutes. Thereafter the gradient increased to 95 % over 15 minutes. This level of 95 % B was kept for another 10 minutes. The conditions were restored to 10 % mobile phase B over 0.5 minutes followed by 12.5 minutes of equilibration time, leading to a total runtime of 40 minutes. In between each injection the needle and fluidics were washed with 200 µL of 80 % acetonitrile and 0.1 % formic acid in water. The pump systems were rinsed every hour with a seal wash solution of 10 % methanol and 0.1 % formic acid in water. All solvents used were of UHPLC-MS grade. The mass spectrometric analysis was performed on a high-resolution tandem mass spectrometer (Q Exactive HF, Thermo Scientific). Analytes were ionised by electrospray ionisation using an EASY-Spray ion source. The applied spray voltage was 1.50 kV during positive polarity and 1.70 kV during negative polarity with a capillary temperature of 250 °C and an S-lens RF level of 50. No sheath, aux, and sweep gas was used.

HRMS acquisition was done in either full scan mode for quantification or iterative data-dependent fragmentation (ddMS²) mode for identification [10]. Both the positive and negative polarity modes were used. Full scan acquisition was recorded using a resolution of 240K at m/z 200, an automatic gain control (AGC) target of 1e6, a maximum injection time of 100 ms, and a scan range of 70-1050 m/z for positive mode and 100-1500 m/z for negative mode. ddMS² acquisition was done using full scan settings with a resolution of 240K, AGC target of 1e6, maximum IT of 100 s, and scan range of 120-1500 m/z at m/z 200 for positive mode, and 100-1500 m/z at m/z 200 for negative mode. ddMS² settings used a resolution of 15K, maximum IT of 50 s, an isolation window of 1.0 m/z, AGC target of 5e4, loop count of 5, and stepped collision energies of 30, 70, and 120 NCE. The acquisition was performed with a dynamic exclusion of 5 s, minimum AGC target of 500, charge exclusion of >2, and an apex trigger between 2-6 s. An estimated chromatographic peak width (FWHM) was set to 3 s. Sub-ppm mass accuracy was ensured by real time calibration of a lock mass of 371.10124 (polysiloxane from air) during positive polarity and 112.98563 (sodium formate cluster) during negative polarisation [11], [12]. Calibration of the mass spectrometer was performed with Pierce™ LTQ Velos ESI Positive and Negative Ion Calibration Solutions (Thermo-Fisher Scientific).

Instrumental performance was ensured by regular monitoring of an in-house laboratory quality control sample prepared from fetal bovine serum.

Appendix 2.2 Gas chromatography high-resolution mass spectrometry

GC-HRMS analysis was achieved using an Orbitrap mass spectrometer (Exactive GC, ThermoFisher Scientific) with a TriPlus autosampler and a TraceGOLD TG-5MS analytical column (30 m, 0.25 µm, 0.25 mm, 5% phenyl - 95% dimethyl polysiloxane phase, ThermoFisher Scientific) installed in a TRACE 1310 GC (ThermoFisher Scientific). As described in the HITLIST2 report, one-microliter sample extract was injected sandwiched with air using a split-splitless mode at 280 °C and 70 mL/min split flow after 60 sec. The column was operated with high purity

helium at 1.00 mL/min and a temperature program; initial 60 °C with 2 min hold and ramped (5 °C/min) to 240 °C and further (10 °C/min) to 300 °C with a final holding time of 16 min. Analytes were transferred using a MS-transferline at 280 °C and ionized using electron impact ionisation (EI) at 70 eV with a 12 minutes filament delay. The Orbitrap HRMS system was operated in full scan mode (m/z 50 to 750) at a 60,000 resolution in centroid mode and an automatic gain-control target of 1e6 ions. The Q Exactive HRMS system was tuned and calibrated on a daily basis using FC43.

