

Research Paper

Microbial defluorination of TFA, PFOA, and HFPO-DA by a native microbial consortium under anoxic conditions



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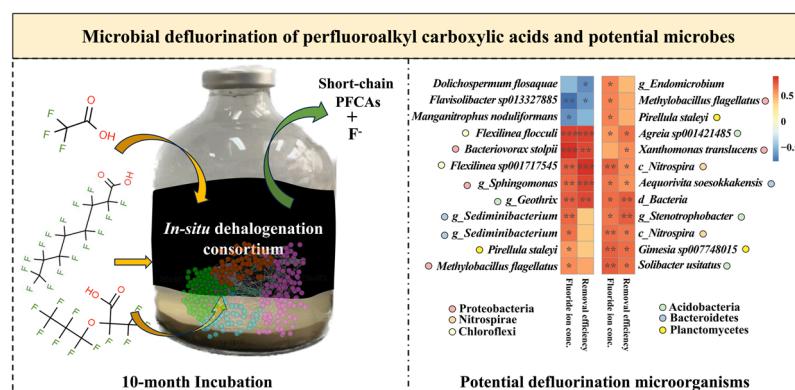
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HIGHLIGHTS

GRAPHICAL ABSTRACT

- The biodegradation of PFASs by a native microbial consortium was assessed under anoxic condition.
- Fluoride production was confirmed in the biodegradation of TFA, PFOA and HFPO-DA.
- Intermediate products of PFOA and HFPO-DA biodegradation were identified and quantitatively determined.
- Species from the phyla of Nitrospirae, Proteobacteria, Actinobacteria, and Chloroflexi might be responsible for biodeflourination.



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ABSTRACT

In this study, the biodegradability of trifluoroacetate (TFA), perfluoroctanoic acid (PFOA), and perfluoro-2-methyl-3-oxahexanoic acid (HFPO-DA) by a native microbial community was evaluated over a 10-month incubation period. The observed microbial defluorination ratios and removal efficiency were 3.46 (± 2.73) % and 8.03 (± 3.03) %, 8.44 (± 1.88) % and 13.52 (± 4.96) %, 3.02 (± 0.62) % and 5.45 (± 2.99) % for TFA, PFOA and HFPO-DA, respectively. The biodegradation intermediate products, TFA and pentafluoropropionic acid (PFA), of PFOA and HFPO-DA were detected in their biodegradation treatment groups. Furthermore, the concentrations of the PFOA metabolites, perfluorohexanoic acid (PFHxA) and perfluoroheptanoic acid (PFHpA), in the aqueous solutions after incubation were quantified to be 0.21 and 4.14 $\mu\text{g/L}$. TFA, PFOA and HFPO-DA significantly reduced the microbial diversity and changed the structure of the community. The co-occurrence network analysis showed that low abundance species, such as *Flexilinea flocculi*, *Bacteriovorax stolpii*, and *g_Sphingomonas*, are positively correlated with the generation of fluoride ion, implying their potential collaborative functions contributing to the observed biodeflourination. The findings in this study can provide insights for

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the biodegradation of perfluoroalkyl carboxylic acids and their emerging alternatives by indigenous microorganisms in the environment.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs), known as "forever chemicals", are defined as fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom) [48,66]. PFASs have been broadly used in various industrial and consumer applications due to their unique properties (e.g., surfactant properties, hydrophobicity, lipophobicity, and chemical/thermal stability) [8]. However, these properties also contribute to their environmental persistence, bioaccumulation, and toxicity. The frequent detections of PFASs in the environment have raised growing concerns for public and ecosystem health [17,58]. Therefore, PFASs have been regulated by countries and institutions around world. For example, perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), and long-chain perfluoroalkyl carboxylic acids (PFCAs), along with their salts and related compounds, were phased out by the Stockholm Convention on Persistent Organic Pollutants [55]. The restricted use of legacy PFASs has led to the applications of their emerging alternatives, such as perfluoro-2-methyl-3-oxahexanoic acid (HFPO-DA), which has been used as a replacement for PFOA [44]. In China, the regulation on emerging contaminants was published this year, and the recommended values for PFOS and PFOA in drinking water were 40 and 80 ng/L, respectively [20].

In recent years, it has been reported that PFOA and HFPO-DA are the dominant PFCAs at industrial and non-industrial areas, with their concentrations up to hundreds of $\mu\text{g}/\text{kg}$ in soil (or $\mu\text{g}/\text{L}$ in groundwater) [56, 65,67,71]. However, there is limited knowledge on the biodegradability of PFASs, particularly for perfluorinated compounds that are of significance for their environmental fate. Due to their persistent nature contributed by the strength of C-F bond, their high negativity and fluoride toxicity, the natural breakdown of PFASs is highly difficult, with little or no current evidence of significant biodegradation.

Microbial populations generally lack the adaptation to these synthetic compounds that have only been released into the environment in the past century [60]. However, there are a handful of organofluorine natural products generated in biologic systems. For example, some plants and certain Gram-positive *actinomycete* species can produce a small number of fluorinated compounds, such as fluoroacetate, ω -fluorinated fatty acids [9]. A fluorinase that incorporates fluoride into organic molecules has been identified and characterized in *Streptomyces cattleya* [9,16]. Recently, Xie et al. [69] found that the alkane degrader *Pseudomonas* sp. strain 273 can break the C-F bond of a natural mono-fluorinated organic compound (1-fluorodecane), most likely through the oxygenolytic enzyme system. Further work revealed that this bacterium can use inorganic fluoride to synthesize fluorinated anabolites and phospholipids that were subsequently incorporated into the lipid bilayer [70]. Hence, the finding that the C-F bond is not solely from anthropogenic sources implies that fluorinated organic compounds have been part of the biosphere and microbes might have evolved over time with functions being able to break and/or generate C-F bonds. In addition, based on thermodynamic calculations, reductive defluorination is energetically favorable under anaerobic conditions and would release more energy than those from sulfate reduction and methanogenesis [51].

To date, exploratory experiments have been conducted to uncover biodeflourination and the associated degradation pathways of PFASs. For example, Visscher et al. [59] reported that trifluoroacetate (TFA), the shortest-chain PFCAs, can be rapidly degraded under anoxic and oxic conditions with a methanogenic microbial community. Furthermore,

TFA was found to be co-metabolically degraded in an engineered anaerobic reactor [30]. However, there is no further study on the microorganisms/enzymes that are responsible for the biodegradation of TFA. Liou et al. [35] utilized five microbial communities to degrade PFOA with different electron donors and co-metabolism substrates incubated for 259 days under anaerobic conditions, and no PFOA biodegradation was observed. On the other hand, Huang and Jaffe [25] demonstrated that *Acidimicrobium* sp. strain A6, in both pure and enriched culture, degraded PFOA/PFOS with efficiencies up to 60% after 100 days of incubation. The degradation was accompanied by an increase of fluoride ion and shorter-chain fluorinated compounds. In addition, the microbial defluorination of unsaturated PFASs containing C=C double bonds under both anaerobic and aerobic conditions was verified via reductive defluorination and/or hydrogenation pathways by organohalide-respiring microbial consortiums and activated sludge communities [73,74]. As put forward by Ernest Gale, the microbial infallibility hypothesis is that if there is energy to be gained from a compound, a microorganism will figure out how to extract it and create a niche for itself [19,34].

