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# Aerobic Biotransformation of $^{14}$C-Labeled 8-2 Telomer B Alcohol by Activated Sludge from a Domestic Sewage Treatment Plant

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This study investigated the biodegradation potential of 3-$^{14}$C,1*H*,1*H*,2*H*,2*H*-perfluorodecanol [$CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$, $^{14}$C-labeled 8-2 telomer B alcohol or $^{14}$C-labeled 8-2 TBA] by diluted activated sludge from a domestic wastewater treatment plant under aerobic conditions. After sample extraction with acetonitrile, biotransformation products were separated and quantified by LC/ARC (on-line liquid chromatography/accurate radioisotope counting) with a limit of quantification about 0.5% of the $^{14}$C counts applied to the test systems. Identification of biotransformation products was performed by quadrupole time-of-flight mass spectrometry. Three transformation products have been identified: $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$ (8-2 saturated acid); $CF_3(CF_2)_6{}^{14}CF{=}CHCOOH$ (8-2 unsaturated acid); and $CF_3(CF_2)_6{}^{14}COOH$ (perfluorooctanoic acid, PFOA), representing 27, 6.0, and 2.1% of the initial $^{14}$C mass ($^{14}$C counts applied) after 28 days, respectively. A transformation product, not yet reported in the literature, has also been observed and tentatively identified as $CF_3(CF_2)_6{}^{14}CH_2CH_2COOH$ (2*H*,2*H*,3*H*,3*H*-perfluorodecanoic acid); it accounted for 2.3% of the mass balance after 28 days. The 2*H*,2*H*,3*H*,3*H*-perfluorodecanoic acid is likely a substrate for $\beta$-oxidation, which represents one of the possible pathways for 8-2 telomer B alcohol degradation. The 8-2 saturated acid and 8-2 unsaturated acid cannot be directly used as substrates for $\beta$-oxidation due to the proton deficiency in their $\beta$-carbon ($C_3$ carbon) and their further catabolism may be catalyzed by some other still unknown mechanisms. The 2*H*,2*H*,3*H*,3*H*-perfluorodecanoic acid may originate either from the major transformation product $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$ or from other unidentified transformation products via multiple steps. Approximately 57% of the starting material remained unchanged after 28 days, likely due to its strong adsorption to the PTFE (poly(tetrafluoroethylene)) septa of the test vessels. No $CF_3(CF_2)_6{}^{14}CF_2COOH$ (perfluorononanoic acid) was observed, indicating that $\alpha$-oxidation of $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$ did not occur under the study conditions. Several $^{14}$C-labeled transformation products that have not yet been identified (each less than 1% of the mass balance) were also observed and together accounted for 7% of the total $^{14}$C mass balance after 28 days. It is not clear whether these unidentified transformation products were resulting from further metabolism of 8-2 saturated acid or 8-2 unsaturated acid. The results suggest that perfluorinated acid metabolites such as perfluorooctanoic acid account for only a very small portion of the transformation products observed. Also, the observed volatility and bioavailability of $^{14}$C-labeled 8-2 TBA for microbial degradation was markedly decreased as a result of the presence of a strongly adsorbing matrix such as PTFE in the experimental systems. It is apparent that the biological fate of 8-2 telomer B alcohol is determined by multiple degradation pathways, with neither $\beta$-oxidation nor any other enzyme-catalyzed reactions as a single dominant (principal) mechanism under the study conditions.

## Introduction

There is growing interest in the environmental fate and effects of fluorinated chemicals in the environment. As a result of the identification and ubiquitous global presence of perfluorooctane sulfonate [$F(CF_2)_8SO_3^-$] in the environment and humans and to a lesser extent perfluorooctanoic acid [$F(CF_2)_7COOH$], the environmental fate and distribution of perfluorinated chemicals (PFCs) is of growing interest in order to understand the sources of these chemicals in the environment (*1*−*6*). Carbon−fluorine bonds are extremely strong (*7*). As a result, it is generally accepted that perfluorocarbon chains do not readily biodegrade and any biodegradation may be limited to hydrocarbon functionality to which a perfluorocarbon moiety is attached.

Interest extends beyond perfluorooctane sulfonate and perfluorooctanoic acid to the broader class of perfluorinated chemicals, which includes fluorotelomer alcohols [FTOHs]. 8-2 telomer B alcohol [8-2 TBA, $F(CF_2)_8CH_2CH_2OH$, CAS Registry No. 678-39-7] is one of the raw material intermediates used in the manufacture of fluorotelomer-based products. Fluorotelomer alcohols are comprised of an even number of fluorinated carbons appended to an ethanol moiety. The fluorotelomer functionality in products delivers unique surface modification properties including water and oil repellency (polymeric products) and wetting and leveling (surfactants) (*8*−*10*).

The environmental fate and routes into the environment of fluorotelomer-based substances is still largely unknown. Biodegradation is one transformation route that will determine the fate of 8-2 TBA in the environment. A limited number of experimental investigations have been conducted to investigate microbial biodegradation of FTOHs and telomer-based substances (*11*−*16*). The same is true regarding metabolism in living systems and pharmacokinetics (*17*, *18*). Potential transformation pathways have been reported from predictive models based upon known abiotic and biotic functional group transformations and their probabilities (*5*). After submission of the initial draft of this study, a new paper was published (*19*) that reports the biodegradation of non-

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radioisotope-labeled 8-2 Telomer B Alcohol by microbial enrichment culture. In this study, the authors quantified three known transformation products (8-2 saturated acid, 8-2 unsaturated acid, and PFOA) and found that 8-2 unsaturated acid is the major metabolite and that PFOA accounts for about 3% of total mass at day 81. The authors conclude that the $\beta$-oxidation is the principal pathway that determines the fate of 8-2 Telomer B Alcohol. In this study, PFOA concentration also decreased significantly from about 6% of total mass at day 51 to 3% at day 81. Because the above studies in general were limited to identification and quantification of transformation products already known from prior literature or predictive models, potential unknown transformation products were not identified or quantified. Hence, the transformation pathway(s) and stable transformation products remain unclear. In addition, while widely believed to be the major transformation product from microbiological transformation of 8-2 TBA, perfluorooctanoic acid ($F(CF_2)_7COOH$) was only identified in small quantities. 8-2 TBA is highly surface active, sorptive (unpublished results by Telomer Research Program), and both hydro- and oleophobic (*20*). Additionally, 8-2 TBA has exhibited unique physical−chemical properties that make it a challenging test substance. As a result, test substance bioavailability and partitioning in the test system present real challenges. Headspace migration, strong, potentially irreversible sorption, and rapid migration to test system surfaces have been reported (*20*, *21*). Finally, the isolation, identification, and accurate quantitation of the transformation product(s) as well as obtaining authentic standards of them has been limited thus far by testing of non-radiolabeled material.

