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1 Occurrence and contamination profile of legacy and emerging per- and

2 polyfluoroalkyl substances (PFAS) in Belgian wastewater using target, suspect

3 and non-target screening approaches

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Abstract

With the growing concern regarding the health risks of per- and polyfluoroalkyl substances (PFAS), there is an increasing demand for the identification of emerging PFAS. This study provides a comprehensive investigation of legacy and emerging PFAS in 16 wastewater treatment plants (WWTPs) in Belgium using target, suspect, and non-target screening methods. Perfluorobutanoic acid (PFBA) and perfluoropentanoic acid (PFPeA) were the dominant compounds in most locations, whereas perfluorooctanoic acid (PFOA) was the most predominant PFAS in WWTP Deurne (Antwerp region). Using a suspect screening approach, 14 PFAS were annotated as confidence level (CL) of 4 or higher and 4 PFAS were annotated as CL 2a and 2b, including aqueous film forming foam (AFFF)-derived PFAS. The compound group of n:3 unsaturated fluorotelomer carboxylic acid was found using non-target screening in the wastewater from WWTP Deurne. Population exposure in a catchment area estimated using population-normalized mass loads (PNML) showed the highest value in the catchment area of WWTP Deurne, implying a potentially higher exposure to PFAS in this community.

Environmental Implication

Per- and polyfluoroalkyl substances (PFAS) are a group of chemicals that have been receiving global attention due to their strong persistence, bioaccumulation potential, and toxicity. This study conducted comprehensive monitoring of a broad range PFAS in Belgian wastewater. Target, suspect, and non-target analytical approaches were used to identify emerging PFAS, examine contamination profiles, and to assess population exposure. The outcome of this study can be utilized to build new monitoring strategies for emerging PFAS and to characterize regions at elevated exposure to protect local health.

1. Introduction

Poly- and perfluoroalkyl substances (PFAS) are a class of synthetic chemicals that have been used in a wide range of industrial and commercial products since the 1950s (Evich et al., 2022; Giesy and Kannan, 2002; Lindstrom et al., 2002; Wang et al., 2017). Due to their unique physicochemical properties (i.e., strong C-F bonds and repellency to water and oil), PFAS have been applied as surfactants, lubricants, mist suppressants, fast-food contact materials, cosmetics, and aqueous film-foaming foams (AFFFs) (Buck et al., 2011). The most well-known PFAS classes are perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs), which are represented by perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), respectively. Due to the strong persistence, bioaccumulation potential, and toxicity (i.e., thyroid dysfunction (Aimuzi et al., 2019), low birth weight (Wikström et al., 2020), and immune function disorders (Chang et al., 2016; Wen et al., 2019)), PFOS, PFOA and their salts were designated as persistent organic pollutants (POPs) under the United Nations Environment Programme (UNEP) Stockholm Convention (UNEP, 2019a, 2009). In line with this regulation, in 2019, the POPs review committee of the Stockholm Convention adopted a new decision to add perfluorohexane sulfonic acid (PFHxS) and its salts to the POPs list Annex A (UNEP, 2019b).

With respect to the global regulation of PFOS and PFOA, industries have claimed to shift their production of fluorinated compounds to less persistent and bioaccumulative PFAS, such as shorter chain PFAS (i.e., perfluorobutane sulfonic acid (PFBS) and perfluorobutanoic acid (PFBA)) and/or fluoroether replacements, such as GenX (hexafluoropropylene oxide dimer acid; HFPO-DA), ADONA (dodecafluoro-3H-4,8-dioxanonanoate), and F-53B (6:2 chlorinated polyfluoroalkyl ether sulfonate). However, shorter chain PFAS and alternatives are still of concern, because they are still recalcitrant, highly mobile or comparably persistent and toxic to PFOS and PFOA (Gaballah et al., 2020; Gordon, 2011; Munoz et al., 2019; Wang et al., 2013). In addition to the known alternatives, fluorine mass balance studies have recently reported that known PFAS (routinely monitored PFAS) only account for 2–44% of extractable organic fluorine (EOF), encouraging paradigm changes in scientific research to broad

screening of PFAS using suspect and/or non-target screening (Cousins et al., 2020; Kwiatkowski et al., 2020; Ruan and Jiang, 2017; Yeung et al., 2013).

High resolution mass spectrometry (HRMS) instruments, such as time-of-flight MS (TOF-MS) and Orbitrap MS, offer the opportunity to discover and identify unknown chemicals by providing resolving power and mass accuracy (Barzen-Hanson et al., 2017a; Liu et al., 2019). Recent studies have found previously unreported PFAS in various matrices, such as wastewater released from a fluorochemical manufacturing park (Liu et al., 2015), AFFFs-impacted groundwater (Barzen-Hanson et al., 2017a), wastewater treatment plant (WWTP) receiving large AFFF-inputs (Houtz et al., 2018), wastewater from a fluorochemical manufacturing park (Wang et al., 2018), fire-fighting foam impacted water (Yukioka et al., 2020), airborne particulate matter (Yu et al., 2018), and wastewater from electronics fabrication facilities (Jacob et al., 2021a) using suspect and non-target approaches. However, to the best of our knowledge, we are only aware of one study has performed a non-target analysis of PFAS in municipal wastewaters (Wang et al., 2020) despite of the important role of WWTPs as an indicator of regional contamination patterns and domestic exposure routes. Municipal wastewater has been successfully utilized to assess human exposure to chemicals by using a population-normalized mass load (PNML) (Choi et al., 2018).

Non-target analysis of PFASs has been performed using mass defect (i.e., < 0.15 or > 0.85) filtering accompanied with homologue analysis by repeating moiety patterns (i.e., CF_2, CF_2CF_2, and CF_2O) or fragment ion flagging (Liu et al., 2015). Non-target screening of PFAS is challenging because of the sophisticated data analysis procedure and absence of generalized workflow. In previous studies, acquired data from HRMS was processed using R packages (e.g., Package 'nontarget' (Loos and Singer, 2017) or in-house script (Wang et al., 2021)), MATLAB, or Excel, which requires specific training and expertise and/or involve time-consuming manual identification.

