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Distinctive biotransformation and biodefluorination of 6:2 versus 5:3 fluorotelomer carboxylic acids by municipal activated sludge

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ABSTRACT

Fluorotelomer carboxylic acids (FTCAs) represent an important group of per- and polyfluoroalkyl substances (PFAS) given their high toxicity, bioaccumulation potential, and frequent detection in landfill leachates and PFAS-impacted sites. In this study, we assessed the biodegradability of 6:2 FTCA and 5:3 FTCA by activated sludges from four municipal wastewater treatment plants (WWTPs) in the New York Metropolitan area. Coupling with 6:2 FTCA removal, significant fluoride release (0.56~1.83 F-/molecule) was evident in sludge treatments during 7 days of incubation. Less-fluorinated transformation products (TPs) were formed, including 6:2 fluorotelomer unsaturated carboxylic acid (6:2 FTUCA), perfluorohexanoic acid (PFHxA), perfluoropentanoic acid (PFPeA), and perfluorobutanoic acid (PFBA). In contrast, little fluoride (0.01~0.09 F-/molecule) was detected in 5:3 FTCA-dosed microcosms, though 25~68% of initially dosed 5:3 FTCA was biologically removed. This implies the dominance of "non-fluoride-releasing pathways" that may contribute to the formation of CoA adducts or other conjugates over 5:3 FTCA biotransformation. The discovery of defluorinated 5:3 FTUCA revealed the possibility of microbial attacks of the C-F bond at the γ carbon to initiate the transformation. Microbial community analysis revealed the possible involvement of 9 genera, such as Hyphomicrobium and Dechloromonas, in aerobic FTCA biotransformation. This study unraveled that biotransformation pathways of 6:2 and 5:3 FTCAs can be divergent, resulting in biodefluorination at distinctive degrees. Further research is underscored to uncover the nontarget TPs and investigate the involved biotransformation and biodefluorination mechanisms and molecular basis.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs) have attracted unprecedented attention given their prevalent detection and adverse health effects (Arvaniti and Stasinakis 2015; Evich et al., 2022; Rahman et al., 2014). PFASs have been applied in a myriad of commercial products (e.g., stain- and water-repellents and firefighting foams) due to their chemical and thermal stability, high surface activity, and hydro-/-lipophobic properties (Buck et al., 2011; Meshri 1986; Wang et al., 2017; Xiao 2017). Unfortunately, toxicological studies have demonstrated that PFAS exposure can elicit hepatotoxicity, neurotoxicity, immunotoxicity, genotoxicity, and reproductive and developmental effects (Butenhoff et al., 2002; Guruge et al., 2006; Lau et al., 2004; Ma et al., 2020a; Pinkas et al., 2010; Ren et al., 2009). Accordingly, primary regulatory attention has been centered on perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), and other PFASs that mostly belong to perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs) (Rahman et al., 2014). Because of the extreme stability of the C-F bond (Scheringer et al., 2014), these PFASs are considered highly recalcitrant in the environment.

Fluorotelomer carboxylic acids (FTCAs) represent an important group of PFASs, consisting of one or more non-fluorinated alkyl carbons between the perfluorinated tail and the carboxylic acid head. FTCAs are frequently found as key intermediates generated from the biotransformation of fluorotelomer (FT)-based PFAS precursors, such as fluorotelomer alcohols (FTOHs) (Kim et al., 2012; Liu et al., 2010b; Tseng et al., 2014a; Wang et al., 2009; Zhao et al., 2013), fluorotelomer sulfonates (FTSs) (Shaw et al., 2019; Zhang et al., 2016), and fluoroalkyl phosphates (PAPs) (Lewis et al., 2016). A portion of the generated FTCAs can be further degraded, leading to the accumulation of downstream shorter chain PFCAs, such as perfluorohexanoic acid (PFHxA), perfluoropentanoic acid (PFPeA), and perfluorobutanoic acid (PFBA)

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(D'Agostino and Mabury 2017; Evich et al., 2022; Lee et al., 2010; Li et al., 2018; Qiao et al., 2021; Wang et al., 2011b; Wang et al., 2012a; Zhang et al., 2013; Zhao et al., 2013).

Recent investigations revealed the dominant detection of FTCAs, particularly 5:3 FTCA and 6:2 FTCA, in landfill leachates and other environmental matrixes (Allred et al., 2014; Fuertes et al., 2017; Lang et al., 2017). Lang et al. reported that 5:3 FTCA accounted for up to 25~57 % of the total mass of PFASs detected in landfill leachates collected across the US, resulting in a median release estimated at nearly 200 kg/yr (Lang et al., 2017). Since landfill leachates are frequently handled at domestic wastewater treatment plants (WWTPs), WWTPs can be considered potential nodes for FTCAs to enter the water cycle (Allred et al., 2014; Hamid et al., 2018; Huset et al., 2011; Lang et al., 2017). A recent study revealed a high mass flow of 6:2 FTCA at 12~15 g/d in the secondary clarifier and effluent at a WWTP of a fluorochemical manufacturing facility (Dauchy et al., 2017). Accordingly, we identified FTCAs and their derivatives with hydrogen substitution or unsaturation bond across the wastewater treatment train at three WWTPs in New Jersey (Wu et al., 2022). FTCAs have also been reported in other aquatic environments, such as rainwater at the concentration range of 0.30~1.00 ng/L (Loewen et al., 2005). Unfortunately, FTCAs exhibit higher bioaccumulating potentials and are at least two orders of magnitude more toxic than their corresponding PFCAs (Phillips et al., 2007; Shi et al., 2017). The median effective concentrations (EC50s) of 6:2 FTCA were 63.0 mg/L for the midge fly *Chironomus tentans* (Phillips et al., 2007) and 26.2 mg/L for the green algae *Chlorella vulgaris* (Mitchell et al., 2011), and the median lethal concentration (LC50) of 6:2 FTCA was 7.33 mg/L for zebrafish embryos (Shi et al., 2017).

