



Stimulating *Acidimicrobium* sp. Strain A6 in iron-rich, acidic sediments from AFFF-impacted sites for PFAS defluorination

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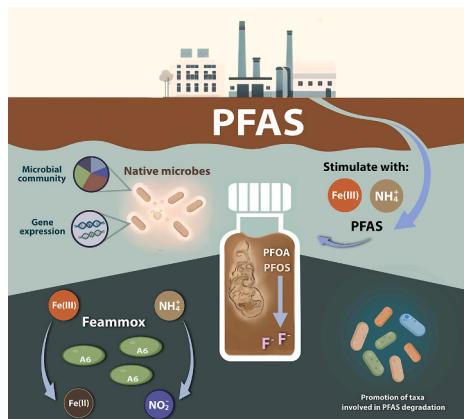
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HIGHLIGHTS

- *Acidimicrobium* sp. Strain A6 (A6) has been shown to defluorinate PFAAs.
- A6 is present at some acidic iron-rich AFFF-impacted sites and can be stimulated.
- This stimulation results in degradation of PFOA/PFOS and production of F⁻.
- It also results in an increased expression of dehalogenase and F⁻ transporter genes.
- The presence of PFAS affects the microbial community composition.

GRAPHICAL ABSTRACT



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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are persistent and bioaccumulative contaminants that are widely used in industrial applications and consumer products and pose significant risks to ecosystems and human health. *Acidimicrobium* sp. Strain A6 (A6), which is common in acidic, and iron rich soils and sediments is capable of both anaerobic ammonium (NH₄⁺) oxidation under iron reduction (Feamox) and defluorination of perfluorinated alkyl substances, such as perfluoroalkyl acids (PFAAs). This study investigates the potential for biostimulating A6 via the supply of NH₄⁺ and ferric iron (Fe(III)) with the goal of defluorinating PFAAs. Sediment samples from acidic, iron-rich, AFFF (aqueous film forming foam) impacted sites were collected and incubated with added Fe (III) and NH₄⁺. Quantitative PCR was used to track A6 numbers as well as dehalogenase and F⁻ ion transporter genes during these incubations; changes in the microbial community structure were tracked through 16S rRNA gene sequencing. The findings reveal that the addition of Fe(III) and NH₄⁺ stimulated the Feamox reaction and A6 growth and enhanced the degradation of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). Results also show a significant presence and activity of the above-mentioned genes in these incubations.

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The insights gained from this study could inform bioremediation strategies for PFAS-contaminated environments, especially in geochemical settings that favor the presence of A6.

1. Introduction

Many studies have focused on investigating soils contaminated with *per-* and polyfluoroalkyl substances (PFAS) to find PFAS-degrading microbes. However, most successful degradation of PFAS has been limited to the less recalcitrant polyfluorinated compounds (LaFond et al., 2023). PFAAs (perfluorinated alkyl acids) such as PFOA (perfluorooctanoic acid) and PFOS (perfluorooctane sulfonic acid) are particularly difficult to degrade (Smorada et al., 2024). These compounds have been linked to health effects (US EPA, 2018), for which the US Environmental Protection Agency has set very low drinking water levels, and remediation requirements (US EPA, 2024). Many other PFAAs monomers have been reported at sites contaminated by aqueous film forming foams (AFFF), these monomers may be part of the original AFFF formulation or might have been produced from the degradation/biotransformation of polyfluorinated alkyl substances (Reinikainen et al., 2022).

Feammox, which couples ammonium oxidation to iron reduction, has been observed globally in iron rich and acidic environments, including wetland sediments, paddy soils, acidic soils, and river sediments (Huang and Jaffé, 2018; Yi et al., 2019; Ahmed et al., 2021; Zhu et al., 2022; Cerdá et al., 2023; Sherman et al., 2023; Ma et al., 2024). In these settings, the Feammox process is thought to play an important role in the biogeochemical cycling of nitrogen (Yang et al., 2012; Ding et al., 2021; Sherman et al., 2023). *Acidimicrobium* sp. Strain A6, which is an organism capable of conducting the Feammox process (Huang and Jaffé, 2015, 2018; Jaffé et al., 2024), has been identified in many acidic, iron rich soils/sediments (Huang et al., 2016; Yi et al., 2019). *Acidimicrobium* sp. Strain A6 has also been shown to defluorinate perfluorinated alkyl substances (Huang and Jaffé, 2019; Jaffé et al., 2021; Ruiz-Urigüen et al., 2021; Huang et al., 2022; Park et al., 2023; Sima et al., 2023; Jaffé et al., 2024). Hence, understanding how to stimulate the Feammox process may become particularly important for remediating soils contaminated with PFAS.