In the present study, we used a newly developed software 'FluoroMatch 2.0' for suspect and non-target screening of PFAS (Koelmel et al., 2020). The FluoroMatch software accurately annotates PFAS in a suspect screening fashion and contains fragmentation rules

for about 7,000 PFAS species across 70 classes. Furthermore, FluoroMatch performs peak

picking, blank feature filtering, mass defect filtering, homologue analysis using CF_2-normalized

Kendrick mass defect (or any other repeating unit), retention time (RT) order within homologue

groups, fragment screening (777 F-containing fragments), and *in silico* MS/MS library matching

applied to the suspect list from US CompTox 'PFAS master list' for annotation. The software

also assigns a confidence level for each feature by its scoring system based on the levels

proposed by Schymanski et al. (2014). With emerging PFAS being a growing concern,

FluoroMatch is a user-friendly and effective approach for the identification and the build-up of

an extensive database on PFAS. In recent studies, FluoroMatch showed good performance

for suspect and non-target analysis of PFAS in comparison with other software (i.e., Compound

Discoverer) (Jacob et al., 2021b; Nason et al., 2021).

This study aims to investigate the occurrence and contamination profile of a broad

range of PFAS in influent wastewater from 16 WWTPs in Belgium using target, suspect, and

non-target screening approaches. Also, the estimation of population exposure in the catchment

area has been used to identify regions at elevated risk. Additionally, the successful application

of FluoroMatch 2.0 for suspect and non-target screening of PFAS in this study will provide

guidance for further use of the software for extensive PFAS research. To the best of our

knowledge, this is the first report on the suspect and non-target analysis of PFAS in Belgian

influent wastewater.

2. Materials and Methods

2.1. Sample Collection

Daily 24-h composite influent wastewater samples were collected from 16 Belgian

WWTPs (names given in English) including Aartselaar, Antwerp (North and South), Boom,

Bruges, Deurne, Dendermonde, Genk, Ghent, Hasselt, Houthalen-Helchteren, Leuven,

Mechelen, Sint-Niklaas, Turnhout, and Brussels-North regions during autumn (September and

October), 2020 (**Figure S1**). For WWTP Brussels-North, influent wastewater samples collected

in 2013, 2015, 2016, and 2017 were available for an overview of contamination pattern

126   changes during the past years. Detailed information on wastewater samples such as sampling

127   months and dates, flow rate, and population equivalents are presented in **Table S1**.

128   Wastewater samples were collected in high-density polyethylene (HDPE) bottles and stored at

129   -20 °C until analysis.

130

131   *2.2. Sample Preparation*

132   A volume of 250 mL of each influent wastewater sample was transferred to pre-

133   cleaned 50 mL polypropylene tubes. Samples were centrifuged for 10 min at 2465 g and an

134   PFAS internal standard mixture (12.5 ng) was added. Detailed information on reference

135   standards of PFAS used in this study are presented in **Table S2**–**S3**.

136   Optimization of the sample extraction method (cartridge selection) and suppliers of

137   chemicals are described in Supporting Information and **Table S4**. Solid phase extraction (SPE)

138   of wastewater samples was performed using Oasis HLB (6 mL, 500 mg) and Oasis WAX (6

139   mL, 150 mg) cartridges. Prior to extraction, HLB cartridges were conditioned sequentially with

140   10 mL of methanol (MeOH) and 10 mL of ultrapure water, while WAX cartridges were

141   conditioned with 4 mL 0.5% (v/v) NH_4OH in MeOH, 4 mL MeOH, and 4 mL of ultrapure water.

142   After connecting the cartridges (HLB top and WAX bottom), wastewater samples (250 mL)

143   were loaded into HLB to WAX cartridges sequentially under vacuum (1 drop/sec), then the

144   cartridges were disconnected and washed with 10 mL of ultrapure water for the HLB cartridge

145   and 4 mL of 25 mM sodium acetate buffer (pH 4) for the WAX cartridge considering the different

146   sorbent types. After reaching dryness under vacuum, cartridges were eluted with 10 mL of

147   MeOH (HLB) or 8 mL 0.5% (v/v) NH_4OH in MeOH (WAX). Extracts were combined and

148   evaporated under a gentle nitrogen stream until dryness (at 30 °C) and then reconstituted in

149   500 µL of H_2O/MeOH (10/90, v/v). Samples were centrifuged for 10 min at 3220 g and

150   transferred to polypropylene vials for instrumental analysis.

151

152   *2.3. Instrumental analysis*

153   For target analysis, an Agilent 1290 Infinity ultra-high performance liquid

154 chromatograph (UHPLC; Agilent Technologies, Santa Clara, CA, USA) interfaced with an

155 Agilent 6495 triple quadrupole mass spectrometer (MS/MS; Agilent Technologies) was

156 operated in electrospray ionization (ESI) negative polarity and target PFAS were monitored

157 with multiple reaction monitoring (MRM) mode. Chromatographic separation was performed

158 by a Zorbax Eclipse Plus RRHD C18 column (2.1 × 100 mm, particle size: 1.8 μm) connected

159 with a guard column (Eclipse Plus C18, 2.1 × 5 mm, 1.8 μm). The mobile phases consisted of

160 (a) 2 mM ammonium acetate in water and (b) MeOH with a flow rate of 0.25 mL/min. The

161 detailed LC conditions, source parameters and MRM transitions are presented in **Table S5**–

162 **S6**.

163   Suspect and non-target analysis was performed with an Agilent 1290 Infinity UHPLC

164 coupled with an Agilent 6560 quadrupole time-of-flight mass spectrometry (QTOF-MS; Agilent

165 Technologies). The UHPLC analytical condition and mobile phases were consistent to target

166 analysis. Data acquisition was accomplished using ESI negative mode in full scan and iterative

167 data-dependent acquisition modes (selection of 10 precursors) operated at a 2 GHz extended

168 dynamic range mode with mass range *m/z* 1700. Detailed information on QTOF-MS acquisition

169 parameters is presented in **Table S7**. During the runs, real-time calibration was performed by

170 infusing internal reference masses (lock mass) using purine (*m/z* 119.0363) and hexakis (1H,

171 1H, 3H-tetrafluoropropoxy) phosphazine (*m/z* 980.0163).

172

173 *2.4. Quality control and quality assurance (QA/QC)*

174   To avoid contamination during sample preparation, all experimental apparatus were

175 cleaned 2–3 times with MeOH in prior to usage. Limit of quantification (LOQ) values and

176 linearity of calibration curves (R^2) applied in target analysis are presented in **Table S6**.