So far, based on the growth and reductive dehalogenase active expression, some unknown phylogenetic groups with low abundance, rather than *Dehalococcoides*, were responsible for the defluorination in the seed cultures [74]. Hence, further exploration of specific microbes responsible for the defluorination of PFASs from the perspective of their chemical structure is needed. In addition, the fate of PFASs in the environment and the potential defluorination ability by indigenous organohalide enriched consortium remain unclear. Therefore, the aim of this study was to explore the biodegradability of three typical perfluorinated compounds, TFA, PFOA, and HFPO-DA by a native microbial community collected from soils being exposed to PFASs and chlorinated solvents for decades. The associated microbial responses in the community were also examined.

2. Materials and methods

2.1. Chemicals and materials

Standard compounds of TFA (CF_3COOH , CAS: 76-05-1, 99.9%, J&K), PFOA ($\text{CF}_3\text{CF}_2\text{CF}_2\text{CF}_2\text{CF}_2\text{COOH}$, CAS: 335-67-1, 99-100%, Aladdin), HFPO-DA ($\text{CF}_3\text{CF}_2\text{CF}_2\text{OCFCF}_3\text{COOH}$, CAS: 13252-13-6, 99.7%, Wellington Lab) were purchased and prepared in stock solution. The basal mineral salt medium consisted of Na_2HPO_4 , KH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$, the Wolin vitamin mix, and trace metals, all of which were analytical reagent grade or higher and purchased from Aladdin (detailed ingredients provided in *Supporting Information*, SI). Liquid chromatography grade methanol and ammonium acetate were obtained from Merck. All stock solutions were prepared by dissolving the chemical agents in sterilized ultrapure water (18.3 $\Omega\text{-cm}$) generated from Milli-Q synthesis A10 system (Millipore, USA).

The aquifer solids were sampled from a fluorochemical plant, which has a history of long-term fluorinated and chlorinated chemicals production [56]. We used a core drilling rig to collect the aquifer solids from 15 to 18 m below ground surface, where high concentrations of PFASs and chlorinated solvents were detected in groundwater. The collected solids were transferred to the laboratory in sterilized bags and stored at 4 °C prior to further biodegradation microcosm assays. The total organic carbon (TOC) content in the aquifer solids were determined using the Walkley–Black method [46] and varied in the range from 0.02% to 0.06%.

2.2. Biodegradation microcosms and experimental setup

In this study, the biodegradability of TFA, PFOA and HFPO-DA by the native microbial community from the above-mentioned aquifer solids was evaluated in serum bottle-based biodegradation assays for over 10 months. The different treatments included a biological group (with or without PFASs labeled with “Biodegradation” and “Blank”, respectively) and an abiotic control (autoclaved three times, then PFASs added, labeled “Sterilized”).

The initial concentrations used in the current study were 10 mg/L for TFA, 5 mg/L for PFOA and HFPO-DA, which were similar to those observed in the field in previous studies, e.g., 6.57 mg/L of PFOA in groundwater at Naval Air Station Fallon [43] and 9.7 mg/L of PFOS in groundwater of primary source sites [29]. Furthermore, the quantitative analyses of fluoride ion (F^-) and the defluorination intermediate products were also considered in the selection of the initial concentrations of TFA, PFOA and HFPO-DA. These concentrations were chosen based on an initial assumption of 1% theoretical defluorination efficiency of TFA, PFOA and HFPO-DA, which would result in the F^- concentrations in the serum bottles that would be high enough so that they can be quantitatively analyzed, but low enough (e.g., < 1 mM reported by Wackett [61]) so that they do not exert a toxic influence on the microbial consortium.

Additionally, to simulate subsurface conditions, the incubation method used in this study was similar to previous procedures for the enrichment and cultivation during the reductive dechlorination of chlorinated aliphatic hydrocarbons [40]. Briefly, about 9 g (wet, 24.22% \pm 0.96%, moisture content) aquifer solids, 36 mL medium, and 36 mL PFCAs stock solution were added separately to maintain the ratios of solid-liquid (5–50%) and headspace (1–1.6:1) in the 120 mL serum bottles. Before the bottles were capped and sealed with blue butyl stoppers, 0.01% resazurin was added as the redox indicator. The observation of a light pink color at the end of 10-month period indicated the presence of oxygen during the incubation period in the current study. The initial pH was adjusted to \sim 7.2 in all groups, and there were slight/non-significant fluctuations of pH in different treatment groups, indicating that neutral conditions were maintained during the incubation period. All cultures were set up in triplicate and incubated at room temperature (\sim 22°C) in the dark without shaking.

2.3. Determination of TFA, PFOA, HFPO-DA, intermediate products and fluoride ion

Aqueous samples were taken during the incubation period for the measurements of TFA, PFOA, HFPO-DA and F^- . Perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), and perfluoroheptanoic acid (PFHpA), the biodegradation intermediates of PFOA, were observed in the studies of *Acidimicrobium* sp. strain A6 [25,26]. Therefore, the potential intermediate products of PFCAs, including C₂-C₇ PFCAs and acetate, were analyzed quantitatively to support the evaluation of TFA, PFOA, HFPO-DA biodegradation.

The PFASs concentrations were quantified by UPLC (ExionLC™ AD, AB Sciex, USA) coupled with a Sciex 5500 triple-quadrupole mass spectrometer operated by negative electrospray ionization (ESI) in a

multiple reaction monitoring mode (MRM). The limits of detection (LOD) were calculated with a signal-to-noise (S/N) ratio of 3, and the LODs for TFA, PFOA, HFPO-DA were 0.2, 0.03 and 0.05 µg/L, respectively. The F^- in the culture was determined by Dionex ICS-5000⁺ Ion-chromatography system Chromeleon 7.1 (Thermo Fisher Scientific, USA). The retention time of F^- was 4.67 min, and its LOD was determined to be 0.006 mg/L. The acetate in the culture was measured by liquid chromatography coupled with C18 column (Thermo Fisher Scientific, USA) and its LOD was 0.5 mg/L (Retention time: 4.51 min, UV 214 nm). Additionally, the concentrations of CO₂ and CH₄ in the headspace of sealed bottles, the end-products of PFCAs degradation and indicators of microbial activities [59,76], were measured at the end of the incubation by gas chromatography (GC-7890A, Wilmington, USA). More details were provided in SI.