In addition to the degradation studies of PFAAs by *Acidimicrobium* sp. Strain A6 mentioned above, only a few studies have to-date provided convincing findings about the degradation of PFAAs in environmental media (Smorada et al., 2024). In an anaerobic incubation with aquifer solids sourced from a fluorochemical plant, Tang et al. (2024) reported 13.52 % removal of PFOA (5 mg/L) supported by fluoride production and buildup of perfluoroheptanoic acid (PFHpA) and perfluorohexanoic acid (PFHxA) during 10-month incubations. At the end of these incubations, Proteobacteria and Bacteroidetes were the most abundant phyla. The study points to decarboxylation and subsequent hydrolytic elimination (Tang et al., 2024). Lorah et al. (2024) conducted anaerobic sediment incubations with a known dehalogenating culture, WBC-2, resulting in 46.4 % removal of PFOS when WBC-2 was incubated with chlorinated volatile organic compounds (cVOC) as co-contaminants. These results indicate that reductive defluorination of PFOS to non-fluorinated compounds or ultrashort-chain PFAS occurred over 45 days. A sediment incubation with added cVOCs, but without the WBC-2 culture, exhibited 25.5 % removal of PFOS. Although the PFOS removal was lower than the incubations with the WBC-2 culture, these sediment incubations suggest PFOS removal may occur naturally with the native microbes in the PFAS-impacted sediment. Fluoride analyses were not performed for these experiments (Lorah et al., 2024). PFOS biodegradation was accompanied by increased abundance of sulfate reducers, suggesting biodegradation was initiated by desulfonation of the sulfur-containing functional group. A small accumulation of perfluorohexane sulfonate (PFHxS), perfluoropentane sulfonate (PFPeS), and perfluorobutane sulfonate (PFBS) was detected in the incubations with WBC-2 and cVOCs (Lorah et al., 2024). In both biodegradation studies,

the bacteria cultures for these experiments were sourced from PFAS-contaminated sites, which are more likely to contain PFAS-degrading bacteria due to selective pressure (Wackett, 2024).

The objective of this study was to determine if *Acidimicrobium* sp. Strain A6 is present at selected iron-rich, acidic, AFFF-impacted Department of Defense (DoD) sites, and if so, if A6's activity can be stimulated via the addition of an Fe(III) source and/or NH₄⁺, and if this biostimulation results in the defluorination of PFAAs, including PFOA and PFOS. Furthermore, despite the increase in PFAS biodegradation studies, a better understanding of the impacts of PFAS impacts on the microbial community is warranted. Hence, an additional goal of this study was to determine how the presence of PFAS from AFFF-impacted soils may affect the microbial community structure.

To address these questions, a series of sediment batch incubations were conducted to track degradation of selected PFAAs, formation of degradation products, functional gene expression for enzymes that have been linked to PFAS degradation, and microbial community shifts in the presence of PFAS. Incubations used six distinct sediments, four from AFFF-impacted DoD sites and two were co-located but not impacted by AFFF. To determine the effect of higher PFAS concentrations and to allow for better tracking of the F⁻ produced, incubations were also performed with these sediments spiked with PFOA or PFOS.

2. Materials and methods

2.1. Sediments used in the incubations

Low pH aquifers, that may favor the presence of A6 are common throughout the Northern Atlantic Coastal Plain, ranging from Long Island, through most of North Carolina. Among 419 groundwater samples collected by the USGS from this aquifer system, 60 % were reported to have pH values of 5.5 or below (Denver et al., 2014). To conduct the proposed incubations, sediment samples were collected by CDM Smith from four different DoD sites located in the Northern Atlantic Coastal Plain (Table 1). Samples were obtained from four AFFF-impacted sites, and from two non-impacted locations near two of the AFFF-impacted sites. Sediment from the non-impacted sites shared similar physiochemical soil properties with the nearby AFFF-impacted sediment. Analyses reveal that the non-impacted sediments had a PFOS concentration of about an order of magnitude lower than the impacted sediments and a PFOA concentration of about 1/3 of the impacted sediments. All sediment samples collected were surface sediments (<2 ft. below ground surface, bgs, and several feet above the water table), and were collected using hand tools. All sediment samples were homogenized (coning and quartering) in the lab upon receipt. Sediment samples were shipped and stored in sealed 5-gal buckets at room temperature.