Appendix 2.3 CapLC-HRMS/MS for PFC analysis

Same system as described under Appendix 2.1 was utilized, however retrofitted with micro-bore UHPLC analytical column for PFC analysis. Chromatographic separation was performed on a Dionex Ultimate 3000 NCS-3500RS high-flow system (Thermo Scientific). Ready samples were stored in plastic vials in a Dionex WPS-3000 TPL RS autosampler at 8°C. Sample were loaded (1 µL) onto an UHPLC column (Phenomenex, C18, 3 µm, 100 Å, 75 µm x 150 mm) equipped with a 20 mm guard column with same material and inner diameter. The flow rate of mobile phases was 1000 nL/min. Chromatographic separation was achieved using a gradient beginning at 10 % mobile phase B (10 mM NH₄Ac in 60 % MeCN) and 90 % mobile phase A (10 mM NH₄Ac in 10% MeCN) kept for 2 minutes. In between each injection the needle and fluidics were washed with 200 µL of 80 % acetonitrile and 0.1 % formic acid in water. The pump systems were rinsed every hour with a seal wash solution of 80 % methanol and 0.1 % formic acid in water. All solvents used were of UHPLC-MS grade. The mass spectrometric analysis was performed as described in Appendix 2.1.

Appendix 2.4 Sample preparation for inorganic element analysis (ICP-MS)

Sample aliquots of 0.2 grams for inorganic element analysis were acid extracted using inverse aqua regia (6 ml Merck Suprapure HNO₃ + 2 ml Merck Suprapure HCl) in an Anton Paar Multiwave 7000 microwave oven according to EPA method 3051A and subsequently diluted with MQ water and analysed by an Agilent 7900 ICP-MS for 61 elements. For quality assessment and control, 3 procedural blanks, 2 duplicate samples (for Roskilde and Herning sludge) and 6 Certified Reference Material samples (3 ERM-CC144 and 3 IMEP-21) were digested and measured with the samples. Elemental analysis under the NOVAVA programme is done by ICP-MS and includes the whole periodic table from lithium (third element) to uranium (92nd element). Elements above uranium; neptunium, plutonium and americium are radioactive but the first two can be found in low concentrations in uranium ores, and the remaining elements from 95 to 118 have only been synthesised in labs or during nuclear fission testing. Some elements is not analysed by ICP-MS due to use as internal standards (typically rhodium, iridium and indium), or used as plasma source and collision cell (argon, helium) or as digestion acids (hydrogen, oxygen, nitrogen, chloride and for total dissolution fluoride and boron). The elements normally monitored in NOVANA are mercury, cadmium, lead, nickel, chromium, arsenic, copper, silver (in biota) and zinc.

Appendix 2.5 Sample preparation for organic micropollutant analysis

Sample aliquots of 0.2 grams were mixed with 5 grams of pre-washed diatomaceous earth and placed in 10 mL pressurized liquid-extraction cells pre-fitting with glass fibre filters. Cells were spiked with internal standards. Capped PLE cells were extracted twice in two extracts; two cycles with methanol:water (1:1) followed by two cycles with dichloromethane:hexane (1:1). Pre-heat time was 5 minutes, purge volume 60% and purge time 60 seconds. Methanol:water extracts were directed towards LC-HRMS analysis, while DCM:hexane extracts were prepared for GC-HRMS analysis. Methanol:water extracts were prior to LC-analysis purified using 500 mg HLB solid-phase material.

Appendix 2.6 Quality control samples

Four types of quality control samples were prepared; 1) laboratory procedural blanks by performing entire sample preparation workflow without adding any sample matrix. These samples would be used for background filtration. 2) A pooled sample by combining all sample extracts. 3) A composite sample were made by aliquoting 0.5 grams of each sample into a pool. 4) Two certified reference materials of ICP-MS analyses; ERM-CC144 and IMEP-21. Calibration standards were prepared by adding 0, 50, 100, 150, and 200 ng internal standards (see Table 7) into five aliquots of 200 μ L total pooled quality control sample respectively. A calibration blank was prepared by adding 50 ng of internal standards into 200 μ L methanol.