2.4. DNA extraction and PacBio sequencing

Community DNA was extracted from 5 mL of mixed culture using the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, USA) according to the manufacturer's protocol, which was consistent with our previous work [56]. To obtain more accurate phylogenetic identification, the full-length 16 S rRNA gene amplicon (27 F: 5'-AGRGTYYGATYMTGGCTCAG-3' and 1492 R: 5'-RGYTACCTTGTACGACTT-3') was sequenced with a PacBio Sequel. PacBio raw reads were processed using the SMRT Link Analysis software version 9.0 to obtain demultiplexed circular consensus sequence (CCS) reads. The detailed PCR amplification, library construction and sequencing were provided in SI.

Raw reads were processed and filtered through SMRT Portal with sequences length between 1301 and 1500 bp, which accounted for 98.51% of the total sequences. The operational taxonomic units (OTUs) were clustered with 98.65% similarity cutoff using UPARSE (version 7.1 <http://drive5.com/uparse/>) and chimeric sequences were identified and removed using UCHIME. The obtained reads were deposited in the NCBI Sequence Read Archive with the project number (PRJNA995193). The phylogenetic affiliation of each 16 S rRNA gene sequence was analyzed by RDP Classifier (<http://rdp.cme.msu.edu/>) against the silva (SSU132) 16 S rRNA database using the confidence threshold of 70%. The OTUs not assigned to the Kingdom Bacteria were excluded from the obtained sequences for each sample, which were then normalized to the equal sequencing depth (8114 sequences) for downstream analysis.

2.5. Data analysis

The biological removal efficiency, defined as the concentration difference between the sterilized group and the microbial treatment groups divided by the concentration in the sterilized group, was calculated using Eq. 1. The defluorination ratios of TFA, PFOA, and HFPO-DA were determined by dividing the maximum fluoride formed by the fluoride number in the sterilized group (Eq. 2). It is worth noting that the biological removal efficiency was calculated based on the assumption that the removal of TFA, PFOA, and HFPO-DA from aqueous solutions could be contributed to both the biodegradation of chemicals (i.e., the breakdown of bonds) and biosorption.

$$\text{Biological removal efficiency}(\%) = \frac{\text{PFAS conc. in (Sterilized group} - \text{Biodegradation group})}{\text{PFAS conc. in sterilized group}} \times 100\% \quad (1)$$

$$\text{Defluorination ratio}(\%) = \frac{\text{Max. fluoride formed}}{\text{PFAS conc. in sterilized group} \times \text{number of } F \text{ in one molecule}} \times 100\% \quad (2)$$

The community abundance, diversity, and coverage were interpreted using Alpha diversity indices calculated from OTUs by Mothur (version v.1.30.1). The one-way analysis of variance (ANOVA) was used to examine the difference in microbial alpha-diversity at a significance threshold of 0.05. The consistency of the microbial community structure in the samples was assessed using principal coordinate analysis (PCoA) based on the Bray-Curtis distances matrix. Rarefaction curves (Fig. S1), Venn diagrams and microbial community composition profiles were created by R (version 4.2.1).

Molecular co-occurrence ecological networks were established to explore the potential defluorination microorganisms in microcosmic samples [18]. The OTUs were filtered and those detected in more than 50% of samples were used as the input of the network following the principle of majority selection [18]. The SparCC method was applied to construct the network using the OTUs selected with the threshold of correlation coefficients (r) > 0.6 and p -value < 0.05 . All correlations with better correlation coefficients and p -values, identified from the pairwise comparison of the abundance of OTUs, formed a correlation network where each node represented one OTU, and each edge represented a strong and significant correlation between two nodes. The interactive Gephi platform was used to visualize the network and to further identify ecological clusters (i.e., modules) of taxa with strong correlations among each other [4]. Furthermore, linear regressions between the ecological clusters and generation of F^- were calculated to obtain the microbial community modules with positive correlations. The correlation heatmap among microbial community modules, the generation of F^- and the removal efficiency of PFASs was then prepared to explore the potential participation of microbes in the ecological clusters.

3. Results and discussion

3.1. Biological removal efficiency of TFA, PFOA, and HFPO-DA in different treatments

The biological removal efficiency of TFA, PFOA and HFPO-DA in the microbial treatments after 10 months incubation, was 8.03 (± 3.03) %, 13.52 (± 4.96) %, and 5.45 (± 2.99) %, respectively (Fig. 1 and Table S1). The result demonstrated that TFA, PFOA, and HFPO-DA can be removed by the microorganisms. However, it is worth noting that the concentrations of PFOA or HFPO-DA in aqueous solutions were significantly lower than the initial concentration in the sterilized treatments (Table S1), whereas the concentration of TFA was slightly lower than its initial concentration. We speculated that this observation was primarily due to the sorption of TFA, PFOA, and HFPO-DA onto or into the aquifer solids, as discussed below.

Previous studies showed that the sorption behaviors of PFASs in soil

are affected by the molecule structure and soil properties [47,68]. The hydrophobicity of PFASs is contributed by CF_2 fragment and PFASs with higher hydrophobicity ($\log K_{ow}$) values have a higher affinity for organic matter and minerals present in the solids [32,68]. Therefore, the different levels of reduced concentration observed in the aqueous solution between PFOA/HFPO-DA and TFA in the sterilized group could be explained by their different hydrophobicity values (i.e., the $\log K_{ow}$ of PFOA is 4.67 [68], is 4.0 for HFPO-DA [24], and 0.5 for TFA [45]). In addition to the structure of PFASs, the soil physico-chemical properties, such as cation exchange capacity, pH, micropore volume, and silt-plus-clay content play important roles in the PFASs sorption [47]. For example, Liu et al. [36] reported that more than 50% of PFOA (2.0 mg/L) could be adsorbed by soils through mechanisms including surface complexation, hydrogen bonding, and electrostatic force. This is similar to the percentage observed for PFOA in this study.

3.2. Defluorination ratio and biodegradation pathway for TFA, PFOA, and HFPO-DA

The release of F^- has been used as the most critical indicator for the degradation of PFASs [25,57,73]. The concentration of F^- and defluorination ratios in the treatments with and without PFASs are shown in Fig. 2 and Table S1. The results revealed that compared to the blank and sterilized treatments, F^- was detected in all the biodegradation treatments. The concentrations of F^- in TFA, PFOA, and HFPO-DA biodegradation groups were 0.166 (± 0.134) mg/L, 0.119 (± 0.023) mg/L, and 0.068 (± 0.013) mg/L, respectively (Fig. 2 and Table S1). The corresponding defluorination ratios of TFA, PFOA, and HFPO-DA were 3.46 (± 2.73) %, 8.44 (± 1.88) %, and 3.02 (± 0.62) %, respectively (Fig. 2 and Table S1).