177 Concentrations below LOQ values were treated as zero to avoid overestimation due to a lower

178 concentration range of PFAS (Baccarelli et al., 2005). In the QTOF-MS analysis, known

179 amounts of a native PFAS standard mixture (0.1, 0.5, 1, 5, and 10 ng/mL) were injected to

180 check the instrumental sensitivity and mass accuracy (**Table S8)**. All compounds in the native

181 standard mixture, including 4 sulfonates (C4, C6, C8, and C10) and 11 carboxylates (C4–C14)

182 were detected with concentrations above 0.1 ng/mL, except for PFBA, which was found above

183 5 ng/mL. To avoid cross-contamination between samples, the injection needle was washed for

184 1 min with 50% isopropanol (IPA) in water (v/v) after every injection and a MeOH solvent blank

185 was injected between wastewater sample injections of each site. Instrumental sensitivity was

186 examined by injection of internal standard mixture (25 ng/mL) after every 15 samples and they

187 showed consistent peak abundances and mass accuracies. The MeOH solvent blank and

188 procedural blank did not show any quantifiable levels of targeted and newly identified PFAS,

189 except for 6:2 fluorotelomer sulfonate (6:2 FTS). The area of 6:2 FTS in blank has been

190 subtracted from the wastewater samples. Detailed QA/QC procedures and results including

191 matrix effect test (**Table S9**, **Figure S2**) are presented in Supporting Information.

192

193 *2.5. Data analysis*

194 *Target analysis.* A total of 34 PFAS were quantified using Agilent MassHunter Quantitative

195 Analysis 10.0 software. Compounds without corresponding labeled internal standard were

196 quantified with the most similar labeled compound considering the molecular weight, RT, and

197 functional group (**Table S6**).

198 *Suspect and non-target screening.* The data analysis workflows for suspect and non-targeted

199 screening of PFASs in wastewater are summarized in **Figure 1** and described in **Supporting**

200 **information** (Data analysis). Prior to data analysis, the workflow was validated by applying

201 native PFAS standard injections to the entire software process and the homologue groups

202 (PFSAs and PFCAs) were successfully annotated and categorized (**Table S10**).

203 Data preprocessing was performed using the open-source software MS-DIAL (version

204 4.60) (Tsugawa et al., 2015) and the detailed parameters are presented in **Table S11**. The

205 feature table obtained from MS-DIAL, which includes peak alignment ID, average *m/z*, average

206 RT, and peak area, and the data-dependent acquisition files for each site were processed with

207 FluoroMatch 2.0 Modular with the following criteria; (1) RT tolerance: ± 0.3 min; (2) ppm window:

208 10 ppm; (3) mass accuracy: 0.01 Da; (4) MS/MS isolation window: 0.05 Da; (5) MS/MS

209   intensity cutoff (minimum signal intensity cutoff): 10; (6) mass defect filtering: -0.11 to 0.12.

210   After processing the dataset, features classified as level 'E' (not likely PFAS) according to the

211   internal scoring system of FluoroMatch 2.0 (**Table S12**) and features with Kendrick mass defect

212   value between 0.15 to 0.85 were excluded. As a result, a total of 8,706 aligned features were

213   classified as potential PFAS.

214   For suspect screening, the *m/z* values of features were matched with the PFAS Master

215   List (included in FluoroMatch) and were sorted by detected frequency (> 60% of the sampling

216   sites). The peak shapes of matched features were examined with MS-DIAL. With the selected

217   features, the isotopic pattern and MS/MS fragmentation pattern were manually inspected with

218   Agilent MassHunter Qualitative Analysis 7.0. Additionally, to include a recent literature finding,

219   the suspect list provided by Liu et al. (2019) which merged previously reported PFAS

220   homologue groups and lists of novel PFAS in recent wastewater studies (Jacob et al., 2021a;

221   Wang et al., 2020, 2018) were also applied to Belgian wastewater.

222   Non-target screening was conducted by investigating assigned homologue groups

223   provided by FluoroMatch 2.0. Homologue groups which had features characterized with

224   diagnostic fragment ions (i.e., SO_3^-, CF_2^-, $C_2F_5^-$, etc.) or consecutive RT for over three

225   compounds were sorted for further investigation. Each feature's peak shape, RT and isotopic

226   patterns were manually checked with MassHunter Qualitative Analysis 7.0.

227   With the compounds identified by suspect and non-target screening, the confidence

228   level (CL) of compound identification was determined based on the recommendation of

229   Schymanski et al. (2014), which consists of CL 1 (confirmed structure by reference standard),

230   CL 2 (probable structure by library matching; 2a or diagnostic evidence; 2b), CL 3 (tentative

231   candidates identified with substructure, class, etc.), CL 4 (unequivocal molecular formula

232   without sufficient structure evidence), and CL 5 (mass of interest) (Schymanski et al., 2014).

233   The semi-quantification has been performed for newly found PFAS based on the

234   method described in Jacob et al. (2021a). Initially, individual newly identified PFAS were

235   matched with target PFAS quantified by LC-MS/MS according to molecular weight, RT, and

236   functional group similarity. As a further step, the ratio of newly identified PFAS peak area

divided by that of matched target PFAS was then multiplied by the concentration of the target PFAS quantified by LC-MS/MS to generate semi-quantitative concentration values.

The population exposure to PFAS in catchment area was estimated using population normalized mass load (PNML). Total mass load (g/day) was defined as the PFAS measured concentration multiplied by the daily service volume on the sampling day for each WWTP and PNML of PFAS in influent wastewater were calculated as total mass load divided by the served population. The inputs used for estimating total mass load and PNML are presented in **Table S1**.

3. Results and discussion

3.1. Target analysis

In total, 34 PFAS including groups of sulfonates, carboxylates, sulfonamides, sulfonamido acetic acids, sulfonamido ethyl alcohols, unsaturated carboxylates, phosphonic acids, fluorotelomer sulfonates, and ether-substituted PFAS alternatives, such as HFPOs, ADONA, and F-53B, were analyzed in Belgian wastewaters. Concentration, mass loads, and contamination patterns of measured PFAS in 16 municipal WWTPs and historical Brussels WWTP influent (2013–2020) are presented in **Table S13**, **Figure 2a, and 2b**. In our study, comparisons of PFAS levels and composition between WWTPs were conducted on a total mass load balance to account for variation in flow rates associated with specific sampling dates.

In all 16 WWTPs, PFBS, PFOS, PFBA, PFPeA, PFOA, PFDA and 8:2 FTS were observed and PFHxS, PFHxA, PFHpA, and PFNA were detected in over 80% of the sampling sites, whereas other compounds showed lower detection frequency (< 5%). These results indicate continuous introduction of legacy PFAS including PFOS and PFOA to Belgian WWTPs in spite of global regulation. The highest mass load of total legacy PFAS was found in the WWTP Deurne (133 g/day), which covers wastewater from the outer districts of Antwerp, followed by WWTPs of Bruges (8.89 g/day), Ghent (5.06 g/day), Antwerp South (3.41 g/day), Dendermonde (2.93 g/day), and Turnhout (2.46 g/day). At the majority of the sites, the dominant PFAS were shorter chain PFAS including PFBA (3.2–54%), PFPeA (5.8–78%), and

265 PFBS (0.2–29%), whereas PFOA represented approximately 80% of total PFAS in WWTP

266 Deurne. This result indicates that there might be specific sources of PFOA or its precursor (i.e.,

267 fluoropolymer, fluorotelomer-based substances such as fluorotelomer alcohols; FTOHs, etc.)