Appendix 2.7 Post-processing

After acquiring NTS raw data for non-target screening, the bottleneck was the identification or assigning the correct chemical structure for the features. Here, the feature (the combination of m/z and retention time) represents a particular compound in the sample. Compound Discoverer version 3.3, a commercial software package developed by Thermo Fisher Scientific, was used for peak detection, retention time alignment and peak picking. The workflow displaying the selected processing nodes and the associated workflow connections is given in Figure 4. The general overview of the NTS workflow used in *Compound Discoverer* for the raw data processing.. The raw files obtained in full scan mode (samples, blanks and pooled QCs) and data-dependent, MS/MS, mode (pooled QCs) were processed. In the workflow, the pooled QCs were labelled as "identification only" which were used as a source of fragmentation data. The main preliminary data processing workflow nodes includes input files, select spectra, align retention times, detect compound and mark backgrounds nodes. The list of features for the ionized compounds present in the samples, blanks and pooled QCs were created by the "Detect Compounds" node. Then, the generated ion list was used by the "Group Compounds" node which combines chromatographic peaks across the raw files by using their molecular weight and retention time. Afterwards, the "Predict compositions" node predicts elemental compositions for all features/compounds, which are subsequently annotated against compounds whose chemical information was previously recorded in mzCloud, ChemSpider (exact mass or formula) and local database searches against Mass Lists (exact mass with or without RT). "Assign compound annotation" node performs spectral similarity search against mzCloud (online database, ddMS2 and/or DIA) and mzVault (inhouse spectral database), for compounds with ddMS2. Finally, the "Mark Background Compounds" node incorporates blanks to trace features/compounds arising from sample preparation.

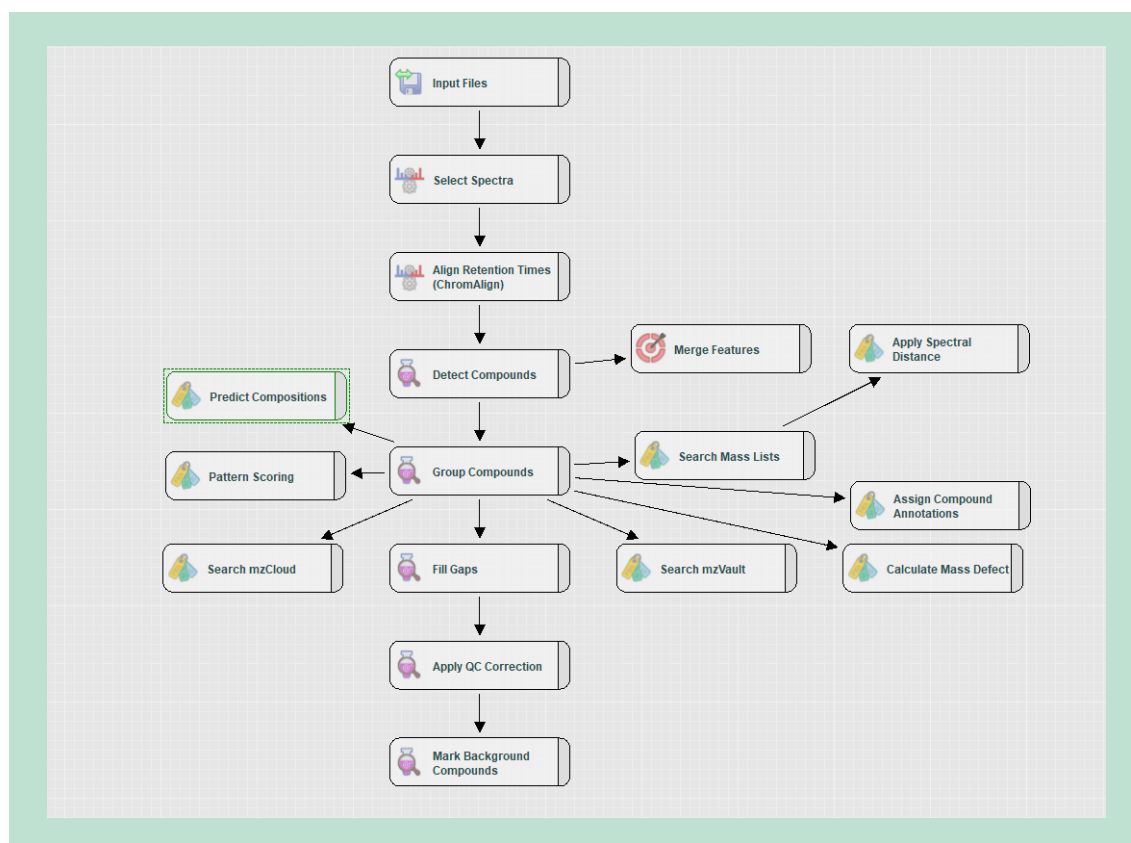


Figure 5. The general overview of the NTS workflow used in *Compound Discoverer* for the raw data processing.

