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Screening for 32 per- and polyfluoroalkyl substances (PFAS) including GenX in sludges from 43 WWTPs located in the Czech Republic -Evaluation of potential accumulation in vegetables after application of biosolids



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## HIGHLIGHTS

• a new method was optimized to analyze an extended group of PFAS consisting of 32 PFAS.

• PFAS contamination of sludge was detected in 43 WWTPs samples from Czechia.

• Some samples contained short-chain PFAS as replacements of PFOA and PFOS.

• GenX was also detected, confirming the suggested trend in the use of new PFAS.

• Sludge application in agriculture can cause contamination of cereals and vegetables.

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## ABSTRACT

Highly persistent, toxic and bioaccumulative per - and polyfluoroalkyl substances (PFAS) represents a serious problem for the environment and their concentrations and fate remain largely unknown. The present study consists of a PFAS screening in sludges originating from 43 wastewater treatment plants (WWTPs) in the Czech Republic. To analyze an extended group of PFAS consisting of 32 PFAS, including GenX and other new replacements of older and restricted PFAS in sludge, a new method was optimized and validated using pressurized solvent extraction, followed by the SPE clean-up step to eliminate the observed matrix effects and LC-MS/MS. The results revealed high PFAS contamination of sewage sludge. reaching values from 5.6 to 963.2 ng  $g^{-1}$ . The results showed that in the majority of the samples (about 60%), PFOS was the most abundant among the targeted PFAS, reaching 932.9 ng g<sup>-1</sup>. Approximately 20% of the analyzed samples contained more short-chain PFAS, suggesting the replacement of long-chain PFAS (especially restricted PFOA and PFOS). GenX was detected in 9 samples, confirming the trend in the use of new PFAS. The results revealed that significantly higher contamination was detected in the samples from large WWTPs (population equivalent > 50,000; p-value <0.05). Concerning the application of sludge in agriculture, our prediction using the respective PFAS bioconcentration factors, the observed concentrations, and the legislatively permitted management of biosolids in Czech Republic agriculture revealed that PFAS can cause serious contamination of cereals and vegetables (oat, celery shoots and lettuce leaves), as well as general secondary contamination of the environment.

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## 1. Introduction

Per- and polyfluorinated alkyl substances (PFAS) belong to a group of highly persistent pollutants with more than 5000 identified substances (Liu et al., 2019a). The structure of PFAS typically consists of a fluorinated hydrocarbon forming the hydrophobic moiety of the molecule and a functional group, which represents the hydrophilic part. According to the functional group, PFAS can be divided into sulfonates (PFSAs), carboxylates (PFCAs), sulfonamides (FASAs), sulfonamido acetic acids (FASAAs), fluorotelomer acrylates and sulfonates (FTAs and FTSs), and others (Buck et al., 2011). The strong carbon-fluorine bond gives PFAS unique physicochemical properties desirable for applications, such as thermal, chemical stability and amphiphilic behavior (Paul et al., 2009). Nevertheless, the strength and stability of the carbon-fluorine bond also make the hydrophobic part of PFAS highly resistant to degradation or biodegradation in the environment (Parsons et al., 2008; Merino et al., 2016). Only some of the functional groups of PFAS may undergo partial biodegradation/biotransformation under appropriate environmental conditions, making the parent molecule a precursor for the formation of daughter products (mainly PFCAs and PFSAs) that do not decompose further and accumulate in the environment (Liu and Avendano, 2013). Despite the fact that PFAS are generally considered to be highly persistent, they are still widely used in products such as firefighting foams, technical lubricants, greaseproof paper, or ski and floor waxes (Wang et al., 2019b).

In the early stages of PFAS research, attention was paid to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) (Nakayama et al., 2019). Due to their high production and persistence, these substances have been detected as the most abundant in the environment compared to other PFAS. PFOA and PFOS toxicity are also the most explored. Recent studies have shown the hepatotoxic effect of PFOS and have indicated that the liver is the main organ of bioaccumulation of this substance and its derivatives (Cui et al., 2009; Sharpe et al., 2010). The endocrine disruptive effects of these substances are also known, as they can interfere with the thyroid and estrogenic and androgenic hormonal pathways (Jensen and Leffers, 2008; Zeng et al., 2019). The persistence, environmental accumulation and adverse health effects of the combination of PFOS and PFOA have led to them both being included in the Stockholm Convention and subjected to restrictions on persistent organic pollutants. Currently, following regulations and restrictions, industrial uses have shifted toward new substitutes, in particular, (ultra)short PFCAs, short PFSAs and permolecular fluorinated of lower ethers weight e.g., perfluorobutanoic acid, PFBA; perfluorobutane sulfonate, PFBS; hexafluoropropylene oxide-dimer acid, HFPO-DA (as well as GenX, that is the industrially used ammonium salt of the respective acid) (Ateia et al., 2019; Munoz et al., 2019). Despite the fact that the toxicity or mechanism of action of these substitutes have not vet been sufficiently investigated, similar properties can be expected due to their analogous structure, and more attention should therefore be paid to these new substitutes (Wang et al., 2019a).