2.2. Setup of sediment incubation experiments

The incubation experiments were conducted using Sediments #I to #VI (Table 1), to study the transformation and degradation of PFOA and PFOS by native microbes during anaerobic incubations, with and without the addition of NH₄⁺ and/or Fe(III), the electron donor and acceptor required by *Acidimicrobium* sp. Strain A6. To conduct these incubations, 5 g of homogenized fresh undried sediment (original moisture content provided in Table 1) and 30 mL of either DI water or a Feammox medium (Huang and Jaffé, 2019) were placed in 50 mL vials. Incubation experiments were divided into four groups as detailed in Table 2: Group 1, Condition A is the background group to which only DI water was added; Group 2, Condition B includes nutrient amendments

with 2.8 mM of NH_4^+ , Condition C includes nutrient amendments with 10 mM of Fe(III), and Condition D with both NH_4^+ and Fe(III); Group 3, Condition E consists of incubations amended with 2.4 μM PFOA and Condition F amended with 2.0 μM PFOS, both of which were also amended with NH_4^+ (2.8 mM) and Fe(III) (10 mM); Group 4 consists of autoclaved controls for each condition, where the sediment and base medium were autoclaved before adding the ferrihydrite, PFAS (PFOA or PFOS), and vitamins.

All vials were vacuumed and flushed with a N_2/CO_2 (80:20) mixture to establish anoxic conditions and were sealed with butyl rubber stoppers and aluminum caps. They were then placed on a shaker at 25 °C for 90 days. Subsamples of the homogenized slurry were taken every 30 days to analyze for Fe(II), NH_4^+ , pH, F^- , and the targeted PFAAs, as well as microbial analysis. For the dissolved phase constituents (NH_4^+ , F^- , dissolved PFOA/PFOS), a supernatant sample was collected and filtered (0.22 μm) prior to analysis. For the PFOA/PFOS analyses, a representative sample (5 mL) of the slurry suspension was collected under strong mixing and extracted as described below. Each treatment was performed in triplicate, and the results were averaged to ensure accuracy and reliability.

2.3. Chemical analyses

For PFAS analyses, 5 mL of slurry sample were collected from each sample vial at each time point. An aliquot of liquid phase was collected after centrifuging at high speed (>15,000 $\times g$ > 5 min) to remove biomass and particulate matter. Then the liquid sample was stabilized in a methanol solution (50 % methanol). PFAS in the solid phase were extracted after centrifugation according to the method reported by Chiavola et al. (2020), for which 10 mL of a solution containing 50 % methanol and 50 % DI water were added to the centrifuged solids. The mixture was then sonicated in a water bath for 15 min at 60 °C, and then filtered through 0.22 μm membrane filters for PFAS analysis. Results of the analyses from the sediment extracts, which includes sorbed and dissolved PFAS were then expressed as mass of PFAS per volume of sediment slurry. The total concentration reported is the sum of the dissolved and sorbed concentration expressed as μmol of PFAS/L of sediment slurry. The method described by Chiavola et al., 2020 has been applied previously to sludge sediments (Huang et al., 2022). The method is based on ASTM D7968–17 and spike recovery % for all analytes met the acceptable range between 70–130%.

PFAS analyses were conducted using an LCMS-2050 Single Quadrupole Mass Spectrometer (Shimadzu), in accordance with EPA Method 537.1 (EPA 2018, Shimadzu, 2023). Filtered aliquots (1 μL) were injected into the LC/MS system, which is equipped with a 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 at 40 °C, enabling the separation of parent compounds (PFOS and PFOA) 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 ammonium acetate in water) and 5 % solvent B (HPLC-grade methanol), maintained for 0.5 min. The concentration of solvent B was then gradually increased to 95 % over 12 min and held steady for 2

Table 2

Conditions for incubation experiments.