Considering the introduction of FTCAs from the discharge of landfill leachates and other domestic sources and their toxicity concerns, it is of great value to investigate the biodegradability of FTCAs by activated sludge. In fact, increased PFCA mass flows over the activated sludge process have emerged as a pressing issue at WWTPs (Kunacheva et al., 2011; Schultz et al., 2006; Sinclair and Kannan 2006; Wu et al., 2022; Yu et al., 2009). Biotransformation of FTCAs and other FT-based precursors to PFCAs during the aeration treatment is widely proposed as a source of PFCAs in WWTPs (Chen et al., 2017; Lee et al., 2010; Sinclair and Kannan 2006; Yu et al., 2009). Based on the target analysis, several studies on biotransformation of FT-based precursors suggested 6:2 FTCA as a key intermediate that can be further degraded to shorter chain PFCAs (e.g., PFHxA, PFPeA, and PFBA) and 5:3 FTCA (Kim et al., 2014; Kleiner and Jho 2009; Lewis et al., 2016; Li et al., 2018; Liu et al., 2010a; Liu et al., 2010b; Merino et al., 2018; Qiao et al., 2021; Royer et al., 2015; Shaw et al., 2019; Shi et al., 2017; Tseng et al., 2014a; Wang et al., 2011b; Zhang et al., 2016; Zhao et al., 2013). Wang et al. reported the aerobic biotransformation of 5:3 FTCA by activated sludge via "one-carbon removal pathways" (Wang et al., 2012a), through which 5:3 FTCA is transformed to 4:3 FTCA or shorter chain PFCAs via the formation of α-OH 5:3 acid and 5:2 FTCA (Wang et al., 2012a). However, limited knowledge is available for a quantitative comparison between 6:2 and 5:3 FTCAs regarding their biotransformation potentials and biodegradation pathways.

In this study, we selected 6:2 and 5:3 FTCAs as representatives and investigated their biotransformation and biodefluorination processes in activated sludge collected from four municipal WWTPs in the New York metropolitan area. Nano-electrospray ionization high-resolution mass spectrometry (Nano-ESI HRMS) was employed for the quantitative analysis of FTCAs and their transformation products (TPs) over 7 days of incubation (Wu et al., 2022). Microbial populations that may contribute to FTCA biotransformation and biodefluorination were investigated by 16S rRNA amplicon-based sequencing and bioinformatics analysis based on the shifting of the relative abundances of these taxa in response to the FTCA exposure. These findings are of significant value to advance our understanding of PFAS biotransformation and biodefluorination pathways and involved microorganisms.

2. Methods

2.1. Chemicals and reagents

Reagents, solvents, and standards used in this study are described and listed in the SI.

2.2. Sample collection

Activated sludge samples were collected from four domestic WWTPs (designated as Sludge R, P, L, and W) located in Northern New Jersey and New York City in October 2019, which serve populations ranging from 6.0×10^4 to 1.4×10^6 and a diversity of local industries (see specifics of these four WWTPs in Table S7). At each WWTP, 500 mL of activated sludge slurry was collected in triplicate in the center of the aeration tank and stored in a sealed 1-L HDPE container with 500 mL headspace. All samples were shipped on ice from the sampling sites to our laboratory at NJIT on the same day. All sludges were then temporarily stored in 4 °C refrigerator before the experiment within 24 h.

2.3. Microcosm study

Four sets of microcosms, including sludge treatment, analytical control, abiotic control, and microbial control, were conducted in triplicate to investigate the biotransformation of 6:2 FTCA and 5:3 FTCA individually. 6:2 FTCA and 5:3 FTCA stock solutions were prepared at the concentration of 40 mM in pure methanol. For sludge treatment microcosms, 100 μL of the 40 mM FTCA stock solution was added to 160-mL amber serum bottles, which were then flushed with nitrogen flow to fully evaporate the methanol solvent. Sludges were well mixed and concentrated via centrifugation at 12,000 rpm, followed by being washed three times with phosphate buffer saline (PBS, 20 mM sodium phosphate, pH 7.0) to remove the dissolved and unbounded organic carbon. After removing the PBS, 1 g of sludge (wet weight) was inoculated into the serum bottles spiked with FTCAs, followed by the addition of 50 mL synthetic wastewater prepared as previously described(Gao et al., 2018) to mimic the BOD (biological oxygen demand), COD (chemical oxygen demand), and ammonia nitrogen condition of domestic sewage. The recipe of synthetic wastewater consists of: 206.4 mg/L d-glucose, 256.0 mg/L $CH_3COONa \cdot 3H_2O$, 35.4 mg/L $(NH_4)_2SO_4$, 11.1 mg/L K_2HPO_4, 14.2 mg/L $CaCl_2$, 0.21 mg/L $MgSO_4 \cdot 7H_2O$, 0.225 mg/L $FeCl_3$, 0.018 mg/L, $MnCl_2 \cdot 4H_2O$, 0.023 mg/L H_3BO_3, 0.018 mg/L $ZnSO_4 \cdot 7H_2O$, 0.005 mg/L $CuSO_4 \cdot 5H_2O$, 1.5 mg/L EDTA, and 0.027 mg/L KI. The final wastewater characteristics were as follows (mean ± standard errors): COD = 415±21 mg/L, NH_4^+-*N* = 7.5 ± 0.4 mg/L, and pH = 7.3 ± 0.1. All bottles were sealed with septa, leaving adequate headspace to maintain aerobic conditions.

In parallel, analytical controls were prepared with synthetic wastewater spiked with either FTCA without inoculating sludges. Abiotic controls were prepared with autoclaved sludge (120 °C for 20 min) to distinguish the abiotic loss of FTCAs, mainly due to the adsorption to the sludge biosolids. Considering the total volume of 50 mL, the initial spiking concentration of FTCAs in these treatments and controls was estimated as 80 μM. Microbial controls containing sludge and synthetic wastewater, but no FTCAs, were prepared as a reference for the microbial community analysis. All microcosms were incubated at room temperature (24±3 °C) while being shaken at 130 rpm. Since aeration tanks at these four WWTPs were operated with the solid retention time between 3 and 24 days, we chose an incubation time of 7 days for the microcosm assays. On days 0, 1, 2, 3, 5, and 7, 4 mL of the liquid sample was collected and centrifuged. The supernatant was used for the fluoride and PFAS analysis, while the biosolids were stored in a −20 °C freezer prior to DNA extraction. Genomic DNA from triplicates was pooled for the microbial community analysis. Experimental details are provided in the SI.

