Journal of Hazardous Materials 480 (2024) 136426

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Defluorination of various perfluoro alkyl acids and selected PFOA and PFOS monomers by *Acidimicrobium* sp. Strain A6 enrichment cultures

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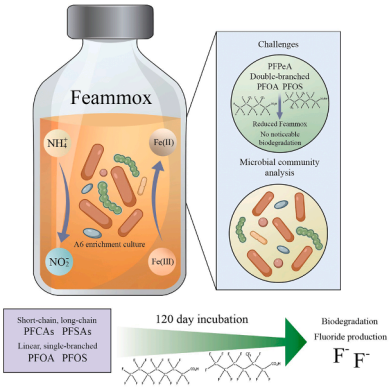
HIGHLIGHTS

- Shorter PFAAs produced during the degradation of PFOA/PFOS by A6 are also degradable.
- Of these shorter PFAAs, PFPeA is degraded by far the slowest.
- Linear and single branched PFOA/PFOS monomers are degradable by A6.
- Double branched PFOA/PFOS monomers are either not degradable or degrade very slowly.
- The presence of PFAAs has a strong impact on the microbial community structure.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Acidimicrobium sp. Strain A6
PFAS biodegradation
PFOA
PFOS
Linear
Branched
Perfluoroalkyl acids
Microbial community

ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) have emerged as a diverse class of environmental pollutants, garnering increasing attention due to their various structural types and potential ecological impacts. The impact of select PFAS on environmental microorganisms and the potential for microbial degradation of certain PFAS are timely research topics. In this study, we conducted a series of batch incubation to investigate the effects of C_4-C_{10} perfluoroalkyl carboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs), as well as linear and branched perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) monomers, on the Feammox reaction and *Acidimicrobium* sp. A6 (A6), a microbe known to degrade PFOA and PFOS. We explored the defluorination ability of A6 cultures with these PFAS, evaluating their response to varying chemical structures. While A6 cultures demonstrated the ability to degrade a wide range of PFAAs (11.5–56.9 % reduction over 120 days), challenges were noted with specific compounds like PFPeA and double-branched PFCAs and PFSAs, which also showed reduced ammonium removal. Additionally, exposure to the selected PFAS resulted in notable shifts in the microbial community within the A6 enrichment cultures, indicating a selective pressure that benefits certain strains (e.g., increased percentages of *Acidimicrobium*, *Paraburkholderia*, and *Desulfosporosinus* in several PFCA, PFSA and PFOA/PFOS monomers enriched cultures). These insights contribute to our understanding of

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https://doi.org/10.1016/j.jhazmat.2024.136426
Received 29 July 2024; Received in revised form 8 October 2024; Accepted 5 November 2024
Available online 6 November 2024

microbial-PFAS interactions and are instrumental in developing bioremediation strategies for PFAS-impacted environments.

1. Introduction

Per- and Polyfluoroalkyl Substances (PFAS) are a group of man-made chemicals that have been used in various industrial and consumer products for their oil and water-repellent properties [1,2]. These compounds, often referred to as "forever chemicals," are characterized by their persistence in the environment and resistance to natural biodegradation processes. Such persistence poses significant challenges and has led to potential environmental concern. Certain PFAS have been detected in various environmental matrices, including water, soil, and air [3–5]. According to recent research, once released into the environment, select PFAS can travel long distances due to atmospheric deposition and transport through water, often reaching remote and pristine areas [6]. The environmental concern is compounded by the bioaccumulative nature of certain PFAS compounds. These substances have been found in various species, including fish, birds, and mammals, potentially posing a risk to ecological systems and human health [7].

The potential impact of PFAS on the environment is increasingly becoming a focal point of research, including their effect on microbial communities. Microorganisms play a crucial role in ecosystem functioning, and the introduction of PFAS may alter the structure and functionality of these microbial communities [8]. Studies have shown that exposure to certain PFAS can lead to reduced microbial diversity and abundance in various environments, including Antarctic coastal waters, soil, wetland sediment, and activated sludge from wastewater treatment plants [9–12]. At higher concentrations (e.g., >100 μg kg^{-1} dw), PFAS may also inhibit the growth of a broader range of microbial taxa, thereby reducing diversity [13]. Conversely, other studies also noted increases in microbial diversity in the presence of PFAS. Specifically, under the long-time exposure to perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), there was an observed increase in the alpha-diversity of microbial communities in soils [14]. A consensus emerging from all these studies is that exposure to certain PFAS can potentially create niches that select for a variety of tolerant species. While certain studies may show that the overall microbial abundance may decrease under the stress of PFAS, there is a notable increase in the abundance of certain bacteria that exhibit a more positive response to certain PFAS. Bacteria such as *Proteobacteria*, *Burkholderiales*, and *Rhodocyclales* in soils, along with *Acinetobacter*, *Pseudomonas*, and *Arthrobacter* in groundwater, were observed to be more abundant in samples exposed to PFAS [9,14]. The response of microbial communities to PFAS also varied depending on the type of PFAS compound and its concentration.

Microbial degradation of non-polymeric PFAS could constitute a crucial aspect of how microorganisms respond to exposure to these compounds. This process, which entails the defluorination and eventual breakdown of PFAS by microbial activity, results from the cleavage of carbon-fluorine (C-F) bonds to release fluoride ions (F^-) and the degradation of parent PFAS molecules. The microbial-mediated cleavage of C-F bonds in PFAS compounds has long been deemed challenging [15]. Recent studies have increasingly provided evidence of microbial degradation and defluorination, with both mixed and pure microbial cultures showing the ability to degrade per- and polyfluoroalkyl substances under both aerobic and anaerobic conditions. Most reported biodegradation or biotransformation studies have utilized mixed cultures degrading shorter-chain (C_4-C_5) fluorinated carboxylic acids derived from activated sludge [16], PFOA from digestion sludge [17], 6:2, 4:2 fluorotelomer alcohols (FTOH), and 2-(trifluoromethyl)acrylic acid from landfill soil [18], and 6:2 fluorotelomer sulfonate (6:2 FTSA) in aerobic and anaerobic sediments [19]. *Pseudomonas* sp. appear to be a promising candidate for the biodegradation of PFOA and PFOS, as well as C_7-C_{10} PFCAs [20–24]. Research conducted by Shaw et al. also revealed that the aerobic *Gordonia* sp. strain NB4–1Y can effectively degrade 6:2 FTAB and 6:2 FTSA, achieving degradation efficiencies of 85% and 88%, respectively, within a week [25].