We report here the first experimental biodegradation study to achieve mass balance which more clearly defines the biological fate of a fluorotelomer alcohol. To achieve mass balance and overcome identification and quantitation problems, a custom synthesized $^{14}$C-labeled 8-2 TBA [$F(CF_2)_7{}^{14}CF_2CH_2CH_2OH$] was used to investigate the biodegradation potential and transformation products after incubation with activated sludge inoculums from a domestic wastewater treatment plant. The use of $^{14}$C-labeled material allowed us to quantify and identify unequivocally potential transformation products down to a limit of quantification (LOQ) of 0.5% of initial $^{14}$C applied to the experimental system. The objective of this study is to assess whether 8-2 TBA can be readily transformed aerobically and, if so, to discern the likely biotransformation pathway(s) and products.

## Experimental Section

**Materials and Methods.** Non-radioisotope-labeled 8-2 TBA ($CF_3(CF_2)_6CF_2CH_2CH_2OH$, 8-2 TBA) was from TRP (Telomer Research Program) and had a 99.9% purity. The $^{14}$C-label 8-2 TBA ($CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$) was custom synthesized with a radiochemical purity of virtually 100%. No $^{14}$C impurity was detected with a LOQ of ∼0.02% as analyzed by HPLC coupled with a $^{14}$C flow-through detector. The specific activity was 54.3 mCi mmol$^{-1}$ based on $^{14}$C counts on a mass basis and was confirmed by gas chromatography/mass spectrometry (GC/MS) analysis to be within ±2% difference. The chemical purity was 93% with $CF_3(CF_2)_6CH_2CH_2OH$ as the major impurity. The $^{14}$C-label 8-2 TBA was dissolved in absolute ethanol (purity = 99.97%, Aldrich Chemical Co., Milwaukee, WI) to about 2.2 mCi mL$^{-1}$ ethanol as the stock solution and was stored at approximately −10 °C. The positive control reference chemical aniline·HCl ($C_6H_7N$·HCl) was from Sigma Chemical Co. (St. Louis, MO) with a purity of 99.6% and uniformly labeled aniline hydrochloride ($^{14}C_6H_7N$·HCl or $^{14}$C(U)-aniline·HCl) was from Moravek Biochemicals (Brea, USA) with a specific activity of 78 mCi mmol$^{-1}$ and radiochemical purity of 99.1%. Three milliliters of 1 g of aniline·HCl L$^{-1}$ aqueous solution was added to 0.28 mCi $^{14}$C(U)-aniline·HCl solids to give a final concentration of approximately 1.2 g L$^{-1}$ as the stock solution. All other solvents used had a purity of >99%, and all other chemicals were reagent grade or higher. The water used throughout the experiment was deionized water (∼17.6 MΩ-cm) from Barnstead E-pure system. Activated sludge (2 L in a 4-L container) was collected the same day the experiment was initiated from the City of Wilmington (Delaware) Municipal Wastewater Treatment Facility, aeration basin 2. After arriving at the lab, the sludge was mixed by shaking to suspend the microorganisms. After settling for approximately 14 min, the upper aqueous phase of the sludge was used as the inoculums or was autoclaved and then used as abiotic control.

**Experimental System.** Because of the semivolatile nature of the starting material (*20*), a sealed test system was used to prevent loss of $^{14}$C-labeled 8-2 TBA due to volatilization. Glass serum bottles (120-mL volume) were used as the test vessels. The mineral medium used in this study was composed of 8.5 mg L$^{-1}$ of $KH_2PO_4$, 21.8 mg L$^{-1}$ of $K_2HPO_4$, 33.4 mg L$^{-1}$ of $Na_2HPO_4{\cdot}2H_2O$, 0.5 mg L$^{-1}$ of $NH_4Cl$, 36.4 mg L$^{-1}$ of $CaCl_2{\cdot}2H_2O$, 22.5 mg L$^{-1}$ of $MgSO_4{\cdot}7H_2O$, and 0.25 mg L$^{-1}$ of $FeCl_3{\cdot}6H_2O$, and the pH was adjusted to 7.0 (*22*). The test and appropriate control media were prepared in 0.5−1 L polypropylene containers and then dispersed with glass pipets into individual serum bottles. Five different treatments were included in the test design to ensure study integrity. (1) Biodegradation test vessels (4 replicates at each sampling time point): contain 30 mL of test medium composed of activated sludge (0.5%), 0.48 $\mu$L $^{14}$C-label 8-2 TBA stock solution, and about two-thirds saturated (∼80 $\mu$g L$^{-1}$) 8-2 TBA solution made in mineral medium. (2) Abiotic control vessels (4 replicates at each sampling time point): autoclaved sludge (0.5%) plus 0.5 mM NaCN were substituted into the live sludge, and the rest of the components were identical as in biodegradation test vessels. (3) Spike recovery vessels (4 replicates at each sampling time point): contain 30 mL of the test medium with activated sludge (0.5%) in mineral medium, 5 mL of which was withdrawn for fluoride analysis before being spiked (dosed) at each sampling time point (days 0, 7, 14, and 28) with 50 $\mu$L of 226 mg L$^{-1}$ $^{14}$C-labeled 8-2 TBA and 0.5 mL of 4 mg L$^{-1}$ freshly prepared standard fluorinated acids, $CF_3(CF_2)_6CF_2CH_2COOH$ (8-2 saturated acid), $CF_3(CF_2)_6CF{=}CHCOOH$ (8-2 unsaturated acid), $CF_3(CF_2)_6COOH$ (PFOA), and $CF_3(CF_2)_4COOH$ (PFHA). The $^{14}$C-labeled spike solution (226 mg L$^{-1}$) was prepared by mixing 2 mL of 150 mg of 8-2 TBA L$^{-1}$ in ethanol and 8 $\mu$L of original $^{14}$C-label 8-2 TBA stock solution (specific activity = 54.3 mCi mmol$^{-1}$). (4) Sample matrix vessels (2 replicates at each sampling time point): contain 30 mL of the test medium with activated sludge (0.5%) in mineral medium to be used as a blank solution for LC/MS/MS analysis of spiked fluorinated acids. (5) $^{14}$C(U)-aniline·HCl positive control vessels (2 replicates at each sampling time point) to assess the inoculum microbial activity over time: contain 30 mL of the test medium with activated sludge (0.5%) in mineral medium plus 30 $\mu$L of 1.2 g of $^{14}$C(U)-aniline·HCl L$^{-1}$ stock solution.