2.4. Target PFAS quantification by Nano-ESI-HRMS

PFAS analysis by Nano-ESI-HRMS was operated by a high-resolution Q Exactive hybrid quadrupole−Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a Nano-ESI injector. The instrument setup and operational parameters were optimized based on our previous publication (Wu et al., 2022). The quantification of FTCAs and their target TPs derived from "one-carbon removal pathways" (as shown in Fig. 2c) was achieved through the calibration using analytical standards that are commercially available (Table S1). As suggested by EPA method 537.1, a mixed internal standard (IS) stock solution consisting of M2–6:2 FTCA and M8-PFOA was prepared at the concentration of 500 μg/L. The collected liquid samples (1 mL) were filtered through a 0.22-μm polyethersulfone (PES) membrane. After a 100-fold dilution in methanol (with 4% Millipore water), the IS stock solution was spiked to reach the concentration of 50 μg/L for the target PFAS quantification. More details (e.g., calibration curves and detection limits) can be found in SI and our publication (Wu et al., 2022).

2.5. Suspect and non-target analysis of novel TPs

In addition to the target analysis, Nano-ESI-HRMS enables the screening of potential TPs that haven't been reported. A list of mass species (m/z) and their intensities was generated as MS1 data exported by Xcalibur, which were used for the following suspect and non-target analysis. The suspect screening was conducted following four steps described in our previous study: local database construction, background noise removal, positive hit screening, and molecular structure validation (Wu et al., 2022).

The non-target analysis workflow is outlined in Figure S1 to identify novel TPs generated from FTCA biotransformation. Positive hits must meet the following criteria: (1) the mass ion occurred in >50 scans per sample, (2) the mass ion was positively detected in at least 2 out of 3 replicates at each sampling time and at 2 or more different sampling intervals for each treatment, (3) the mass ion was positively detected in at least 2 different treatments (e.g., with different sludge sources or spiked with different FTCAs), and (4) its average relative intensity (RI) in samples was at least 100 times higher than those in reagent blanks and control samples. All positive hits were gathered as a list and ranked by RI. Then, possible formulae were assigned by matching with the local database or fitting with the restriction of (1) element filter: C 1–8, H 0–18, F 1–13, O 0–10, S 0–3, N 0–3, (2) unsaturated degree in the range between 0 and 8, (3) mass error <5 ppm, and (4) formula validation via public chemical online databases, including SciFinder, PubChem, and Chemspider.

2.6. Terms and indices

In this study, we define the total fluorine (TF) as the molar sum of fluorine in organofluorine (OF, organic molecules that contain at least one C-F bond) and free fluoride.

$$TF = \sum \left(C_{OF} * \frac{fluorine\ number}{molecule} \right) + C_{F^-}$$

Fluorine mass recovery (FMR) is estimated as the percent ratio of the sum of TFs in sludge treatments and abiotically removed FTCA (the difference between analytical control and abiotic control) as compared to the TF in analytical control on day 7.

$$FMR = \frac{TF_{sludge\ treatment} + \left(TF_{analytical\ control} - TF_{abiotic\ control}\right)}{TF_{analytical\ control}} * 100\% = \left(1 - \frac{TF_{abiotic\ control} - TF_{sludge\ treatment}}{TF_{analytical\ control}}\right) * 100\%$$

3. Results

3.1. Biotransformation of 6:2 FTCA coupled with fluoride liberation

Concentrations of 6:2 FTCA and its target TPs were monitored by Nano-ESI-HRMS in treatment and control microcosms over 7 days of incubation as shown in Fig. 1c and Table S2. The increase of 6:2 FTCA concentrations on day 1 reflected the dissolution equilibrium of the dosed 6:2 FTCA necessitated ~24 h to reach in the microcosm setup. On day 7, 6:2 FTCA concentrations were 76.8 ± 1.8 μM in analytical controls, and 18.9 ± 1.7 μM (50.3 ± 5.4 μM), 36.7 ± 3.3 μM (45.9 ± 1.7 μM), 29.6 ± 0.8 μM (50.7 ± 3.9 μM), and 14.4 ± 1.9 μM (47.1 ± 4.1 μM) in FTCA treatments (and their corresponding abiotic controls) inoculated with Sludge P, L, R, and W, respectively. On day 7, 6:2 FTCA concentrations in abiotic controls were all similar at an average of 48.5 μM, indicating an abiotic loss of ~37% 6:2 FTCA as compared to the analytical control, probably due to the adsorption to the sludge biosolids. Biotic 6:2 FTCA removal varied greatly (12~43%) among four activated sludge treatments, following the rank: Sludge *W*> Sludge *P* > Sludge *R*> Sludge L (Fig. 1c and S2), suggesting the sources and microbial community structures and activities can affect 6:2 FTCA removal performance at different municipal WWTPs.

Significant defluorination was observed in all treatments of 6:2 FTCA with activated sludge from four WWTPs (Fig. 1a). After 7 days of incubation, 18.2 ± 1.0 μM, 11.6 ± 1.3 μM, 36.4 ± 2.1 μM, and 27.0 ± 1.5 μM of fluoride were released in microcosms inoculated with Sludge P, L, R, and W (Table 1), respectively. In contrast, no fluoride accumulation (<1 μM) was observed in analytical or abiotic controls, indicating the fluoride release was attributed to the biotransformation of 6:2 FTCA rather than abiotic processes. On average, 0.61~1.92 $F^-$was released per molecule of 6:2 FTCA that was biodegraded (Table 1).