However, PFAS might end up in anoxic environments, like relatively saturated soils, sediments, groundwater, where breakdown processes would have to rely on anaerobic microorganisms. A study by Huang and Jaffé (2019) demonstrated via a rigorous mass balance that PFOA and PFOS were degraded anaerobically by *Acidimicrobium* sp. Strain A6 (A6) [26]. A6 is an autotroph that uses either NH_4^+ or H_2 as the electron donor and ferric iron phases as the electron acceptor [27] in a process that has been termed Feammox. Degradation of perfluoroalkyl acids (PFAAs) by A6, with the production of smaller (i.e., shorter carbon-chain) PFAAs and F^-, has now, in addition to the initial study by Huang and Jaffé (2019), been observed in multiple incubations and different settings, including in biosolids [28], in microbial electrolysis cells [29], and in the presence of ferrihydrite treated with polyacrylic acids [30], which enhanced the overall Feammox and PFOA degradation activity. Biodegradation/biotransformation of various polyfluorinated compounds (6:2 FTS, 8:2 FTS, 6:2 FTOH, 8:2 FTOH, 8:2 diPAP) by the A6 enrichment culture has also been observed [31], although the role of A6 in these processes has not been investigated further. Kinetics of defluorination of PFOA and PFOS by A6 showed that the defluorination rate is directly proportional to the rate of NH_4^+ oxidation and first order with respect to the PFOA/PFOS concentration [32]. In addition to the production of smaller PFAAs, the production of H-PFOA during the degradation of PFOA was also observed [33]. Based on the incubation results with the A6 enrichment cultures, it is hypothesized that after A6 removes a fluorine atom from PFOA, other microbes, likely heterotrophs, can then further degrade the partially defluorinated compounds. This underscores the importance of further investigating how different organisms within the microbial community (including the A6 enrichment culture) interact and contribute to the PFAS degradation process.

There are many ongoing efforts to understand the underlying mechanisms of all the different non-polymeric PFAS degradation processes. While various hypotheses for biodegradation mechanisms remain somewhat unclear (with some showing fluoride ion production, others intermediate products, and some none), experimental results have indicated the potential for biodegradation of several PFAS. This is encouraging for further research and development of novel biodegradation technologies.

Our various studies to-date have demonstrated that A6 is capable of degrading PFAAs such as PFOA, PFOS, and PFHxS [26,28–30,33]. The objectives of this work were to:

(i) Determine whether the shorter carbon-chain PFAAs that are produced during the degradation of PFOA/PFOS are also degradable by the A6 enrichment culture.
(ii) Determine how the structure of the PFOA/PFOS monomers (i.e., linear, single branched, double branched) affects the degradation of these compounds by A6.
(iii) Examine the impact of the presence of PFAAs on the abundance and structure of the microbial community within the A6 enrichment culture, and identify organisms in that enrichment culture that have been associated with the degradation of polyfluorinated alkyl substances.

2. Methods and materials

2.1. Chemicals

The degradation by the *Acidimicrobium* sp. A6 enrichment culture of 11 different perfluoroalkyl compounds plus different PFOA and PFOS monomers (see Table S1 for detailed information) was investigated in this study. These compounds were purchased from Wellington, Inc., Sigma-Aldrich, and Manchester Organics, Ltd. Stock solutions of the various PFAAs investigated were prepared in DI water/HPLC grade methanol at concentrations of ~2 ppm for PFOA/PFOS monomers, and ~10 ppm for the individual perfluoroalkyl carboxylic acids (PFCAs), and perfluoroalkyl sulfonates (PFSAs). All the stock solutions were stored at 4 °C.

2.2. Setup of the anaerobic incubation experiments

PFAA degradation by *Acidimicrobium* sp. A6 has been observed for both pure and enrichment cultures, but since *Acidimicrobium* sp. A6 is more active in enrichment cultures, indicating a synergistic interaction with other organisms, incubations described here were done with an *Acidimicrobium* sp. A6 enrichment culture. The enrichment culture was maintained under anaerobic conditions in an inorganic Fe(III)-NH_4^+ enrichment medium [26,27], and acclimated to 10 ppm PFOA for 180 days. To avoid carryover of PFOA, the culture was centrifuged and then the supernatant was replaced, which was repeated twice before the culture was added to the serum bottles containing the growth medium and the selected PFAAs at their specific concentrations.

In the study examining various PFOA/PFOS monomers, the *Acidimicrobium* sp. A6 enrichment culture, containing about 10^6 copies/mL of *Acidimicrobium* sp. strain A6 as determined using qPCR, was mixed with the inorganic Fe(III)-NH_4^+ medium in a 1:9 vol ratio. This mixture was then incubated in a series of 15 mL and 60 mL vials, maintaining 50% headspace. In the 15 mL vials, each PFOA/PFOS monomer was added to the culture to reach a final concentration of ~100 ppb. Similarly, for incubations involving C_4-C_{10} PFCAs and PFSAs in 60 mL vials, the respective compounds were introduced to achieve a final concentration of approximately 10 ppm. For the incubations described herein, the initial concentration of Fe(III) and NH_4^+ was around 365 mg/L and 50 mg/L, respectively. Additionally, the background concentration of as F^- was about 60 ppb.

Then all the vials were sealed with butyl rubber stoppers, and a N_2/CO_2 (80:20) gas mixture in the headspace to achieve anoxic conditions and to provide additional CO_2 for the growth of A6. Abiotic and heat-inactivated biomass controls were set up in parallel for all these incubations, and in the same manner, as described above. A biotic control, but without added PFAA was also run in parallel to determine if the presence of various PFAA had a negative effect on the Feammox activity by A6. Each treatment was repeated in triplicate, and the results were averaged. During the 120-day incubations, which were conducted at ambient temperature (25 °C), three vials were sacrificially sampled every 30 days and the chemical and microbial characteristics were analyzed.

