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Article

Microbial Defluorination of Unsaturated Per- and Polyfluorinated Carboxylic Acids under Anaerobic and Aerobic Conditions: A Structure Specificity Study

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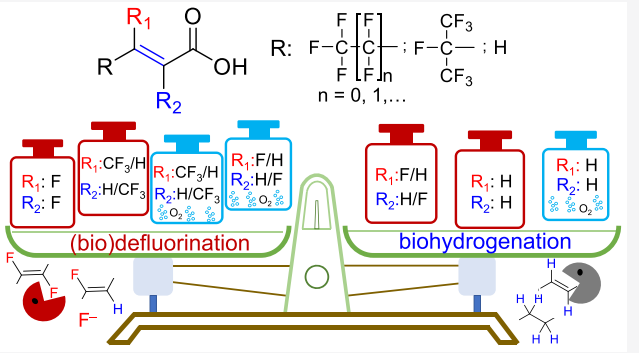
ABSTRACT: The recently discovered microbial reductive defluorination of two C_6 branched and unsaturated fluorinated carboxylic acids (FCAs) provided valuable insights into the environmental fate of per- and polyfluoroalkyl substances (PFASs) and potential bioremediation strategies. However, a systematic investigation is needed to further demonstrate the role of C═C double bonds in the biodegradability of unsaturated PFASs. Here, we examined the structure-biodegradability relationships of 13 FCAs, including nine commercially available unsaturated FCAs and four structurally similar saturated ones, in an anaerobic defluorinating enrichment and an activated sludge community. The anaerobic and aerobic transformation/defluorination pathways were elucidated. The results showed that under anaerobic conditions, the α,β-unsaturation is crucial for FCA biotransformation via reductive defluorination and/or hydrogenation pathways. With sp^2 C−F bonds being substituted by C−H bonds, the reductive defluorination became less favorable than hydrogenation. Moreover, for the first time, we reported enhanced degradability and defluorination capability of specific unsaturated FCA structures with trifluoromethyl ($-CF_3$) branches at the α/β-carbon. Such FCA structures can undergo anaerobic abiotic defluorination in the presence of reducing agents and significant aerobic microbial defluorination. Given the diverse applications and emerging concerns of fluorochemicals, this work not only advances the fundamental understanding of the fate of unsaturated PFASs in natural and engineered environments but also may provide insights into the design of readily degradable fluorinated alternatives to existing PFAS compounds.



KEYWORDS: *organofluorines, PFASs, reductive dehalogenation, bioremediation, β-oxidation, α-oxidation*

INTRODUCTION

Organofluorines, particularly per- and polyfluoroalkyl substances (PFASs), have been widely used as surfactants and oil/water-repelling materials due to their unique properties (e.g., hydrophobicity, lipophobicity, and thermal stability).[1−3] The highly stable carbon−fluorine (C−F) bonds[4] in PFASs make them very recalcitrant in the environment. Because of their environmental persistence, bioaccumulation, and toxicity,[1,5] the frequent occurrence of PFASs in various environments[6−10] has caused emerging concerns to public health and ecosystems. So far, tremendous efforts have been made on PFAS regulations and treatment approaches, yet most of the efforts are spent on the legacy PFASs listed in the Unregulated Contaminant Monitoring Rule (UCMR 5) by the U.S. EPA.[11] Consequently, fluorochemical manufacturers have been phasing out those legacy PFASs and developing fluorinated alternatives.[1,12] The concentrations and diversities of alternative PFASs in the environment have rapidly increased compared to the legacy PFASs.[13−16] However, some PFAS alternatives (e.g., perfluoroalkyl ether carboxylic acids or short/branched-chain PFASs) revealed unpredictable toxicities and even higher recalcitrance and mobilities than the legacy PFASs.[17−20] As some PFAS applications are essential and currently have not established fluorine-free alternatives,[2] it is urgent to design novel fluorinated alternatives with enhanced degradability while maintaining similar functional properties.

The biodefluorination of fluorinated compounds (mostly the polyfluorinated structures) has been previously reported.[21−36] The defluorination was typically initiated by HF elimination at the α and β positions for polyfluorocarboxylic acids[25,29,32,37] or by desulfonation for polyfluorosulfonic acids.[23,28,30,35] Compared to biotransformation of polyfluorinated compounds,

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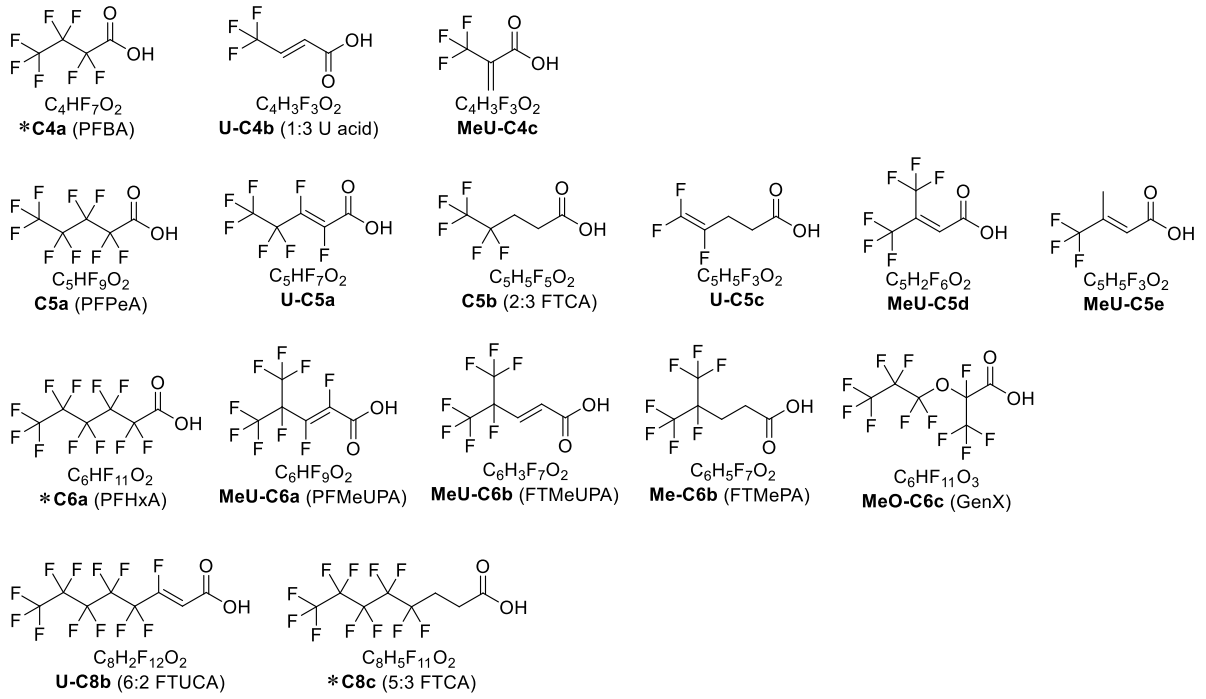