**Test Conditions and Sampling.** After each test vessel was filled with appropriate test medium, the bottle was crimp-sealed with a PTFE septum (prewashed with methanol and sterile water)/aluminum cap. It should be noted that the biodegradation system in this study contained only about 13 mg L$^{-1}$ organic carbon, which included the microbial inoculum and the ethanol as a cosolvent to disperse the starting material. This low amount of organic carbon plus enough headspace (90 mL air) assured aerobic conditions in sealed serum bottles. The diluted sludge (0.5%, 200-fold dilution) contributed to about 1 mg of organic carbon L$^{-1}$. The inoculum level was also low (estimated to be approximately $10^5$ bacterial cells per bottle), so that the test

conditions were comparable to the "Ready Biodegradation" test according to the study guide provided by the Organization for Economic Co-Operation and Development (*22*). All the glass serum bottles were shaken at 250 rpm in an environmental incubator in the dark and at room temperature (range of 25−27.7 °C monitored by a Dickson chart recorder during 28 days). The test vessels from different experimental treatments were sampled at days 0, 7, 14, and 28 for extraction and analysis.

**Sample Extraction and Analysis.** To quantify the initial concentration of the starting material, triplicate 30 mL of medium from the 1 L polypropylene bottles for treatments 1 and 2 were extracted with 50 mL of MTBE−0.1 M $H_2SO_4$ for 1.5 h, and the MTBE phase (top phase of the extract) was used for quantification of 8-2 TBA and $^{14}$C-labeled 8-2 TBA by GC/MS as described (*23*). On days 0, 7, 14, and 28, 5 mL of the test medium from treatments 1−4 above was withdrawn from each of the serum bottles with a syringe through a 26-gauge needle and was treated with 50 $\mu$L of 5N NaOH first and then neutralized with 42 $\mu$L of 6N $H_2SO_4$ for fluoride analysis. The remaining 25 mL of medium in each bottle was extracted with 45 mL of acetonitrile (injected into the sealed bottle) for approximately 1 h at room temperature (first extraction). Due to possible injection wound (piercing damage) to the septa, some of the bottles were then re-sealed with fresh PFTE septa/aluminum cap to ensure an airtight seal during long-term storage at approximately −10 °C. The acetonitrile extract from each sample bottle was used for liquid scintillation counting of $^{14}$C, for quantification and identification of $^{14}$C-labeled transformation products using LC/ARC, and for LC/MS/MS analysis of fluorinated acids. To measure the mineralization of $^{14}$C(U)-aniline·HCl by the inoculum (treatment 5), 0.1 mL of aliquot from the serum bottles was mixed with 0.2 mL of 0.1 M HCl to remove $^{14}CO_2$ remaining in the medium, and 5 mL of scintillation cocktail was added to the 0.3 mL of mixed solution for $^{14}$C counting by a liquid scintillation counter.

It was found later that initial $^{14}$C counts of the acetonitrile extract (from first extraction) did not account for all $^{14}$C applied as $^{14}$C-labeled 8-2 TBA at day 0. For this reason, a second sequential extraction was carried out for the serum bottles and septa separately. Each sample bottle was re-extracted with 10 mL of acetonitrile for 3−4 h at room temperature after rinsing 2 times with deionized water, and each PTFE septum from individual sample serum bottles, if saved, was re-extracted with 10 mL of acetonitrile 4−5 times at 50 °C (each extraction lasted 4−7 days) and $^{14}$C activity was measured for mass balance calculation. Also, some of the acetonitrile solution was pooled from each sample for LC/ARC analysis to confirm whether extended extraction at 50 °C caused any degradation of $^{14}$C-labeled 8-2 TBA. As mentioned above, some of the original septa (piercing damaged) were not saved for the re-extraction.

Fluoride ion was measured by fluoride-selective electrode after mixing the sample with equal volume (5 mL) of Thermo Orion TISABII buffer (*15*) using 5−500 $\mu$g of NaF $L^{-1}$ as standard calibration. Fluorinated acids in the spiked samples were quantified by LC/MS/MS in negative electrospray ionization mode by a Waters 2795 HPLC/Micromass Quattro Micro system. The 10−50 $\mu$g $L^{-1}$ calibration standards were made in sample matrix blank (from Treatment 4) and the injection volume was 10 $\mu$L. The HPLC column used is a reverse-phase Zorbax $C_8$ (150 × 2.1 mm, 5 $\mu$m particle size). Mobile phase was 0.15% acetic acid/acetonitrile in a gradient with a flow rate of 0.4 mL $min^{-1}$. Multiple reaction monitoring (313 > 269 for $CF_3(CF_2)_4COOH$, 413 > 369 for $CF_3(CF_2)_6COOH$, 457 > 393 for $CF_3(CF_2)_6CF{=}CHCOOH$, and 477 > 393 for $CF_3(CF_2)_6CF_2CH_2COOH$) was used for compound quantification.

**Analysis of $^{14}$C-Labeled 8-2 TBA and $^{14}$C-Labeled Transformation Products by LC/ARC.** Original acetonitrile-extracted samples from Treatment 1 and 2 in sealed glass GC vials were diluted 1:1 with 2 mM ammonium acetate in water. The diluted samples were analyzed by LC/ARC to quantify $^{14}$C-labeled 8-2 TBA and $^{14}$C-labeled transformation products by integrating radioactivity of apparent (baseline-resolved) chromatographic peaks during the separation. The LC/ARC system (AIM Research Company, Newark, DE) utilizes advanced stop flow counting technologies to accurately detect and quantify radioisotope with a sensitivity of several counts per minute (cpm) above background level (*24*). The initial analysis (LC/ARC Method 1) used a Zorbax $C_{18}$ column (4.6 mm × 150 mm, 5 $\mu$m particle size), and mobile phase was 2 mM ammonium acetate/acetonitrile in a gradient with a flow rate of 1.0 mL $min^{-1}$. The injection volume was 0.5 mL, the fraction size was 10 s, and the $^{14}$C counting time was 120 s for each fraction. The initial analysis (LC/ARC Method 1) was unable to separate $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$ from $CF_3(CF_2)_6{}^{14}CF{=}CHCOOH$ to give individual transformation product peaks. A second chromatographic method (LC/ARC Method 2) was used, with 0.15% acetic acid/acetonitrile as mobile phase, to further separate and quantify the two $^{14}$C-labeled transformation products.

**Identification of $^{14}$C-Labeled Transformation Products by LC/MS Analysis.** A HPLC (Alliance HT, Model 2795, Waters)/Q-TOF (Micromass) system in negative electrospray ionization mode was used to identify the transformation products observed with the LC/ARC system. The chromatographic conditions of the LC/MS analysis were kept essentially the same as for LC/ARC analysis in order to match the retention times of the observed signals in the LC/MS and LC/ARC analysis as closely as possible. The LC/MS analysis was done off-line from the LC/ARC system. A Zorbax $C_{18}$ (2.1 mm × 150 mm, 5 $\mu$m particle size) column was used with a gradient mobile phase (0.15% acetic acid/acetonitrile) and a flow rate of 0.25 mL $min^{-1}$; the lower flow rate was used to compensate for column diameter change from 4.6 to 2.1 mm and was necessary for proper electrospray operation. Other MS parameters were as follows: capillary voltage of 2.5 kV, cone voltage of 10 V, source temperature of 120 °C, and desolvation temperature of 350 °C. The transformation product identification was based on matching the retention time, observed molecular ion, daughter ion spectrum, and accurate mass measurement of the observed transformation product with that of respective standards. The retention time regions of the LC/MS chromatograms where the LC/ARC peaks were observed were carefully examined to identify transformation products that could not be matched with available standards.