Although the hepatotoxicity and endocrine disruptive effects of PFOS were documented at relatively high doses (mg L<sup>-1</sup>) using human cell lines, the bioaccumulative potential of PFAS, as well as the immunotoxicity results of recent studies, demonstrate the risk of long-term human exposure to these substances even at very low concentrations (Grandjean, 2018; Zeng et al., 2019). With increasing information on PFAS, especially on their mechanism of toxicity and degradation resistance, regulatory limits are being introduced or tightened worldwide. In 2018, the European Food Safety Authority (EFSA) adopted limits for the "tolerable" weekly intake of PFOS and PFOA in food (6 and 13 ng kg<sup>-1</sup> of body weight, respectively). Despite the presence of other PFAS, the intake of PFOS

from agricultural plants probably plays a crucial role in human exposure (Klenow et al., 2013; Liu et al., 2019b). Many studies have shown the uptake of PFAS by agricultural plants grown in artificially contaminated soil or soil fertilized by contaminated sludge (Ghisi et al., 2019).

Accordingly, the contamination of sewage treatment sludge by PFAS should be monitored and evaluated prior to its agricultural application. Most of the global data from wastewater treatment plants (WWTPs) originate from the USA, Asia, and European countries. Concerning the concentration of PFAS in European WWTPs, data are available for Germany, Spain, Greece, Switzerland, the Netherlands, Denmark, and Sweden (Arvaniti and Stasinakis, 2015). In contrast, data from the rest of Europe, where up to 80% of the produced sludge is reused in agriculture, are completely missing (Hudcova et al., 2019).

In the majority of recent screening studies, only a few representative PFAS have been monitored (i.e., PFCAs, PFSAs, and FASAs) in sludge. The current analytical methods, including extraction and matrix effect elimination of sludge samples, typically consist of up to 22 compounds (Arvaniti and Stasinakis, 2015). However, the increasing use of short-chained PFAS and other alternatives of PFOS emphasize the need for monitoring an extended set of these compounds. Despite the fact that several authors have recently detected new replacements of PFOS in several environmental matrices, none of the authors analyzed GenX in sludge (Munoz et al., 2019). Optimized and validated methods for the extended analysis of PFAS, including GenX and other replacements, in sludge will allow the monitoring of one of the main sources that allow these compounds to enter the environment. Moreover, there is a lack of information about the fate (e.g., removal efficiency, sorption on sludge) of new PFAS, e.g. replacements of PFOA and PFOS in WWTPs.

The main purpose of this study was to evaluate the level of PFAS contamination in sludges originating from selected PFAS in 43 WWTPs in the Czech Republic. For this purpose, an analytical screening method for 32 PFAS representatives, including new substitutes (e.g., GenX, sodium dodecafluoro-3H-4, 8-dioxanonanoate - NaDONA), in soil and sludge was developed and validated. Attention was paid to the elimination of the LC-MS ionization matrix effects caused by sludge and soil matrix compounds in the extracts. To evaluate the risks of the agricultural use of WWTP sludge commonly used as a fertilizer, human exposure to PFAS from different vegetables grown in soil potentially fertilized by real contaminated sludge in the Czech Republic was calculated.

## 2. Materials and methods

## 2.1. Materials

The LC column XSelect CSH C18 (75 × 2.1 mm, 2.5  $\mu$ m) and the XSelect CSH C18 (2.5  $\mu$ m) precolumn were purchased from Waters (USA). Milli-Q water was prepared by a Direct-Q® water purification system (18.2 M $\Omega$  cm<sup>-1</sup>; Merck, Germany). Methanol for extraction (HPLC grade) was obtained from VWR (Czech Republic), and acetonitrile (LC-MS grade) was purchased from Honeywell Company (USA). The SPE Supelclean<sup>TM</sup> ENVI-Carb<sup>TM</sup> columns were from Merck (Germany). Standards of PFAS were purchased as analytical grade chemicals (>97% purity) from Apollo Scientific (UK) or in stock solutions of 10  $\mu$ g mL<sup>-1</sup> from Wellington Laboratories (Canada). GenX (FRD-902) was obtained from HPC Standards (Germany).

#### 2.2. Sample collection

Samples of sewage treatment sludge were collected during a

two-year sampling period (2018–2019) from 43 different WWTPs located in the Czech Republic that were artificially divided into 4 categories based on population equivalents (see Table 1). To avoid contamination by PFAS originating from sampling containers, glass bottles were used.