Figure 1. Structures of the 16 FCA standards used in this study, including the 13 FCAs used as parent compounds and three asterisks (*) used as the analytical standards of transformation products. "U" stands for the unsaturated structures, "Me" stands for the structures with either $-CF_3$ or $-CH_3$ branches, and "O" stands for the ether structure.

convincing reports on microbial cleavage of C−F in perfluorinated structures are even fewer.[24,38,39] Based on fluoride formation and transformation product identification, our recent study has reported microbial reductive defluorination of two branched unsaturated per- and polyfluorinated carboxylic acids in an anaerobic enrichment.[38] It suggested that the biotransformation and biodefluorination of fluorinated carboxylic acids (FCAs) might be structure-dependent and that the C═C double bond could play an essential role in the reductive defluorination of FCA. However, since only two unsaturated structures were investigated in the previous study,[38] the structure specificity of FCA biotransformation by the anaerobic defluorinating enrichment has not been well demonstrated.

In this study, we aimed to fill this knowledge gap by investigating the transformation and defluorination capabilities of all commercially available unsaturated FCAs (linear and branched), as well as the structurally similar saturated ones by the same anaerobic defluorinating enrichment. We identified the major transformation products (TPs), elucidated the transformation pathways, and examined the microbial preference of defluorinating and nondefluorinating pathways. Based on the pathway preference under anaerobic conditions, we further investigated the aerobic biotransformation and biodefluorination of the unsaturated FCAs and compared them to the anaerobic condition. We proposed specific structures that may have enhanced (bio)degradability in both anaerobic and aerobic conditions. These findings expand our knowledge of the fate and transport of PFASs in natural and engineered environments, which could provide insights into the PFAS source tracking, bioremediation, and the structural design of more readily degradable fluorinated PFAS alternatives.

MATERIALS AND METHODS

Chemicals. Sixteen FCA standards, which were used in this study as parent compounds in the biotransformation experiments or analytical reference compounds for TPs, were purchased from SynQuest Laboratories (Alachua, FL) and used without further purification (Figure 1). For all authentic standards, 10 mM stock solutions were prepared anaerobically in autoclaved Milli-Q water in 160 mL sealed serum bottles and stored at room temperature until use. The limit of quantification (LOQ) for each standard compound was determined as the lowest concentration of calibration standards with a detection variation within ±20%. The detailed compound information is provided in Table S1.

Maintenance of the Anaerobic Defluorinating Enrichment. As previously described,[38] the defluorinating enrichment was maintained in 160 mL sealed serum bottles containing 90 mL of sterile anaerobic basal medium with 100 μg/L vitamin B_{12} and the reducing agent cysteine-sulfide (48 mg/L cysteine and 96 mg/L sodium sulfide). The headspace contains 60 mL of Ar/CO_2 (75:25, v/v). For routine maintenance, 5 mM lactate and 75 μM **MeU-C6a** (PFMeUPA) were added as the primary electron donor and sole electron acceptor, respectively. Lactate was reamended upon depletion. After the first dose of **MeU-C6a** was completely transformed with no F^- further released (~11% total defluorination), cultures were subsequently transferred (5%, v/v) into 90 mL of fresh basal medium containing 5 mM lactate and 75 μM **MeU-C6a**. The defluorination activity of the subcultures was verified by the continuous formation of fluoride and complete removal of the parent compound.

FCA Biotransformation by the Anaerobic Defluorinating Enrichment. After the consumption of the **MeU-C6a**, 5 mL of the enrichment culture was inoculated into 90 mL of sterile anaerobic medium the same as described above.

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Environ. Sci. Technol. 2022, 56, 4894−4904

Approximately 75 μM individual FCA was added into the subcultures as the sole electron acceptor with 5 mM lactate as the primary electron donor. For each batch of the experiment, a **MeU-C6a**-added positive control was set up to verify the defluorinating activity of the subcultures. Heat-inactivated biomass controls were set up by inoculating 5 mL of autoclaved (at 121 °C for 20 min for two cycles) culture in the same basal medium with the parent compound amended in the same way as described above to determine the abiotic degradation and potential adsorption of each compound. Samples were taken subsequently during the incubation period for the measurement of the parent compound, TPs, and F^-. Briefly, at each sampling time, 3 mL of aqueous suspension was centrifuged at 16,000 × g for 30 min (4 °C). Two milliliters of the supernatant was used for F^- measurement, and the remaining supernatant (~1 mL) was stored at 4 °C in the dark for LC-HRMS/MS measurement. All experimental samples and controls were performed in triplicate. Sufficient incubation time was given, and the incubation ended when either all of the parent compound was depleted, or the fluoride/TP formation reached a plateau. The same process was used for the aerobic biotransformation experiment.

Abiotic Defluorination of FCAs with Reducing Agents. To test the FCA reactivity with the reducing agent (i.e., cysteine-sulfide), individual FCAs (~75 μM) that were degraded in the anaerobic defluorinating enrichment were separately added into 90 mL of sterile anaerobic Milli-Q water with the addition of 48 mg/L cysteine and 96 mg/L sodium sulfide. Milli-Q water amended with only individual FCAs and without the reducing agent was set up as the control. Experiments were performed in triplicates. Three milliliters of the supernatant was periodically taken via centrifugation for F^-, the parent compound, and TP measurements. To avoid any further abiotic degradation of the parent compound in the sample vials, we measured the samples immediately after sampling.