PFHpA, 6:2 FTUCA, 5:3 FTCA, PFHxA, PFPeA, and PFBA were observed as the major target TPs of 6:2 FTCA (Fig. 2a), which together accounted for 14.5% (Sludge P), 56.7% (Sludge L), 61.0% (Sludge R), and 7.3% (Sludge W) of biotic 6:2 FTCA molar removal. Fluorine mass recovery (FMR) was estimated as the total fluorine (TF) in sludge treatments and abiotically removed FTCA normalized to the TF in analytical control on day 7. The FMR of four treatments inoculated with Sludge P, L, R, and W were 68.0%, 91.3%, 92.5%, and 60.9%, respectively (Table 1). The molar discrepancies between target TPs generation and biotic 6:2 FTCA removal (Table S2), as well as FMR of 61~93%, supported the existence of unknown F-containing TPs, which accounted for 7~39% of TF in analytical controls. Furthermore, if we assume the fluorine difference between 6:2 FTCA and its target TPs was all released as free fluoride, the theoretical fluoride release amounts based on all detected target TPs were only 5~77% of the corresponding fluoride release detected over 6:2 FTCA biotransformation (Table 1). This suggested the significant contribution of unknown TPs to fluoride release during the biotransformation of 6:2 FTCA by activated sludge. These unknown TPs are unlikely derived from the one-carbon removal pathways shown in Fig. 2c, implying the possibility of different defluorination pathways.

3.2. Biotransformation of 5:3 FTCA with minimal fluoride release

Different from 6:2 FTCA, liberation of free fluoride was minimal during 5:3 FTCA transformation in microcosms inoculated with all four types of activated sludge (Fig. 1b). After 7 days of incubation, only 4 ± 0.2 μM fluoride was released by Sludge P, and no significant accumulation of fluoride was observed in Sludge L, R, or W. However, significant 5:3 FTCA removal was observed as shown in Fig. 1d. On day 7, concentrations of 5:3 FTCA in treatments dropped to 32.3 ± 1.7 μM (Sludge P), 23.8 ± 0.4 μM (Sludge L), 29.5 ± 3.1 μM (Sludge R), and 57.3 ± 1.5 μM (Sludge W) as compared to the initial dosage of 80 μM on day 0. Unlike 6:2 FTCA, adsorption to biosolids was minimal for 5:3 FTCA since its concentrations remained high at 74.3 ± 10.0 μM

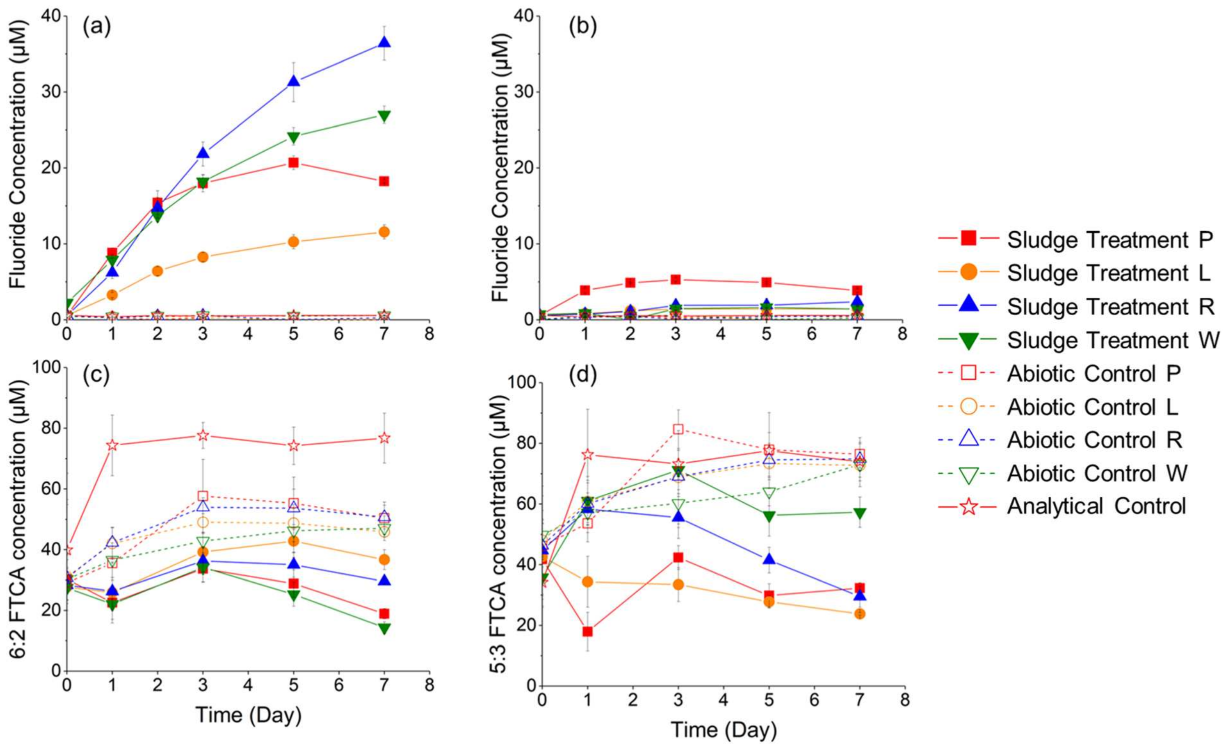

Fig. 1. Fluoride release and FTCA removal in treatment and control microcosms spiked with 6:2 FTCA (a and c) and 5:3 FTCA (b and d) at a theoretical initial dosage of 80 μM.

Table. 1
FTCA removal and generation of target TPs and free fluoride in 6:2 FTCA treatments on day 7.