2.3. Chemical analyses

A 1 mL slurry sample from both day 0 and day 120 was extracted using a 50:50 methanol solution. Extracts were then centrifuged for 2 min at 4000 rpm, and the supernatant was then filtered through 0.22 a μm membrane filter. PFAA analyses were then performed on a liquid chromatograph coupled to a single quadrupole mass spectrometer (LCMS-2050 Shimadzu), in accordance with EPA Method 537.1 [34,35]. Filtered aliquots (1 μL) were injected into the LC/MS system, which is equipped with a Shimpak GIST C18 (delay column, 5 μm, 3.0 × 50 mm) and Shimpak Velox C18 column (I.D. 2.1 mm, length 50 mm, particle size 2.7 μm) from Shimadzu. This was done at a flow rate of 0.4 mL min^{-1} at 40 °C, enabling the separation of parent compounds and their tentatively identified metabolites among shorter-chain PFAAs. The gradient for the mobile phase in liquid chromatography was programmed as follows: Initially, the mobile phase consisted of 95% solvent A (5 mM ammonia acetate in water) and 5% solvent B (pure methanol), maintained for 0.5 min. The concentration of solvent B was then gradually increased to 95% over 12 min, held steady for 2 min, subsequently reduced to 10% in 1 min, and finally maintained for an additional 4 min. The mass spectrometer operated in negative-ion electrospray mode. Quantitative analysis relied on certified standards for native compounds and isotope-labeled internal standards, obtained from Wellington Laboratories, Inc., with a purity of 98% or higher. LC/MS identification of PFAS depended on matching retention times with certified standards (Table S2). Also, 2 mL subsamples collected on day 0, 30, 60, 90, were centrifuged filtered through a 0.22 μm membrane filter, then the quantitative analysis of PFAAs was performed using High-Performance Liquid Chromatography coupled to tandem Mass Spectrometry (HPLC-MS-MS: Agilent 1290–6430 A), by SGS-CSTC Standards Technical Services Co., Ltd (China) according to well-established methods [36,37], to assess the trend in PFAS concentration changes. 2 mL liquid samples were filtered (0.2 μm) and then NH_4^+ were analyzed via Ion Chromatography (IC-3000, Dionex), with an CS-16 column (flow rate = 1.00 mL min^{-1}, detection limit = 0.01 mg/l) [26]. F^- was measured via IC using an AS-22 column (flow rate = 1.20 mL min^{-1}, detection limit = 0.012 mg/l). Analyses were then repeated using an AS-18 column (flow rate = 1.00 mL min^{-1}, detection limit = 0.01 mg/l). Dionex™ Retention Time Standards for all the anion analysis were used. As an additional confirmation, F^- was also analyzed using a perfectION™ combination electrode (Mettler-Toledo, USA) [26]. For Fe analysis, slurry sample were extracted with 0.5 mM HCl for 24 h, then Fe(II) concentration was measured spectrophotometrically with ferrozine at 560 nm [38].

2.4. 16S rRNA Sequencing on Illumina Miseq platform and data processing

DNA was extracted from 1 mL sub-samples, following the procedure described in the manual of the Fast DNA spin kit (Bio 101, Qbiogene Inc. CA, USA). The concentration and purity of the isolated total DNA were checked using a Qubit@ 2.0 Fluorometer (Thermo Scientific). The V4 region of the 16S rRNA gene of bacteria was amplified using primer-set 515f-806r [39]. All PCR reactions were carried out with a Phusion® High-Fidelity PCR Master Mix (New England Biolabs), and the PCR products were quantified and purified before sequencing. Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina MiSeq platform at Novogene Co. (Beijing, China) and 250 bp paired-end reads were generated.

Raw data were analyzed using QIIME2 version 2022.11 [40]. Briefly, forward and reverse paired-end reads were trimmed for quality and the Illumina adaptors were removed using Dada2 [41], as implemented in QIIME2. Subsequently, denoised data were clustered and classified against the Greengenes2 database [42] to assign the appropriate taxonomy for every Amplicon Sequence Variant (ASV). For certain ASVs of interest, manual classification using NCBI's nucleotide Basic Local Alignment Search Tool (BLAST, 40) was performed. Diversity data were computed directly in QIIME2, while ASV counts at the genus level were used for subsequent analyses. Sequences are available from the NCBI Sequence Read Archive under BioProject PRJNA1140605.

2.5. Real-time quantitative PCR (qPCR) analysis

qPCR analyses were used to quantify total bacterial numbers, and *Acidimicrobiaceae* bacteria, with primers 1055 f(5′-ATGGCTGTCGTCAGCT-3′) −1392r(5′-ACGGGGCGGTGTGTAC-3′) and acm_v1F (5′-

GGCGGCGTGCTTAACACAT-3′) and acm_v1R (5′-GAGCCCGTCCCA-GAGTGATA-3) [43–45], respectively. The qPCR analysis of each gene was done on a 96-well StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA), followed detailed qPCR protocol as well as quality control were as described previously [44,45]. The PCR amplification efficiencies were 91–105%, and coefficient of determination (R^2) for all assays were > 0.99.

2.6. Statistical methods

Significance for all tests was accepted at the 0.05 level and was performed using SPSS v12. Alpha diversity and richness indices (Observed-species, Chao1, Shannon, Simpson, ACE, Goods-coverage) were estimated using QIIME2 version 2022.11 [40].