FCA Biotransformation by Aerobic Activated Sludge. As described in our previous study,[40] activated sludge communities were taken from the aeration tank in a local municipal WWTP the same day of the experiment. Fifty milliliters of activated sludge was inoculated into a 150 mL glass bottle. To maintain the activity of the activated sludge community during the incubation period, we added methanol (~47 mg/L) and NH_4–N (~14 mg/L) every other day after 3 days, as described in our previous study.[40] The activated sludge community showed similar defluorination (>80%) of trifluoropropionate as previously reported.[40] All reactor bottles were loosely capped and shaken at 150 rpm at room temperature, with the dissolved oxygen maintained at 6.5–7.0 mg/L during the entire incubation period. For the biotransformation experiments, ~50 μM of individual FCA was added into the bottles inoculated with 50 mL of activated sludge. Abiotic controls were set up using an autoclaved sludge filtrate to determine the abiotic defluorination of the investigated FCAs. Briefly, activated sludge was filtered through a 0.22 μm filter (Durapore Membrane Filters), and the filtrate was then autoclaved at 121 °C for 40 min. The same amount of FCAs and nutrients (methanol and NH_4–N) was added to each abiotic control bottle. Besides, biomass-only controls were set up by inoculating the same amount of activated sludge and nutrients without adding any FCAs. No fluoride increase was observed from the biomass-only control (Figure S1), indicating no background F-containing chemicals contributed to the observed defluorination in the FCA-added experimental groups. All experiments were set up in triplicate. After mixing for 5 min, 3 mL of the aqueous suspension was taken as 0-day samples in the same way as described above. Subsequent samples were taken during the incubation period of 14–21 days, for the measurement of F^-, parent compounds, and TPs. Each reactor was weighed every day, where the average water evaporated was <0.1 mL/d (i.e., a max. total water loss <2.1 mL out of 50 mL), thus water loss was neglected in those measurements.

Fluoride Measurement. F^- concentrations in the supernatant were measured by an ion-selective electrode (ISE, HACH, Loveland, CO) connected to an HQ30D Portable Multi Meter (HACH), as previously described.[38,40] Briefly, before each measurement, the ISE was calibrated according to the manufacturer's instructions. For the sample measurement, 100 μg of fluoride ionic strength adjustment powder (HACH) was dissolved into 2 mL of the supernatant. The F^- concentration was then determined by the ISE-Multi Meter system. The detection limit was 0.01 mg/L (ca. 0.5 μM). The ISE method has been previously cross-checked by ion chromatography (IC) in the two matrices used in this study, the anaerobic medium and the activated sludge filtrate, where the two methods showed less than 10% difference.[38,40] The F^- released from FCA was determined by subtracting the initial F^- concentration (due to carryover or background F^-) from F^- detected at a later time point. For FCAs with impurities being abiotically defluorinated, the F^- released from FCA was corrected by subtracting the F^- release in the abiotic control from that in the experimental group. The transformation percentage and defluorination degree of FCAs were calculated as follows:

$$\text{Transformation percentage (\%)} = \frac{\text{Removed FCA } (\mu\text{M})}{\text{Total added FCA } (\mu\text{M})} \times 100\%$$

$$\text{Defluorination degree (\%)} = \frac{F^- \text{released from FCA } (\mu\text{M})}{\text{Removed FCA } (\mu\text{M}) \times \#\text{ of F in one molecule}} \times 100\%$$

Ultrahigh-Performance Liquid Chromatography Coupled to High-Resolution Tandem Mass Spectrometry (UHPLC-HRMS/MS) Analysis. The FCAs were analyzed by UHPLC-HRMS/MS (Q Exactive, Thermo Fisher Scientific) as described previously.[40] For UHPLC analysis, a 2 μL sample was loaded onto a Hypersil GOLD column (particle size 1.9 μm, 100 × 2.1 mm, Thermo Fisher Scientific) and eluted with nanopure water (A) and methanol (B) (both amended with 10 mM ammonium acetate) at a flow rate of 300 μL/min, with the following gradient: 95% A: 0–1 min, 95%–5% A: 1–6 min, 5% A: 6–8 min, and 95% A: 8–10 min. For HRMS, mass spectra were acquired in full scan mode at a resolution of 70,000 at m/z 200 and a scan range of m/z 50–750 in the negative mode of electrospray ionization (ESI). To elucidate TP structures, a data-dependent MS^2 scan was performed at a resolution of 17,500 at m/z 200. Xcalibur 4.0 (Thermo Fisher Scientific) was used for data acquisition and analysis.

TP Identification. Both suspect and nontarget screening were conducted to identify TPs as previously described[38,41,42] with a slight modification. For suspect screening, the TP suspect lists were generated by a self-written automatic metabolite mass prediction script,[43] which was modified and specifically used to predict the potential TPs of the investigated

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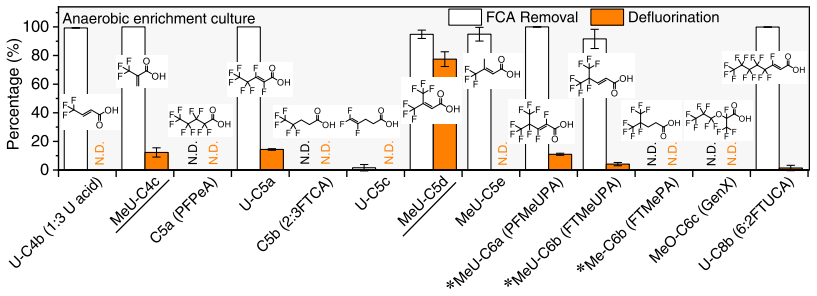

Figure 2. Parent compound removal and defluorination degree of 13 FCAs that underwent biological transformation/defluorination in the anaerobic defluorinating enrichment. *: data acquired from our previous study using the same enrichment culture;[38] ___: abiotic transformation and defluorination. A sufficient incubation period (18−150 days) was performed until all of the parent compound was depleted or the fluoride/TP formation reached a plateau, and the end time point data were shown in the figure. N.D.: not detected; n = 3.