Kim et al. [30] indicated that TFA could be completely degraded with a 100% release of F^- through a co-metabolism process in an anaerobic bioreactor after a 630-day incubation. However, there has been no further report on the successful biodeflourination of TFA. Similarly, there is no robust evidence of PFOA biodeflourination except by *Acidimicrobium* sp. strain A6, which can achieve ~11% or ~36% defluorination ratios in pure culture or its enrichment culture [25]. Furthermore, to the best of our knowledge, there have been no reports on the successful biodegradation of HFPO-DA (Table S2). Therefore, the current results provided the strongest evidence to date for the biodeflourination of TFA, PFOA, and HFPO-DA. Further calculation of fluorine content balance revealed that the total fluorine content of the selected PFASs in biodegradation groups showed lower values than their corresponding sterilized groups (Table S3). The lower calculated fluorine contents in the biodegradation groups can be attributed to the unaccounted intermediates, e.g., the fluorine in TFA and PFA, which were detected, but not quantified, hence not accounted for in the calculation.

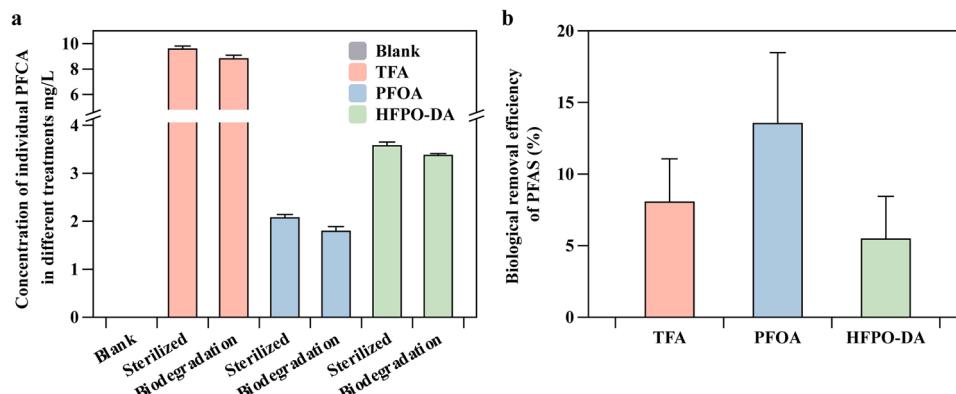


Fig. 1. The biological removal efficiency of TFA, PFOA and HFPO-DA groups after 10 months incubation. Note: the values represented the average and standard deviations of triplicate samples.

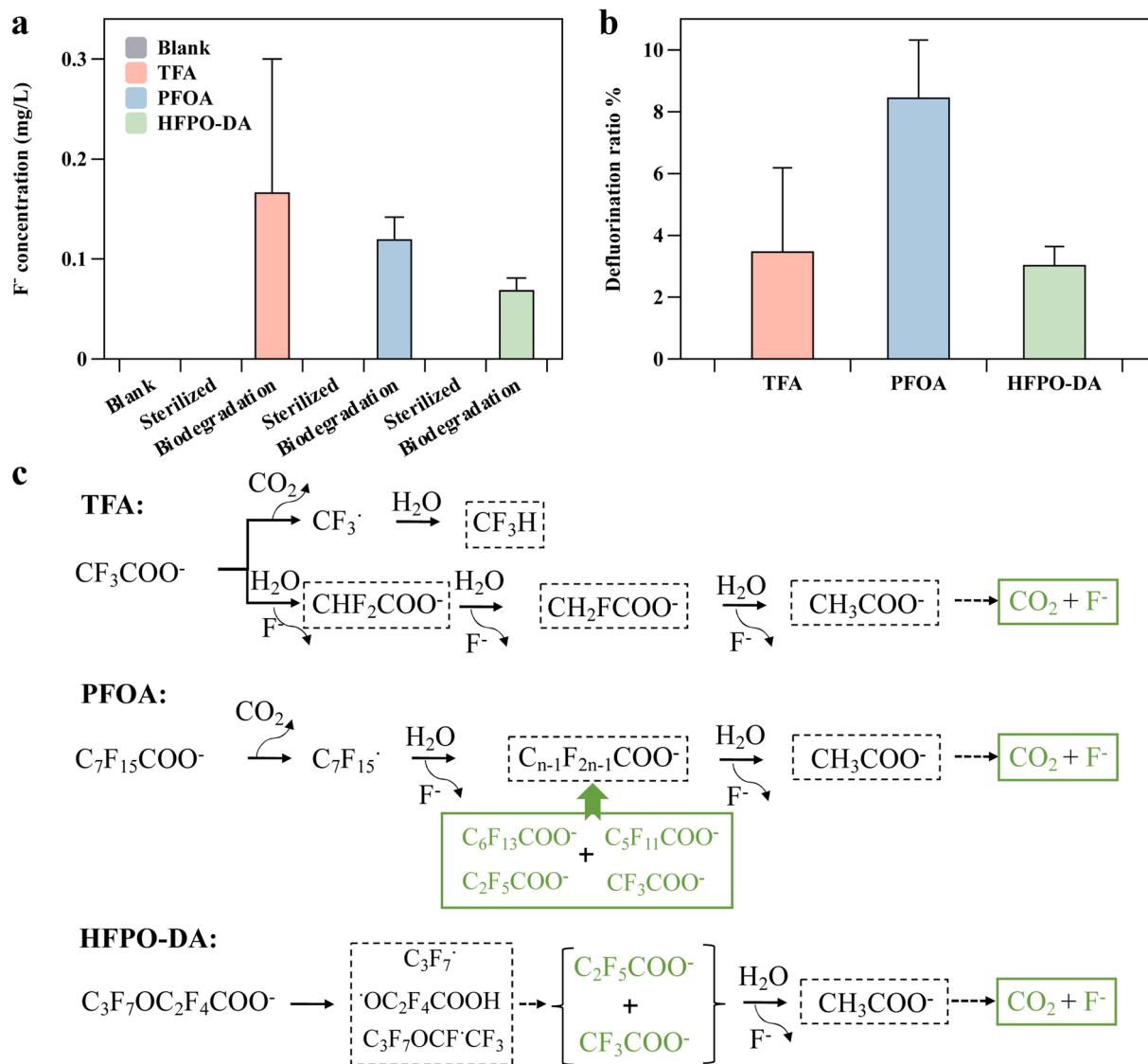


Fig. 2. Concentration of F(a), defluorination ratios (b) and proposed biodegradation pathways (c) for TFA, PFOA, and HFPO-DA based on the intermediates identified in this study and previous studies. Note: the green color labeled chemicals have been detected as intermediate products in the current study and the chemicals in the box with dashed lines are potential intermediates based on previous studies.

In addition, the potential intermediate products were identified and summarized in the current study (Fig. S2) and inputs from previous studies as shown in Fig. 2c [3,13,14,25,30,49,59]. TFA and PFOA were detected as the intermediate products in both the PFOA and HFPO-DA biodegradation groups. In addition, concentrations of PFHxA and PFHpA were quantified to be 0.21 and 4.14 µg/L in the aqueous solutions of PFOA biodegradation group after the 10-month incubation period. Note that the Σ₁₇PFASs concentrations in the aquifer solids from the sampling site ranged from 7.65 ng/g dw to 20.2 ng/g dw at various depths [15] and were much lower than the detected concentrations in this microcosm study.