#### 2.3. Sample extraction and purification

The collected sludge samples were freeze-dried, thoroughly homogenized and extracted by pressurized liquid extraction (PLE) using an Accelerated Solvent Extractor ASE 200 (Dionex, France) according to the following protocol. Prior to extraction, 1 g of a homogenized sludge sample was mixed with 10 g of sea sand and placed in a stainless steel cell. Different temperatures (60 °C-150 °C) at a pressure of 1500 psi were tested (see the results section), and the final extraction program consisted of 3 extraction cycles using methanol at 150 °C and 1500 psi, with 5-min static periods. The methanolic extracts of the sludge samples were concentrated to a final volume of 5 mL under a gentle stream of nitrogen. For comparison, different methods of purification were tested using Captiva EMR lipid columns (Agilent Technologies, USA), Supelclean<sup>™</sup> ENVI-florisil<sup>®</sup> columns (Sigma-Aldrich, Czech Republic), OASIS®HLB 6 cc columns (Waters, Czech Republic), and QuEChERS (Sigma-Aldrich, Czech Republic) following the manufacturer protocols and their modified versions. Nevertheless, all the methods were not sufficient to remove the matrix, and finally, SPE Supelclean<sup>™</sup> ENVI-Carb<sup>™</sup> columns were employed. After the PLE extraction, 1 mL of the concentrated extract was acidified by the addition of 10 µL of formic acid and passed through a Supelclean<sup>TM</sup> ENVI-Carb<sup>™</sup> column (6 mL; 500 mg). To achieve the best recovery, especially of longer PFAS, the analytes were eluted from the SPE cartridge by acidified methanol ( $4 \times 2.5$  mL; methanol:formic acid, 99:1), and the eluent was evaporated to 1 mL prior to LC-MS analysis. Finally, the results obtained by LC-MS were then recalculated to 1 g of sample dried weight (dw). Each sample has been extracted and analyzed in triplicates and the procedural blanks (uncontaminated sea sand) were performed every 10 samples to ensure quality control.

## 2.4. LC-MS analysis

For the analysis of 32 PFAS in the sludge extracts, an LC-MS/MS method was optimized. The method included the detection of 11 PFCAs, 4 PFSAs, 3 FOSAs, 3 FOSAAs, 3 FTAs, 4 FTSs and 4 recent replacements of PFOS and PFOA. The mixture of 32 PFAS was

#### Table 1

Overview of WWTPs together with their respective capacities expressed as population equivalents.

separated using a Shimadzu Nexera 2 LC equipped with the XSelect CSH C18 column ( $75 \times 2.1 \text{ mm}$ ,  $2.5 \mu\text{m}$ ). The flow rate was set at 0.6 mL min<sup>-1</sup>, and the column temperature was held constant at 40 °C. An aliquot of 5  $\mu$ l was used for injection, and separation was achieved by gradient elution combining two mobile phases: 99.5:0.5 acetonitrile:formic acid (A) and 79.5:20.0:0.5 water:-acetonitrile:formic acid (B). The chromatographic run started at 80% A, and the gradient conditions were as follows (min, %A): 0, 80; 0.5, 80; 5, 10; 12, 10; 15, 5; 16, 0; 20, 0; 25, 80; 30, 80.

The separated PFAS were detected by a Sciex 4500 mass spectrometer equipped with electrospray ionization and operated in the negative mode. The source temperature was set at 650 °C, and the ion source voltage was set at -5500 V. Detection of the targeted compounds was performed in the multiple reaction monitoring (MRM) mode in 60-sec acquisition windows for each compound (scheduled MRM). Two transition reactions were monitored: the most intensive transition was used for quantification, and the second served for identity confirmation (Table 2). For quantification of each analyte, an external calibration curve was used. Moreover, to ensure quality control, LC-MS solvent blank and the PFAS standard solution (50 ng ml<sup>-1</sup>) was analyzed after each 20 injections.

## 2.5. Evaluation of matrix effects

To evaluate the effect of different matrices on PFAS ionization, four different PLE matrix extracts were artificially contaminated by a standard PFAS mixture (1–100 ng mL<sup>-1</sup> of each compound). Accordingly, fortified sand, soil and two different samples of sludge were analyzed by LC-MS without any additional clean-up step. The influence of the matrix on ionization – matrix effects (ME) – was calculated according to a previously published formula (Eq. (1)), where a<sub>matrix</sub> and a<sub>solvent</sub> represent the slopes of the calibration curves constructed using matrix-affected standard solutions and pure solvent solutions of chemical standards, respectively (Zhang et al., 2010). Ion suppression or enhancement is then expressed as a percentage, where a negative value represents a decrease in ionization of targeted PFAS and, correspondingly, a positive value represents ion stimulation by coeluting matrix compounds.