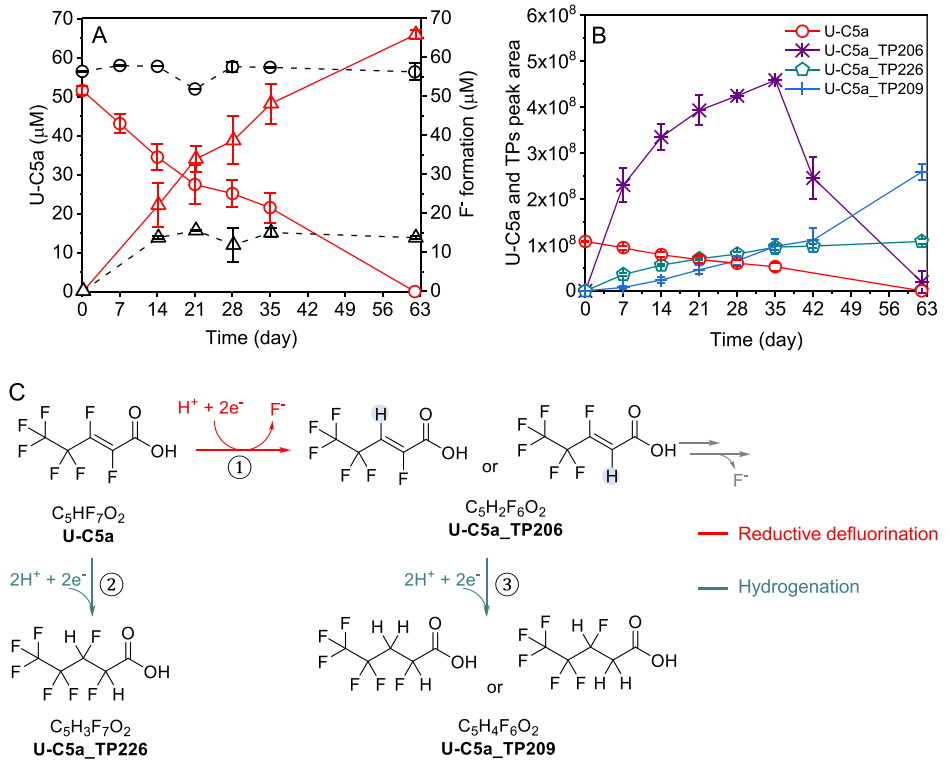

Figure 3. Biotransformation of **U-C5a** in the anaerobic defluorinating enrichment. A: parent compound removal and the F^- release, red: biological samples, black: heat-inactivated controls, circles: parent compound, triangles: fluoride formation, n = 3. B: temporal trends of the parent compound and the TPs. TPs identified in the biological samples were not detected in the heat-inactivated control. Note: due to different ionization efficiencies, the peak area only indicates the degradation/formation trend of each compound but not their relative abundances. C: proposed biotransformation pathways of **U-C5a**.

FCA structures. Plausible TPs were identified using Compound Discoverer 3.1 (Thermo Fisher Scientific) based on the following criteria: (i) mass tolerance < 5 ppm; (ii) isotopic pattern score > 90%; (iii) peak area > 10^5; (iv) peak area showing either an increasing trend or first an increasing and then a decreasing trend over time; (v) no formation in the heat-inactivated biomass and abiotic controls; (vi) not detected as an in-source fragment of the parent compound or other identified TPs. For nontarget analysis, all detected features that met the above criteria were picked by Compound Discoverer 3.1. The formula of identified TPs was further validated by Xcalibur 4.0. MS^2 fragments were used for TP structure elucidation. The confidence level of the structure elucidation for each TP was assigned based on the criteria set up by Schymanski et al.[44] ChemDraw Professional 20.0 and MarvinSketch (version 19.20.0, ChemAxon, http://www.chemaxon.com) were used for drawing, displaying, and characterizing chemical structures.

RESULTS AND DISCUSSION

***α,β*-Unsaturation Is Crucial for FCA Biotransformation in the Anaerobic Defluorinating Enrichment.** To demonstrate the structure specificity of FCA biodegradability and the role of C=C double bonds and branched structures, we used the same anaerobic defluorinating enrichment culture that was reported to reductively defluorinate two branched and unsaturated FCAs, i.e., **MeU-C6a** (PFMeUPA) and **MeU-C6b** (FTMeUPA).[38] We investigated the biotransformation/defluorination feasibilities of a comprehensive set of commercially available unsaturated FCAs, together with the structurally

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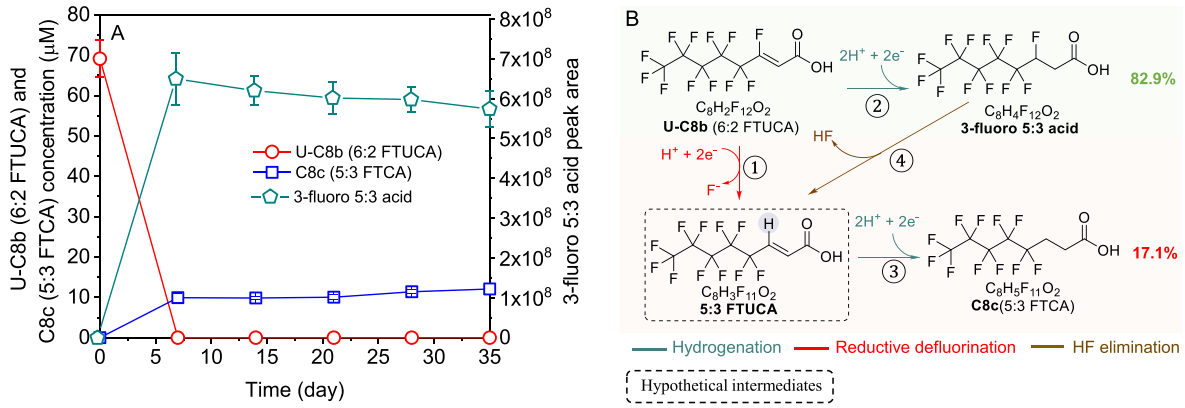

Figure 4. Biotransformation of the **U-C8b** in the anaerobic defluorinating enrichment. A: parent compound removal and TP formation. Note: in the heat-inactivated control, **U-C8b** did not show any removal, and the two TPs identified in biological samples were not detected. B: proposed anaerobic biotransformation pathways of **U-C8b**. The percentage indicates the molar ratio of the parent compound subject to the specific route: 82.9% for hydrogenation and 17.1% for defluorination. The dashed box indicates the transient intermediate not detected by LC-HRMS.

similar saturated ones. The tested FCAs also have linear/branched structures with various chain lengths and fluorine substitution degrees. Among the nine unsaturated FCAs, the six structures with α,β-unsaturation (−C═C−COOH) (i.e., **U-C4b** (1:3 FTUCA), **U-C5a**, **MeU-C5e**, **MeU-C6a**, **MeU-C6b**, and **U-C8b** (6:2 FTUCA)) showed nearly complete biological transformation/defluorination (no degradation in the heat-inactivated controls). In contrast, the structurally similar saturated FCAs (i.e., **C5a**, **C5b**, and **Me-C6b**) revealed no biodegradability (Figure 2). Moreover, the tertiary sp^3 C−F bond in the branched moiety of MeO-C6c (GenX) was not microbially cleaved like the one in **MeU-C6b**,[38] although the bond dissociation energy of the tertiary sp^3 C−F bond in GenX is low (ca. 432.2 kJ/mol[20]). It was likely due to the saturated structure of GenX, which made it less biodegradable. **U-C5c**, which has a terminal C═C bond rather than α,β-unsaturation, was not biotransformed, either (Figure 2). The other two unsaturated FCAs (**MeU-C4c** and **MeU-C5d**) showed abiotic transformation and defluorination, which will be discussed in the following section. The linear unsaturated perfluorinated FCA (i.e., **U-C5a**) exhibited similar defluorination activity to the branched and unsaturated perfluorinated structure (i.e., **MeU-C6a**), indicating that it is the C═C double bond that determined the anaerobic microbial defluorination of the two structures. Collectively, the α,β-unsaturation in FCA was crucial for the initiation of anaerobic microbial defluorination by the investigated defluorinating enrichment, which seemed independent of fluorine substitution degrees and the linear/branched structures.