Furthermore, the detections of only PFHpA and PFHxA as the intermediate products were different from the PFOA biodegradation by *Acidimicrobium* sp. strain A6 in which all short-chain PFCAAs, i.e., PFHpA, PFHxA, PFPeA, and PFBA, were produced during the Feammox process [25]. We speculate that the lack of detections of PFBA and PFPeA might be attributed to the degradation of PFBA and PFPeA by the consortium, since PFBA and PFPeA were known to be more readily degraded than TFA as indicated by previous advanced reduction process of hydrated electron reactions [6,50]. Moreover, we speculate that the synergistic

functions of different microorganisms in the consortium vs. the pure culture of *Acidimicrobium* sp. strain A6 may contribute to the discrepancy observed in the two studies. For the HFPO-DA biodegradation group, no other short-chain PFCAAs were detected, instead, there was an unidentified intermediate product (*m/z*: 212.9/169.9) detected (Fig. S2). Furthermore, the biodegradation of TFA has been a major challenge, and there is no further study on the microorganisms/enzymes that are responsible for the biodegradation of TFA, which was reported by Visscher et al. [59] and Kim et al., [30]. However, it is worth noting that Alexandrino et al., [2] confirmed the generation of mono-fluoroacetate, a metabolite resulted from the anaerobic biodegradation of TFA, which underwent biodegradation with the release of fluoride ions by microbial cultures with different origins. Therefore, as supported by the conclusions in previous studies [25,74], it was inferred that decarboxylation, hydrolytic elimination and H/F exchange might be the main pathways to achieve defluorination of selected PFASs (Fig. 2c).

Additionally, acetate was considered as a key byproduct of degradation of PFASs (Fig. 2c), as evidenced in previous studies [25,30]. However, we were not able to quantify the concentration of acetate in

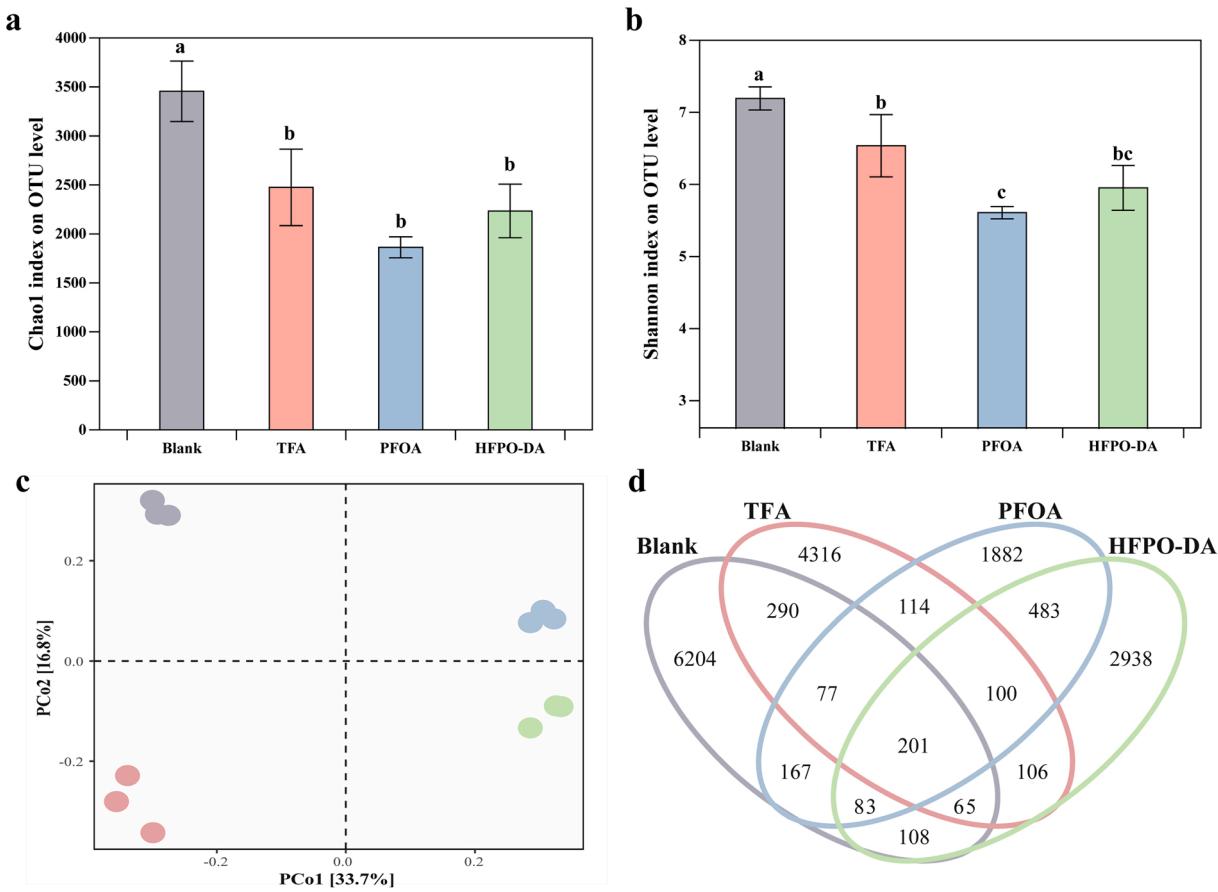


Fig. 3. Alpha diversity indices (a and b) and Beta diversity of the microbial communities (principal coordinates analysis based on Bray-curtis distance of OTUs level, c), and Venn diagrams of core OTUs among four treatments (d). Different letters represent significant differences based on one-way ANOVA with LSD's comparisons.

the samples due to an unknown peak (Retention time: 2.75 min, UV 214 nm) that exhibited significantly higher intensity in the biological groups (Blank and Biodegradation groups) (Fig. S3). Considering the same absorbance of 214 nm, we speculate that the unknown compound might be a fatty acid originated from the aquifer solid. Hence, though the identification and quantitative determination of intermediate products provided a second line of evidence to support the occurrence of PFOA and HFPO-DA biodegradation, more mechanistic studies are warranted for this indigenous microbial consortium.

3.3. Characterization and activity of the defluorination microbial community

3.3.1. Richness and diversity variation of the microbial communities

Fig. 3 and Table S4 compared the richness and diversity of the microbial communities among these treatments. The community richness and evenness were assessed using the Ace (a) and Shannon (b) indices, with higher values suggesting a greater number and diversity of microbial species. Compared with the Blank group, both the Ace and Shannon indices at OTUs level significantly decreased after the addition of TFA, PFOA, and HFPO-DA (Fig. 3a & b). The exposure of TFA, PFOA, and HFPO-DA might have inhibited the growth and activities of microorganisms and led to reduced species richness and diversity, even though the indigenous microbial community had been exposed to relatively high concentrations of PFASs and chlorinated solvents for decades. These results provide additional support for the lower microbial diversity observed after being exposed to PFASs in the field in previous investigations [53,56]. In this study, the reduction of microbial diversity caused by PFOA was significantly greater than that caused by TFA and

HFPO-DA, even though the TFA concentration at 10 mg/L was twice that of PFOA. Similarly, Liu et al. [37] reported that PFOA and HFPO-DA could restrict diversification of rhizosphere soil bacterial communities, and single or mixed PFAS had different influences on the diversity.