Group 1	A	5 g sediment +30 mL DI water
Group 2	B	5 g sediment +30 mL medium +2.8 mM NH_4^+
	C	5 g sediment +30 mL medium +10 mM Fe(III)
Group 3	D	5 g sediment +30 mL medium +2.8 mM NH_4^+ and 10 mM Fe(III)
	E	5 g sediment +30 mL medium +2.8 mM NH_4^+ and 10 mM Fe(III) + 2.4 μM PFOA
Group 4	F	5 g sediment +30 mL medium +2.8 mM NH_4^+ and 10 mM Fe(III) + 2.0 μM PFOS
	G	Autoclaved control for each condition, which was also spiked with PFOA or PFOS

min, was subsequently reduced to 10 % in 1 min, and was 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, when applicable, depended on matching retention times with certified standards, detectable signals of quantifier (QT) and confirmation (CT) MS/MS transitions, and matching QT/CT ratios (Table S1). Four day 0 subsamples from Sediments #II and #III, under two different treatments—Condition A (DI water added) and Condition D (nutrient amendments with 2.8 mM of NH_4^+ and 10 mM of Fe(III))—were sent to SGS AXIS in Canada for analysis using EPA Method 1633. The results from this analysis were then, as described below, compared to those obtained through the methanol extraction method described above.

Anions and cations (NO_3^- , NO_2^- , F^- and NH_4^+) were analyzed using a Dionex™ Ion Chromatograph (IC3000) with an AS-18 and CS-16 column, respectively. As an additional confirmation, F^- was also analyzed using a perfectION™ combination electrode (Mettler-Toledo, USA). The Fe(II) concentration was measured spectrophotometrically with ferrozine at 560 nm, and Fe(III) was evaluated as the difference between the Fe(II) concentrations before and after reduction with excess hydroxylamine hydrochloride, following the method described by Lovley and Phillips (1987).

2.4. Microbial community analyses

Total DNA and RNA was extracted from each subsample, as described in the manual of the Fast DNA™ spin kit for soil (MP Biomedicals) or RNA PowerSoil® Total RNA Isolation 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 (Caporaso et al., 2012). 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

Table 1

Physicochemical properties of the sediments from four DoD sites from the Northern Atlantic Coastal Plain.

	NH_4^+ (mg/kg)	Fe(II) (mg/kg)	Fe(III) (mg/kg)	pH	moisture %	TOC (mg/kg)	A6 (10^6 copies/g)
Sediment # I. - Site 1, AFFF impacted	16.34	53.21	77.45	6.11	38.2	179.6	0.51
Sediment # II. - Site 2, AFFF impacted	5.08	67.02	61.19	6.04	41.5	117.8	0.43
Sediment # III. - Site 3, AFFF impacted	5.34	23.10	20.30	6.57	26.3	23.50	0.22
Sediment # IV. - Site 4, AFFF impacted	3.56	19.34	21.45	7.05	33.7	105.3	–
Sediment # V. - Site 2, non-AFFF impacted	6.87	72.32	85.34	6.05	32.5	98.21	0.27
Sediment # VI. - Site 3, non-AFFF impacted	5.41	23.14	33.70	6.03	21.3	86.32	0.33

Note: sediments from site 3 were very sandy, while the other sediments had higher clay contents.

platform at Novogene Co. (Beijing, China) and 250 bp paired-end reads were generated.

A total of 32,143–73,821 sequences were obtained for the 16S rRNA gene for each sample. Operational taxonomic units (OTUs) were clustered with a 97 % similarity cut-off using the Uparse software (Uparse v7.0.100, <http://drive5.com/uparse/>) (Edgar, 2013), which resulted in 2091–4680 Operational Taxonomic Units (OTUs). A representative sequence for each OTU was screened for further annotation. The taxonomy of each 16S rRNA gene sequence was analyzed with mother (version v.1.30.1) against the Silva SSUrRNA database using a confidence threshold of 0.8–1. Sequences are available from the NCBI Sequence Read Archive under BioProject PRJNA1130055.

2.5. qPCR analyses

qPCR analyses were used to quantify total bacterial numbers, *Acidimicrobiaceae* bacteria, dehalogenase genes, and F[−] transporter genes with specific primers and annealing temperatures (Table S2). The qPCR analysis of each gene was done on a 96-well StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA), following the detailed qPCR protocol as well as quality control described previously (Huang and Jaffé, 2018). The RNA recovery rate for these samples ranges between 67.2 % and 89.4 %. mRNA copy numbers of functional genes were examined by RT-qPCR using the One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa, Japan) following the manufacturer's instructions. The PCR amplification efficiencies were 92–112 %, and the correlation coefficients (R^2) for all assays were > 0.99.

2.6. Statistical analyses

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 Qiime (Version 1.7.0). Principal coordinate analysis (PCoA) of microbial community composition were performed to illustrate the similarity of the microbial community in different samples, and the significance was tested by permutational multivariate analysis of variance (Adonis) via R.