6:2 FTCA Treatment	Sludge P	Sludge L	Sludge R	Sludge W	Abiotic Control	Analytical Control
Total Δ6:2 FTCA (μM)	57.9	40.0	47.2	62.4	28.2	–
Biotic Δ6:2 FTCA (μM)	29.6	11.8	19.0	34.2	–	–
Target TPs (μM)	4.6	5.2	12.9	2.4	0.2	0.7
Theoretical fluorine release from Target TPs (μM)	8.9	8.6	27.9	1.3	–	–
F- (μM)	18.2	11.6	36.4	27.0	1.7	0.6
F- / Biotic Δ6:2 FTCA	0.61	0.98	1.92	0.79		
FMR (%)	68.0	91.3	92.5	60.9		

(average) among four abiotic controls, which were comparable to the analytical controls (74.0 ± 8.3 μM). This was probably due to the lower hydrophobicity of 5:3 FTCA (Log P = 4.19, predicted by ACD/Labs platform, same below) than 6:2 FTCA (Log P = 5.72). Overall, these results indicated that 25~68% of the initially dosed 5:3 FTCA was removed via biotransformation, while such process did not generate free fluoride. Though variance existed in different sludge treatments, a consensus could be drawn that the 5:3 FTCA biotransformation was dominated by non-fluoride-releasing pathways.

Accordingly, PFHxA was detected as the dominant non-defluorinated TP for 5:3 FTCA biotransformation (Fig. 2b). On day 7, PFHxA concentrations ranged between 1.54 and 7.69 μM, accounting for 3.7~40.2% of the 5:3 FTCA biotic removal. As shown in Fig. 2c, the transformation of 5:3 FTCA to PFHxA can involve a series of dehydrogenation, hydroxylation, and other oxidation processes for the cleavage of the two alkyl carbons without the breakdown of any C-F bond. Alternatively, 5:3 FTCA may behave similar to its fatty acid analog, octanoic acid, and enter β oxidation considering the availability of both α and β carbons (Wang et al., 2012b). Unfortunately, none of key target TPs (e.g., non-defluorinated 5:3 FTUCA in Fig. 2c and 5:2 sFTOH) were detected in our sludge treatments to discern the pathways for PFHxA formation.

In 5:3 FTCA treatments, a noticeable accumulation of PFBA and PFPeA indicated the breakdown of C-F bonds in 5:3 FTCA. PFBA (1.09~2.04 μM) and PFPeA (up to 0.33 μM) occurred in minor concentrations across four sludge treatments (Fig. 2b), though no other target TPs in Fig. 2c were detected. However, free fluoride was barely detected. As shown in Table 1, the theoretical fluoride release based on these target TPs was greater than the fluoride detected in all four sludge treatments. Further, the TF of target TPs and F^{-} only accounted for 6.4%, 18.6%, 12.5%, and 48.3% of biotic 5:3 FTCA removal observed in treatments with Sludge P, L, R, and W, respectively (Table 2 and S5). As compared to 6:2 FTCA treatments, FMR of four 5:3 FTCA treatments were much lower in general, with 47.4%, 44.9%, 47.5%, and 88.6% for Sludge P, L, R, and W, respectively (Table 2). These findings corroborate the existence of non-fluoride-releasing processes (e.g., adduct formation and conjugation) that contribute significant to 5:3 FTCA biotransformation, underscoring future investigation.

3.3. *Biodefluorination of 5:3 FTCA at the γ carbon*

Coupling with 5:3 FTCA biotransformation, a less-fluorinated 5:3 FTUCA intermediate (IUPAC name: 4,5,5,6,6,7,7,8,8,8-decafluorooct-3-enoic acid) was first detected as an important metabolite. This intermediate was detected on day 3 and day 5 and fully vanished on day 7 in all treatments, though with the low detection at RIs in the range between 0.012 to 0.069 (equivalent to <0.5 μM when estimated using the calibration of 5:3 FTCA). Compared to 5:3 FTCA, this intermediate has one