**$^{14}$C Radioactivity Measurement By Liquid Scintillation Counting.** Throughout the study, 5 mL aliquots of liquid scintillation cocktail (Packard BioScience Ultima Gold XR) were mixed with processed samples for $^{14}$C radioactivity counting by a liquid scintillation counter (Beckman LS 5000TD). The counting time was normally 10 min to reduce counting error to within ±2% except for samples with only background or close to a background level of $^{14}$C radioactivity. Typical counting efficiency was >95%, and the final radioactivity was automatically calculated and reported as DPM based on: (CPM/counting efficiency) = DPM.

## Results and Discussion

The measured starting concentration of the parent material [$CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ plus nonlabeled $CF_3(CF_2)_6CF_2CH_2CH_2OH$] was 337 ± 13 $\mu$g $L^{-1}$ for the test vessels and 306 ± 9 $\mu$g $L^{-1}$ for abiotic control vessels, which is about 2.3-fold above 8-2 TBA solubility limit (*20*). The purpose of using the concentration above the solubility of the test chemical is to increase the sensitivity for detecting the $^{14}$C-labeled trans-

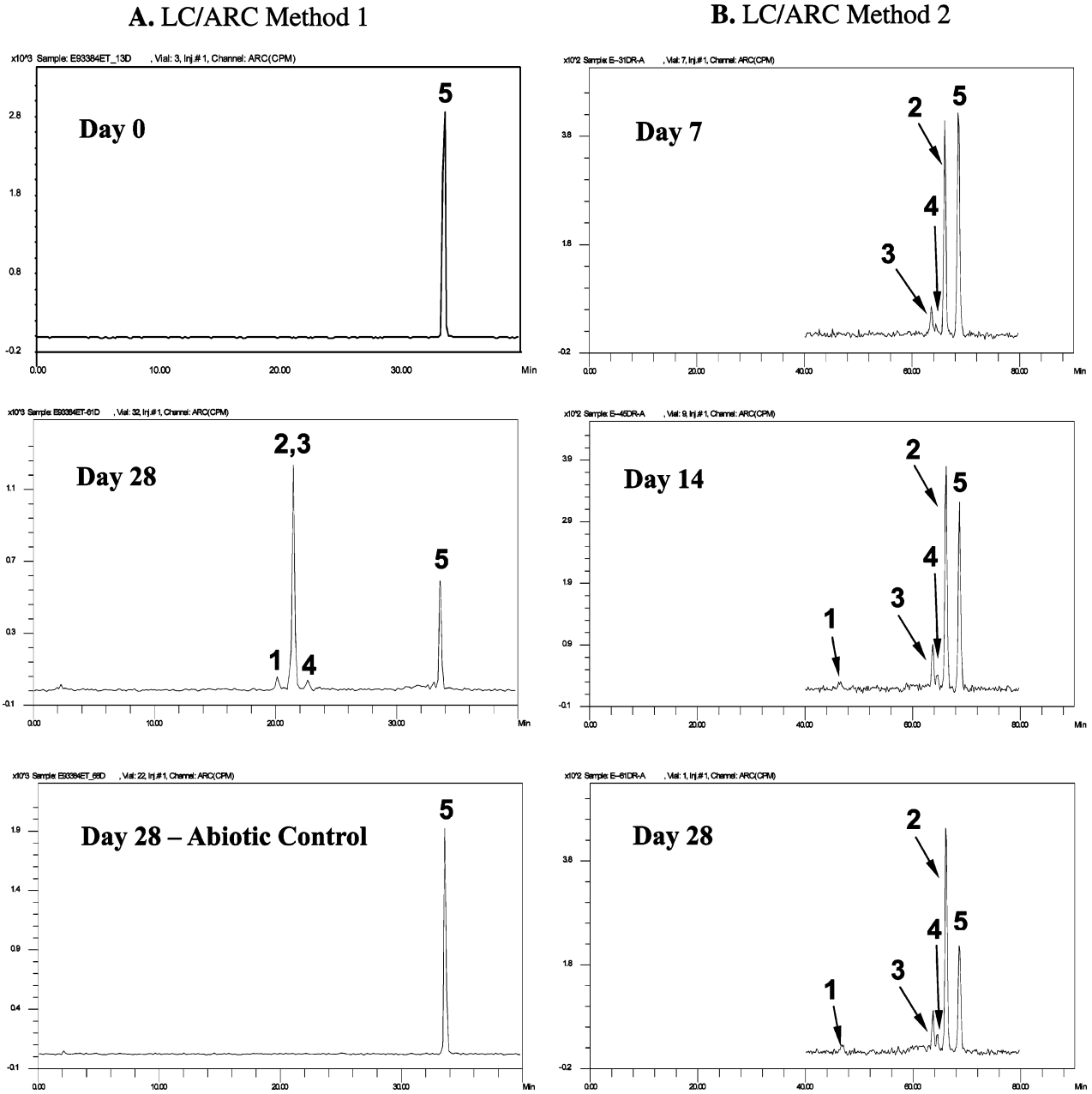


**FIGURE 1. LC/ARC chromatograms of the parent, $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ (peak 5) and $^{14}C$-labeled transformation products (peaks 1−4: peak 1, $CF_3(CF_2)_6{}^{14}COO^-$; peak 2, $CF_3(CF_2)_6{}^{14}CF_2CH_2COO^-$; peak 3, $CF_3(CF_2)_6{}^{14}CF{=}CHCOO^-$; peak 4, $CF_3(CF_2)_6{}^{14}CH_2CH_2COO^-$) at different sampling time points using LC/ARC method 1 (A, left column) and method 2 (B, right column).**

formation products. Based on the amount of $^{14}C$ counts available to each test vessel after the sample extraction, the LOQ for $^{14}C$-labeled transformation products was approximately 0.5% of the initial $^{14}C$ counts applied. The recoveries of the starting material and fluorinated acid standards that had been spiked (dosed) into the test medium were acceptable. The recoveries for spiked test medium averaged 92 ± 7% for $^{14}C$-labeled 8-2 TBA, 100 ± 20% for $CF_3(CF_2)_6CF_2CH_2COOH$, 81 ± 21% for $CF_3(CF_2)_6CF{=}CHCOOH$, 104 ± 14% for $CF_3(CF_2)_6COOH$, and 100 ± 20% for $CF_3(CF_2)_4COOH$ ($n$ = 16 for day 0, 7, 14, and 28 samples).