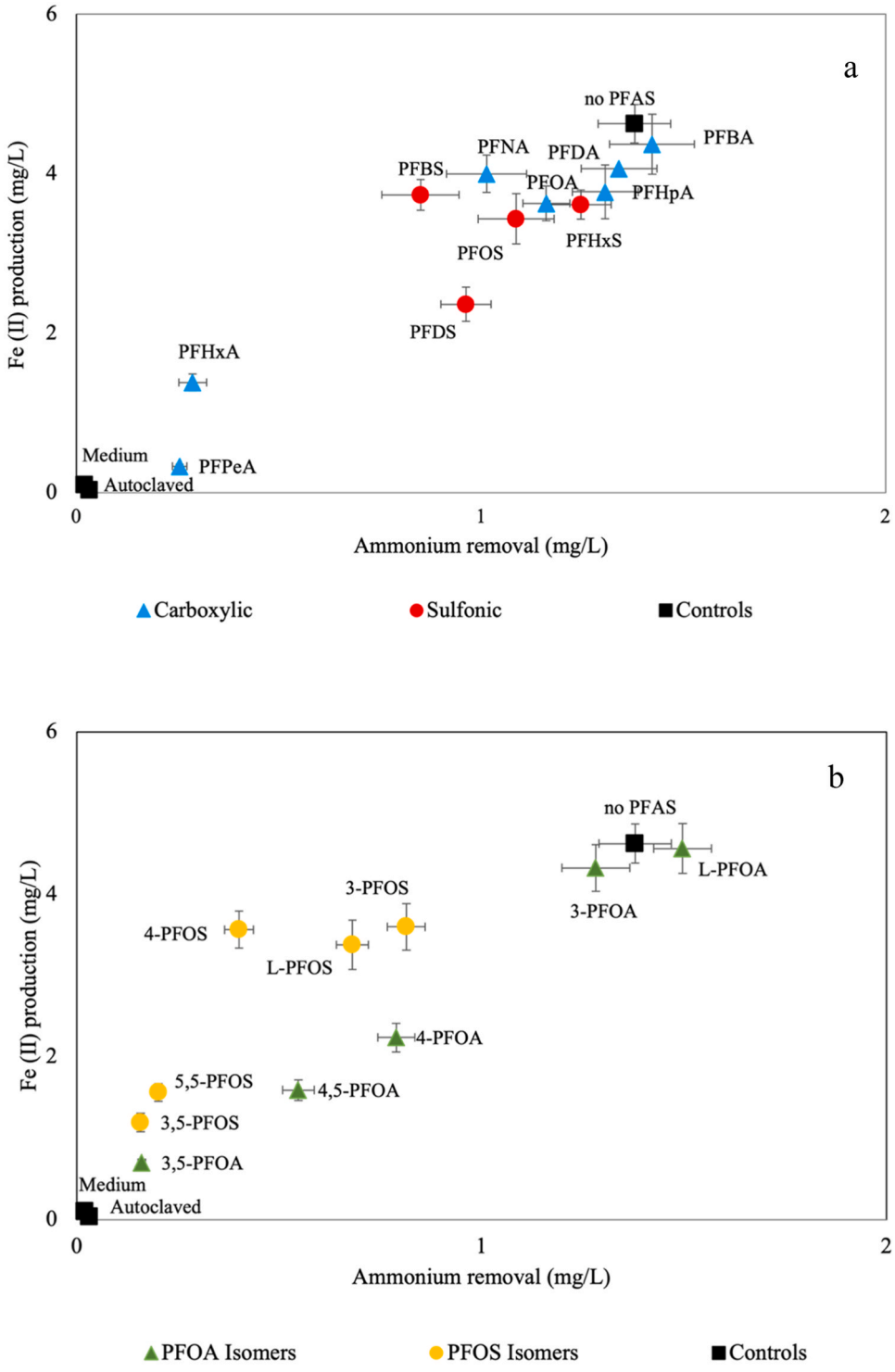

Fig. 1. NH_4^+ oxidation and Fe (II) production after 120-day incubations with various PFAS.

3. Results and discussion

3.1. Feammox activity in A6 enrichment incubations with various PFAAs

Feammox activity was evaluated by tracking NH_4^+ oxidation and iron reduction (production of Fe (II)), as well as the changes in the abundance of *Acidimicrobium* sp. A6 during the incubations [27]. In the incubations without PFAAs, about 51.1%–62.2% of NH_4^+ was removed during the 120-day incubations (Fig. 1c). In the incubations amended with 10 ppm C_4-C_{10} PFCAs or PFSAs, the NH_4^+ removal ranged from 28.3% to 49.6% after 120 days of incubation, except for the incubations spiked with PFHxA and PFPeA, which exhibited a lower NH_4^+ removal of 11.7% and 9.9%, respectively (Fig. 1a). In cultures amended with the various PFOA/PFOS isomers at concentrations of 100 ppb, 29.3%–56.7% NH_4^+ was removed during incubations, except for incubations spiked with 3,5-PFOA, 3,5-PFOS, and 5,5-PFOS, which had NH_4^+ removal of less than 10% (Fig. 1b). The background concentration of Fe (II), which was due to carryover from the inoculation with the A6 enrichment culture, ranged from 3.9 mg/L to 11.8 mg/L and was determined prior to initiating the incubations. After 120 days of incubation, Fe(II) increased by more than an order of magnitude, up to 198.2 mg/L-300.9 mg/L in the no PFAA incubations, and 77.2 mg/L-287.4 mg/L in samples spiked with the different PFAAs. In the abiotic controls, neither NH_4^+ removal nor Fe(II) production was observed over the incubation time (Fig. 1a,b).

Comparing the total microbial abundance in cultures with different treatments after 120 days shows that cultures with PFAAs generally had lower overall microbial abundance than the no PFAS controls. After inoculation on day 0, the initial A6 numbers in the incubations ranged from 0.1 x 10^7 copies/mL to 0.6 x 10^7 copies/mL, with A6 consisting of 56.1% ~ 65.2% of the total bacterial population (Fig. 2). During the 120-day incubation, changes in the number and proportion of A6 varied between experiments with different PFAA treatments. Overall, the percentage of A6 in total microbial abundance increased, especially in incubation with PFAAs, as shown in Fig. 2. This increase in both the quantity and proportion of A6 in all treatments indicates that, despite PFAS impacting Feammox activity, A6 continues to thrive and becomes the predominant species in this enrichment culture when exposed to these compounds. This suggests that PFAAs may have a positive impact on the overall numbers of certain microorganisms that can tolerate them, with A6 being a prime example of such resilience.

3.2. Potential Defluorination of C_4-C_{10} PFCAs and PFSAs

Whereas sorption of PFOS onto ferrihydrite is important in DI water, it was shown to be negligible in the Feammox growth medium [46], and no loss due to sorption in the incubation vials with the Feammox medium was observed for PFOA and PFOS in the incubations described by Huang and Jaffé , 2019 [26]. Additionally, the removal of PFAAs was quantified by comparing their concentrations in the biologically active vials to those in vials containing medium and ferrihydrite but lacking an active Feammox process. After 120 days of incubation, the concentration of PFCAs and PFSAs did not show a significant change, and no significant increases ($p > 0.1$) in F^- were observed in the samples from the abiotic control groups (Fig. 3), indicating that these PFAAs did not degrade abiotically under the conditions tested in this experiment. Although not shown here, previous incubations with *Geobacter sp*. in a Feammox medium with acetate, showed that as the ferrihydrite phase was being reduced and slowly converted to magnetite, there were no abiotic transformations of PFOA/PFOS, nor changed in sorption [26] in that specific growth medium. In the incubations with the active enrichment culture, the concentrations of the various C_4-C_{10} PFCAs decreased over the 120-day anaerobic incubations (Fig. 3), except for PFPeA, for which the concentration did not show a statistically significant decrease in concentration with time. In these PFCAs degradation experiments, the concentration of the long-chain PFCAs (PFDA and PFOA, Fig. 3) decreased the most during the 120-day incubation (6.63 mg/L and 5.91 mg/L, respectively), followed by PFHpA (5.63 mg/L) (Fig. 3a). Of the PFSA incubations, the PFOS concentration decreased 3.99 mg/L after 120 days of incubation, which was higher than the decrease in the concentration of PFBS and PFHxS (1.89 mg/L and 1.49 mg/L, respectively) (Fig. 3b).