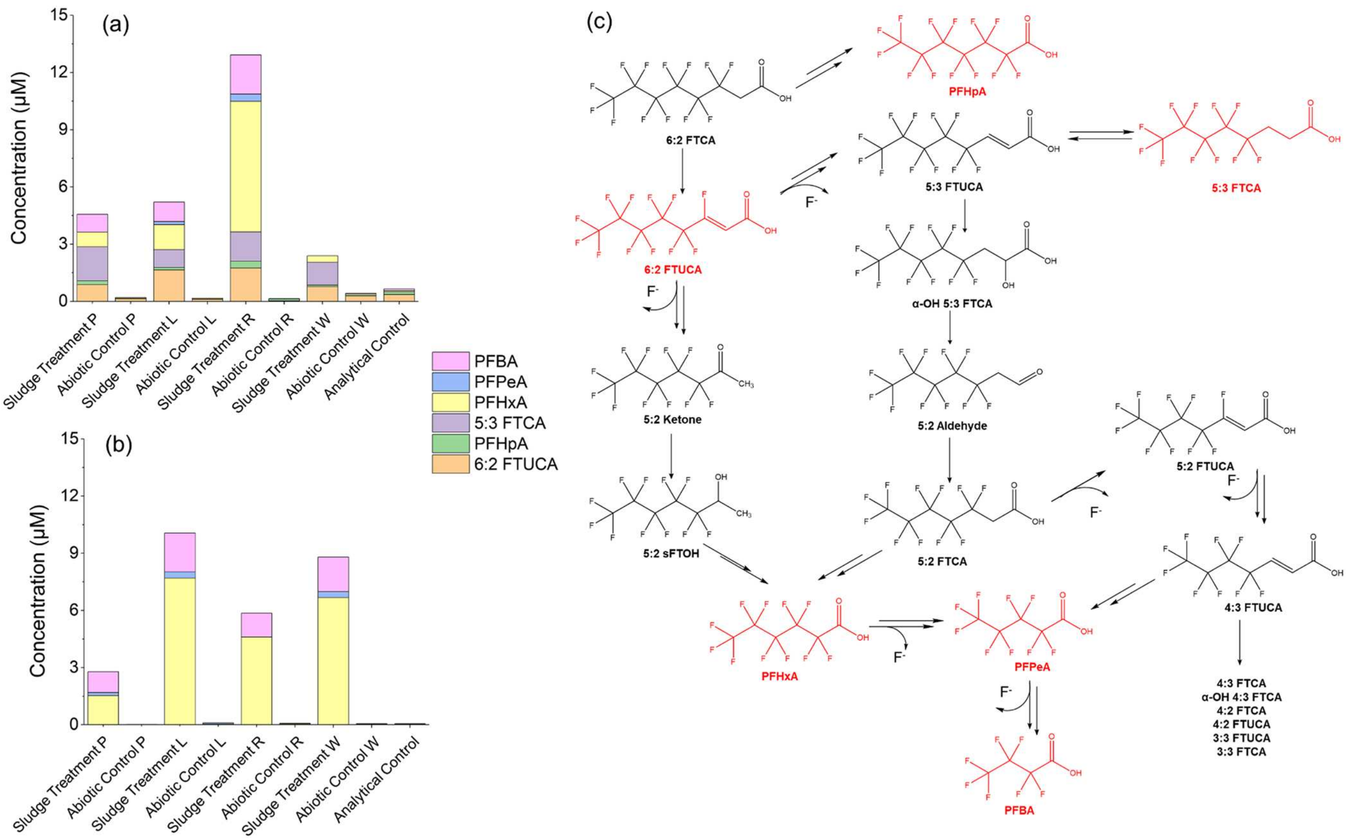

Fig. 2. Detection of target TPs in (a) 6:2 FTCA and (b) 5:3 FTCA treatment and control microcosms. (c) FTCA biotransformation pathways that form target TPs based on "one-carbon removal pathways" and previous literature. Double arrows indicate multiple transformation steps. PFASs highlighted in red were detected in this study.

Table. 2
FTCA removal and generation of target TPs and free fluoride in 5:3 FTCA treatments on day 7.

5:3 FTCA Treatment	Sludge P	Sludge L	Sludge R	Sludge W	Abiotic Control	Analytical Control
Total Δ5:3 FTCA (μM)	41.6	50.2	44.4	16.6	0.0	–
Biotic Δ5:3 FTCA (μM)	41.6	50.2	44.4	16.6	–	–
Target TPs (μM)	2.8	10.1	5.8	8.8	0.1	0.00
Theoretical fluorine release from Target TPs (μM)	4.7	8.8	5.0	7.9	–	–
F- (μM)	1.4	3.9	1.4	2.4	0.9	0.6
F- / Biotic Δ5:3 FTCA	0.01	0.06	0.01	0.09	–	–
FMR (%)	47.4	44.9	47.5	88.6		

less fluorine and hydrogen, forming a double bond between the fluorinated β and non-fluorinated γ carbons possibly via dehydrogenation (Fig. 3). This intermediate is possibly formed from different microbial dehydrogenases as those initiated the "one-carbon removal pathways" in Fig. 2c, in which a non-defluorinated 5:3 FTUCA was formed via the dehydrogenation of two non-fluorinated α and β carbons of 5:3 FTCA. It is plausible different dehydrogenases attack different carbons in 5:3 FTCA, leading to the variance in defluorination levels and subsequent biotransformation pathways. Low detection of this less fluorinated 5:3 FTUCA and PFBA (Fig. 2b) may explain the minimal fluoride release (Table 2) during 5:3 FTCA biotransformation, demonstrating the minor contribution of well-received fluoride-releasing pathways.

3.4. Shifting of sludge communities by FTCA exposure

Genomic DNA in the microcosm assays was extracted on day 5 to assess the responses of microbial populations to FTCA exposure. 16S rRNA amplicon-based sequencing of 12 samples resulted in 42,168 to 99,533 total qualified and valid sequences, which were assigned as 7783 OTUs. Student *t*-test was used to evaluate the significance of FTCA exposure influence on the dominant OTUs with maximum relative abundance (RA) >1‰ by comparing their normalized relative abundances (NRAs) among 6:2 FTCA treatments ($n = 4$), 5:3 FTCA treatments ($n = 4$), and microbial controls ($n = 4$). As depicted in Fig. 4, 12 OTUs were significantly influenced by the exposure to FTCAs (see their taxonomic details in Table S9), falling into three groups according to their NRA responses. Group 1 contains only 1 OTU that presented unique opposite trends in 5:3 FTCA and 6:2 FTCA treatments (Fig. 4), Group 2 contains 8 OTUs that presented a correlated increasing trend in both FTCA treatments (Figure S4), and Group 3 contains 3 OTUs that presented a consistent decreasing trend with the presence of either FTCA (Figure S5). All 12 OTUs are commonly found and isolated in activated sludge (Chen et al., 2020; Gao et al., 2016; Layton et al., 2000; Ma et al., 2020b; Martineau et al., 2015) and their RA data are presented in Table S8.

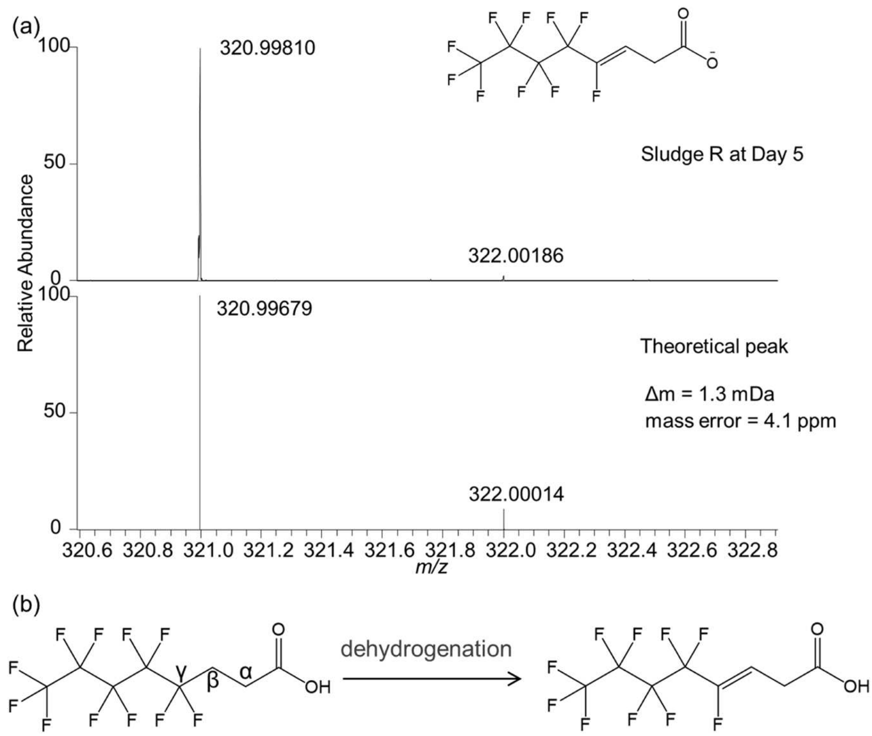

Fig. 3. Detection of a 5:3 FTUCA metabolite (4,5,5,6,6,7,7,8,8,8-decafluorooct-3-enoic acid) in 5:3 FTCA treatments inoculated with Sludge R on day 5 as compared to its theoretical spectrum (a) indicating the biodefluorination of 5:3 FTCA at the γ carbon (b).