The reference compound, $^{14}C$-labeled aniline·HCl with a starting concentration of approximately 1.2 mg $L^{-1}$, degraded over time, with 45% of total $^{14}C$ counts remaining at day 7 compared with that of day 0, 41% at day 14, and 29% at day 28 ($n$ = 2 samples), indicating that more than 70% of the $^{14}C$-labeled aniline·HCl had been mineralized. $CO_2$ evolution in excess of 50% of theoretical is interpreted as complete or near-complete biodegradation of a test chemical because a substantial portion of $^{14}C$ applied was incorporated into the structural components of the microbes (*25*). This suggests that the sludge inoculums used in this study were metabolically active during the test.

**Formation of Biotransformation Products.** Under the test conditions with live microbial inoculums, the starting material (parent) was readily transformed to various transformation products (Figure 1). The LC/ARC Method 1 (Figure 1A, left column) was initially used to separate and quantify the $^{14}C$-labeled transformation products. As shown in Figure 1A, at day 28, one major peak with a retention time ($t_n$) of 21.6 min emerged in addition to the parent (peak 5, $t_n$ = 33.6 min), and two additional smaller peaks (peaks 1 and 4) with retention times of approximately 20 and 22.6 min are clearly visible. For the abiotic control, only the parent was observed at day 28. These results demonstrate that the formation of transformation products was due to microbial activity. An

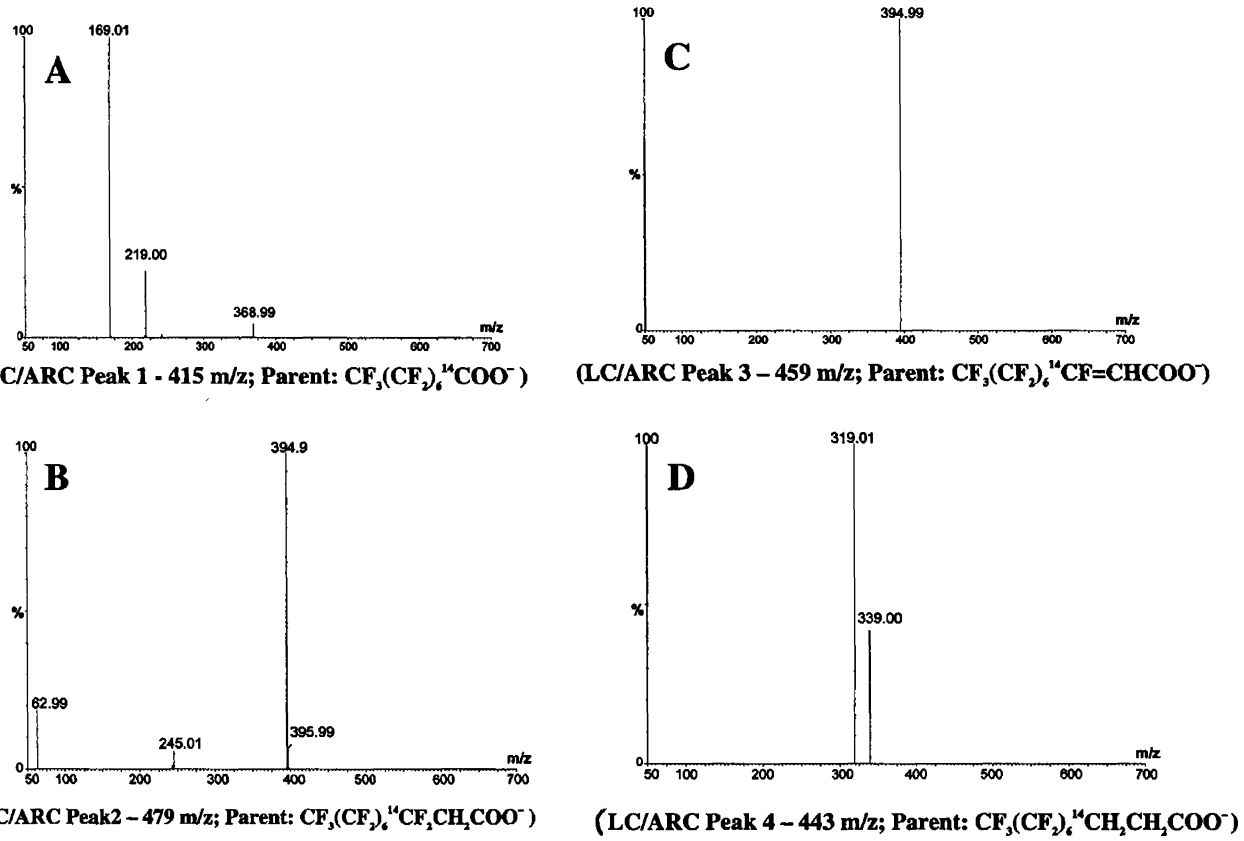


**FIGURE 2. Daughter ion spectra of deprotonated molecular ions: *m/z* 415 (A), 479 (B), 459 (C), and 443 (D) obtained for transformation products observed in a day 28 sample.**

**TABLE 1. Accurate Mass Measurements of Observed Transformation Products**

| determined mass | $^{14}$C-labeled transformation product ion | elemental composition | calculated mass | error in ppm |
|---|---|---|---|---|
| 478.9803 | $CF_3(CF_2)_6{}^{14}CF_2CH_2COO^-$ (peak 2) | $^{12}C_9{}^{14}CH_2O_2F_{17}$ | 478.9816 | −2.6 |
| 458.9764 | $CF_3(CF_2)_6{}^{14}CFCHCOO^-$ (peak 3) | $^{12}C_9{}^{14}CHO_2F_{16}$ | 458.9753 | +2.3 |
| 414.9771 | $CF_3(CF_2)_6{}^{14}COO^-$ (peak 1) | $^{12}C_7{}^{14}CO_2F_{15}$ | 414.9691 | +19.3 |
| 443.0037 | $CF_3(CF_2)_6{}^{14}CH_2CH_2COO^-$ (peak 4) | $^{12}C_9{}^{14}CH_4O_2F_{15}$ | 443.0004 | +7.4 |

LC/MS analysis using analogous chromatographic conditions revealed that the major peak ($t_n$ = 21.6 in Figure 1A) contained two $^{14}$C-labeled transformation products. A second LC/ARC method, Method 2, was developed that allowed efficient separation and quantification of all observed transformation products (Figure 1B, right column).