3. Results

3.1. Sediments characteristics from the AFFF-impacted sites

The chemical properties of the four samples from AFFF-impacted sediments and the two from non-impacted sediments are shown in Table 1. All the samples except those from Site 4 were acidic, with Fe(III) levels ranging from 20 mg/kg to 85 mg/kg. Slurry PFOS concentrations reported by SGS AXIS using the EPA1633 method were, for Sediments #II Condition A = 972.4 ng/g, and Condition D = 547.4 ng/g, and for Sediments #III Condition A = 916.4 ng/g, and Condition D = 1221.0 ng/g. These sediments were also analyzed for PFOS using the methanol extraction method described above. The results obtained were, for Sediments #II Condition A = 930.5 ng/g and for Condition D = 764.5 ng/g, and for Sediments #III Condition A = 1750.4 ng/g and for Condition D = 1407.6 ng/g. Comparing these results shows an average variation of ~14.1 % in PFOS concentration between methodologies. For PFOA, SGS AXIS reported concentrations for Sediments #II, Condition A = 5.6 ng/g and Condition D = 26.8 ng/g, and for Sediments #III, Condition A = 6.7 ng/g and Condition D = 6.5 ng/g. Using the methanol extraction method, analysis showed PFOA concentrations for Sediments #II, Condition A = 19.4 ng/g and Condition D 38.3 ng/g, and for Sediments #III, Condition A = 27.8 ng/g and Condition D = 37.3 ng/g. These results show an average variation of about 43.9 % in PFOA concentrations between the two methods, which is larger than that for PFOS. This larger difference for PFOA might be explained, at least in part, in that PFOA sorbs less than PFOS and the methanol extraction

method included both the dissolved and sorbed fraction, while the analyses by SGS were done only for the solid fraction and hence does not include the dissolved amount. Hence, we conclude that, for these specific experiments and sediments, the methanol extraction method yields PFAS analyses results that are useful for assessing trends in the experiments described below, particularly when performed in triplicate to account for variability.

The presence and quantity of *Acidimicrobium* sp. Strain A6 in these sediment samples was examined using qPCR with primer set v1–4 (Ruiz-Urigüen et al., 2018; Chester et al., 2023). The PCR products were then sequenced to ensure successful targeting of *Acidimicrobium*. As shown in Table 1, the presence of A6 could be detected (detection limit ~10² cells/g of sediment) in all sediment samples except Sediment #IV, which was the sample with a pH > 7.

3.2. Sediment incubation results

As discussed above, incubation experiments were carried out in four groups, as shown in Table 2. Group 1, Condition A, consists of sediment samples that were incubated with DI water as a control to compare the response of the system to stimulation with NH₄⁺ and/or Fe(III). Group 2, Conditions B, C and D, were set up to determine if A6 and the Feammox reaction responds to stimulation with an additional electron donor (NH₄⁺) and/or acceptor (Fe(III)). Conditions E and F in Group 3 were incubations supplemented with 2.4 μM PFOA or 2.0 μM PFOS as well as NH₄⁺ and Fe(III), to allow for an easier tracking of F[−] production. Group 4, Condition G, is an autoclaved control with added PFOA and PFOS.

3.2.1. Stimulation of the Feammox process during the sediment incubations

The Feammox reaction (anaerobic NH₄⁺ oxidation coupled to Fe(III) reduction) takes place under anoxic conditions. Under anoxic conditions, Fe(III) reduction in environmental samples may also be carried out by heterotrophic iron reducers. Hence, in these incubations we are tracking NH₄⁺ oxidation as an indicator of the Feammox process instead of iron reduction. Under anoxic conditions there could also be NH₄⁺ removal via annamox, which requires NO₂[−] as electron acceptor. Since in these sediments NO₂[−] and NO₃[−] were below detection (0.2 ppm), NH₄⁺ removal via annamox, if any, would be negligible in these incubations. Furthermore, the microbial community analyses did not show the presence of known anammox bacteria such as *Ca. Kuenenia*, *Ca. Jettenia* and *Ca. Brocadia*.