The production of F^- is an indicator for the PFAA defluorination process (Fig. 3). For experiments where the pH was controlled carefully, and intermediates measured, a fluorine balance has been achieved during the degradation of PFOA and PFOS [26]. No additional fluorine balance was performed here since intermediates were not measured in all incubations and the pH was not controlled. Although a comprehensive analysis of the F^- balance was absent, this experiment showed a clear correlation between F^- production and PFAA decrease. A more substantial increase in F^- was observed in the incubation containing long-chain PFAAs. These findings also reveal that within the A6 enrichment culture, most C_4-C_{10} PFAAs were subjected to differing

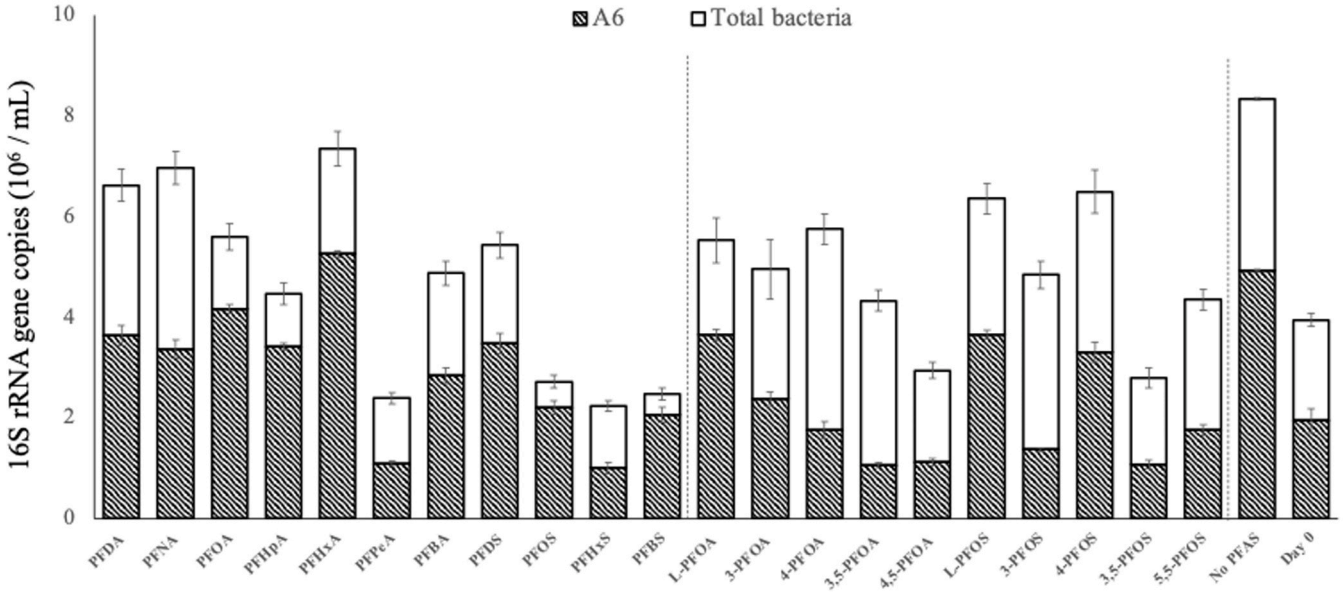

Fig. 2. A6 numbers after 120-day incubations in the presence of different PFAS. (Results of day 0 and the no PFAS control were included).

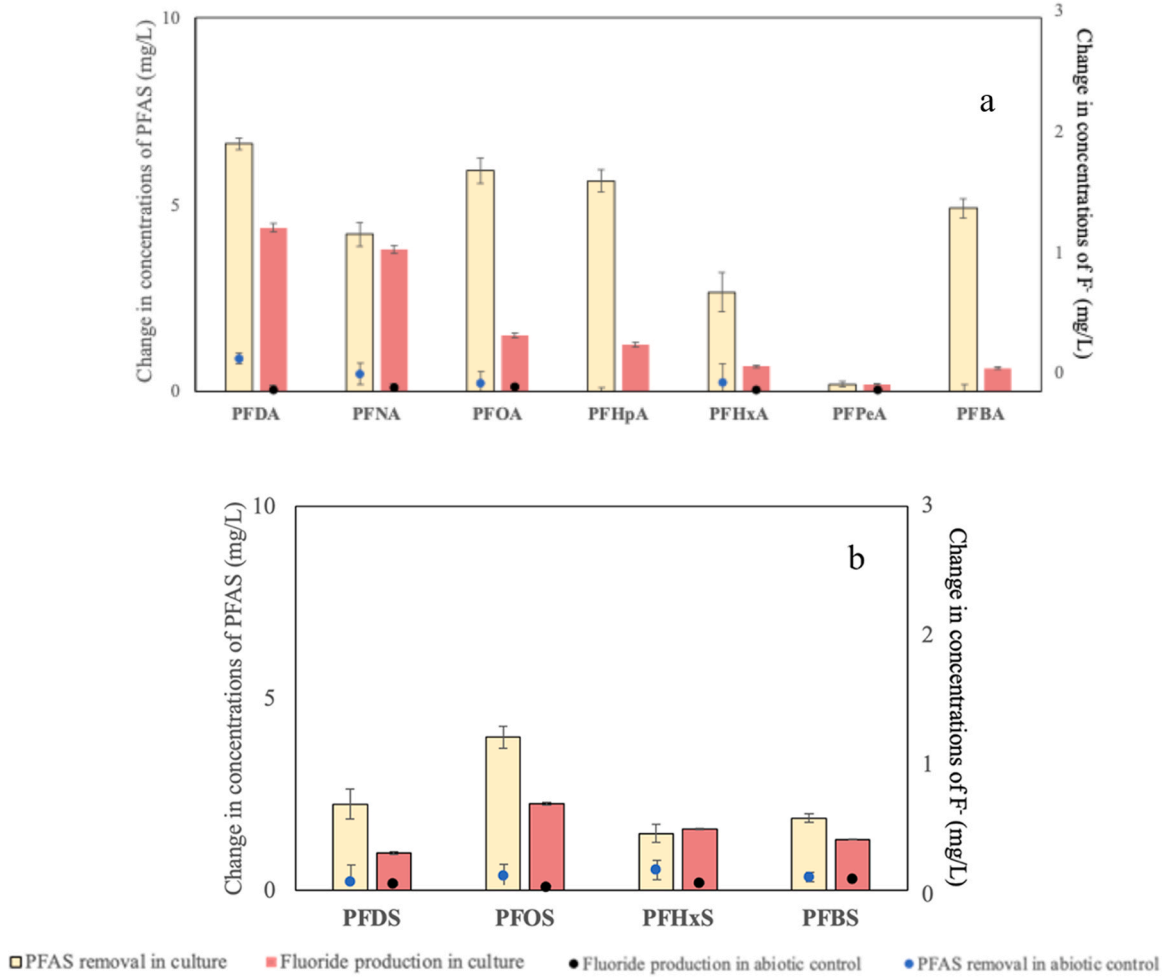

Fig. 3. Change in concentration of PFAS and F^- after 120-day incubations with PFCAs or PFSAs.

extents of degradation or removal. Additionally, this suggests that the shorter carbon-chain PFAAs generated during the degradation of PFOA/PFOS are likely intermediates and not final products.