Equation 1. Matrix effects calculation according to Zhang et al., (2010).

$$ME_{ionization} \ [\%] = \left(\frac{a_{Matrix}}{a_{Solvent}} \ x \ 100\right) - 100$$

Large WWTPs (PE > 50,000)		Medium WWTPs (PE 10,000 -50,000)		Medium-9 	Small WWTPs (PE 5000	Small WWTPs (PE < 5000)		
Site N°	Population equivalents	Site N°	Population equivalents	Site N°	Population equivalents	Site N°	Population equivalents	
1	110,000	9	10,000	17	8000	32	10	
2	53,000	10	21,000	18	8800	33	3500	
3	192,000	11	13,000	19	8800	34	280	
4	88,000	12	31,000	20	5200	35	1400	
5	59,000	13	10,000	21	5900	36	120	
6	99,000	14	14,000	22	5000	37	200	
7	100,000	15	31,000	23	8400	38	150	
8	100,000	16	13,000	24	5400	39	1400	
				25	7000	40	3000	
				26	8000	41	1100	
				27	9000	42	100	
				28	5000	43	4500	
				29	6000			
				30	5000			
				31	5000			

#### Table 2

Selected dMRM transitions for PFAS analysis; the first transition was selected as a quantifier and the second as a qualifier.

Name	Abbreviation	Q1	Q3	RT [min]	DP [V]	CE [V]	CXP [V]
perfluoro-1-butanoic acid	PFBA	213	169	1.4	-40	-12	-13
		213	69	1.4	-40	-58	-19
perfluoro-1-pentanoic acid	PFPeA	263	219	2.2	-40	-10	-11
		263	69	2.2	-40	-52	-7
perfluoro-1-hexanoic acid	PFHxA	313	269	3.1	-45	-12	-7
		313	119	3.1	-45	-24	-5
perfluoro-1-heptanoic acid	PFHpA	363	319	3.9	-45	-14	-7
		363	169	3.9	-45	-22	-13
perfluoro-1-octanoic acid	PFOA	413	369	4.8	-50	-14	-15
		413	169	4.8	-50	-24	-5
perfluoro-1-nonanoic acid	PFNA	463	419	6.2	-60	-14	-9
and the second second		463	219	6.2	-60	-24	-9
periluoro-1-decanoic acid	PFDA	513	469	7.8	-65	-18	-11
perfluere 1 undecanoic acid	DELICA	562	219 510	7.8	-05	-24	-15
perindolo-1-undecanoic acid	FIOUA	563	319	9.5	-05	-10	-11
perfluoro_1_dodecanoic acid	PEDoA	613	569	114	-65	-24 -18	-13 -13
	TIDON	613	319	11.4	-65	-26	-13
perfluoro-1-tridecanoic acid	PFTrDA	663	619	13.6	-65	-20	-13
		663	169	13.6	-65	-34	-5
perfluoro-1-tetradecanoic acid	PFTeDA	713	669	15.8	-70	-18	-17
		713	169	15.8	-70	-34	-5
perfluoro-1-butanesulfonate	PFBS	299	80	2.5	-30	-62	-7
		299	99	2.5	-30	-54	-5
perfluoro-1-hexanesulfonate	PFHxS	399	80	3.7	-90	-88	-7
		399	99	3.7	-90	-72	-9
perfluoro-1-heptanesulfonate	PFHpS	449	80	4.4	-90	-94	-5
		449	99	4.4	-90	-84	-5
perfluoro-1-octanesulfonate	PFOS	499	80	5	-110	-106	-7
		499	99	5	-110	-96	-9
n-methyl-perfluoro-1-butanesulfonamide	MeFBSA	312	219	2.3	-80	-22	-13
	7004	312	65	2.3	-80	-52	-7
perfluoro-1-octanesulfonamide	FOSA	498	/8	3.4	-115	-82	-5
n othul porfluoro 1 octonocultonomido	E+EOCA	498	64 160	3.4	-115	-130	-9
n-ethyi-perhuoro-i-octanesunonannue	ELFUSA	520	210	4.1	-95	-34	-/
fluorinated telomer acid (3.3)	3·3 FTA	520 241	177	4.1	-95	-32	-15
	5.5 FIA	241	177	1.4	-55	-10	_7
fluorinated telomer acid (5·3)	5·3 FTA	341	236	25	-65	-18	_9
	5.5111	341	217	2.5	-65	-32	_9
fluorinated telomer acid (7:3)	7:3 FTA	441	337	3.4	-75	-16	-7
		441	317	3.4	-75	-28	-7
potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	9-Cl-PF3ONS	531	351	5.6	-80	-36	-11
		531	83	5.6	-80	-78	-7
potassium 11-chloroeicosafluoro-3-oxaundecane-1-sulfonate	11-Cl-PF3OUdS	631	451	7.4	-80	-40	-11
		631	83	7.4	-80	-88	-7
sodium dodecafluoro-3H-4, 8-dioxanonanoate	NaDONA	377	251	3.9	-50	-16	-9
		377	85	3.9	-50	-66	-7
2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoic acid	HFPO-DA (Gen-X)	329	285	3.3	-35	-8	-11
	50044	329	169	3.3	-35	-18	-11
perfluoro-I-octanesulfonamidoacetate	FOSAA	556	498	3.5	-80	-38	-13
n mothyl porflyoro 1 octopocylfonomidoocotato	MAEOSAA	570	419	3.5	-80	-30	-11
n-methyi-pernuoio-i-octanesunonamuoacetate	MERUSAA	570	419	2.9	-65	-20	-15
n_ethyl_perfluoro_1_octanesulfonamidoacetate	F±FΩS Δ Δ	584	405	J.5 // 1	-85	-22	-11
in entyr permuoro-r-octanesunollalliluoacetate	LUOJIM	584	526	4.1	_80 _80	-26 -26	-13 -13
fluorinated telomer sulfonate (4:2)	4·2 FTS	327	307	2	_70	-26	_11
masimated teromer sunonate (1.2)		327	80.9	2	-70	-56	-5
fluorinated telomer sulfonate (6.2)	6:2 FTS	427	407	3.4	-70	-32	-11
		427	81	3.4	-70	-68	-7
fluorinated telomer sulfonate (8:2)	8:2 FTS	527	80	4.9	-120	-108	-5
. /		527	81	4.9	-120	-94	-5
fluorinated telomer sulfonate (10:2)	10:2 FTS	627	607	8	-85	-42	-15
		627	81	8	-85	-100	-7