**Identification of Biotransformation Products.** The identification of biotransformation products was conducted using negative ion electrospray ionization on an LC/Q-TOF MS system and applying chromatographic conditions matching those used on the LC-ARC system. Figure 2 shows the daughter ion spectra of major transformation products (LC/ARC peaks 1−4 of Figure 1B). The identity of transformation products (metabolites) 1−3 was elucidated by matching the retention times and daughter ion spectra of the respective authentic standards [$CF_3(CF_2)_6COOH$, $CF_3(CF_2)_6CF_2CH_2COOH$, and $CF_3(CF_2)_6CF{=}CHCOOH$] with those of the $^{14}$C-labeled transformation products. The peaks 1−3 were identified as $CF_3(CF_2)_6{}^{14}COOH$, $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$, and $CF_3(CF_2)_6{}^{14}CF{=}CHCOOH$, respectively. The daughter ion spectra of identified transformation products are presented in Figure 2A−C. The identification was based on that the daughter ion spectrum of a metabolite from the sample extract is essentially identical to that of a corresponding standard. The match of the spectrum of an unknown metabolite and a standard is evaluated by the comparison of observed masses of fragment ions in the daughter ion spectrum and their relative intensities in the spectrum. Taking into account that observed metabolites contain one $^{14}$C atom and, therefore, some of the resulting fragment ions would be shifted two mass units. Full agreement was observed between the ions in the daughter ion spectrum of metabolites and nonlabeled standards as well as their relative intensities.

Further conformation of the observed transformation products structures was obtained by accurate mass measurements using LC/Q-TOF MS (Table 1). The measured masses agreed well with the calculated masses for the proposed elemental composition of transformation products, especially for the two transformation products (LC/ARC peaks 2 and 3 of Figure 1B) observed at higher concentration. The accurate mass measurement for $CF_3(CF_2)_6{}^{14}COOH$ exhibited highest error, outside of the typically accepted level of 5 ppm. The signal for this transformation product was very weak, which in turn affected the accuracy of the mass measurement. The above three transformation products had also been reported in blood plasma of male rats after a single oral dose (*11*), in mixed bacterial cultures (*15*), and in enrichment culture dosed with 8-2 TBA (*19*) and are also predicted by the CATABOL software (*5*). This suggests that common biodegradation pathways for 8-2 TBA may be shared between animals and microorganisms.

Accurate mass measurement for the ion *m/z* 443 (LC/ARC peak 4 of Figure 1B) pointed to an elemental composition of the deprotonated molecular ion containing four hydrogens

**TABLE 2. Mass Balance of $^{14}$C-Labeled 8-2 TBA and Quantified Transformation Products**

| compound | % of total initial mass[a] day 0 | day 7 | day 14 | day 28 |
|---|---|---|---|---|
| **Test Vessels** | | | | |
| first acetonitrile extraction | | | | |
| $CF_3(CF_2)_6{}^{14}CF_2CH_2COO^-$ (peak 2) | nd[b] | 20.7 ± 2.4 | 24.8 ± 1.7 | 26.5 ± 3.6 |
| $CF_3(CF_2)_6{}^{14}CF{=}CHCOO^-$ (peak 3) | nd[b] | 3.0 ± 1.1 | 4.6 ± 0.7 | 5.9 ± 0.2 |
| $CF_3(CF_2)_6{}^{14}COO^-$ (peak 1) | nd[b] | 0.8 ± 0.2 | 1.1 ± 0.1 | 2.1 ± 0.4 |
| $CF_3(CF_2)_6{}^{14}CH_2CH_2COO^-$ (peak 4) | nd[b] | 0.8 ± 0.1 | 1.6 ± 0.1 | 2.3 ± 0.2 |
| unidentified transformation products | nd[b] | 3.9 ± 0.8 | 6.0 ± 0.4 | 6.9 ± 0.6 |
| $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ (parent; peak 5) | 99 ± 0.3 | 47.4 ± 4.9 | 27.0 ± 1.8 | 16.0 ± 2.6 |
| second acetonitrile extraction | | | | |
| $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ (adsorbed to septa) | 0.3 ± 0.2 | 23.5 ± 3.1 | 34.3[c] | 40.8[c] |
| $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ (adsorbed to glass) | 0.7 ± 0.3 | 0.8 ± 0.3 | 0.6 ± 0.1 | 0.5 ± 0.0 |
| sum (total label) | 100 | 101 | 100[c] | 100[c] |
| sum of $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ (parent) | 100 | 72 | 62 | 57 |
| **Abiotic Control** | | | | |
| first acetonitrile extraction | | | | |
| $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ | 99 ± 0.3 | 74.7 ± 1.9 | 61.1 ± 4.0 | 49.2 ± 3.6 |
| second acetonitrile extraction | | | | |
| $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ (adsorbed to septa) | 0.1 ± 0.0 | 27.0 ± 1.9 | 37.5 ± 3.3 | 51.4[c] |
| $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ (adsorbed to glass) | 0.5 ± 0.3 | 0.5 ± 0.2 | 0.3 ± 0.1 | 0.4 ± 0.1 |
| sum (total label) | 100 | 102 | 99 | 100[c] |

[a] The mass balance was calculated based on the $^{14}$C counts for the parent or individual transformation products at each sampling time points vs the $^{14}$C counts applied initially at day 0. All values represent mean ± SD (standard deviation) for $n = 4$ samples unless otherwise stated. [b] Not detected. [c] Because the original septa after the first acetonitrile extraction were not saved for re-extraction (second extraction), no direct $^{14}$C counts data are available. However, available $^{14}$C counts data indicated a virtually 100% mass balance for day 7 test vessels and abiotic samples and day 14 abiotic samples. Thus, a 100% mass balance was reasonably assumed for the rest of samples, and $^{14}$C counts in these septa were calculated based on a 100% mass balance.

(Table 1). Further evidence of the presence of four hydrogens in the molecule was obtained from the daughter ion spectrum of ion $m/z$ 443 (Figure 2D). The ions observed in the daughter ion spectrum of $m/z$ 443 can be rationalized as follows: $m/z$ 339 represents loss of $CO_2$ (molecular weight 44) and of 3 HF ($3 \times 20 = 60$) from the deprotonated molecular ion ($m/z$ 443) and $m/z$ 319 represents additional loss of HF from ion $m/z$ 339. The fragmentation pattern of $m/z$ 443 closely resembles the fragmentation patterns observed for standards $CF_3(CF_2)_6$-$CF_2CH_2COOH$ and $CF_3(CF_2)_6CF{=}CHCOOH$ that would predominantly form ions resulting from losses of $CO_2$ and HF from the deprotonated molecular ion. However, it is not possible from the daughter ion spectrum of $m/z$ 443 alone to assign the placement of the four hydrogens in the carbon chain of the molecule. The structure for peak 4 was postulated as $CF_3(CF_2)_6{}^{14}CH_2CH_2COOH$, 2*H*,2*H*,3*H*,3*H*-perfluorodecanoic acid, a transformation product that has not been reported in the literature previously.