The decrease in PFPeA concentration, shown in Fig. 3a, was not statistically significant throughout the 120-day incubation period, although there was a minor production of F^- observed (~60 ppb). The distinct degradation pattern of PFPeA, in contrast to other examined PFCAs, prompted further investigation into the structure of the PFPeA monomer. To confirm its linearity, F NMR analysis was conducted, verifying that the PFPeA monomer used was linear. The specific pathways of PFPeA degradation remain unclear. Previous research has shown that the degradation of PFOA can result in the formation of various shorter-chain PFAAs, with PFBA frequently appearing at similar or greater concentrations compared to PFPeA [26,29,30,33]. Given the slower degradation rate of PFPeA relative to PFBA reported here, this suggests that PFBA formation might not solely occur through sequential carbon chain shortening but could also involve cleavage within the PFOA carbon chain. To determine the location of the PFOA carbon that is being defluorinated, samples from incubations where H-PFOA production was observed were analyzed via a method that combines nano electrospray ionization and high-resolution mass spectrometry (Nano--ESI-HRMS) [47]. Results of these analyses indicate that the defluorination of PFOA by A6 may occur at the α carbon as well as the ε or δ carbons, which are also the carbons with the lower C-F energy bonds [48]. The weaker signal for defluorination at the α carbon than that for the ε or δ carbons could mean that either less defluorination occurred on that carbon or that this H-PFOA was more reactive and hence its concentration was lower.

It was hypothesized, based on previous experimental results comparing PFOA and PFOS defluorination by a relatively pure A6 culture and an A6 enrichment culture, that A6 can partially defluorinate a PFAA molecule, after which other organisms can then degrade this partially defluorinated compound further [31]. Then, based on the findings that defluorination of PFOA can occur at the α carbon, the partially defluorinated compound could undergo a decarboxylation via breakage of the respective carbon-carbon bond, followed by a carboxylation of this seven-carbon intermediate compound, producing PFHpA. At the same time the PFOA molecule is also defluorinated at the ε or δ carbon, resulting in the production of PFBA, which would explain the higher buildup of PFBA than PFPeA. As shown in the results presented here, PFPeA degrades much slower than PFBA, and a higher buildup of PFBA could not be explained via a gradual decrease in the carbon chain length. As of now, we have not been able to detect intermediate compounds that are able to verify this pathway, and further research is needed to demonstrate this mechanism.

Although the location of the carbon that was defluorinated was not investigated, the production of H-PFHpA, H-PFHxA, H-PFPeA, and H-PFBA was also observed in incubations with mixtures of PFHpA, PFHxA, PFPeA, and PFBA [48]. Hence, we hypothesize that in A6 enrichment culture incubations, as described above, PFAAs can gradually undergo defluorination on the α carbon, followed by decarboxylation and then carboxylation, resulting in the next shorter carbon-chain length PFAA.

3.3. *Defluorination of branched vs. linear PFOA and PFOS*

During the 120-day incubations of various PFOA isomers at a

concentration of 100 ppb, cultures supplemented with the 3,5- and 4,5-PFOA isomers did not show a change in concentration of these PFOA monomers, with no statistical difference in the PFOA concentrations between the biologically active vials and their respective autoclaved and medium controls. Conversely, in incubations that involved linear and single-branched PFOA isomers, a decrease in the PFOA concentration from 17.2% to 46.2% was observed over the 120-day incubation period, as shown in Fig. 4a. The results for the PFOS incubations mirrored the observations made with PFOA to some extent. PFOS removal ranged from 14.5% to 40.1% over a 120-day period for incubations, as illustrated in Fig. 4c, and as was the case for the double-branched PFOA monomers, the double-branched PFOS isomers, 3,5- and 5,5-PFOS did not exhibit a significant decrease in concentration over the incubation time, nor when compared to the abiotic controls by the end of the incubation period, as shown in Fig. 4d. When conducting experiments with lower PFAA concentrations, particularly those focused on the different linear vs. branched PFOA and PFOS monomers, it was observed that the F^- production remained below 60 ppb. This value is close to the F^- background concentration, thus rendering accurate quantification of F^- challenging, although the measured F^- production may still be used as an additional indicator of PFOA and PFOS defluorination. In these incubations, it is observed that the concentrations of individual PFOA or PFOS isomers remained unchanged after a 120-day incubation in the abiotic control groups. As depicted in Figs. 4b and 4d, this consistency emphasizes that under the conditions tested, no notable abiotic degradation or transformation of either PFOA or PFOS occurred.

In summary, these results indicate that linear and single-branched PFOA and PFOS isomers undergo degradation over time, which is supported by the observed decrease in concentration and concomitant F^- production. However, the double-branched PFOA and PFOS isomers remain notably stable throughout the 120-day incubation period, exhibiting no significant production of F^-. The underlying reason for this stability in double-branched isomers remains elusive. Yet, it's noteworthy that the Feammox reaction exhibited diminished activity in the presence of these double-branched isomers compared to their linear counterparts, as evidenced in Fig. 1. Interestingly, the biodegradation of PFOS observed in this study was similar for both linear and single-branched isomers of PFOS (Fig. 3). This is different from the typically abiotic degradation pathway of PFOS, where linear isomers typically remain largely unchanged in comparison to their branched counterparts [49,50].

3.4. *Effect of PFAAs on the microbial community dynamics in these enrichment culture incubations*

To investigate how the presence of different PFAAs affects the microbial communities of A6 enrichment cultures, and whether different PFAAs have different effects on their compositions, high throughput 16S rRNA gene sequencing was performed for both the control without PFAAs and the incubations with various PFCAs/PFSAs at different times.