2.6. Calculation of hypothetical human exposure through vegetables grown in soil fertilized by sludge

To evaluate the potential risks associated with the agricultural use of WWTP sludge, the following approximation was used. Using bioconcentration factors (concentration in edible plant parts/ concentration in soil) documented in previously published studies (Stahl et al., 2009; Blaine et al., 2013, 2014) and the maximal allowed amount of sludge used for soil fertilization in the Czech Republic (10 t per ha), a theoretical total weekly intake of PFOS from 1 kg of selected agricultural plants was calculated (The Ministry of Environment of the Czech Republic; Decree 382-2001). Due to

maximal tilling of up to 30 cm, we considered this soil layer to be possibly affected by PFAS during sludge reuse in agriculture (maximal dilution). Considering a theoretical average soil density of 1.5 g mL<sup>-1</sup>, the weight of the soil in 1 ha was calculated, and using the theoretical amount of 10 t of sludge per ha, the PFOS final concentrations were estimated. This worst-case scenario was used to calculate the possible amounts of PFOS in edible parts of plants using previously published bioconcentration factors. The formulas used for the approximation of the concentration in soil after fertilization by sewage sludge treatment and the theoretical accumulation in edible plant parts are displayed inSupplemental Figure S1.

#### 2.7. Data analysis and presentation

The LC-MS/MS data as well as the construction of the calibration curves used for ME determination were processed by Analyst 1.6.3 software. MS Excel was used to graphically present the results employing the levels of PFAS in sludge and the calculated human intake in exposed vegetables. Statistical and correlation analyses were processed by OriginPro 9 software. Prior to one-way ANOVA, the normal distribution of the data was tested by the Shapiro–Wilk test using the same software.

## 3. Results and discussion

## 3.1. Extraction efficiency

The most commonly used methods for the extraction of solid and biosolid samples are supported liquid extractions with organic solvents employing shaking, sonication or heat (Jahnke and Berger, 2009; Nakayama et al., 2019). Despite the fact that pressurized liquid extraction (PLE) has only been employed in a limited number of studies dealing with PFAS, the technique is often considered the most efficient extraction method, and its automatization enables high-throughput use with a decreased extraction time (Schroder, 2003; van Leeuwen and de Boer, 2007). On the other hand, as a possible disadvantage, the typical high content of matrix compounds in PLE extracts can be considered to be a corollary of the powerful PLE extraction conditions. Accordingly, our newly developed extraction method using heated and pressurized methanol showed very promising results. The solvent was selected according to the literature, where methanol is used in other extraction methods and due to its general compatibility with PLE and high recoveries for various analytes (Nieto et al., 2010). Artificially contaminated sea sand was used for validation. Five different extraction temperatures were tested; however, all the conditions reached similar recoveries (data not shown), and finally, the highest temperature was selected for further testing. A satisfactory recoverv of 70–129% was reached for the majority of PFAS (see Fig. 1). Only MePFBSA showed poor recovery, from 25 to 30%. Several studies have employed PLE to extract a similar or smaller group of PFAS from different matrices, such as fish tissue, human hair, passive samplers or indoor dust (Liu et al., 2009; Llorca et al., 2009; Li et al., 2012b; Yao et al., 2018). To the best of our knowledge, PLE has never been used or validated for the extraction of such a wide range of PFAS from sludge.