The output of this was a feature list, i.e. a table with m/z and retention time pairs (features) and their peak area as well as other necessary parameters depending on the nodes used. The feature was subject to peak prioritization for the purpose of identification and structural elucidation of contaminants. Peak intensity threshold, blank subtraction, reasonable peak symmetry (sharp peak apex), reasonable elemental composition predicted from the exact mass and the isotopic pattern as well as structural similarity match were among the feature prioritization criteria used (nontarget data processing workflow, Figure 4). Every step of the nontarget workflow leads from lower identification confidence (level 5) to higher (level 1) suggested by Schymanski et al., 2014. Briefly, the identification journey was started with the HRMS features with exact masses (level 5), whose unequivocal chemical formulas were computed based on the isotopic pattern of peaks and adducts (level 4). The plausibility of computed formulas was evaluated by searching against the online chemical data (e.g. ChemSpider) and in-house-mass list. To move on to level 3, the tentative chemical structure was searched against the online spectral library through compound discoverer (e.g. mzCloud) and manual search against MetFrag, SIRUIS and MassBank), and in-house library (mzVault) using triggered MS2 fragmentation data. Here, tentative candidates that match MS1 accurate mass and the MS2 fragmentation spectra were identified. The diagnostic MS/MS fragment masses and/or ionization behaviour together with the information on parent compounds were used to categorize the tentative candidates to the plausible/probable chemical structure (level 2). Then, the identity of the compound was confirmed by comparing it with MS/MS fragmentation spectra of the analytical reference standard (level 1).

GC EI HRMS were search against NIST and NORMAN libraries. Substances with total spectrum scores above 70 were assigned as level 2 identifications, while scores below this value are assigned as level 3. Substances without chemicals formulas were assigned as level 5.

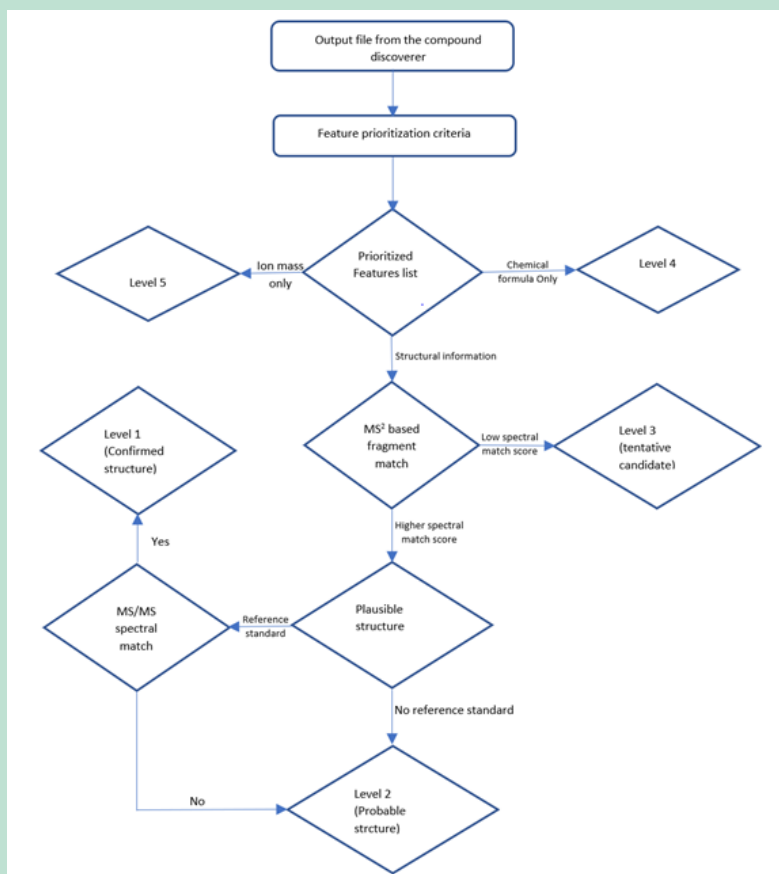


Figure 6. Nontarget data processing workflow for the categorization and confirmation of features to identification confidence levels.