268 nearby WWTP Deurne. However, due to the limited information on the type of wastewater (i.e.,

269 industrial) input to WWTPs, we could not conduct in-depth investigation on the possible

270 sources of PFOA. Consistent with our results, Phong Vo et al. (2020) reported the dominance

271 of shorter chain PFAS in European countries, United States, and Australia associated with the

272 regulation effect, while PFOS and PFOA are still a major PFAS in developing countries (Lenka

273 et al., 2021; Phong Vo et al., 2020).

274   In the WWTP Brussels-North, the largest WWTP in Belgium, retrospective time point

275 samples collected in 2013, 2015, 2016, 2017, and 2020 were analyzed to estimate the

276 temporal changes of PFAS contamination pattern. The total PFAS mass loads varied

277 throughout the sampling years and ranged from 13.2 g/day to 69.7 g/day. The relative

278 contribution of individual PFAS showed a slight decreasing trend of PFOS, PFHpA, and PFOA

279 from 2013 to 2020, yet those trends were not statistically significant (**Figure S3**). The PFAS

280 contamination pattern changed between the year of 2015 and 2016, from the dominance of

281 PFOS (32–46%) in 2013 and 2015 wastewaters to PFPeA and PFBA (11–74%) in 2016, 2017,

282 and 2020 (**Figure 2b**) implying that the sources of PFAS in Brussels region may have changed

283 during those years. However, due to the limited sample size, the result might not reflect the

284 actual PFAS contamination trend in WWTP Brussels. To validate the time trend of PFAS

285 contamination, additional retrospective wastewater samples should be analyzed.

286

287 *3.2. Suspect Screening of PFAS*

288   Molecular formulas, matching scores (obtained by software), compound names

289 (matched name with suspect list), and identification confidence levels of PFAS that identified

290 by suspect screening are presented in **Table 1 and Table S14**. In total, 14 compounds were

291 annotated as CL 4 or higher based on exact mass matching, detection frequency (> 60%),

292 isotopic pattern, and available MS/MS fragmentation patterns. Among them, 4 compounds

were classified as CL 2a and 2b and 4 compounds were identified as CL 3 while others remained as CL 4.

   CL 2a and 2b. In Belgian influent wastewaters, two compounds with chemical formulas of $C_{15}H_{18}F_{13}NO_5S_2$ (*m/z* 602.0339; 6:2 fluorotelomer sulfinyl amido sulfonic acid; 6:2 FtSOAoS) and $C_{15}H_{18}F_{13}NO_6S_2$ (*m/z* 618.0288; 6:2 fluorotelomer sulfonyl amido sulfonic acid; 6:2 FtSO$_2$AoS) were identified as CL 2a. The diagnostic MS/MS fragments for each compound were [M-H]$^-$, [$C_7H_{14}NO_5S_2$]$^-$, [$C_7H_{12}NO_4S$]$^-$, and [$C_4H_{10}NO_3S$]$^-$ for $C_{15}H_{18}F_{13}NO_5S_2$ (**Figure S4a**), and [M-H]$^-$, [$C_7H_{12}NO_4S$]$^-$, [$C_4H_{10}NO_3S$]$^-$, and [O_3S]$^-$ for $C_{15}H_{18}F_{13}NO_6S_2$ (**Figure S4b**). Previous studies reported the occurrence of $C_{15}H_{18}F_{13}NO_5S_2$ and $C_{15}H_{18}F_{13}NO_6S_2$ in U.S. WWTPs (focusing on AFFF-derived PFAS) (Houtz et al., 2018), AFFF, and Commercial Surfactant Concentrates (D'Agostino and Mabury, 2014). The MS/MS fragmentation pattens obtained from our study were consistent to previous findings, which enables compound annotation as CL2a.

   A compound with formula $C_3H_2F_6O_4S$ (*m/z* 246.9498; 1:2 H-substituted perfluoroalkyl ether sulfonate) was identified based on the findings in Chinese municipal wastewater (Wang et al., 2020). The author provided the potential name of this compound as 1:2 H-substituted perfluoroalkyl ether sulfonate (1:2 H-PFESA) and the structure was confirmed by fragment ions of [$C_3F_5O_4S$]$^-$, [C_3HF_6O]$^-$, [C_3F_5O]-, [C_2F_5O]-, [HO_3S]$^-$, and [CF_3]$^-$ which were consistent with our findings (**Figure S5a**). In the study of Wang et al. (2020), 1:2 H-PFESA was identified by homologue analysis and the major compound of that homologue group is 6:2 H-PFESA (dominant transformation product of 6:2 Cl-PFESA), however, those compounds were not observed in Belgian wastewater. Wang et al. (2020) also mentioned that $C_3H_2F_6O_4S$ might not have consistently a H atom position with other homologues observed. Based on these concerns, despite of the similarity in major MS/MS fragmentation pattern, this compound remained as CL 2b. Compound $C_8HF_{18}NO_4S_2$ (*m/z* 579.8974; bis(1,1,2,2,3,3,4,4,4-nonafluoro-1-butanesulfonyl)-imide; **Figure S5b**) was also identified to CL2b by confirming the structure with fragmentation ions of [M-H]$^-$, [$C_4F_9NO_2S$]$^-$, [FO_2S]$^-$, and [NO_2S]$^-$. Until now, only one study have reported the detection of homologue group of bisperfluoroalkane sulfonylimides including

13

321    $C_8HF_{18}NO_4S_2$ from the mice dosed with an AFFF primarily containing electrochemically

322   fluorinated PFAS (McDonough et al., 2020). To the best of our knowledge, this is the first report

323   on environmental occurrence of bis-(1,1,2,2,3,3,4,4,4-nonafluoro-1-butanesulfonyl)-imide.

324   *CL 3.* Four compounds were assigned as CL 3 with the characterization of

325   distinguishable MS isotopic patterns, such as Cl or Br ions. The MS/MS fragments were not

326   obtained due to a lower peak abundance. $C_5H_3Cl_2F_3O$ (*m/z* 204.9467; 2-chloro-2,3,3-

327   trifluorocyclobutane-1-carbonyl chloride), $C_6H_5Cl_2F_3O$ (*m/z* 218.9589; 2-chloro-2,3,3-trifluoro-

328   1-methylcyclobutane-1-carbonyl chloride), $C_5H_6BrF_4NO_2$ (*m/z* 265.9419; 2-bromo-2,3,3,3-

329   tetrafluoro-N-methoxy-N-methylpropanamide) and $C_8H_4BrF_5O$ (*m/z* 288.9277; 1-bromo-4-

330   (pentafluoroethoxy) benzene) were characterized by unique MS isotopic patterns derived by

331   chlorine and bromine, with the highest isotopic pattern matching score over 90 (**Figure S6**).