## 3.2. Matrix effects

To determine and quantify the matrix effects of different solid matrices, samples of sea sand, soil, and two different WWTP sludges were extracted by PLE first. Subsequently, the methanol extracts containing matrix compounds were fortified with a mixture of 32 PFAS at 7 different concentration levels  $(1-100 \text{ ng mL}^{-1})$  and were directly analyzed by a LC-MS/MS system equipped with an electrospray that was operated in the negative mode. Calibration curves were constructed using Analyst software, and the matrix effects were calculated based on the slope of the curve (see 2.5. Evaluation of matrix effects). The results, expressed as increments and decrements of the calibration curve slopes (standard solutions in HPLC grade methanol were used as the no matrix effect samples), are shown in Fig. 2. The results of the matrix effects clearly demonstrate that the quantitative determination of all the PFAS in more complex matrices (i.e., soil and sludge) was highly affected and could lead to under- or overestimation of the targeted analyte quantities. Interestingly, all the PFCA results suffered from the suppression of ionization in soil and sludge matrices. Overall, the strong influence of the matrix on the negative ionization of PFAS documents the necessity of a clean-up step prior to analysis.

Suppression or stimulation in the ion source caused by matrix effects is a major problem during LC-MS/MS analysis of PFAS and has been observed by many authors (Li et al., 2009, 2012a; Hu and Yu, 2010; Zhang et al., 2010). There are several methods to avoid matrix effects and to achieve the correct results, including standard addition, the use of isotopically labeled internal standards, and sample clean-up prior to analysis. The isotopically labeled standards of PFAS are economically demanding, and their commercial availability is limited only to original representatives and does not cover all new fluorinated alternatives. The use of standard addition requires more than one injection of each sample and is therefore very time consuming. Moreover, during both of the mentioned approaches, the injected nonpurified sample could decrease the precolumn/column life, and frequent MS cleaning is often needed. For these reasons, in the present study, sample clean-up prior to LC-MS analysis was used to eliminate the matrix effects (the ENVI-Carb<sup>TM</sup> column and 4 washing steps with acidified methanol).

#### 3.3. Sample clean-up and method validation

Solid-phase extraction (SPE) is typically used for the clean-up of sample extracts prior to LC-MS/MS analysis. In recent years, SPE columns with C18, weak anion exchangers, and activated carbon have been used to monitor sludge/biosolid contamination by several PFAS (Guo et al., 2010; Alder and van der Voet, 2015; Boiteux et al., 2016). In particular, Supelclean™ ENVI-Carb™ was shown to eliminate the matrix effects from more complex extracts, including sludge, and provided great recovery for several PFCAs, PFSAs and other PFAS representatives. Therefore, this sorbent was selected for the PLE extract purification to be employed for PFAS sludge screening in the Czech Republic. The clean-up step using the ENVI-Carb<sup>™</sup> column and 4 washing steps with acidified methanol showed great recovery ranging from 84 to 117% (see Fig. 3) for all 32 typical representatives of PFAS, including their new substitutes. After the clean-up step, ME reached values from -15 to +16% for all 32 PFAS representatives (see Supplemental Figure S2).

The instrumental limit of detection (ILOD) was calculated as the concentration of an analyte that produces a minimal signal-tonoise ratio (S/N) of 3:1, and correspondingly, the instrumental limit of quantification (ILOQ) was calculated as a concentration producing a S/N of 10:1. The ILOD for all of the targeted PFAS ranged between 0.01 and 0.25 ng mL<sup>-1</sup>, and the ILOQ values were in the range of 0.05–0.7 ng mL<sup>-1</sup>. The entire method limit of quantification (MLOQ) for 1 g of a freeze-dried sample was 0.1–0.7 ng g<sup>-1</sup> for PFCAs, 0.2–1.6 ng g<sup>-1</sup> for PFSAs, 1.4–2.5 ng g<sup>-1</sup> for FOSAs, 0.8–2.0 ng g<sup>-1</sup> for FOSAAs, 4.2–9.6 ng g<sup>-1</sup> for FTAs, and 0.4–3.6 ng g<sup>-1</sup> for FTSs. The MLOQs of the new replacements of PFOS were 0.3, 0.3, 0.7, and 0.3 ng g<sup>-1</sup> for 9-CI-PF3ONS, 11-CI-PF3OUdS, NaDONA, and HFPO-DA, respectively. The respective



Fig. 1. Recovery of PFAS reached by PLE of artificially spiked sea sand.



Fig. 2. Matrix effects observed during analysis of artificially contaminated PLE extracts using 4 different matrices.



Fig. 3. Recovery of the cleanup procedure using artificially contaminated methanol and SPE Supelclean<sup>TM</sup> ENVI-Carb<sup>TM</sup>.

detection limits and other validation parameters for each PFAS are provided in Supplemental Table S1 in details. It is worth to note, that the procedural blank (using noncontaminated sea sand) contained a trace amount of PFOA and PFBS (above ILOD); however, the concentrations were below the respective ILOQs.