PCoA based on Bray-curtis distance was applied to evaluate the overall patterns of variations in the microbial community profiles after the addition TFA, PFOA, and HFPO-DA (Fig. 3c). The microbial community profiles were clustered into four separate groups according to the treatments. In particular, the distance between selected PFASs groups and the Blank varied with the structure of PFCAs. This result further suggested that the exposure and structure of PFASs can alter the structure and compositions of microbial community. In addition, the clusters corresponding to PFOA and HFPO-DA are more closely related to each other than to the cluster associated with TFA. It has been reported that PFOA has adverse effects on the soil ecosystem [12,37], considering the close distance between PFOA and HFPO-DA, the use of HFPO-DA as an alternative should be assessed carefully.

The four separate groups' OTUs derived from PCoA were compared to understand the variability of microbial communities (Fig. 3d). The total OTUs after exposure of PFCAs were lower than the Blank. There were 6204, 4316, 1882 and 2938 unique OTUs in each of these four individual groups, respectively, with a total of 201 core OTUs shared by all treatments. Given that the difficulty of biodeflourination, these shared OTUs might contain putative microorganisms capable of degrading PFCA and the specific microbes in these groups need to be explored. Collectively, the diversity of the microbial community decreased notably after exposure with TFA, PFOA, and HFPO-DA despite the presence of shared microorganisms that might play important roles in defluorination.

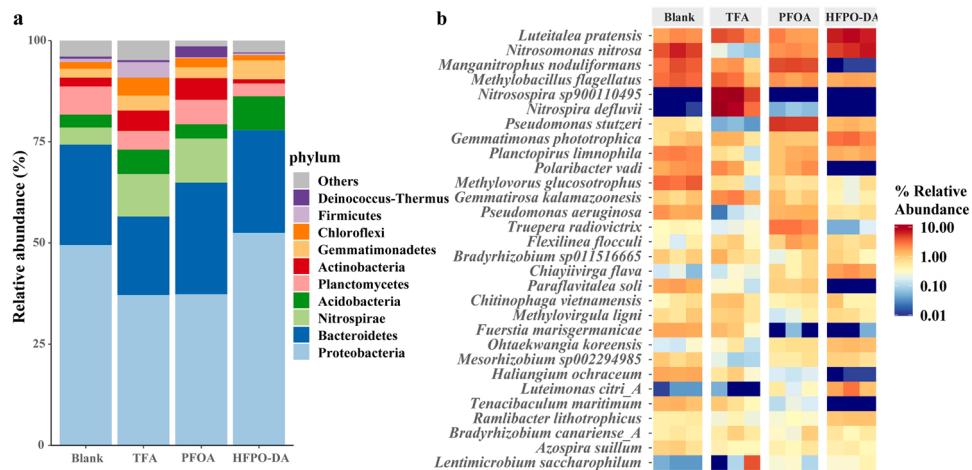


Fig. 4. Compositions and abundance of the microbial community at the phylum(a), and species levels (b). Relative abundance of phylum is presented by the mean value and the top 30 species in each sample were showed in the heatmap.

3.3.2. Microbial community compositions

Fig. 4 depicts the microbial community distribution and variation at the phylum and species levels after incubation with PFASs. The major bacterial phyla in Blank were Proteobacteria and Bacteroidetes, which accounted for 74.27% (Fig. 4a). This aligns with our previous field investigation, where Proteobacteria was the dominant phylum with relative abundance among 61.58–87.23% in PFASs and chlorinated solvents contaminated groundwater [56]. It indicates that microbial community maintained a similar profile in aquifer solids, and microcosmic incubation did not alter the major niche for the ecosystem. After incubation with selected PFASs, Proteobacteria and Bacteroidetes remained the prevailing phyla, constituting 56.52%, 64.91%, and 77.85% in the TFA, PFOA, and HFPO-DA groups, respectively.

Proteobacteria and Bacteroidetes have been identified as the major secretors of extracellular polymeric substances, which act as a cell protective barrier, electron acceptor, and nutrient source [33,64]. In addition, many colonies in these two phyla were identified with the functionalities of organic pollutant biodegradation as well as the carbon, nitrogen, and sulfur cycling [42,54]. Chen et al. [11] recently observed that Proteobacteria became the dominant phylum after 60 days of PFOA exposure. Besides these two tolerant phyla, some phyla were enriched by the addition of TFA, PFOA, and HFPO-DA, specifically. For example, Nitrospirae, Acidobacteria, Actinobacteria and Chloroflexi exhibited higher abundance in TFA and PFOA groups, while no phyla with higher abundance were observed in the HFPO-DA group, except of Acidobacteria and Gemmatimonadetes. Although it was challenging to link defluorination with the changes of these phyla, the enriched phyla were reported with the abilities to degrade complex organic compounds such as alkanes, aromatic and halogenated hydrocarbons as well as carboxylic compounds [22,38].

The variations of microbial community compositions and relative abundance at the species level (top 30) were analyzed to provide further insight into the functional microorganisms after PFASs exposure (Fig. 4b). Some species, such as *Luteitalea pratensis*, *Manganitrophus noduliformans*, *Methylobacillus flagellatus*, and *Methylovorus glucosotrophicus*, were at high abundance in the PFASs groups. Though none of them was reported to be associated with PFASs biodegradation, these species were involved in carbon cycling such as the oxidation of CH₄, autotrophic CO₂ fixation, and assimilation processes [23,39,72]. For example, Liu et al. [39] reported that *Luteitalea pratensis* played a vital role in the syntrophic pathway for manganese-dependent anaerobic oxidation of CH₄ to CO₂ by mediating extracellular electron transfer and participating in the microbial manganese reduction. *Manganitrophus noduliformans* was found to be responsible for coupling extracellular

manganese oxidation to aerobic energy conservation and CO₂ fixation into cellular biomass [72].

Additionally, some species, such as *Nitrosospira sp900110495* and *Nitrosospira defluvii* in TFA group, *Pseudomonas stutzeri* and *Truepera radiovictrix* in PFOA group, and *Nitrosomonas nitrosa* in HFPO-DA group, were enriched. Strains of these species have been identified and linked with the key processes of the nitrogen cycle, such as ammonia oxidation and nitrification/denitrification [31,41]. Furthermore, the genome of these species carries antibiotic-resistance genes and can co-metabolically transform fluorinated antibiotics through defluorination reactions [78]. The effects of selected species enrichment by PFCAs in the current study were similar to those observed in previous studies carried out by Chen et al. [11] and Chen et al. [12], in which denitrifying bacteria were significantly enriched, possibly due to the antibiotic-resistant genes harbored under the stress of PFOA exposure. However, there was no follow-up investigation between the denitrifiers and biodefluorination of PFASs. Hence, future investigations are warranted to determine correlation among the biodefluorination, denitrification and antibiotic-resistant genes.