As shown in Fig. 1, over the 90-day incubation period, most sediment samples showed significant NH₄⁺ removal ($p < 0.05$), although there was variability for different sediments and incubation conditions. In all the abiotic controls, the NH₄⁺ concentration remained stable, suggesting that in the biotic incubations NH₄⁺ removal is microbially mediated. Sediments #I and #III showed relatively greater decreases in NH₄⁺ across all conditions, including when only deionized water was added (Condition A), achieving NH₄⁺ removal ranging from 0.11 to 2.61 mM, or approximately 40.3 % - 98.5 % over 90 days. In the case of Sediment #III, which contained much less Fe(III) than Sediments #I and #II, there was a noticeable impact from the addition of Fe(III) (Condition D vs. F). Sediment #IV (pH > 7, no detectable A6) showed the least NH₄⁺ removal, ranging from 0 % to 16.1 %, with a maximum of 0.42 mM. There was no significant difference in NH₄⁺ removal ($p > 0.1$) between the conditions with PFAS addition (Conditions E and F) and the condition without PFAS (Condition D). The highest NH₄⁺ removal was always observed in incubations to which Fe(III) was added.

Another key indicator that the Feammox reaction is proceeding, is the presence and growth of the proper Feammox microorganism, such as *Acidimicrobium* sp. Strain A6, although the presence of other possible unknown Feammox microorganisms in these environmental samples cannot be ruled out. Numbers of *Acidimicrobium* sp. Strain A6 for the different incubations are shown in Fig. S1. Results show that the addition of Fe(III) (with or without addition of NH₄⁺), Conditions C, D, E, and F resulted in an increase in A6 numbers over the incubation time.