Sequencing of the 16S rRNA produced a total of 926057 reads, and more than 86% of the sequences were retained from each sample after quality trimming and chimera checking (Table S3). Microbiome analyses showed *Ferrimicrobium* as the dominant genus for most conditions, especially at 120 days, with relative abundances ranging from 64% for the no-PFAA incubation, up to 87% for the enrichments with PFBS (Fig. 5). BLAST of this *Ferrimicrobium* ASV against the one from *Acidimicrobium* sp. A6 (NCBI ID MG589453, [27]) showed 100% sequences' similarity, confirming the importance of A6 as primary member of the microbial community involved in PFAA degradation. Additionally, A6 was the ASV with the highest statistical correlation (P-value = 0.006) with F^- production. At the same time, several other genera were enriched during the course of the incubations, sometimes only transiently, especially at 60 days. Among these, the genus *Paraburkholderia* was often present at ~10%–15% relative abundance in most of the enrichments, except the ones with C_4-PFAA (PFBS and PFBA) and was significantly

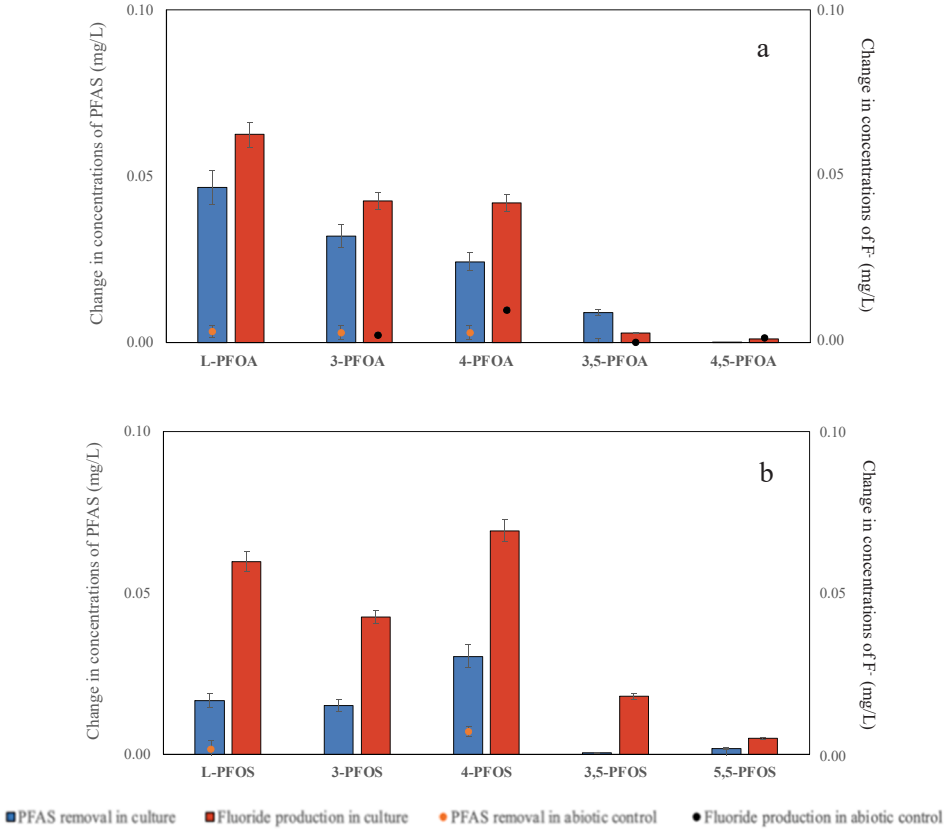

Fig. 4. Change in concentration of PFAS and F^- after 120-day incubations with PFOA and PFOS isomers.