3.3.3. Concentration changes of CO₂ and CH₄ in different treatments

At the end of 10-month incubation, the concentrations of CO₂ in all biodegradation treatments were higher than those in the sterilized treatments (Fig. 5a). The production of CO₂, one of the final products for organic pollutant biodegradation, has been observed in aerobic oxidation and anaerobic fermentation of chemicals containing carboxylic acids. For example, microbes can gain energy from organic carboxylic acids through Krebs cycle or decarboxylation reaction, which can result in the production of CO₂ [63].

Previous studies indicated that microorganisms might prefer to attack at non-fluorinated moieties, such as carboxylates or sulfonates, as the initial reaction point during the metabolic transformation for PFASs rather than a direct attack on the C-F bonds [10,62,77]. Yet, so far, only TFA biodegradation was verified by Visscher et al. [59], and no other study reported that PFCAs can be decarboxylated or mineralized by microbes. On the other hand, the generation of CO₂ was verified in PFCAs chemical degradation (i.e., non-biological) and decarboxylating was considered as the rate-limiting step [14,57,76]. With the above mentioned, the increased CO₂ in the biodegradation treatments might have been contributed by the biodegradation of TFA, PFOA, and HFPO-DA. However, the microbial oxidation and/or fermentation of organic matter, which can be TOC (0.02–0.06%) from aquifer solids or the biomass in the system, played a more substantial role in the increased production of CO₂ and might conceal the contribution of

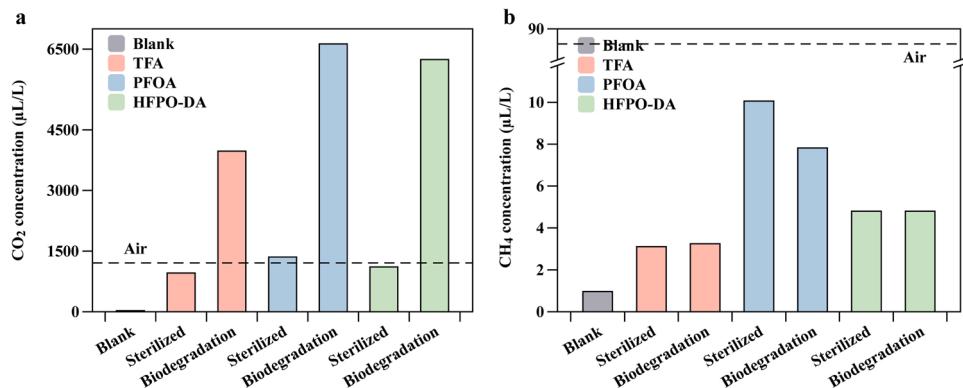


Fig. 5. Concentrations of CO₂ (a) and CH₄ (b) in different treatments. The dotted line represents concentration in the air at the end of 10-month incubation period.

PFCAs biodegradation.

Additionally, the concentration of CO₂ in the blank treatment was significantly lower compared to those in the sterilized treatments. Previous studies indicated that autotrophic and heterotrophic microbes can fix CO₂ through six well-known pathways, such as Calvin cycle and reductive citrate cycle [5] and cellular metabolism [7]. Recent studies found that some microbes can utilize or capture CO₂ and convert it to organic compounds, such as biomass and desired metabolites [1,27]. Therefore, the reduction of CO₂ in the Blank group might be related to the microbial sequestration of CO₂ via pathways such as the microbial carboxylation reaction by the microbial consortium [1,52]. The identification of autotrophic and heterotrophic microorganisms, such as

Manganitrophus noduliformans, *Methylobacillus flagellatus*, *Mesorhizobium*, *Haliangium ochraceum*, in the community further supported this observation (Fig. 4b).

Concentrations of CH₄ in all treatments were lower than the concentration in air (Fig. 5b). The result indicated that there is no substantial CH₄ production in the system, consistent with the observation of a light pink color at the end of 10-month period, which indicated the presence of oxygen during the incubation period in the current study. The microbial defluorination of TFA and the production of CH₄ were verified in an earlier study [59], and it was concluded that the methanogenic conditions might be favorable for its biodegradation.