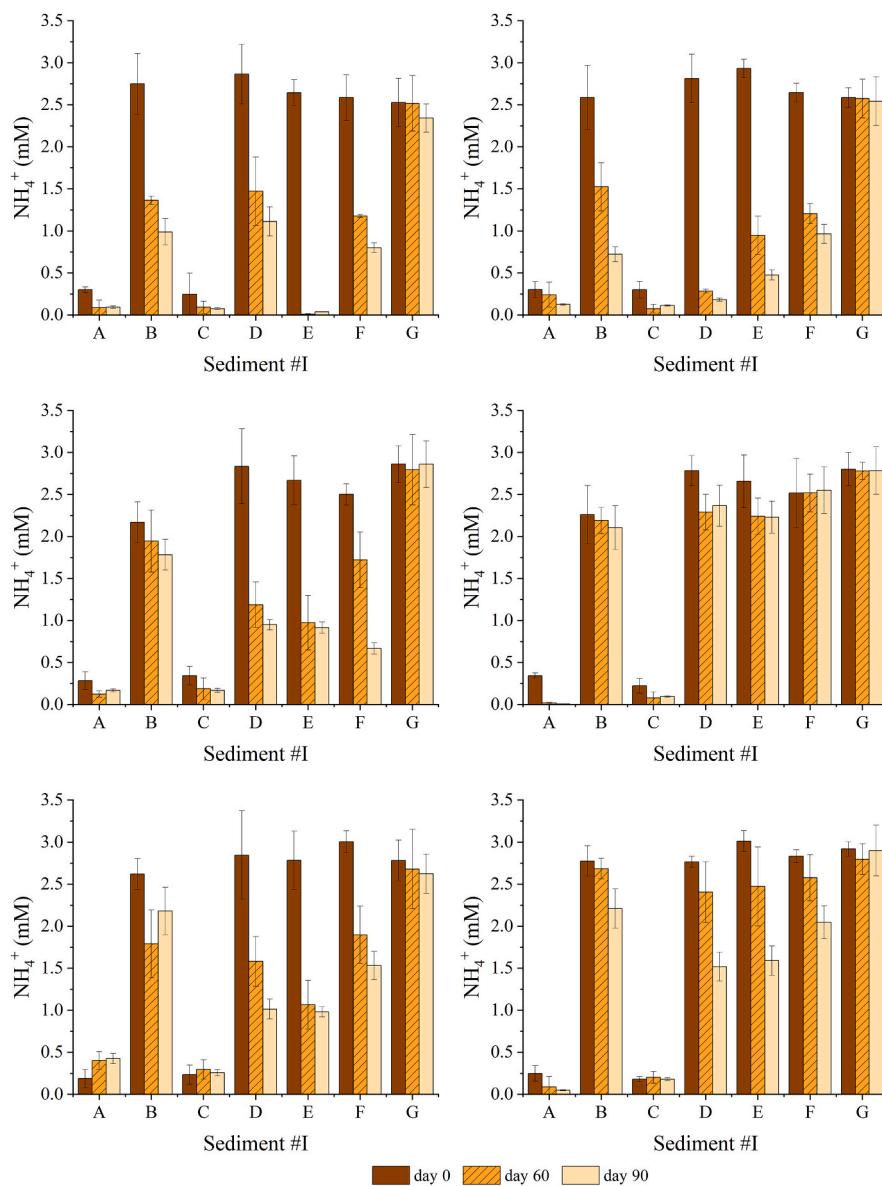


Fig. 1. Concentrations of NH_4^+ during incubation with different sediment slurries. (A = DI; B = medium + NH_4^+ ; C = medium + Fe(III); D = medium + NH_4^+ + Fe(III); E = medium + NH_4^+ + Fe(III) + 2.4 μM PFOA; F = medium + NH_4^+ + Fe(III) + 2.0 μM PFOS; G = autoclaved control spiked with 2.4 μM PFOA (not shown is the autoclaved control spiked with 2.0 μM PFOS). Sediments V and VI are non-PFAS impacted sediments near to Sediments II and III, respectively.

Incubations with Sediments #I and #II showed the most growth of A6 after 90 days of incubation, particularly when the samples were supplemented with NH_4^+ and Fe(III), where the A6 numbers increased from an average of 0.56×10^6 and 0.51×10^6 copies/g to 3.40×10^6 and 3.37×10^6 copies/g, respectively (Fig. S1). For Sediment #V, the increase in A6 numbers was minor in the absence of PFAS but much more pronounced when the sediments were spiked with PFOA or PFOS. Whereas the addition of NH_4^+ alone did not positively affect A6 numbers in any of the incubations. It is important to note that while an increase in A6 with PFAS addition was observed here, incubations with A6 and PFAS conducted to date have not shown that A6 benefits from PFAS degradation, when neither NH_4^+ nor Fe(III) are limiting. The stimulation of A6 in the presence of PFOA or PFOS observed here may be due to other factors present in the complex sediment environment, and further research is needed to clarify this effect. *Acidimicrobium* sp. Strain A6 was below detection at the beginning and end of the incubations with Sediment #IV (not shown).

3.2.2. PFAS degradation under different sediment incubation conditions

As shown in Figs. 2 and 3, over the 90-day incubation experiments, when Fe(III) was added to the sediments, a decrease in PFOA and PFOS could be observed in the incubations of Sediment #I, #II, and #III, which are the AFFF-impacted sediments where A6 was detected and could be stimulated. The incubations with the more alkaline Sediment #IV did not show a statistically significant decrease ($p > 0.1$) in PFOA or PFOS concentrations and A6 was not detected in these incubations. For Sediment #I, Condition A, where no additional nutrients were added to stimulate the Feammox reaction, a significant PFOA decrease ($p < 0.05$) was observed during that incubation. Sediment #I, had the highest NH_4^+ concentration of the sediments studied (by a factor of ~ 3), and among the higher Fe(III) concentrations, which may be contributing to Feammox activity without biostimulation, and hence may explain this decrease in PFOA, although we do not see a commensurate increase in F^- , nor a decrease in PFOS. The non-impacted Sediments #V and #VI, with 2.4 μM PFOA and 2.0 μM PFOS added, exhibited modest decreases in PFAA concentrations, while the AFFF-impacted Sediments #II and