Biotransformation Pathway and the Pathway Preference of the Biotransformed Unsaturated FCAs in the Anaerobic Defluorinating Enrichment. For the biotransformed FCAs, we elucidated the anaerobic biotransformation and biodefluorination pathways based on the F^- formation and the identified TPs. Consistent with what was previously reported for **MeU-C6a** (PFMeUPA) and **MeU-C6b** (FTMeUPA),[38] the other biotransformed unsaturated FCAs also underwent two major pathways: (i) reductive defluorination (−F+H) and (ii) hydrogenation (+2H) and exhibited different pathway preferences correlated with the number of fluorine substitutions on the unsaturated carbons. By multiplying the defluorination degree by the total number of F in the molecule, we were able to determine how many fluoride ions would have been released from one molecule of the parent compound if all had undergone defluorination. If the defluorination degree was less than one over the number of F in the molecule, the parent compound would also undergo nondefluorinating pathways. Thus, we used the defluorination degree to evaluate the preference of reductive defluorination over the nondefluorinating hydrogenation pathway for all biotransformed unsaturated FCAs. The two perfluorinated unsaturated structures (i.e., **U-C5a** and **MeU-C6a**) showed higher defluorination degrees than polyfluorinated ones. Similar to **MeU-C6a**,[38] **U-C5a** (Figure S2) was completely biotransformed, corresponding to at least the 52 μM F^- release after taking the abiotic F^- formation into account (Figure 3A). It is worth noting that the abiotic F^- formation in the heat-inactivated control was from the impurities of the **U-C5a** standard, as no removal of **U-C5a** or formation of any TPs was observed during the entire incubation period (Figure 3A). We assumed that the maximum F^- released from the impurities in the biotransformation group was the same as detected in the heat-inactivated control. Then, we gave a conservative estimation of the F^- released from **U-C5a** by subtracting the F^- formed in the heat-inactivated control from that in the biotransformation group, resulting in 52 μM, which corresponds to a defluorination degree of 14.3% (Figure 3A), as if the parent compound exclusively underwent the first C−F cleavage via reductive defluorination (reaction 1 in Figure 3C). The detection of the hydrogenation product (**U-C5a_TP226**) (reaction 2 in Figure 3C, Figure 3B, and Figure S3) demonstrated that only a portion of **U-C5a** underwent reductive defluorination, forming the first C−F cleavage product (**U-C5a_TP206**) (Figure 3B and Figure S4). A second C−F cleavage should also occur, perhaps after the decarboxylation of the primary defluorination product as reported for **MeU-C6a**.[38] However, the primary defluorination product (**U-C5a_TP206**) seemed to largely undergo hydrogenation, as its rapid decrease was in line with the increase of the hydrogenation product (Figure 3B and Figure S5). The TP formation trend and the overall defluorination degree suggest that the second C−F bond cleavage was not as significant as the first one. In comparison, **MeU-C6b**, which has no F substitution on the unsaturated carbons, only showed a defluorination degree of 4% via the defluorination at the tertiary sp^3 C−F bond. It was less than 14% (1 out of 7 F in the molecule),[38] indicating that more than half of the parent compound underwent the hydrogenation pathway. For **U-C4b** (1:3 U acid), which has neither fluorine substitutions on the

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Environ. Sci. Technol. 2022, 56, 4894−4904

unsaturated carbons nor the tertiary sp^3 C−F bond in a branched moiety as the one in **MeU-C6b**, it only underwent hydrogenation with the formation of the corresponding product, 1:3 FTCA (Figure S6).

The effect of fluorine substitutions on the pathway preference between reductive defluorination and hydrogenation was further demonstrated by the biotransformation of **U-C8b** (6:2 FTUCA) (Figure S7), which is the only commercially available FCA structure with α,β-unsaturation and one fluorine substitution on the unsaturated carbon. It was rapidly biotransformed, together with the release of fluoride, which reached a plateau within the first week of incubation (Figure 4A and Figure S8). The hydrogenation (reaction 2 in Figure 4B) also occurred, forming 3-fluoro 5:3 acid (Figure 4A and Figure S9), which was subject to very slow secondary biotransformation (reaction 4 and then reaction 3 in Figure 4B) corresponding to the slight formation of F^- and 5:3 FTCA after 7 days (Figure 4A and Figure S10). Thus, we inferred that the reductive defluorination of the sp^2 C−F bond in the parent compound (reaction 1 in Figure 4B) was the major defluorination pathway. However, the reductive defluorination product, 5:3 FTUCA, was not detected, likely due to its fast turnover to **C8c** (5:3 FTCA) via hydrogenation (reaction 3 in Figure 4B), which was also observed in anaerobic digester sludge.[29] Due to the abiotic F^- formation from impurities of the **U-C8b** standard (Figure S8), we alternatively used the formation of 5:3 FTCA to determine the defluorination degree and the percentage of **U-C8b** that underwent defluorination. Since 5:3 FTCA formed from 3-fluoro 5:3 acid was minor, we considered that nearly all 5:3 FTCA formed (12 μM) was from the reductive defluorination pathway, corresponding to 17.1% (12 μM/70 μM) of the parent compound that underwent defluorination. The remaining 82.9% of the parent compound underwent the hydrogenation pathway (Figure 4B), suggesting that this pathway was favored. The anaerobic biotransformation products of 6:2 FTUCA detected here were consistent with those found in an anaerobic digester sludge community by Zhang et al.[29] In that study, 6:2 FTUCA was also quickly removed within a week, forming the low-level transient intermediate 5:3 FTUCA and the two semistable/stable products, 3-fluoro 5:3 acid and 5:3 FTCA. Different from the very slow transformation of 3-fluoro 5:3 acid observed in this study, after 6:2 FTUCA was depleted, Zhang et al. observed a substantial decrease of 3-fluoro 5:3 acid, corresponding to a continuous increase of 5:3 FTCA with no formation of 5:3 FTUCA. It indicates that the microorganisms carrying out the biotransformation of 3-fluoro 5:3 acid in the two communities had different abundances or activities. We reanalyzed the TP formation reported in the Supporting Information by Zhang et al. and found that more than half of the total detected 5:3 FTCA was formed after 6:2 FTUCA depletion. Given the quick turnover of 5:3 FTUCA to 5:3 FTCA (reaction 3 in Figure 4B), 5:3 FTCA formation after 6:2 FTUCA depletion should be attributed to 5:3 FTUCA formed from the intermediate 3-fluoro 5:3 acid (via HF elimination, reaction 4 in Figure 4B), rather than the parent compound (via reductive defluorination, reaction 1 in Figure 4B). Thus, microbial preference of the hydrogenation pathway to the reductive defluorination pathway in the anaerobic biotransformation of 6:2 FTUCA was also reflected by the result of another study on a different community.[29] Collectively, the reductive defluorination of structures with one fluorine substitution on the unsaturated carbon was less favorable than the perfluorinated unsaturated structures in the anaerobic defluorinating enrichment. Instead, the hydrogenation pathway became more favorable as more sp^2 C−F bonds were replaced with C−H bonds. Effective inhibition of the enzymes/microorganisms carrying out the hydrogenation pathway is desired to improve the overall defluorination degree of the unsaturated FCA structures. Even though the hydrogenation could be inhibited, the improvement of the overall defluorination could still be limited to the cleavage of the sp^2 C−F bonds and the tertiary sp^3 C−F bond (for branched unsaturated structures). Introducing fluorinated branches, especially those at the α/β positions, might enhance the overall defluorination.