A standard for the postulated structure is not commercially available. Further support of the proposed structure was obtained from studies of a commercially available analog: 2*H*,2*H*,3*H*,3*H*-nonanoic acid ($CF_3(CF_2)_5CH_2CH_2COOH$). Detailed comparison of $^{19}$F NMR spectra, LC/MS daughter ion spectra, and electron impact (EI) GC/MS spectra of methyl and trimethyl silyl (TMS) derivatives of the transformation product (peak 4) derived from biodegradation studies conducted with nonlabeled 8-2 TBA and the analogue 2*H*,2*H*,3*H*,3*H*-nonanoic acid clearly supports the proposed structure for the unknown peak 4 (unpublished results). The quantities of the unknown compound (peak 4) generated in this study were not sufficient to obtain the above data. However, a very good match was obtained for the retention time and the daughter ion spectrum of the metabolite obtained from a nonlabeled 8-2 TBA biodegradation study and the peak 4 data from this study. However, matching all the spectral data obtained for the peak 4 metabolite with that of an authentic standard ($CF_3(CF_2)_6CH_2CH_2COOH$) is still required for a final structural confirmation of this new transformation product.

Among the unidentified $^{14}$C-labeled transformation products, no baseline resolved LC/ARC peak corresponding to $^{14}$C-labeled perfluorononanoic acid ($CF_3(CF_2)_6{}^{14}CF_2COOH$) was visible, suggesting that α-oxidation (*26*) of $CF_3(CF_2)_6{}^{14}$-$CF_2CH_2COOH$, the major transformation product, did not occur. This is consistent with the result of Dinglasan et al. (*19*), in which $CF_3(CF_2)_6CF_2COOH$ and $CF_3(CF_2)_4CF_2COOH$ were not detected in the experiment system. This conveys the notion that potential biodegradation of fluorotelomers in the environment may not lead to the formation of significant amount of odd-numbered perfluorinated carboxylic acids such as perfluorononanoic acid due to microbial α-oxidation.

**Mass Balance.** Compared with day 0, the total $^{14}$C radioactivity in the acetonitrile extract (from the first extraction) decreased continuously over 28 days in both the biodegradation test vessels and abiotic controls (Table 2). It was initially thought that the decrease was due to both adsorption to the test vessel surface and volatilization of the parent. However, after analyzing the $^{14}$C counts recovered from the glass surface and PTFE septa, it was shown that the decrease was actually caused by the strong adsorption of the parent to the PTFE septa of the sealed vessels. The $^{14}$C recovered from the septa was 8-2 TBA. After taking into account the $^{14}$C counts recovered from the septa, the mass balance of the parent plus all transformation products was nearly 100% (Table 2). This observation demonstrates that the potential volatilization of 8-2 TBA from the experimental systems was minimized at the presence of a strongly adsorbing matrix such as PTFE; otherwise, it is impossible to achieve a near 100% mass balance. The adsorption of $^{14}$C-labeled 8-2 TBA to the glass was minimal or at least was reversible as it can be easily recovered by acetonitrile at room temperature (Table 2). In this regard, although some of the original septa (days 14 and 28 for the biodegradation test vessels and day 28 for the abiotic control) were not saved after the first acetonitrile extraction for subsequent re-extraction and $^{14}$C counting, it is reasonable to assume that most of the $^{14}$C counts not recovered from the first extraction

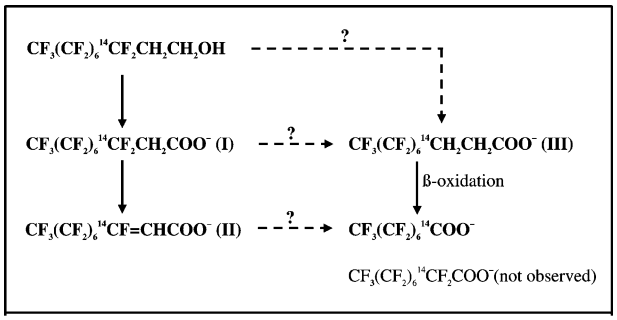


**FIGURE 3. Proposed biotransformation pathways of $^{14}$C-labeled 8-2 telomer B alcohol. The solid arrows indicate proposed transformation steps. The dotted arrows indicate potential transformation steps which may or may not be occurring.**

also reside in these septa. Also, available septum $^{14}$C counts data (Table 2) indicate that the degree of septum adsorption of 8-2 TBA increased over time. The adsorption to the PTFE septa was so strong that it took about 4 weeks at 50 °C to recover most of the $^{14}$C counts from the septa using acetonitrile. The parent, $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$, accounted for virtually all $^{14}$C counts recovered from the septa as revealed by LC/ARC analysis, indicating that 8-2 TBA was remarkably stable abiotically in aqueous solution under moderately high temperature. On the basis of the $^{14}$C counts of individual transformation products versus the total $^{14}$C applied at day 0, $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$ is clearly the most abundant transmation product accounting for about 27% of the mass balance at day 28 (Table 2). The $CF_3(CF_2)_6{}^{14}CF{=}CHCOOH$ was the second major transformation product accounting for about 6.0% of the mass balance at day 28. In contrast, $CF_3(CF_2)_6CF{=}CHCOOH$ was found to be the predominant metabolite in mixed bacterial cultures (*15*) and in microbial enrichment culture (*19*), in which a much higher bacterial density (microbial loading) in the test media and more favorable conditions may accelerate the conversion of $CF_3(CF_2)_6CF_2CH_2COOH$ to $CF_3(CF_2)_6CF{=}CHCOOH$, in comparison with the very low bacterial loading and organic carbon supply used in this study. The newly identified transformation product $CF_3(CF_2)_6{}^{14}CH_2CH_2COOH$ accounted for about 2.3% and $CF_3(CF_2)_6{}^{14}COOH$ accounted for about 2.1% of the mass balance at day 28. The parent still contributed about 57% of the mass balance at day 28, about 41% of which resulted from adsorption to the septa. It appears that the strong adsorption of the parent to the PTFE septa during the test reduced its bioavailability for microbial biodegradation.

**Defluorination of $^{14}$C-Labeled 8-2 TBA during the Biotransformation.** Under the test conditions, the increase of fluoride ion due to defluorination of the parent was not discernible from the background level of fluoride present in the test vessels. The level of calculated fluoride increase (0.9 $\mu$g L$^{-1}$ at day 7, 1.4 $\mu$g L$^{-1}$ at day 14, and 2.0 $\mu$g L$^{-1}$ at day 28 as compared with day 0) based on the biotransformation products formed is low as compared with that of the background level (day 0 level) of fluoride in the test vessels (13.7 $\pm$ 1.0 $\mu$g L$^{-1}$ for $n = 4$ samples) and, thus, made the increase indistinguishable from the background. This observation was consistent with the result that $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$ is the most abundant transformation product whose formation does not involve defluorination. On the other hand, a significant increase of fluoride ion concentration was observed when $CF_3(CF_2)_6CF{=}CHCOOH$ became a major transformation product, which involved defluorination of the nonlabeled 8-2 TBA (*15*).