Appendix 2.8 Quality assurance of level 1 data

Before compounds were confidently annotation at level 1, these entries were manually curated according to the workflow below:

1. A signal-to-noise ratio greater than 5 between at least 1 sample and a corresponding procedural blank.
2. Matching retention time ($\Delta t_R < 0.1$ min) between the compounds identified in a quality control sample and spiked quality control sample as well as a larger peak area in the spiked quality control sample compared to the unspiked quality control sample.
3. Matching MS² data between sample and the spectral reference entry.
4. No ambiguity between the compound of interest and isobaric compounds. In the case where a compound peak cannot be precisely defined on the basis of both retention time and MS² spectrum due to isobaric interferences, its annotation must be downgraded to at least level 2.

Appendix 2.9 Semi-quantitative predictions

The concentration of identified compounds was estimated by employing the Semi-Quantification tool. The ionization efficiency prediction approach that accounts for structural similarity with standard compounds was used. The CSV file containing SMILES, retention time, signal (peak area), and standard compounds with known concentrations were subject to the semi-quantification software. Here, a representative sample (a quality control sample) with a high number of detects was selected for the concentration estimation. Candidate compounds not detected in the selected quality control sample were not quantified. Thereby, a total of 513 compounds were subjected to quantification. The concentration for unknown compounds in

the samples was calculated based on the estimated concentration for the quality control sample using equation (1).

$$\frac{C_{est}}{A_{max}} = \frac{C_{ind}}{A_{ind}} \quad \text{equation (1)}$$

Where concentration estimated by Semi-Quant, max peak area used for concentration estimation, concentration of the compound in the individual sample and peak area of the compound in the sample.

Appendix 3. Internal standards

For use in semi-quantification and as quality assurance of the implemented acquisition workflows, several isotope labelled extraction and internal standards were used in the study. An absolute amount of 50 ng of each isotope labelled extraction standards (see Table 4) were added to each of the 15 PLE packed soil samples before extraction. Only 10 ng of GC-EI standards were added. After PLE, an additional 50 ng of internal standards (Table 5) were added to the extracts of all samples. A pooled composite sample was used as post, pre, and non ES-spiked sample respectively to calculate total (PLE+SPE) extraction recoveries.

Table 6. Recoveries of isotope labelled extraction standards. N.D., not detected.

Platform	Standard	Retention time (min)	Recovery (%)
GC-EI	1,3,6,8-Tetrachloro(13C12)dibenzo-p-dioxin	40.69	50%
	2,3,7,8-Tetrachloro(13C12)dibenzo-p-dioxin	42.35	50%
	1,2,3,7,8-Pentachloro(13C12)dibenzo-p-dioxin	45.44	49%
	1,2,3,4,7,8-Hexachloro(13C12)dibenzo-p-dioxin	48.14	11%
	1,2,3,6,7,8-Hexachloro(13C12)dibenzo-p-dioxin	48.24	19%
	1,2,3,7,8,9-Hexachloro(13C12)dibenzo-p-dioxin	48.46	22%
	1,2,3,4,6,7,8-Heptachloro(13C12)dibenzo-p-dioxin	50.91	<1%
	*Octachloro(13C12)dibenzo-p-dioxin	N.D.	
	2,3,7,8-Tetrachloro(13C12)dibenzofuran	41.90	43%
	1,2,3,7,8-Pentachloro(13C12)dibenzofuran	44.54	58%
	2,3,4,7,8-Pentachloro(13C12)dibenzofuran	45.17	57%
	1,2,3,4,7,8-Hexachloro(13C12)dibenzofuran	47.45	58%
	1,2,3,6,7,8-Hexachloro(13C12)dibenzofuran	47.56	31%
	1,2,3,7,8,9-Hexachloro(13C12)dibenzofuran	48.00	56%
	2,3,4,6,7,8-Hexachloro(13C12)dibenzofuran	48.72	33%
	1,2,3,4,6,7,8-Heptachloro(13C12)dibenzofuran	49.98	25%
	1,2,3,4,7,8,9-Heptachloro(13C12)dibenzofuran	51.3	40%
	Octachloro(13C12)dibenzofuran	53.51	10%
	3,3',4,4'-Tetrachloro(13C12)biphenyl	39.19	42%
	3,4,4',5'-Tetrachloro(13C12)biphenyl	39.57	44%
	2,3,3',4,4'-Pentachloro(13C12)biphenyl	40.33	49%
	2,3,4,4',5-Pentachloro(13C12)biphenyl	40.48	47%
	2',3,4,4',5-Pentachloro(13C12)biphenyl	40.83	45%
	2,3',4,4',5-Pentachloro(13C12)biphenyl	41.37	42%
	2,3',4,4',5-Pentachloro(13C12)biphenyl	42.62	48%
	2,3,3',4,4',5-Hexachloro(13C12)biphenyl	43.24	44%
	2,3,3',4,4',5'-Hexachloro(13C12)biphenyl	44.02	47%
	2,3',4,4',5,5'-Hexachloro(13C12)biphenyl	44.21	49%
	3,3',4,4',5,5'-Hexachloro(13C12)biphenyl	45.41	54%
	2,2',3,3',4,4',5-Heptachloro(13C12)biphenyl	44.62	41%