Abiotic Transformation and Defluorination of Unsaturated FCAs with a Trifluoromethyl ($-CF_3$) Branch at the α/β Carbon in the Presence of Reducing Agents. Among the three commercially available α/β-branched unsaturated FCAs (i.e., **MeU-C4c**, **MeU-C5d**, and **MeU-C5e**), the two with a $-CF_3$ branch (i.e., **MeU-C4c** and **MeU-C5d**) exhibited abiotic transformation and defluorination in the presence of cysteine and sulfide, the commonly used reducing agents in anaerobic culture media. In comparison, no transformation or defluorination was observed when no reducing agent was provided (Figure S11). The similar defluorination degrees of **MeU-C5d** in the anaerobic defluorinating enrichment and the anaerobic sterile Milli-Q water indicate that the defluorination was mainly an abiotic process. Notably, the $-CF_3$ branch in **MeU-C5d** significantly enhanced the degradability and defluorination (Figures S11−S15) compared to the $-CH_3$ branch at the same position in **MeU-C5e**, which only underwent slow microbial hydrogenation (Figures S16−S18). The 60−80% defluorination degree of **MeU-C5d** corresponds to a release of 4−5 F from the molecule if all **MeU-C5d** could have undergone defluorination. As some **MeU-C5d** underwent a nondefluorinating cysteine conjugation pathway (Figures S12 and S15), for the defluorinated portion of **MeU-C5d**, more C−F bonds, perhaps all the six, could be cleaved. However, no less-fluorinated intermediates were detected, perhaps due to a simultaneous cleavage of all C−F bonds and the formation of small products that cannot be detected by LC-HRMS.

Similarly, **MeU-C4c** with an α-CF_3 branch became more vulnerable to degradation and defluorination than its linear isomer **U-C4b** (Figures S11 and S19). However, compared to **MeU-C5d** with two β-CF_3 branches, **MeU-C4c** showed a much lower defluorination (10%, less than one F released per molecule), perhaps due to the position and number of $-CF_3$ branches. At least one C−F bond could be cleaved from the defluorinated parent compound, given that a nondefluorinating cysteine conjugation product was also formed (Figure S19). The abiotic defluorination could be spontaneous when the redox potential was lowered below a threshold by the reducing agents. Alternatively, it could be a reaction between those compounds and the reducing agents. Further studies are needed to examine the abiotic defluorination mechanisms.

The aforementioned abiotic defluorination of impurities in the heat-inactivated controls of **U-C5a** and **U-C8b** was also attributed to the presence of reducing agents because F^- was not released when no reducing agents were added (Figure S11). Interestingly, the total F^- formation from **U-C8b** in the biological samples was even lower than the abiotic F^- formation from the **U-C8b** impurities in the abiotic control (Figure S8). **U-C8b** can be biodefluorinated (Figure 4). If the

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Environ. Sci. Technol. 2022, 56, 4894−4904

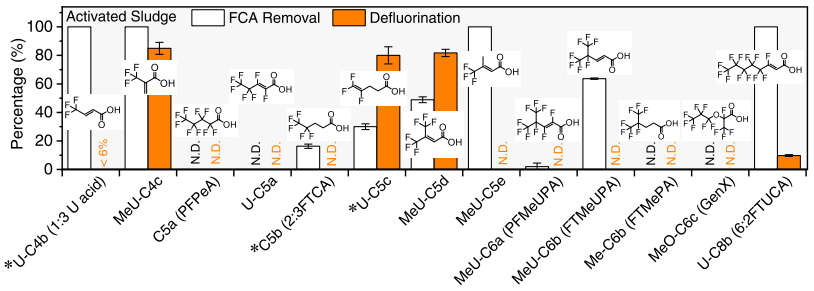

Figure 5. Parent compound removal and defluorination degree of all tested structures in the activated sludge community. *: data acquired from our previous study using the activated sludge community from the same WWTP under the same incubation conditions.[40] All compounds were incubated for 14 days, except for **U-C5c**, which was incubated for 72 h when it reached the maximum removal and defluorination after 24 h.[40] N.D.: not detected; n = 3. Note: there was no abiotic degradation or defluorination for the investigated FCAs during the incubation period.