## 3.4. Occurrence of PFAS in WWTP sludge

The screening of 43 WWTPs revealed extensive contamination of sludge in the Czech Republic, and the results are shown in Fig. 4. All the sludge samples were contaminated by PFAS in a concentration range from 5.6 to 963.2 ng g<sup>-1</sup> (sum of PFAS). Out of the 32 monitored PFAS, 9 compounds, including EtFOSA, MeFOSA, 3:3 FTA, 9-CIPF3ONS, 11CIPF3OUdS, NaDONA, FOSAA, and 4:2 FTS, were not detected across the whole series of samples. In the case of 26 samples ( $\approx 60\%$ ), the major representative of PFAS was PFOS, reaching concentrations up to 932.9 ng g<sup>-1</sup> of dry sludge (see Fig. 4). Regarding the PFAS composition in the samples, 9 samples ( $\approx 20\%$ ) contained dominantly short-chain representatives and 35 samples long-chain representatives. HFPO-DA (GenX), the new replacement for PFOA, was detected in 7 samples at concentrations ranging from 0.3 to 1.2 ng g<sup>-1</sup> dw.

Even after the addition of PFOS to the Stockholm Convention in 2009 (decision SC-4/17) and the subsequent restrictions and regulations, its contamination of the environment remains. Currently, contaminated wastewater is very likely one of the main sources of PFOS pollution in aquatic as well as terrestrial environments (Arvaniti and Stasinakis, 2015: Gallen et al., 2018: Stroski et al., 2020). The PFOS concentration in 7 samples was greater than 100 ng  $g^{-1}$  dried sludge, which represents the limit value for the use of sludge as a fertilizer in agricultural applications in Germany (Alder and van der Voet, 2015). During wastewater treatment, several functional groups undergo biotransformation. There are many known precursors (e.g. perfluorooctane sulfonamidoethanol, FOSA, FOSAA, etc.), which could be microbially transformed to recalcitrant PFOS not only during wastewater treatment but also in soil (Rhoads et al., 2008; Liu and Avendano, 2013; Avendano and Liu, 2015). Moreover, it documented, that FTOHs and FTSs could be transformed to short PFCAs and PFSAs by resident microbial species in activated sludge under aerobic as well as anaerobic conditions (Wang et al., 2011; Li et al., 2018).

The concentrations of the PFOS detected in WWTP sludge in the Czech Republic are similar to the results from other European countries. In Germany, Switzerland, and Spain, the concentrations of PFOS reached values up to 7600, 2440, and 1790 ng g<sup>-1</sup> of dried sludge, respectively (Campo et al., 2014; Alder and van der Voet, 2015; Ulrich et al., 2016). However, in Denmark, Sweden, the Netherlands, and Greece, PFOS has been detected at lower concentrations (up to 74.1, 290, 48, and 11.3 ng  $g^{-1}$  of dw, respectively) (Bossi et al., 2008: Esparza et al., 2011: Olofsson et al., 2013: Stasinakis et al., 2013). The differences in sludge contamination across various countries could be caused by various factors such as population density, contrasting lifestyle, different industry and PFAS legislation or diverse wastewater technology. In addition to ineffective wastewater treatment, which leads to the discharge of PFOS (and other PFAS) to surface water, its strong affinity to WWTP sludge may lead to secondary pollution of agricultural soil, which will in turn also contaminate groundwater.

The point sampling used in the present study does not cover seasonal trends; however, some patterns in the PFAS composition were observed. The composition of the detected PFAS corresponds with previous studies from Switzerland and Korea, where PFOS was the predominant perfluorochemical detected in WWTP sludge (Huset et al., 2008; Guo et al., 2010; Alder and van der Voet, 2015). Nevertheless, several authors have noted a decreasing trend in sludge contamination by longer PFAS (PFOS and PFOA especially) in Germany (2008–2013) and Switzerland (2008 and 2011) during recent years, which could be explained by the use of new shortchain replacements with similar properties (Alder and van der Voet, 2015; Ulrich et al., 2016). Accordingly, in approximately 20% of our samples, the distribution of PFAS shifted to shorter compounds (i.e., PFBA, PFPeA, PFHxA, PFHpA, PFBS, and PFHxS), which corresponds with the replacement trend (Ateia et al., 2019). The detection of HFPO-DA (GenX) also confirms this tendency. Due to the insufficient data regarding the toxicity and/or bioaccumulation potential of GenX and other fluorinated replacements, their fate should be monitored to ensure environmental safety (Munoz et al., 2019; Wang et al., 2019b). The PFAS contents in WWTPs divided into groups based on their size (i.e., population equivalents) were statistically analyzed. One-way ANOVA showed that large WWTPs produce sludge with a higher concentration of PFAS. Based on the results of Tukey's test, only the group of large WWTPs differed significantly from the others in the PFAS concentration in the sludge at the 95% confidence level (see Fig. 5).