	2,2',3,4,4',5,5'-Heptachloro(¹³ C ₁₂)biphenyl	45.6	36%
	2,3,3',4,4',5,5'-Heptachloro(¹³ C ₁₂)biphenyl	46.65	42%
nLC-pos	¹³ C ₃ -Caffeine	14.47	88%
	¹³ C ₆ -Thiabendazole	13.81	69%
	DEET-D ₇	22.80	68%
	Diuron-D ₆	23.13	82%
	Imidacloprid-D ₄	17.87	87%
	Pirimicarb-D ₆	16.07	76%
nLC-neg	2,4-D-D ₃	22.76	94%
	Dicamba-D ₃	21.10	<1%
	Diuron-D ₆	23.20	105%
	Imidacloprid-D ₄	17.84	88%
	Mecoprop-D ₃	24.08	109%
cLC-neg	Perfluoro-n-(2,3,4-[¹³ C ₃])butanoic acid (¹³ C ₃ -PFBA)	4.93	<1%
	Perfluoro-n-(1,2-[¹³ C ₂])octanoic acid (¹³ C ₂ -PFOA)	5.70	76%
	Perfluoro-n-1-(1,2,3,4-[¹³ C ₄])octanesulfonate (¹³ C ₄ -PFOS)	6.44	148%
	Perfluoro-n-(1,2-[¹³ C ₂])decanoic acid (¹³ C ₂ -PFDA)	6.45	174%

Table 7. Signal stability (%RSD) of isotope labelled internal standards. The instrumental %RSD is calculated from peak areas of repeated injections (n=12) of quality control samples, measured every 4th injection throughout the instrument acquisition.

Platform	Standard	Retention time (min)	%RSD
nLC-pos	¹³ C ₃ -Testosterone-2,3,4	23.78	2.67
	¹³ C ₄ -15N ₂ -Riboflavin	14.89	5.60

Appendix 3.1 Signal stability of extraction and internal standards

The signal stability was calculated from the total average of normalised values of all standards measured in each sample:

$$\frac{1}{N} \sum_{j=1}^N \left(\frac{x_i - \mu}{x_{\max}} \right)_j$$

Where x_i is the peak area of standard j in sample i , μ is the mean area of standard j across all samples respectively, x_{\max} is the maximum peak area for standard j across all samples, and N is the number of standards identified.