332   *CL 4.* Six compounds were identified as CL 4 by molecular formula extraction with a

333   higher compound matching score (average > 80 and highest > 90). Compounds with the

334   formulas $C_4H_2F_8O_3S$ (m/z 280.9517) and $C_7H_6F_9NO_4S$ (*m/z* 369.9808) were found with a

335   suspect screening list of newly found PFAS reported in recent non-target PFAS studies (Liu et

336   al., 2019). The potential names of those compounds are 1,1,2,2,3,3,4,4-octafluorobutane-1-

337   sulphonic acid (H-PFSA) and methylperfluorobutane sulfonamidoacetic acid (MeFBSAA;

338    $C_7H_6F_9NO_4S$), respectively. In previous studies, the formulae $C_4H_2F_8O_3S$ and $C_7H_6F_9NO_4S$

339   were observed from downstream of manufacturing facilities near Decatur (Newton et al., 2017)

340   and $C_4H_2F_8O_3S$ was also detected in AFFF formulations (Barzen-Hanson et al., 2017) and river

341   and drinking water near a fluorochemical production plant in the Netherlands (Gebbink et al.,

342   2017). This finding indicates that the continuous update of PFAS suspect list is necessary

343   based on recent scientific findings to investigate broader range of emerging PFAS.

344   With the FluoroMatch workflow, four other CL 4 compounds, $C_8HF_{15}O_3S$ (*m/z*

345   460.9323; perfluoro-4-(perfluoroethyl)-cyclohexylsulfonic acid), $C_{11}H_{12}F_{11}NO_4S$ (*m/z* 462.0265;

346   ethyl   N-ethyl-N-(1,1,2,2,3,3,4,4,5,5,5-undecafluoropentane-1-sulfonyl)glycinate,

347    $C_{15}H_{19}F_{13}N_2O_4S$ (*m/z* 569.0777; 6:2 fluorotelomer sulfonamide betaine; 6:2 FTAB) and

348    $C_{15}H_{18}F_{13}NO_4S_2$ (*m/z* 586.0378; 6:2 fluorotelomer thioether amido sulfonic acid; 6:2 FtTAoS)

14

were identified. The formula $C_8HF_{15}O_3S$ was also annotated as unsaturated (double-bond) perfluoroalkyl sulfonic acid in the Barzen-Hanson et al. (2017), however, due to the unclear MS/MS spectra derived from low peak abundance, we could not confirm the final molecular structure.

In previous studies, 6:2 FTAB was observed in wastewater influent from a regional city catchment of Australia (Gallen et al., 2022), urban surface water in Canada (Agostino and Mabury, 2017), river and seawater samples in China (Chen et al., 2020), and sewage sludge, biowaste compost, and municipal solid wastes (Munoz et al., 2021) implied the widespread detection of AFFF-derived PFAS in the environment. Especially, 6:2 FTAB is known to induce developmental toxicity in zebrafish embryos (Shi et al., 2018) and 6:2 FTAB and 6:2 FtTAoS are known as a PFCAs precursors (D'Agostino and Mabury, 2014; Pan et al., 2018) which requires additional research on source and environmental fate. Due to a lower peak intensity, MS/MS spectra could not be acquired resulting in a CL 4 annotation confidence for those compounds. Also, it should be noted that the investigation of presence of isomer for CL 4 compounds was limited due to the absence of MS/MS fragments.

3.3. Non-target Screening of PFAS

In influent wastewater samples from Belgium, seven PFAS homologue groups were identified which contained 2–3 compounds within a consecutive RT series (**Figure 2c**). Among these groups, 22 compounds from four homologue groups were identified with CL 1–3, whereas others remained at CL 5, because MS/MS spectra were not obtained (**Table S15**).

Group 1–3. Seven compounds were identified as PFSA group (group 1, **Figure S7a**) which comprises of trifluoromethanesulfonic acid (TFMS), perfluoropropanesulfonic acid (PFPrS), PFBS, perfluoropentanesulfonic acid (PFPeS), PFHxS, PFHpS, and PFOS (mass error: -0.5 to 6 ppm). The MS/MS fragmentation pattern of PFBS confirmed that these compounds are classified within the PFSA group. Shorter chain PFSA, such as TFMS, PFPrS, PFPeS, and PFHpS were found by homologue analysis without reference standards. Seven compounds were confirmed as PFCA group (group 2, **Figure S7b**) including

377   perfluoropropanoic acid (PFPrA), PFBA, PFPeA, PFHxA, PFHpA, PFOA, and PFNA (mass

378   error: -0.5 to -9.3 ppm). The MS/MS fragmentation pattern of PFOA confirmed that these

379   compounds are included in PFCA group. PFPrA was newly identified by homologue analysis.

380   Group 3 included four compounds of the fluorotelomer sulfonates (FTS) group (**Figure S7c**),

381   which consisted of 4:2, 6:2, 8:2, and 10:2 FTS and the MS/MS fragmentation pattern was

382   confirmed with 6:2 FTS. Considering the PFSA, PFCA, and FTS groups as commonly

383   monitored PFAS in the environmental matrices, our results showed that the non-target

384   screening broadens the compound monitoring scope.

385   *Group 4.* Four compounds were identified as group 4 (potential name: n:3

386   fluorotelomer unsaturated carboxylic acid (n:3 FTUCA or n:3 Uacids); C4–C7; mass error: -6.2

387   to 1.2 ppm) characterized by the successive losses of CO_2 and C_2H_2 and the presence of fully

388   fluorinated carbon chain $[C_nF_{2n+1}]^-$ ions (**Figure S7d**). The MS/MS fragmentation patterns were

389   obtained from $C_8H_3F_{11}O_2$ (*m/z* 338.9852, -4.35 ppm) and $C_{10}H_3F_{15}O_2$ (*m/z* 438.9813, -0.63 ppm)

390   which confirmed their inclusion in the same homologue group. In previous studies, n:3 FTUCA

391   has reported as an intermediate environmental transformation product (e.g., microbial

392   biodegradation, biotransformation, etc.) of FTOHs such as 6:2 FTOH and 8:2 FTOH which are

393   the principal materials for manufacturing FTOH-based products (Prevedouros et al., 2006;