**Biotransformation Pathways of $^{14}$C Labeled 8-2 TBA.** Several pathways may be available to convert the parent, $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$, to various observed transformation products (Figure 3). The first pathway for $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ transformation is conversion to $CF_3(CF_2)_6{}^{14}COOH$ most likely via HF elimination (*19*) and monooxygenase-mediated reactions. First, the parent can be oxidized to the major transformation product, $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$ (**I**), via alcohol and aldehyde dehydrogenase reactions. No $^{14}$C-labeled fluoroaldehyde ($CF_3(CF_2)_6{}^{14}CF_2CH_2CHO$) was detected with a LOQ of 0.5% of total mass, indicating that the fluoroaldehyde is either unstable or may be quickly oxidized by an aldehyde dehydrogenase before day 7 sampling. The fluoroaldehyde was detected as a transient intermediate (*19*), and its level cannot be quantified, indicating that the observed level may be low. After forming $CF_3(CF_2)_6{}^{14}CF_2CH_2COOH$, this acid may be further converted in multiple steps or via HF elimination to form $CF_3(CF_2)_6{}^{14}CF{=}CHCOOH$ (**II**) and then potentially to $CF_3(CF_2)_6{}^{14}COOH$ perhaps catalyzed by monooxygenase-mediated reactions, as also predicted by CATABOL (*5*). Both **I** and **II** are the principal stable transformation products and do not rapidly degrade under the test conditions.

A second transformation pathway may involve the $\beta$-oxidation of the newly identified transformation product, $CF_3(CF_2)_6{}^{14}CH_2CH_2COOH$ (**III**). First, the $^{14}$C-labeled 8-2 TBA parent may be converted in multiple enzymatic reactions to form $CF_3(CF_2)_6{}^{14}CH_2CH_2COOH$, although the mechanisms of these reactions are currently unknown. Incubation of 2 mg L$^{-1}$ of $CF_3(CF_2)_6CF_2CH_2COOH$ in mixed bacterial cultures resulted in the formation of only trace amount of $CF_3(CF_2)_6CH_2CH_2COOH$ (N. Wang and B. Szostek, unpublished results), suggesting that some other transformation products may also contribute the formation of $CF_3(CF_2)_6{}^{14}CH_2CH_2COOH$ via multiple reactions. Because $CF_3(CF_2)_6{}^{14}CH_2CH_2COOH$ is a likely fatty acid analogue, it can be further oxidized via $\beta$-oxidation reactions. However, direct experimental evidence is still needed to further confirm this hypothesis.

Although long-chain fluorinated carboxylic acids can induce the proliferation of peroxisomal $\beta$-oxidation in rats and mice (*27*), it is not clear whether such acids can be directly used as substrates for the $\beta$-oxidation reactions (*28*, *29*). It has been proposed that 8-2 saturated acid and 8-2 unsaturated acid can be used as substrates for $\beta$-oxidation to form PFOA and that the $\beta$-oxidation pathway may be a principal fate of fluorotelomers such as 8-2 telomer B alcohol (*19*). However, based on current knowledge regarding the enzymology of this pathway, a direct $\beta$-oxidation of 8-2 saturated acid and 8-2 unsaturated acid cannot occur. The $\beta$-oxidation pathway for fatty acids is well-understood and is illustrated by Nelson and Cox (*29*). As Nelson and Cox have stated, in one pass through the $\beta$-oxidation sequence, one molecule of acetyl-CoA, two pairs of electrons, and four protons (from $C_2$ and $C_3$ carbons) are removed from the long-chain acyl-CoA, shortening it by two carbon atoms. Because 8-2 saturated acid contains no H atoms in its $\beta$-carbon ($C_3$ carbon) and 8-2 unsaturated acid contains only one H atom, $\beta$-oxidation of these two acids cannot occur because the proton deficiency prevents the proton/electron shuffling that is essential for the completion of the reactions. Also, since the $\beta$-carbon of 8-2 saturated acid is already highly oxidized with two fluorine atoms attached, it is unlikely that any direct oxidation of this carbon can occur under aerobic conditions. On the other hand, the $\beta$-oxidation may occur via the newly discovered metabolite, 2*H*,2*H*,3*H*,3*H*-perfluorodecanoic acid.

The third possible transformation pathway may be related to the formation of unidentified transformation products. Since each of the unidentified transformation products in general contributed less than 1% of the mass balance, it is extremely difficult to identify their molecular structures. However, formation of these transformation products may implicate some new pathways not yet understood. Compared with perfluorinated acids, the ethanol spacer ($-CH_2CH_2OH$) of $CF_3(CF_2)_6{}^{14}CF_2CH_2CH_2OH$ makes this molecule more

flexible than a perfluorinated acid and could enhance its molecular accessibility to an active site of a given enzyme. This, in turn, could lead to the formation of certain unique metabolites that would not normally occur when a fully fluorinated acid is being used as a starting material for microbial biodegradation. In mixed bacterial cultures, $CF_3(CF_2)_6CF_2CH_2CH_2OH$ can be converted to perfluorohexanoic acid [$CF_3(CF_2)_4COOH$] with about 0.4% of the mass balance (*15*), implying a mechanism for defluorination from the perfluorinated portion of C−F bonds. When $CF_3(CF_2)_6$-$^{14}CF_2CH_2CH_2OH$ was incubated with a high concentration (330 mL of sludge $L^{-1}$ medium) of activated sludge from the same source as for this study, $^{14}CO_2$ release contributed to about 0.4% of the mass balance, indicating a decarboxylation mechanism from the fluorinated $\beta$-carbon. In microbial enrichment culture (*19*), all the metabolites (8-2 saturated and 8-2 unsaturated acids plus PFOA) together accounted for 55% of total mass balance at day 81. The portion of the missing part (45%) may include the metabolites that resulted from the degradation of 8-2 unsaturated acid by some other unknown mechanisms. Also, the PFOA level reduced significantly at day 81 (3% of total mass) as compared with at day 52 (approximately 6% of total mass) (*19*). Does this indicate that PFOA may have been further transformed by the microbial culture? All above evidences so far raise the possibility that alternative pathways may be available for degradation of 8-2 TBA beyond PFOA. Therefore, multiple pathways and enzymes, such as $\beta$-oxidation of 2*H*,2*H*,3*H*,3*H*-perfluorodecanoic acid, HF elimination and monooxygenase-catalyzed oxidation of 8-2 saturated and 8-2 unsaturated acids and other unknown mechanisms may determine the biological fate of 8-2 telomer B alcohol under the study conditions. This report indicates that additional work needs to be done to clarify the prevalence and availability of the pathways identified and their products in a range of microbial cultures, conditions, and time frames to truly define the ultimate, stable, microbial biodegradation products.

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