# 3.5. Local and global risks associated with the agricultural use of activated sludge contaminated with PFAS

In the Czech Republic, approximately 30% of sewage sludge is reused in agriculture as a fertilizer (Hudcova et al., 2019), emphasizing the importance of evaluating its chemical composition. Moreover, many representatives of PFAS have bioaccumulation potential and could be taken up from soil by agricultural plants (Ghisi et al., 2019). Therefore, using previously determined bioaccumulation/biouptake factors, a theoretical human exposure resulting from 1 kg of different vegetables (celery and lettuce) and cereals (oats) was calculated (Stahl et al., 2009; Blaine et al., 2013, 2014). For the calculation, the maximal amount of sludge that could be applied in the Czech Republic according to the legislation limit (i.e., 10,000 kg per ha) was used as the theoretical worst-case scenario.

The results of the approximation of the theoretical concentrations in vegetables (Table 3, dw) showed that this pathway could be very important in the human intake of PFAS. Moreover, the theoretical consumption of 1 kg of vegetables grown in soil fertilized by the sludge with the highest concentration of PFOS detected in this study (Sample No. 5) within a week could exceed the EFSA tolerable weekly intake (TWI for PFOS: 450 ng per 75-kg person) recommendation by several times. On the other hand, plant uptake and the distribution of PFAS to the edible parts of plants is influenced by many factors, such as individual contaminants, soil organic carbon content or differences between species (Navarro et al., 2017; Ghisi et al., 2019; Liu et al., 2019b). These results, therefore, only note the possible negative effects of real contaminated sludge application in agriculture. Nevertheless, the repeated application of sludge as a fertilizer could result in soil degradation, as was already documented in Germany, where the contamination of agricultural land reached up to 1 mg kg<sup>-1</sup> of PFOA and PFOS (Wilhelm et al., 2008). Similar results were observed in the USA, where contamination by fluorochemicals in farmlands was up to 6 mg  $kg^{-1}$ (Washington et al., 2010).

#### 4. Conclusions

The data presented in the current study document serious PFAS contamination of WWTP sludge in the Czech Republic, as documented by the screening of 43 WWTPs of various sizes. Application of the novel optimized analytical procedure for the extended group of PFAS 32 compounds, including the new types of PFAS as replacements, confirms, that shorter-chain PFAS, as well as GenX, are

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Fig. 4. Individual concentrations (column A) and the normalized distribution (column B) of the detected PFAS in 43 WWTPs separated according the respective capacity (large: > 50,000 PE; medium 50,000–10,000 PE; medium-small 10,000–5000 PE; < 5000 PE), the values represent means of 3 individual replicates with standard deviation lower than 20%.

already detectable in the environment. On the other hand, the old problematic PFAS are still detectable, even mostly at the highest concentrations among the monitored PFAS, despite that the compounds (PFOA and PFAS) are listed under the Stockholm Convention on Persistent Organic Pollutants. The results of this study suggest that PFAS can be detected in all the types of WWTPs. The data also document high variability among the pollution levels even in the municipal WWTPs in the Czech Republic. Significantly higher concentrations (p-value <0.05) were detected in larger WWTPs associated with agglomerations with more than 50,000 inhabitants. These results emphasize the need for further studies to track the sources of pollution regarding the local industry and possible other sources of contamination. Although only a few percent of the total mass flow of PFAS is adsorbed on the solid organic matter (sludge) during wastewater treatment processes and that the majority remains in the effluent entering receiving water, the amounts of PFOS and PFOA in sludge can represent a certain risk during its agricultural application as a fertilizer. Applying a simple approximation using previously published bioconcentration factors, our predicted concentrations in oat and



**Fig. 5.** Box plot showing the concentrations of PFAS in sludge samples originating from 44 WWTPs in the Czech Republic with respect to the capacities of the WWTPs (large: > 50,000 PE; medium 50,000–10,000 PE; medium-small 10,000–5000 PE; small < 5000 PE).

#### Table 3

Predicted theoretical concentrations in vegetables grown in soil fertilized by sludge from large WWTPs.

Sludge N°	Concentration of PFOS in sludge [ng g <sup>1</sup> ]	Concentration in vegetable [ng kg <sup>1</sup> of edible parts dw]		
		Oat grains	Celery shoots	Lettuce leaves
1	543	270	1678	2015
2	439	219	1357	1630
3	135	67	416	500
4	16	8	50	60
5	933	464	2882	3462
6	22	11	68	82
7	7	4	23	28
8	11	6	36	43
median	78	39	242	291

vegetable products (celery shoots and lettuce leaves) suggest that biosolids application could be disputable due to possibly exceeding the tolerable weekly intake by the EFSA. Agricultural application of sludge can potentially also contaminate groundwater; however, the downstream environment of large WWTPs could be affected even more.

## Credit author statement

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## **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.

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#### Appendix A. Supplementary data

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