394   Zhang et al., 2013). In the earlier stage of degradation/transformation pathway of 6:2 FTOH

395   and 8:2 FTOH, 5:3 FTUCA and 7:3 FTUCA are generated and PFCAs such as PFBA, PFPeA,

396   PFHxA, PFHpA, and PFOA are known to be formed as final products. (Wang et al., 2005;

397   Zhang et al., 2013; Zhao et al., 2013) In WWTP Deurne, the peak abundances of formulae

398    $C_8H_3F_{11}O_2$ (5:3 FTUCA) and $C_{10}H_3F_{15}O_2$ (7:3 FTUCA) were approximately 10 times higher than

399   other homologues which may be associated a higher concentration of PFCAs in WWTP

400   Deurne. This result implies that there might be an input of FTOH or FT-based chemicals to

401   WWTP Deurne, even though other degradation/transformation products were not observed.

402   *In-source fragmentation.* Recently, Kang et al. (2021) raised a concern that ions

403   generated from the in-source fragmentation of PFCAs might have been classified as a new

404   homologue group, such as unsaturated perfluoroalcohol (UPFA) (Kang et al., 2021; Wang et

16

al., 2018; Yu et al., 2021). The UPFA homologue group was also found in influent wastewater of Belgium, however, we found the possibility that these compound groups are closely associated with in-source fragmentation which could be induced during electrospray ionization. This phenomenon was observed through the comparison of injection results between native standard and wastewater sample from Deurne which showed the highest concentration of PFCAs (**Figure S8–S9**). As presented in **Figure S8–S9**, the peaks of PFCA ($C_nHF_{2n-1}O_2$) and UPFA (loss of CF_2O from PFCA) were present at the same retention time as from native standard injection and WWTP Deurne. These data imply that the findings of UPFA from previous studies might have resulted from in-source degradation of PFCAs. Although Yu et al. (2021) suggested that UPFA might be generated as PFAS degradation intermediate with electrochemical oxidation or ultraviolet-generated hydrated electrons (Yu et al., 2021), careful investigation of PFCA-related homologue group will be needed in further studies.

3.4. Spatial trends of emerging PFAS

The semi-quantified result for newly found PFAS is presented in **Table S16**. The mass load and relative contribution of 4 individual PFAS (annotated as CL 2a and 2b) and 1 PFAS group (n:3 FTUCA) identified by suspect and non-target screening from Belgian WWTPs are shown in **Figure 2d** and **2e**. Similar to legacy PFAS, WWTP Deurne (117 g/day) showed the highest mass load of emerging PFAS followed by WWTPs of Leuven (9.69 g/day), Ghent (3.45 g/day), and Bruges (2.69 g/day). In all sites, 1:2 H-PFESA and AFFF-derived PFAS such as 6:2 FtSOAoS and 6:2 FtSO$_2$AoS were detected indicating wide distribution of those compounds in influent wastewaters of Belgium. Overall, the dominant emerging PFAS in Belgian wastewater was 1:2 H-PFESA which represented 41% (WWTP Boom) to 99% (WWTP Mechelen) of total emerging PFAS, except for WWTP Deurne (0.8%). The distribution of other emerging PFAS was varied among sites, for instance, a higher contribution (38%) of bis(1,1,2,2,3,3,4,4,4-nonafluoro-1-butanesulfonyl)-imide was observed in WWTP Leuven, whereas n:3 FTUCA (group 4) were found as being the dominant emerging PFAS (99%) in WWTP Deurne. In WWTPs of Boom, Bruges, and Genk, 6:2 FtSOAoS and 6:2 FtSO$_2$AoS were

higher than other PFAS which made up 48–60% of total emerging PFAS and which requires further in-depth investigation on contamination source (e.g., AFFF-impacted water) in those regions. Similar PFAS trends were found among time-trend samples in WWTP Brussels which were dominated by 1:2 H-PFESA indicating there might be less specific additional PFAS sources during the sampling periods.

3.5. Estimated population exposure

To estimate population exposure to investigated PFAS in study areas of Belgium, population-normalized mass load (PNML; $\mu g \cdot day^{-1}$ person^{-1}) of legacy (sum of PFBS, PFHxS, PFOS, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, 8:2 FTS, and 10:2 FTS) and emerging PFAS (sum of 1:2 H-PFESA, 6:2 FtSOAoS, 6:2 FtSO$_2$AoS, bis(1,1,2,2,3,3,4,4,4-nonafluoro-1-butanesulfonyl)-imide, $C_7H_3F_9O_2$, $C_8H_3F_{11}O_2$, $C_9H_3F_{13}O_2$, and $C_{10}H_3F_{15}O_2$; compounds annotated as CL 2a and 2b in suspect screening and n:3 FTUCA) were calculated among 16 WWTPs (**Figure 3, Table S17**). Overall, the PNML of legacy PFAS (8.7–626 $\mu g \cdot day^{-1}$ person^{-1}) were in a higher range than that of emerging PFAS (0.7–550 $\mu g \cdot day^{-1}$ person^{-1}) considering all sites, implying that people in the studied areas are more exposed to legacy PFAS than emerging PFAS. The highest PNML of legacy and emerging PFAS was found in WWTP Deurne (626 $\mu g \cdot day^{-1}$ person^{-1} for legacy PFAS and 550 $\mu g \cdot day^{-1}$ person^{-1} for emerging PFAS) which implied that populations in the catchment region of Deurne might be exposed to elevated PFAS levels compared to other regions.

Estimation of human exposure to PFAS using PNML has limitations because there is a lack of clear metabolic biomarker for PFAS, which means that human and other environmental sources (i.e., industrial) are indistinguishable (Choi et al., 2018). Therefore, the PNML values calculated from wastewater might not reflect the population exposure alone, but also the input from other sources. In addition to this, the semi-quantification approach used to calculate concentrations of emerging PFAS could result in under- or overestimation of exposure risks derived from the uncertainties of this method (i.e., differing response factors between compounds and/or instruments). Nevertheless, the monitoring of PFAS using

461   wastewater gives an opportunity to identify regions with relatively higher exposure risks and

462   specific source input to develop further monitoring and management schematics.