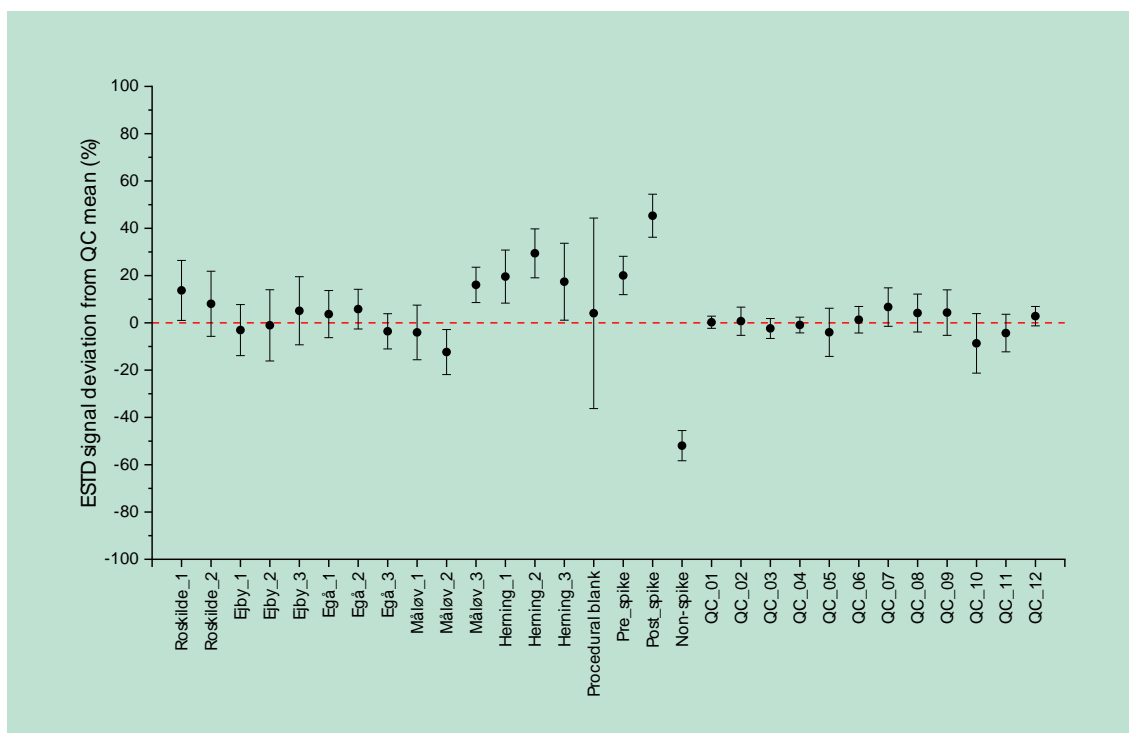


Figure 9. Average signal difference of the six isotope labelled extraction standards in each sample from the mean of twelve QC injections on the LC-pos system ($^{13}\text{C}_3$ -caffeine, DEET-D7, $^{13}\text{C}_6$ -thiabendazole, diuron-D6, pirimicarb-D6, and imidacloprid-D4). Error bars correspond to 1σ .