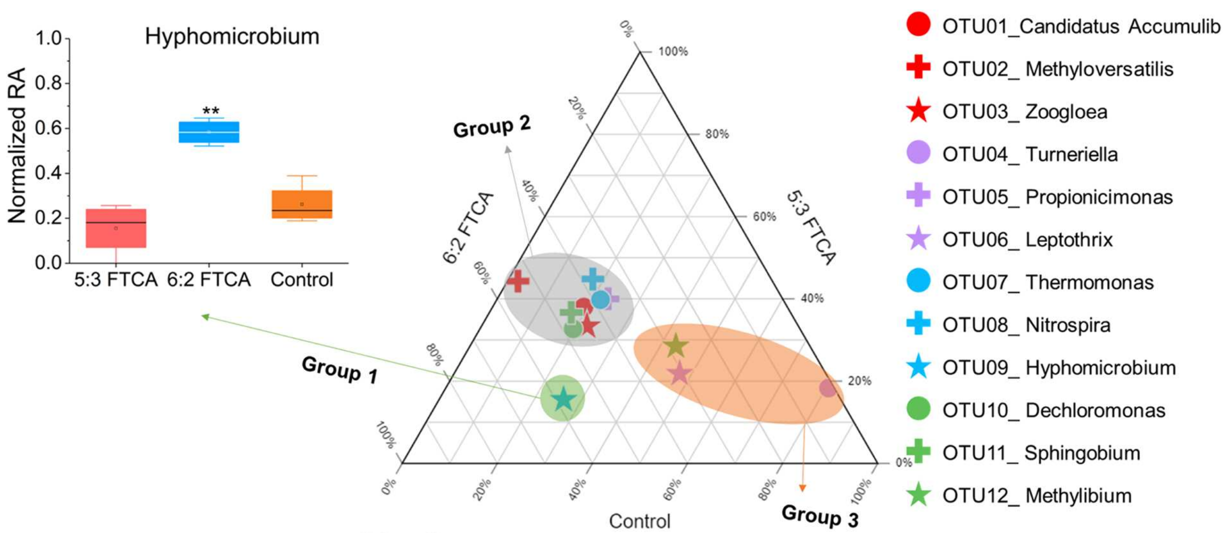

Fig. 4. Ternary plot depicting the normalized relative abundance (NRA) of 12 OTUs that shows significant responses to FTCA exposure. The boxplot shows the normalized RA of OTU09_*Hyphomicrobium* in treatment and control microcosms. Statistical analysis was done by student *t*-test (*: $p < 0.05$, **: $p < 0.01$).

3.4.1. *Hyphomicrobium possibly responsible for 6:2 FTCA biotransformation*

OTU09 assigned to the genus of *Hyphomicrobium* in Group 1 (Fig. 4) was significantly increased in 6:2 FTCA treatments (RA 1.0~1.8‰, $p < 0.01$) but decreased in 5:3 FTCA treatments (RA 0~0.5‰) when compared to the microbial controls (RA 0.3~0.7‰) by student *t*-test. Since *Hyphomicrobium* was the only dominant OTU presenting inconsistent responses to two FTCAs, it may contribute to initiating the degradation pathways unique for 6:2 FTCA but not 5:3 FTCA and participate in the fluoride-releasing reactions during 6:2 FTCA biotransformation. *Hyphomicrobium* spp. are widely identified as restricted facultative methylotrophs (Layton et al., 2000; Martineau et al., 2015). Different from obligate methylotrophs, *Hyphomicrobium* can utilize a limited range of more complex organic compounds (Madhaiyan et al., 2009), including dichloromethane(Kohler-Staub et al., 1995). Though *Hyphomicrobium* members can express inducible oxidative dichloromethane dehalogenase(Kohler-Staub et al., 1995), their capability for defluorination remains uncertain.

3.4.2. *8 OTUs with potential contribution to FTCA biotransformation*

RAs of 8 OTUs were increased in both FTCA treatments, including *Candidatus Accumulibacter, Zoogloea, Methyloversatilis, Propionicimonas, Dechloromonas, Sphingobium, Nitrospira,* and *Thermomonas.* Members of these relevant genera, such as *Methyloversatilis* (Cai et al., 2011) and

Propionicimonas (Song et al., 2018), are associated with organochlorine biodegradation, implying their potential contribution to the FTCA biotransformation. More biochemical and physiological properties for Group 2 members are stated in SI.

3.4.3. Inhibition to three OTUs

As shown in Figure S15, three OTUs were found significantly decreased in at least one FTCA treatment, including *Turneriella* (both FTCAs), *Leptothrix* (5:3 FTCA), and *Methylibium* (6:2 FTCA). The decrease might be related to the toxicity of FTCAs (Shi et al., 2017). The information on the function and environmental roles of genera *Turneriella, Leptothrix,* and *Methylibium* remains limited to date. However, the occurrence of FTCAs might impact the performance of activated sludge by inhibiting functional genera.