463

4. Conclusions

465   In our study, various legacy and emerging PFAS groups were monitored in municipal

466   influent wastewater from Belgium located in the most populated areas and as a result,

467   information on levels, patterns, spatio-temporal distribution, and population-normalized mass

468   loads in each WWTP were provided. The major findings of our study are (1) the dominance of

469   PFBA and PFPeA in the majority of Belgian wastewaters, (2) higher contribution of PFOA in

470   WWTP Deurne (potential specific source indication), (3) occurrence of AFFF-derived PFAS in

471   all Belgian WWTPs, and (4) the detection of n:3 FTUCA group (degradation/transformation

472   product of FTOHs) in WWTP Deurne. The observation of n:3 FTUCA may imply the input of

473   FTOHs in WWTP Deurne. Throughout the study, we confirmed that wastewater is a good

474   indicator in investigating the contamination patterns and application of suspect and non-target

475   screening to wastewater gives an opportunity to early-warning of emerging PFAS exposure to

476   specific regions where people might have higher risks. The developed workflow can be applied

477   to identify other groups of PFAS (i.e., zwitterionic) to extend the monitoring scope. Suspect

478   and non-target screening in wastewaters can be effectively used to determine the site which

479   needs further *in-depth* investigation as well as establishment of future regulations and/or

480   management strategies on chemicals of emerging concern.

481

495

496   **CRediT authorship contribution statement**

497   **Yunsun Jeong**: Conceptualization, Methodology, Validation, Formal analysis, Investigation,

498   Data Curation, Writing–Original Draft. **Katyeny Manuela Da Silva**: Methodology, Software,

499   Data Curation, Writing–Review & editing.   **Elias Iturrospe**: Methodology, Data Curation,

500   Writing–Review & editing. **Yukiko Fuiji**: Methodology, Validation, Investigation, Writing–

501   Review & editing. **Tim Boogaerts**: Conceptualization, Investigation, Writing–Review & editing.

502   **Alexander L.N. van Nuijs**: Conceptualization, Investigation, Writing–Review & editing,

503   Supervision. **Jeremy Koelmel**: Methodology, Software, Writing–Review & editing. **Adrian**

504   **Covaci**: Conceptualization, Methodology, Validation, Investigation, Writing–Review & editing,

505   Supervision.

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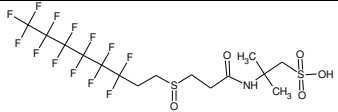697   https://doi.org/10.1021/acs.est.8b02492
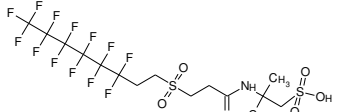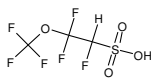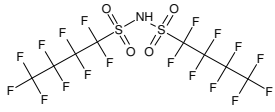
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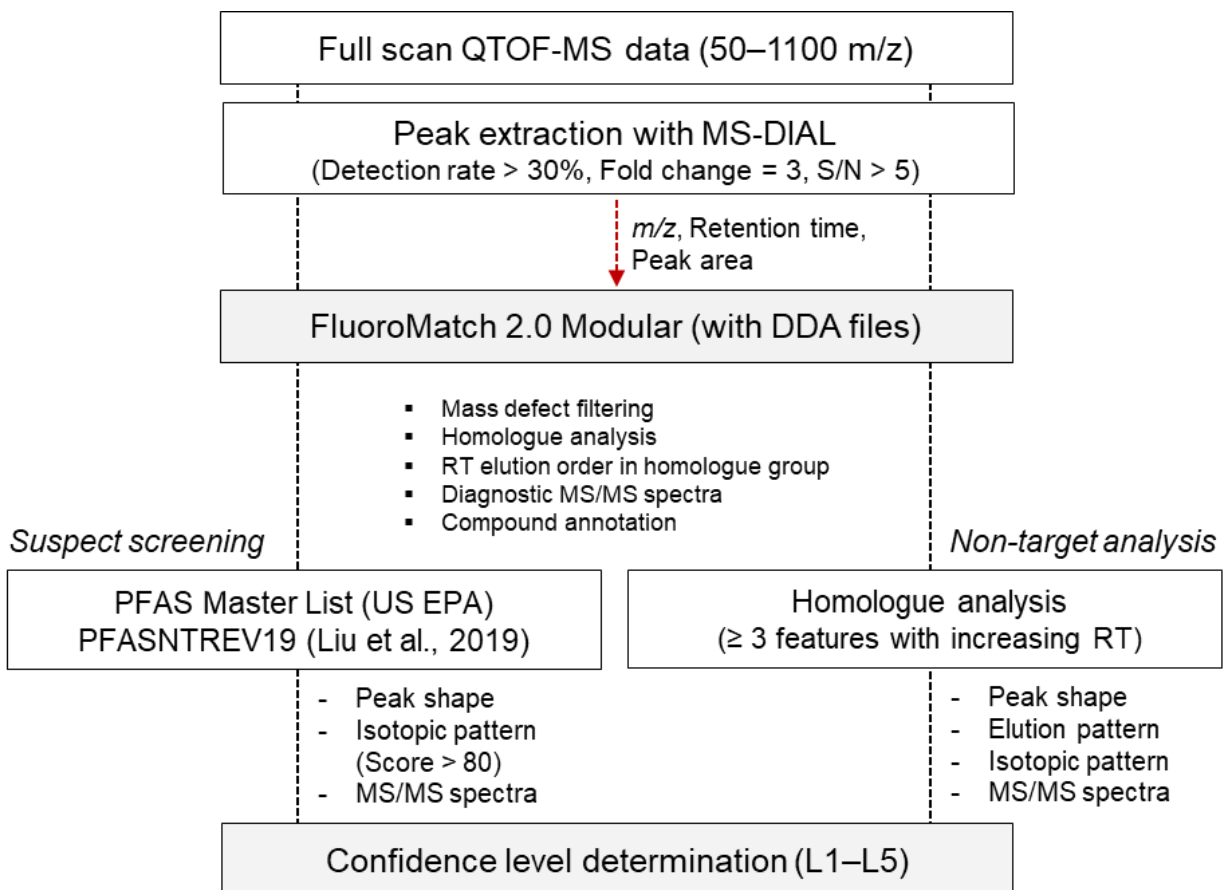712   domestic wastewater treatment plants. Chemosphere 92, 464–470.

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714 **Table 1.** List of molecular formula, *m/z*, retention time (RT), identification score, diagnostic MS/MS fragments, confidence level, detection frequency (DF), and
715 matched suspect list of compounds found in Belgian influent wastewaters.