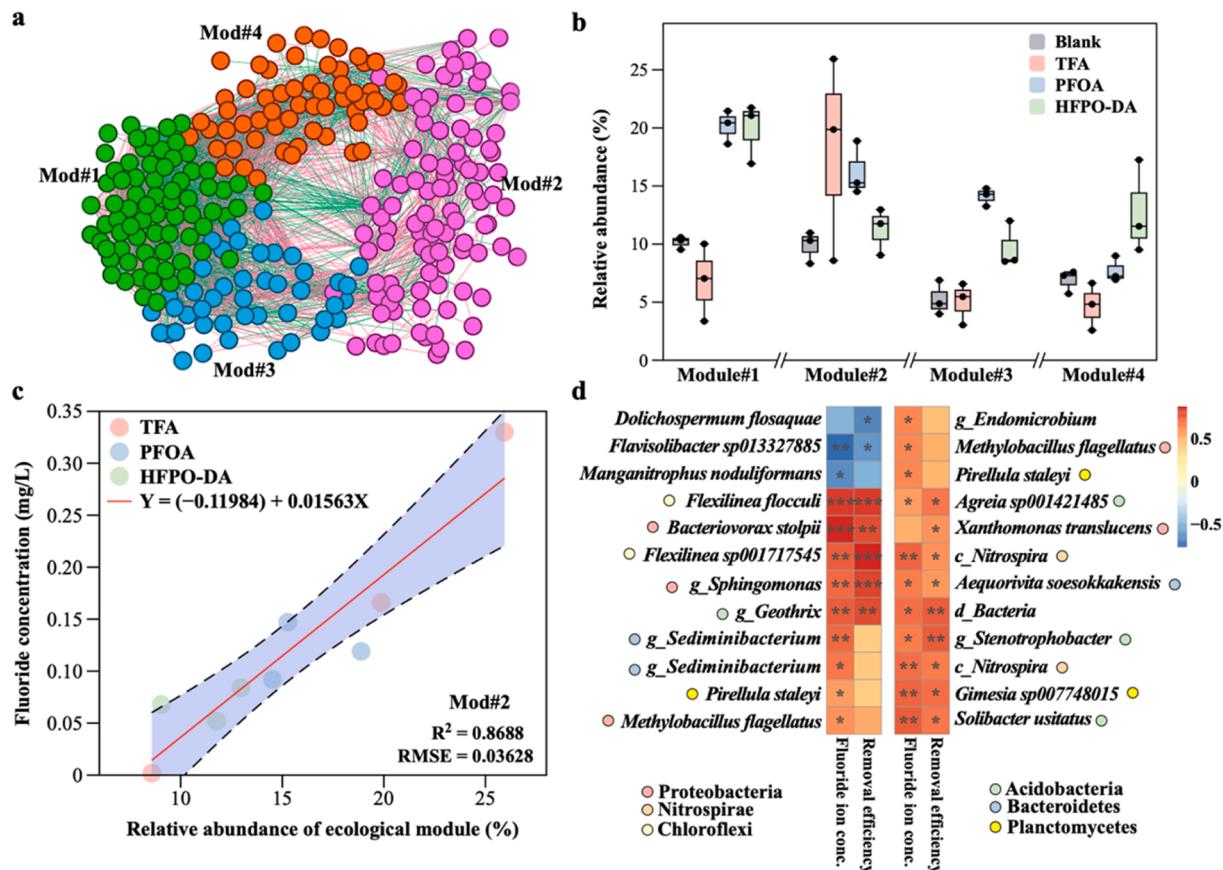


Fig. 6. Microbial co-occurrence network analysis and potential defluorination microbes. Network diagram with nodes colored according to the four ecological clusters, Module #1–4 (a), the relative abundance of the four modules in the different treatments (b), the linear relationship between F⁻ concentration and the relative abundance of ecological Module #2 in selected groups (c), the relationships between species of Module #2 and the generation of F⁻, as well as the removal efficiency of TFA, PFOA and HFPO-DA (d). * **p ≤ 0.001; * *0.001 < p ≤ 0.01; * indicating 0.01 < p ≤ 0.05.

3.4. Co-occurrence network: potential defluorination microbes

The molecular co-occurrence network, constructed using 4650 associations among the shared 279 bacterial taxa and spearman correlation, was combined to further explore potential defluorination microbes (Fig. 6). Microbes potentially contributing to the removal of TFA, PFOA and HFPO-DA were grouped into four ecological modules, generated according to the correlation matrix ($r > 0.6$ and $p\text{-value} < 0.05$) of the microbial communities in Fig. 6a. Four modules comprised multiple taxa, and the total relative abundance of each module are presented in Fig. S4 and Fig. 6b. With the exception of Module #2, the PFOA and HFPO-DA groups exhibited higher total relative abundance in the remaining three ecological clusters. While the relative abundance of each treatment varied across the ecological clusters, the taxa composition within each module exhibited consistent patterns. For instance, Bacteroidetes occupied a dominant niche ($71.28 \pm 13.40\%$, mean) in all four groups from Module #1, whereas Proteobacteria emerged as the dominant taxa ($65.02 \pm 28.85\%$, mean) in Module #3.

Additionally, Module #2 and Module #4, composed of Proteobacteria, Bacteroidetes, and Nitrospirae, exhibited higher taxa diversity and evenness. The relationships between different ecological modules and the generation of F⁻ in selected groups were further calculated and are shown in Fig. 6c and Fig. S5. Only the relative abundance of Module #2 is positively correlated to the generation of F⁻ ($R^2 = 0.8688$, RMSE = 0.03628) after the incubation with TFA, PFOA and HFPO-DA. This suggests that microorganisms in Module #2 are likely to participate in the defluorination process.

Correlations between the relative abundance of species in Module #2 and the generation of F⁻ in TFA, PFOA and HFPO-DA groups, as well as the corresponding removal efficiency were depicted in Fig. 6d. After removing the interrelationships with $p > 0.5$ or $|r| < 0.8$ among the 96 species within Module #2, there are 24 remaining significant interrelationships. The majority of species are positively correlated with the removal efficiency of chemicals and the production of F⁻. The correlations show consistency between the removal efficiency of TFA, PFOA and HFPO-DA and the production of F⁻. The species involved in these positive correlations may play essential roles in the degradation process of TFA, PFOA and HFPO-DA.

As outlined in 3.3.2, certain species enriched during the biodegradation process carried genes related to carbon utilization, nitrogen cycling, and antibiotic resistance. Specifically, *Bacteriovorax stolpii*, *g.Sphingomonas* and *Methylobacillus flagellatus*, belonging to Proteobacteria; *Nitospira defluvii* and *c.Nitospira*, belonging to Nitrospirae; *Flexilinea flocculi*, belonging to Chloroflexi; *Solibacter usitatus*, belonging to Acidobacteria, were identified in associated with these processes [21,23, 28,41,75]. Moreover, these species exhibited a significant positive correlation with the generation of F⁻ as well as the removal efficiency of TFA, PFOA and HFPO-DA. Therefore, some species, such as *Flexilinea flocculi*, *Bacteriovorax stolpii*, and *g.Sphingomonas*, might be responsible for the defluorination through their potential collaborative functions. However, the corresponding relative abundance of these species showed that all of them have low abundance within the microbial community (Fig. S6). Therefore, more advanced microbial technologies, such as metagenomic binning and flow cytometer, are needed to characterize and identify these members of low abundance within the microbial community.

4. Conclusions

The objective of this study was to evaluate the biodegradability of TFA, PFOA and HFPO-DA by a native microbial community collected from PFASs and chlorinated solvents co-contaminated site and to explore the microbes in the community potentially participating in the biodegradation process. The generation of F⁻ and intermediate short-chain PFCAs, including TFA, PFA, PFHpA and PFHxA, were the key lines of evidence for the biodegradation of TFA, PFOA and HFPO-DA by

the indigenous microbial consortium. The co-occurrence network analysis showed that some low abundance species, such as *Flexilinea flocculi*, *Bacteriovorax stolpii*, and *g.Sphingomonas*, are positively correlated with the defluorination ratios and might participate in the biodeflourination processes through their potential collaborative functions. These findings provided insights in understanding the fate of PFCAs and their emerging alternatives in soil and groundwater. However, further studies are needed to address the limitations of the current study. Examples of such studies include the dynamics of the microbial communities beyond the incubation period of 10 months, the mechanisms explaining the lack of certain intermediates such as PFBA and PFPeA, and the exploration of unconfirmed compounds as well as how the functional species collaboratively play a role in the process. Furthermore, extensive studies are needed for future applications to the real-world environmental concerns.

Environmental Implication

Per- and polyfluoroalkyl substances (PFASs) have emerged as a group of contaminants of concern due to their persistence in the environment, bioaccumulation, and potential toxicity to human health and ecosystems. However, the degradation of PFASs poses significant challenges owing to the strong C-F bonds in their chemical structure. This study investigates the biodegradation of three representative PFASs by a native microbial consortium. The findings contribute valuable insights into the biodegradability of PFASs, enhancing our understanding of their potential degradation in the environment.

CRediT authorship contribution statement

Zhiwen Tang: Conceptualization, Data curation, Methodology, Writing - original draft, Investigation, Visualization. **Timothy M. Vogel:** Writing - review & editing, Validation, Methodology. **Qing Wang:** Methodology, Investigation, Resources. **Changlong Wei:** Methodology, Visualization, Software. **Mukhtiar Ali:** Methodology, Visualization, Investigation. **Xin Song:** Conceptualization, Funding acquisition, Validation, Writing - review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.133217.

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