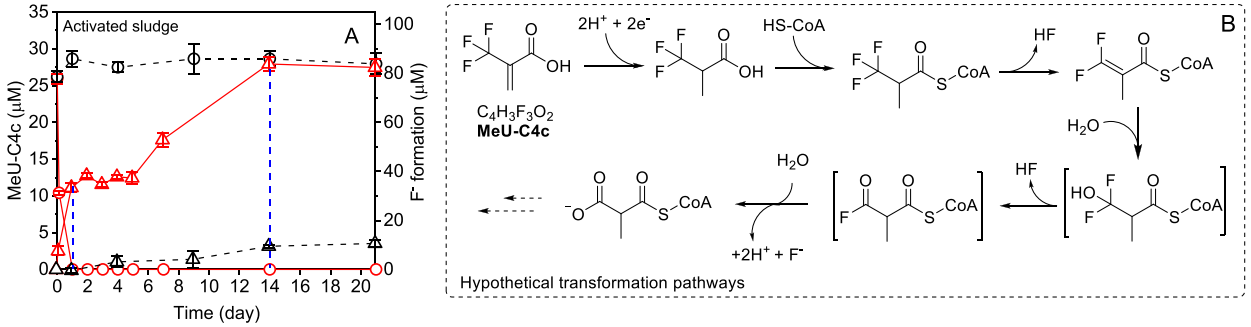

Figure 6. Aerobic biotransformation of **MeU-C4c** in the activated sludge community A: parent compound removal and fluoride release, red: biological samples, black: autoclaved sludge filtrate controls, circles: parent compound, triangles: fluoride, n = 3. B: the hypothetical transformation pathway of **MeU-C4c**. Note: In brackets are unstable intermediates; the dashed box indicates that none of the proposed intermediates were detected.

same amount of F^- was released from the impurities in the biological samples, the total F^- should be higher instead of lower. It suggests that the impurities might have unsaturated structures, which were more likely to be microbially hydrogenated than abiotically defluorinated in the enrichment culture. The hydrogenation pathway was exclusively enzyme-mediated, as no hydrogenation product was detected during the abiotic transformation of **MeU-C5d** (Figure S12C). It, again, implies that microbial hydrogenation of unsaturated structures (particularly those with no sp^2 C−F bonds) was more favorable than the (bio)defluorination pathways under anaerobic conditions.

Aerobic Biotransformation and Biodefluorination of the Unsaturated FCAs. Although the aerobic incubation in the activated sludge community did nothing to all tested perfluorinated structures, it complementarily enhanced the biotransformation and biodefluorination of the unsaturated polyfluorinated FCAs, which showed low removal and defluorination in the anaerobic enrichment culture. For example, **U-C5c** with a terminal C═C bond, which did not show any anaerobic biotransformation, exhibited 30% aerobic removal with ∼80% defluorination of the removed portion (Figure 5) via a 2-F release pathway.[40]

Moreover, **MeU-C4c**, which underwent complete abiotic transformation but only with ∼10% defluorination in the anaerobic condition, exhibited a complete aerobic removal within a day and a complete defluorination within 2 weeks (Figure 6A). The continuous defluorination after the depletion of **MeU-C4c** indicates the F^- release from the intermediate. Since hydrogenation was the major aerobic biotransformation pathway for the two unsaturated structures, **MeU-C5e** (Figure S20) and **MeU-C6b** (Figure S21), we inferred that the complete aerobic biotransformation and biodefluorination of **MeU-C4c** likely started from the hydrogenation, forming 2-methyl-trifluoropropionate, which could undergo a similar defluorination pathway (Figure 6B), as reported in our recent study (Figure S22).[40] Interestingly, we observed a 4-day plateau of fluoride release after the first-day incubation (∼43.8% defluorination) (Figure 6A), likely because the first step of defluorination via HF elimination was relatively fast, while the next two steps needed a longer acclimation for the microbes. However, no intermediates were identified by the LC-HRMS, probably because they were mainly in CoA forms in microbial cells, which were difficult to extract and detect, leaving relatively low levels of the acid forms in culture suspension. In the abiotic control, a low level (<10 μM) of F^- was released with no removal of **MeU-C4c** (Figure 6A), indicating aerobic abiotic defluorination of **MeU-C4c** impurities. Since the F^- released from impurities was not as significant as from the parent compound, it did not affect the biodefluorination analysis.

Besides, **MeU-C5d**, which showed complete abiotic transformation with 77.5% defluorination in the anaerobic condition, also underwent aerobic microbial defluorination with a higher defluorination degree (82%), while the removal was incomplete (50%) after 21 days (Figures 5 and 7). Three defluorination intermediates were identified, including the two defluorinating TPs (**MeU-C5d_TP139** and **MeU-C5d_TP121**) and the hydrogenation product (**MeU-C5d_TP209**) (Figure 7B,C). **MeU-C5d_TP139** was likely further biotransformed to **MeU-C5d_TP121**, as it slightly decreased after 9 days corresponding to an increase of **MeU-**

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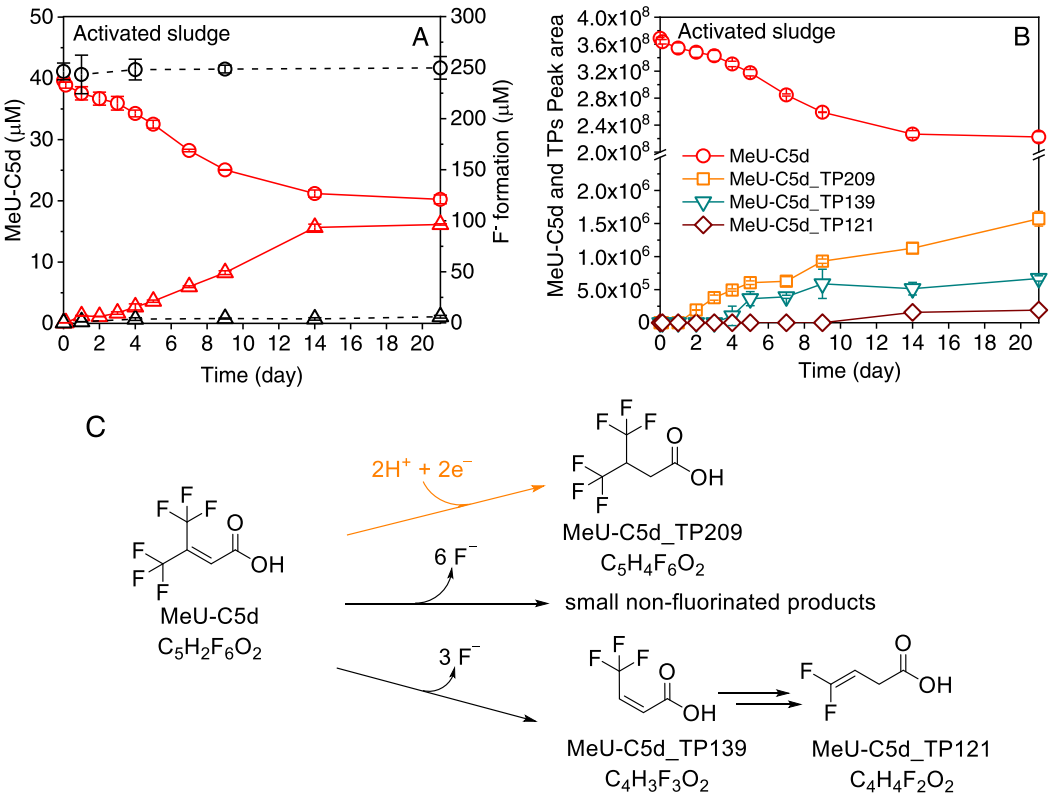