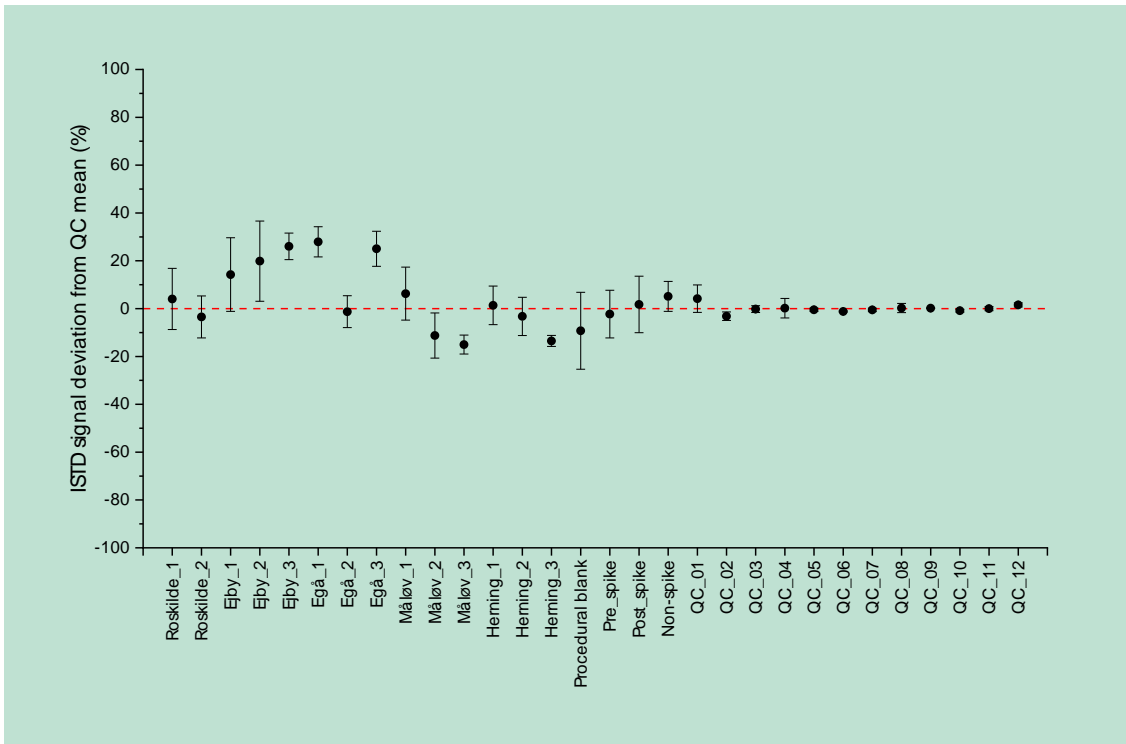


Figure 10. Average signal difference of the two isotope labelled internal standards in each sample from the mean of twelve QC injections on the LC-pos system ($^{13}\text{C}_3$ -Testosterone-2,3,4 and $^{13}\text{C}_4$ -15N₂-Riboflavin(-)). Error bars correspond to 1σ .

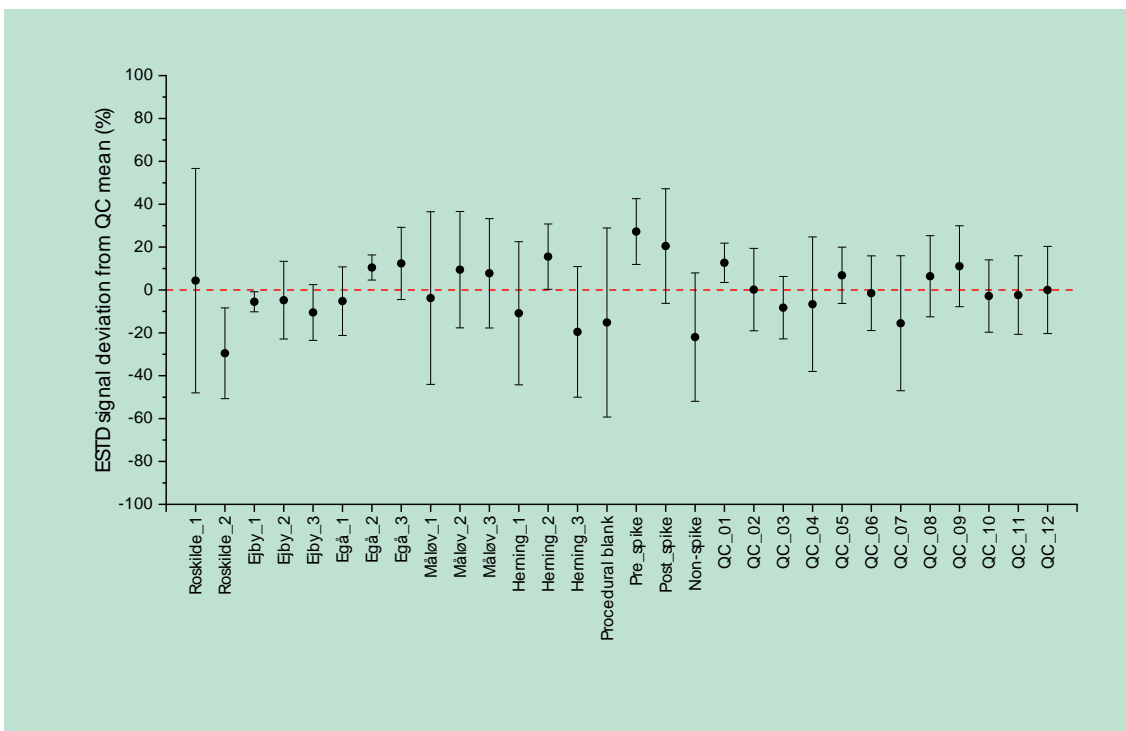


Figure 11. Average signal difference of the four isotope labelled extraction standards in each sample from the mean of twelve QC injections on the LC-neg system (Diuron-D6, imidacloprid-D4, mecoprop-D3, and 2,4-D-D3). Error bars correspond to 1σ .

Appendix 4. ICP-MS dataset

A complete ICP-MS dataset for 61 elements is available via ...MST.dk.

Appendix 5. NTS dataset

The complete NTS dataset is available via ...MST.dk.

File "Appendix 5-HITLIST4-GCEI.xlsx" contains all substances from the GC-EI-HRMS dataset.

File "Appendix 5-HITLIST4-LC.xlsx" contains all substances from the LC-HRMS/MS datasets (nLC and cLC).

HITLIST4: Non-targeted and suspect screening of sewage sludge



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Protection Agency
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