4. Discussion

FTCAs contain one or more non-fluorinated carbons, enabling microbial attacks for biodefluorination. In this study, we observed distinctive biodefluorination potential between 6:2 and 5:3 FTCAs by activated sludge even though the only difference in these two molecules is the fluorination of the β carbon. The defluorination degrees of 6:2 FTCA were between 5~15%. This is in good agreement with previous biotransformation studies on 6:2 FTOH (16%) (Kim et al., 2014; Tseng et al., 2014b) and 6:2 FTS (9%) (Che et al., 2021; Shaw et al., 2019; Wang et al., 2011a), implying the terminal functional group doesn't affect the biodefluorination extent. On average, approximately 1 to 2 fluorines can be detached from these C8 PFAS molecules.

In contrast, defluorination of 5:3 FTCA was minimal (<1%). The absence of fluoride release when the β carbon is non-fluorinated was also reported for aerobic biodegradation of 1:3 FTCA and its unsaturated or less fluorinated analogs (Che et al., 2021). Further molecular work identified the medium-chain acyl-CoA synthetase from the soil bacterium *Gordonia* sp. strain NB4–1Y can catalyze the formation of CoA adducts of 2:3 FTCA, the two fluorine-eliminated 2:3 FTUCA, and 1:4 FTCA (Mothersole et al., 2023). These findings corroborate that the availability of C—H bonds on both α and β carbons may promote the entry of n:3 FTCAs to the β oxidation, forming CoA adducts or other conjugates without the liberation of fluoride. These molecules may mimic the behaviors of non-fluorinated analogs in eukaryotic and prokaryotic cells and may be further transformed to building blocks that can be integrated into macromolecules, such as phospholipid fatty acids as recently reported (Xie et al., 2022). Fluorinated macromolecules may pose imminent health concerns and stimulate further investigations on their chronic effects on humans and natural biota.

The discovery of target TPs over FTCA biotransformation supports the possibility of "one-carbon removal pathways", even though they were minor in our sludge treatments. We also identified an alternative biodefluorination pathway that eliminate the H and F at the β and γ carbons in 5:3 FTCA. This finding of this new PFAS TPs does not only advance our knowledge of the diversity of microbial pathways that may contribute to the breakdown of the recalcitrant C-F bond (Wackett 2022), but also improves the mass recovery analysis for future PFAS biotransformation studies. Though the combination of HRMS and non-target analysis is the predominant tool for PFAS metabolite screening (Bangma et al., 2021; Jin et al., 2023; Manz et al., 2023), the discovery of the new TP underscores the need for further analytical efforts that synergize MS-based approaches with other techniques (e.g., fluorine nuclear magnetic resonance spectroscopy [^{19}F-NMR] (Camdzic et al., 2021; Camdzic et al., 2023; Mifkovic et al., 2022), particle-induced gamma-ray emission [PIGE] (Mifkovic et al., 2022), and Raman spectroscopy (D'Amico et al., 2021)) to validate their structures (e.g., positional and stereo-isomerism) and conduct absolute quantification with appropriate internal and external standards for each molecule of interest.

The microbial community analysis revealed several key bacteria that may contribute to the biotransformation and biodefluorination of FTCAs, though this approach can be biased due to the choice of primers and difference in amplification efficiency. Four sludge communities investigated in this study were all from the New York Metropolitan Area in the Northeast of the US. It is uncertain if activated sludge from other regions with distinctive influent sources (e.g., urban vs industrial vs agricultural) and geographical features (e.g., tropical vs temperate vs arctic) would respond similarly to FTCAs. Thus, further studies at the pure culture and community levels are needed to investigate their ability to degrade FTCAs and other PFASs. Several bacteria in the sludge were inhibited by exposure to FTCAs. Thus, it would be of important value to assess the impacts of FTCAs on the performance of activated sludge, particularly under environment-relevant concentrations. Furthermore, transcriptomics and other approaches can be useful to developing data for the investigation of enzymes involved in PFAS biotransformation (Bottos et al., 2020; Khan and Murphy 2023; Méndez et al., 2022; Merino et al., 2023; Shaw et al., 2019).

When compared with typical volatile PFASs (e.g., 6:2 FTOH with $K_H = 1.28$ (David Hanigan 2022) and pKa = 15.76, predicted by Chemicalize, same below), 6:2 FTCA and 5:3 FTCA present lower volatility (K_H=0.06 and 0.66) and stronger deprotonation ability (pKa =1.56 and 1.82). Therefore, this study primarily focuses on investigating the anion metabolites that are soluble and can be ionized by ESI. The mass recovery can be improved by the inclusion of FTCAs and the metabolites that are volatile or adsorbed or attached to the sludge. TPs of neutral or positive charge (e.g., CoA adducts aforementioned) may also account for a significant portion, necessitating further analytical efforts. Nonetheless, our study revealed the dominance of non-fluoride-releasing pathways for 5:3 FTCA and other PFAS precursors when both α and β carbons are not fluorinated by municipal activated sludge, calling for further investigation on these biotransformation processes and the associated impacts of their TPs.

5. Conclusions

This study revealed the distinctive biodefluorination potentials between 6:2 and 5:3 FTCAs, two of the most concerning PFASs in water and other environments. These two FTCAs were only differentiated by the fluorination of their β carbons. With two fluorine attached to the β carbon, 6:2 FTCA showed significant defluorination and liberate an average of up to 2 fluoride per molecule. In contrast, without a fluorinated β carbon, 5:3 FTCA was transformed with little fluoride release. These findings indicated the fluorination of β carbons in FTCAs governs the biodefluorination extent and associated transformation pathways by municipal activated sludge. Microbial community analysis facilitated the identification of possible FTCA degraders (e.g., *Hyphomicrobium* and *Dechloromonas*) in activated sludge. Future studies are underscored to investigate FTCA biotransformation and biodefluorination at a pure culture level to untangle the key metabolites, pathways, and responsible enzymes. This is of significant value as it will advance our understanding of the fate of FTCAs in the environment and promote the development of green defluorinating techniques for site cleanup.

Code availability

The scripts for non-target PFAS analysis were developed in Python and are available from the authors on reasonable request.

CRediT authorship contribution statement

Chen Wu: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Sandra Goodrow:** Writing – review & editing, Resources. **Hao Chen:** Resources, Methodology. **Mengyan Li:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121431.

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