Figure 7. Aerobic biotransformation of **MeU-C5d**. A: parent compound removal and fluoride release in the activated sludge community, red: biological samples, black: abiotic controls, circles: parent compound, triangles: fluoride, $n = 3$. B: temporal trends (as peak areas) of the parent compound and TPs. C: the proposed biotransformation pathway. Note: the retention time of TP139 (0.87 min) was different from that of **U-C4b** (2.36 min), suggesting a different structure.

C5d_TP121 (Figure 7B). Given that the formation of **MeU-C5d_TP209** did not contribute to the defluorination, the actual defluorination degree could be even higher. For example, all six C−F bonds in the molecule were cleaved simultaneously as in the anaerobic condition.

The linear unsaturated structure, **U-C8b** (6:2 FTUCA), also exhibited an enhanced defluorination in the aerobic condition compared to the anaerobic condition (10% vs 1%) (Figures 6 and 2). It was because that more of the parent compound underwent defluorinating pathways in the aerobic condition. The 3-fluoro 5:3 acid formed from the preferred hydrogenation pathway in the anaerobic defluorinating enrichment was not detected during the aerobic biotransformation (Figure S23B−D). 6:2 FTUCA is a key biotransformation intermediate of 6:2 fluorotelomer alcohol (6:2 FTOH, a component of coating material in commercial products) under aerobic conditions by various microbial communities and microbial species.[30−33,36,45−47] Many of the previously reported TPs were also detected here (Figure S23B−D), and among them, **C6a** (PFHxA) and **C5a** (PFPeA) were two major ones. We also detected two new TPs (2H-PFHpA and 2H-PFHxA) (Figures S23D and S24), which were not reported in previous studies on 6:2 FTOH.[23] However, a similar product, 2H-PFOA, was detected from the aerobic soil biotransformation of 8:2 FTOH.[48] We did not detect 5:2 ketone or 5:2 sFTOH, two important TPs of 6:2 FTUCA reported in previous studies.[23,32] It could be due to differences in the parent compound (6:2 FTOH vs 6:2 FTUCA) and the community compositions between previous studies and this study. We summarized the pathways, including reductive defluorination, hydrogenation, and β- and α-oxidation, which led to all the detected TPs in this study (Figure S23E). The reductive defluorination of the sp^2 C−F bond in 6:2 FTUCA was a critical step leading to chain-shortening reactions via β- and α-oxidation-like pathways in the aerobic condition. When α-oxidation followed the reductive defluorination, it entered the one-carbon shortening loop as previously reported,[31] forming 5:2 FTUCA and 4:2 FTUCA. The β-oxidation of n:2 FTUCA formed PFHxA, PFPeA, and PFBA. The α-oxidation of n:2 FTUCA led to 2H-PFHpA and 2H-PFHxA. The formation of those shorter-chain acids released additional F^- via HF elimination, thus contributing to the higher defluorination degree than the anaerobic biotransformation.

Environmental Implications. The F^- release has been used as a necessary indicator of defluorination activities. For biodefluorination assays, it is worth noting that some impurities in the purchased standard compound may be defluorinated, and the abiotic defluorination of impurities could be suppressed by other competing enzymatic pathways in biological samples, such as biohydrogenation of unsaturated structures. If the F^- released from impurities were at significant levels, it would render an inaccurate assessment of the biodegradability of the target organofluorine molecule, either an overestimation (for **U-C5a**) or underestimation (for **U-C8b**). Thus, besides F^-, comprehensive analyses of the target parent compound and TPs in the biotransformation experimental groups, as well as various abiotic control groups, must be included to ensure an accurate assessment of biodefluorination.

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The structure-biodegradability relationships revealed in this study emphasize that FCAs with the same signature structures or functional groups exhibited similar biotransformation performance in the same microbial community. More specifically, the α,β-unsaturation rather than the $-CF_3$ branch is the required structure for anaerobic biotransformation by the anaerobic defluorinating enrichment when the fluorinated compound was provided as the sole electron acceptor. With the α,β-unsaturation, the tertiary sp^3 C−F bond can also be anaerobically defluorinated. The fluorine substitution degree and the chain length in the α,β-unsaturated FCAs did not affect biodegradability. However, the reaction preference (defluorination vs hydrogenation) was affected by the fluorine substitution degree on the C=C bond. The more F being substituted by H, the more favorable the hydrogenation will be over the defluorination in the anaerobic defluorinating enrichment. It implies that although perfluorinated structures were exclusively defluorinated anaerobically, anaerobic conditions may not be suitable for polyfluorinated structures with fewer fluorine substitutions, which could be better defluorinated aerobically. Thus, the combination of anaerobic and aerobic conditions can be implemented in PFAS bioremediation to degrade a broader range of PFAS structures and achieve deeper defluorination.

Moreover, we demonstrated that introducing one α/β $-CF_3$ branch in unsaturated FCAs could significantly enhance the degradability via anaerobic abiotic defluorination or aerobic biodefluorination. It provides essential insights into the environmental fate of PFASs and opens new avenues for designing novel fluorinated PFAS alternatives that are readily (bio)degradable. We acknowledge that due to the unavailability of authentic standards for some specific FCA structures, only a limited number of unsaturated FCA structures were tested. The generality of the finding still needs to be further validated when more desired structures become available. Collaborations between researchers from academia and fluorochemical industries would be needed to examine the structure-biodegradability relationship using custom-synthesized structures[31] and discover fluorinated PFAS alternatives with retained functionality, maximized environmental degradability, and minimized adverse impact to the environment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c05509.

Detailed information on investigated FCAs, background fluoride levels in activated sludge community, LC-HRMS/MS results of parent compounds and transformation products, anaerobic abiotic transformation of unsaturated FCAs, and proposed aerobic and anaerobic transformation pathways (PDF)

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Notes

The authors declare no competing financial interest.

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