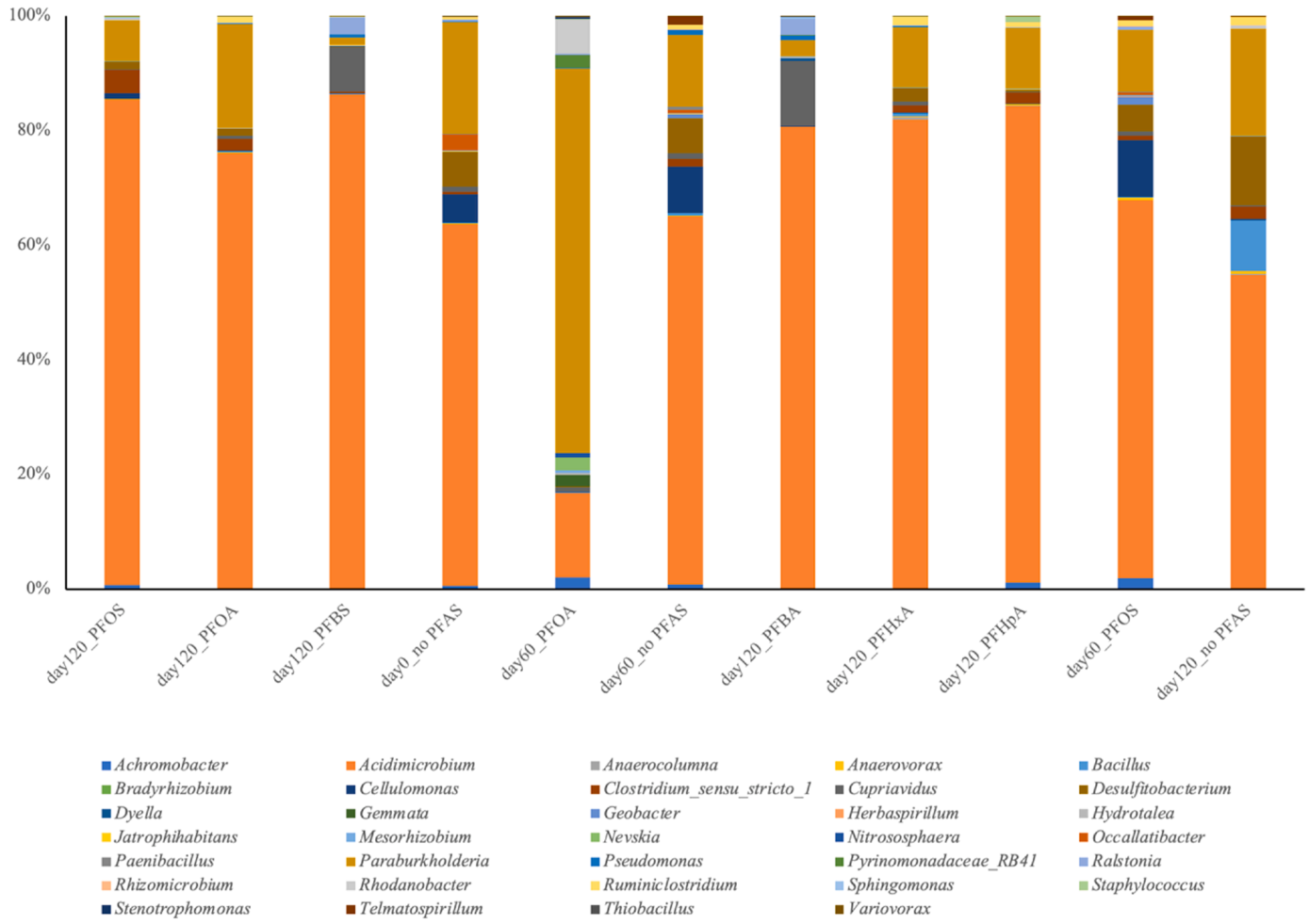

Fig. 5. The 20 most abundant bacterial genera in A6 enrichment cultures amended with 10 ppm PFAS for 120 days incubations.

correlated with F^- production ($P = 0.009$). Moreover, *Paraburkholderia* became dominant, reaching 56% of relative abundance in the enrichments with PFOA at 60 days. Interestingly, the genome of the genus *Paraburkholderia* contains genes coding for 2 different dehalogenases, including a (S)−2-haloacid dehalogenase, which has been recently found to be involved in PFOA degradation in *Delftia acidovorans* [51], as well the gene *crcB*, coding for an ion channel linked to F^- export (https://www.genome.jp/dbget-bin/www_bget?bxb:DR64_551), plus other genes specific for haloacid transport [52]. The enrichment with PFOA at 60 days was also characterized by a generally higher diversity (Table S3) and by the appearance of specific lineages, such as *Rhodanobacter* (~6 % relative abundance), which was recently reported among 11 genera to show significant correlations ($P < 0.05$) with soil PFAS levels [53], and in our study showed a high correlation with F^- production ($P = 0.008$). As above mentioned, C_4-PFAAs did show minimal presence of *Paraburkholderia* but, instead, where characterized by higher relative abundance (7–10 %), of the genus *Cupriavidus*. Intriguingly, a recent study by Liu et al. [54] showed the strain W12 of this genus involved in F^- precipitation through Microbially Induced Calcium Carbonate Precipitation. What makes this observation quite remarkable, is that the strain W12 was cultured in Liu et al. work with succinate as carbon source, possibly pointing to a high affinity for this genus to C_4 compounds, eventually in synergy with A6 defluorination capabilities [51]. Additional relevant genera include the Firmicutes sulfate reducer *Desulfosporosinus*, and the biomass degrader *Cellulomonas*, both particularly enriched (up to 11 % and 9 % relative abundance, respectively) in the no PFAA controls as well as in the PFOS enrichments, especially at 60 days. The enrichment without PFAAs at 60 days also show the genus *Neobacillus*, normally below 1 % in the other enrichments, raising to 8 % in relative abundance, possibly confirming the role of *Bacillales* as generalists in xenobiotic degradation [55]. Finally, the genus *Achromobacter*, which was recently reported among the members of a microbial community from PFAS-impacted soil amended with iron [56] was retrieved up to 2 % in relative abundance especially in the 60 days enrichments with PFOS and PFOA, and also showed significant correlation with F^- production ($P = 0.02$).

4. Conclusions

In this study, we conclude that *Acidimicrobium* sp. A6 enrichment cultures are capable to biodegrade and biodefluorinate a wide range of PFAAs as demonstrated by the decrease in PFAA concentration and increase in F^- concentration over time as compared to the abiotic controls. These results also show that the shorter carbon-chain PFAAs produced during the degradation of PFOA/PFOS are likely intermediates and not final products. However, certain PFAAs, such as PFPeA, double-branched PFCAs and PFSAs presented challenges in biodegradation by the A6 enrichment culture, thereby indicating the necessity for further investigation into the underlying enzymatic mechanisms. Furthermore, the incubation with PFAAs led to a noticeable shift in the microbial community, characterized by an increase in heterotrophic populations. This suggests that some microorganisms might utilize energy from oxidizing the carbon-carbon bonds in partially defluorinated PFAAs. It also opens the possibility that specific bacteria may directly engage in or facilitate pathways leading to PFAS degradation. This is evidenced by the presence of multiple organisms that become more dominant in the

enrichment culture and that have been associated with PFAS degradation or with PFAS impacted sites.

Employing a multifaceted evidence approach, which integrates target measurements (i.e., PFAS), geochemistry (i.e., ammonium oxidation, iron reduction, fluoride), and molecular biological tools (e.g., qPCR targeting the 16S rRNA gene or reductive dehalogenases), enhances the accuracy in detecting ongoing biological processes and improves the monitoring of biodegradation or biodefluorination at the field-scale, as previously described [57–59]. However, to address the challenges posed by lower environmental concentrations (ng/L to μg/L) and complex environmental matrices in the field, there is a pressing need to refine these methodologies and assays. Despite these challenges, utilizing such a comprehensive approach strengthens the interpretation of laboratory data and outlines potential strategies for monitoring these biological processes in situ.

Environmental implications

It has been shown that *Acidimicrobium* sp. Strain A6 (A6) is capable of degrading PFAAs such as PFOA and PFOA and that the production if shorter carbon chain PFAAs is being observed during this process. Here we show that these shorter carbon-chain PFAAs are also degradable and do not build up, indicating the potential for complete degradation of PFOA/PFOS by A6. Either much slower or no degradation was observed for PFPeA and double branched PFOA/PFOS monomers, indicating that these PFAAs will be much more challenging to degrade in potential A6 based bioremediation schemes.

CRediT authorship contribution statement

Shan Huang: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Peter R Jaffe:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Giovanni Pilloni:** Writing – review & editing, Methodology, Formal analysis. **Trent A. Key:** Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Peter Jaffe reports financial support and writing assistance were provided by ExxonMobil Technology and Engineering Company. Shan Huang reports was provided by Princeton University. Peter Jaffe reports a relationship with ExxonMobil Technology and Engineering Company that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Partial funding for this effort was provided by a grant from ExxonMobil Environmental and Property Solutions Company.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.136426.

Data Availability

DNA sequences are available from the NCBI Sequence Read Archive under BioProject PRJNA1140605. Other data will be made available on request.

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