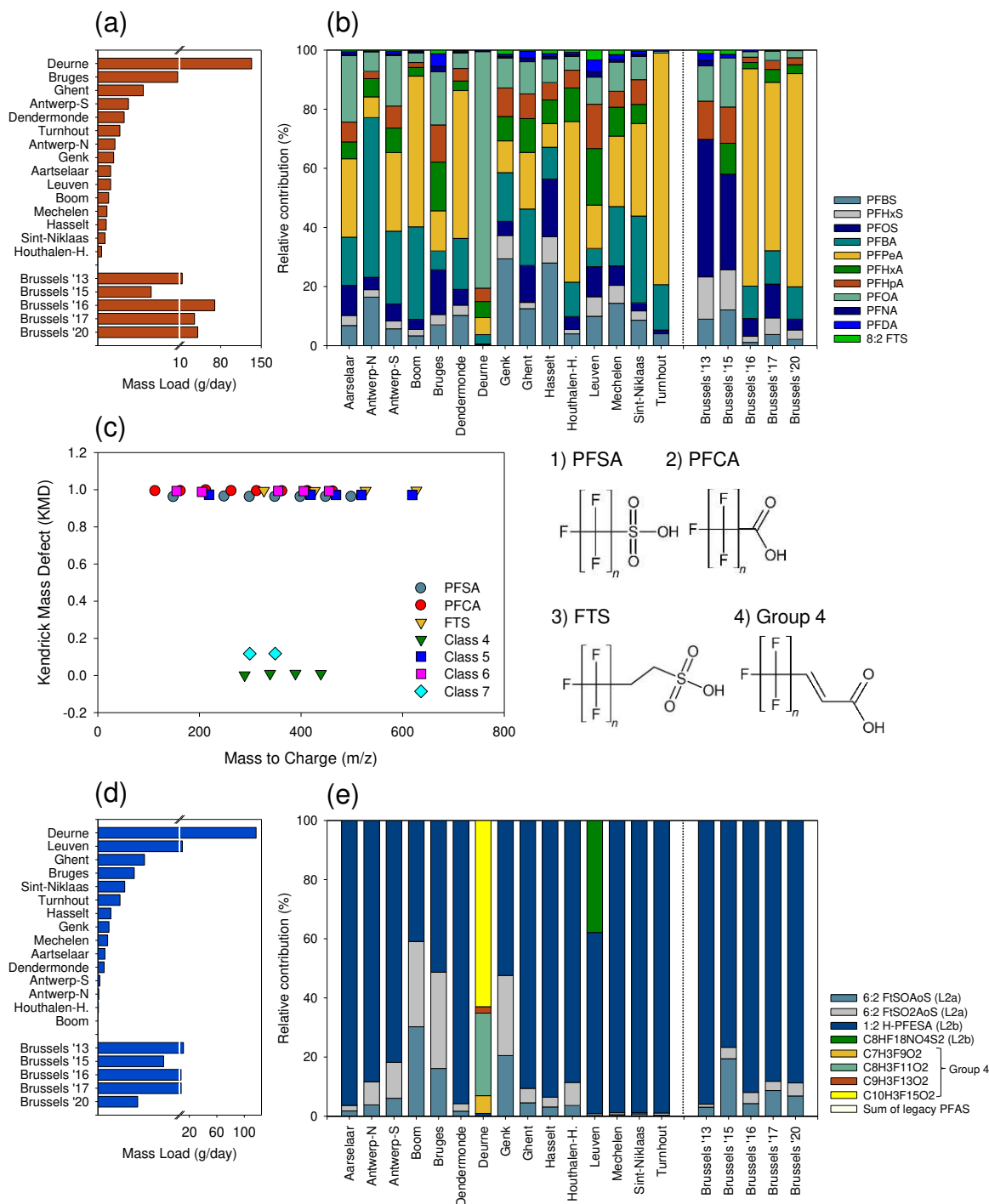| No	Formula[a]		*m/z*	RT	Score[b] (highest)	MS/MS fragments (Diagnostic ion)	Confidence Level	DF (%)	List[c]
1	$C_{15}H_{18}F_{13}NO_5S_2$		602.0339	8.18	92 (99)	SI[d]	CL2a	100	*
2	$C_{15}H_{18}F13NO_6S_2$		618.0288	8.27	93 (99)	SI	CL2a	100	*
3	$C_3H_2F_6O_4S$		246.9498	4.68	85 (99)	SI (68.9, 146.9)	CL2b	100	***
4	$C_8HF_{18}NO_4S_2$		579.8974	8.30	95	SI	CL2b	5	*
5	$C_5H_3Cl_2F_3O$		204.9467	6.59	80 (93)	N.A. (Cl$_2$ confirmed)	CL3	100	*
6	$C_6H_5Cl_2F_3O$		218.9589	3.83	81 (93)	N.A. (Cl$_2$ confirmed)	CL3	62	*
7	$C_5H_6BrF_4NO_2$		265.9419	5.06	81 (92)	N.A. (Br confirmed)	CL3	76	*
8	$C_8H_4BrF_5O$		288.9277	5.99	90	N.A. (Br confirmed)	CL3	5	*
9	$C_4H_2F_8O_3S$		280.9517	5.15	90	N.A. (79.96)	CL4	43	***
10	$C_7H_6F_9NO_4S$		369.9808	7.41	80 (95)	N.A.	CL4	81	**
11	$C_8HF_{15}O_3S$		460.9323	8.16	78 (95)	N.A.	CL4	76	*, ***
12	$C_{11}H_{12}F_{11}NO_4S$		462.0265	6.01	80 (88)	N.A.	CL4	62	*
13	$C_{15}H_{19}F_{13}N_2O_4S$		569.0777	8.66	95	N.A.	CL4	5	*
14	$C_{15}H_{18}F_{13}NO_4S_2$		586.0378	8.43	90 (99)	N.A.	CL4	100	*

716 [a]Formula used for find by formula option in Agilent MassHunter Qualitative Analysis 7.0; [b]Average Score obtained by Agilent MassHunter Qualitative Analysis
717 7.0; [c]Matched suspect list (*PFAS Master List; **PFASNTREV19; ***Previous studies); [d]Supporting Information.

Figure 1. Schematic diagram of data analysis workflow applied for suspect and non-target analysis of PFAS in Belgian wastewaters.

Figure 2. Concentration and contamination profiles of legacy and emerging PFAS in influent wastewater from 16 Belgian wastewater treatment plants (WWTPs) collected in 2020 and time-trend samples from WWTP Brussels (2013–2020). For the calculation of mass loads and contamination profiles of emerging PFAS, semi-quantification method was used. (a) mass load of legacy PFAS; (b) relative contribution of legacy PFAS; (c) PFAS homologue groups found by non-target screening; (d) mass load of emerging PFAS; (e) relative contribution of emerging PFAS.

Figure 3. Calculated PNMLs (population normalized mass loads; $\mu g \cdot day^{-1} person^{-1}$) in 16 Belgian WWTPs. Blue and yellow bars indicate the sum of PNML values for legacy (sum of 12 PFAS) and emerging PFAS (sum of 8 PFAS), respectively. Red dots and dashed lines indicate the location and name of WWTPs. Detailed values are presented in SI (Ant-N: Antwerp North, Ant-S: Antwerp South).