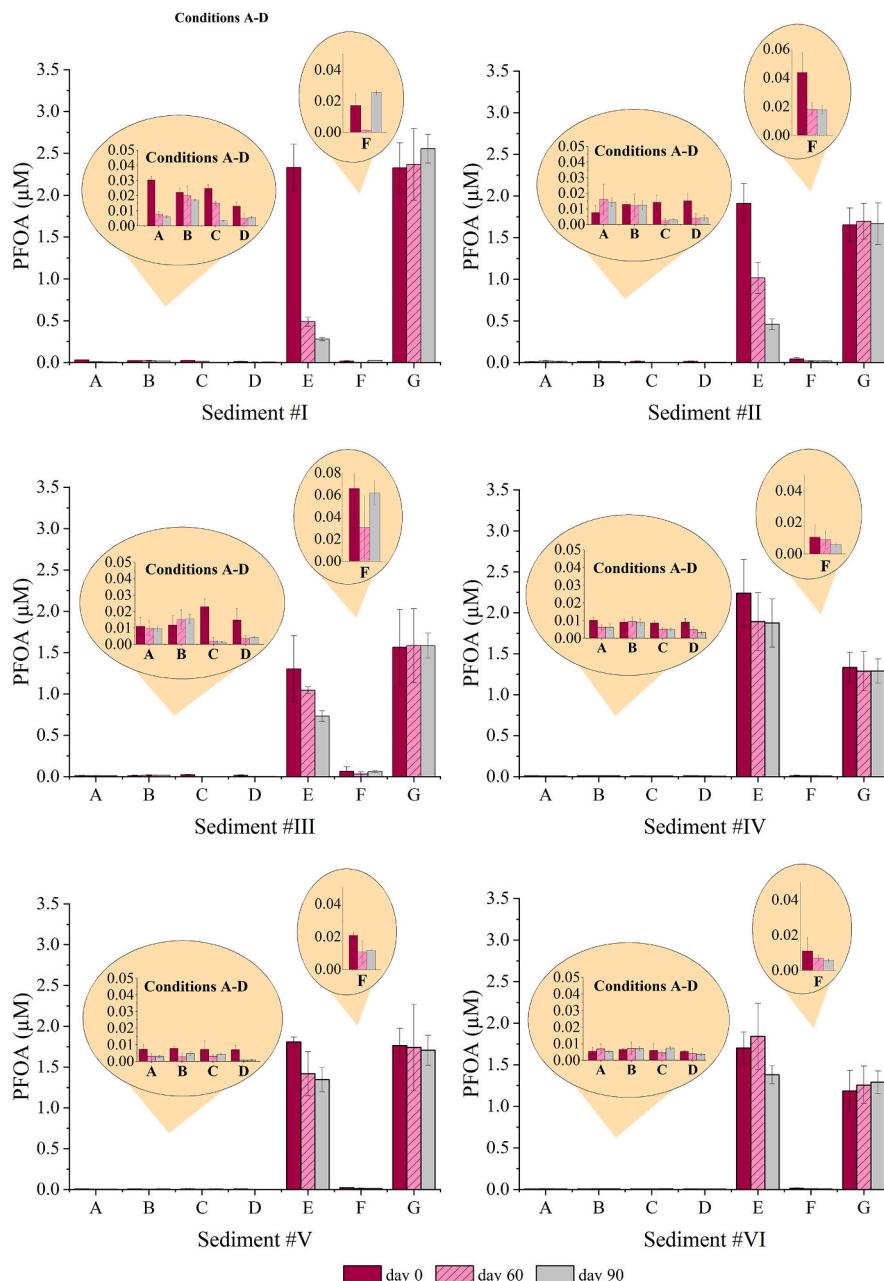


Fig. 2. Concentrations of PFOA during incubation with different sediment slurries. (A = DI; B = medium + NH_4^+ ; C = medium + Fe(III); D = medium + NH_4^+ + Fe (III); E = medium + NH_4^+ + Fe(III) + 2.4 μM PFOA; F = medium + NH_4^+ + Fe(III) + 2.0 μM PFOS; G = autoclaved control, spiked with 2.4 μM PFOA. Sediments V and VI are non-PFAS impacted sediments near to sediments II and III, respectively.

#III showed significantly higher ($p < 0.05$) removal. Notably, while the average fraction of PFOA degraded across these sediments remained relatively constant, the fraction of PFOS degraded increased when the sediments were amended with this PFAA. The reason for this difference is attributed to the difference in sorption of these two components in these slurry experiments. While dissolved-phase PFOS in the unamended sediments were at or below detection, once the sediments were amended with 2.0 μM PFOS, about 50 % of the mass remained in solution. Hence, a larger fraction of PFOS is degraded as its total mass, and therefore dissolved fraction, increased. In contrast, the difference between extracted (total) and dissolved PFOA mass is minor, indicating little sorption and less effect of the concentration on the total fraction degraded.

Fluoride (F^-) production during these incubations exhibited similar trends (Fig. S2). A substantial increase in F^- was observed in samples

spiked with 2.4 μM PFOA or 2.0 μM PFOS vs. that of the non-spiked samples, suggesting that the microorganisms present in these bio-stimulated sediments have the capability to defluorinate PFOA and PFOS. There was only a minor increase in F^- in the incubations of Sediment #IV in response to spiking these samples with PFOA or PFOS, which also did not show a statistically significant ($p > 0.1$) decrease in the concentration of these constituents during the incubations, although results indicate some decrease in the PFOA concentration that would be consistent with the small observed increase in F^- . There was no increase in F^- in the incubations with autoclaved sediment samples. A fluorine mass balance for the samples spiked with 2.4 μM PFOA or 2.0 μM PFOS (Conditions E and F, after correcting for the amount produced under Condition D), accounts for 69.5 % to 82.8 % of the fluorine in the system, assuming complete defluorination of PFOA and PFOS (shorter carbon chain PFAAs are likely to have been produced but were not quantified

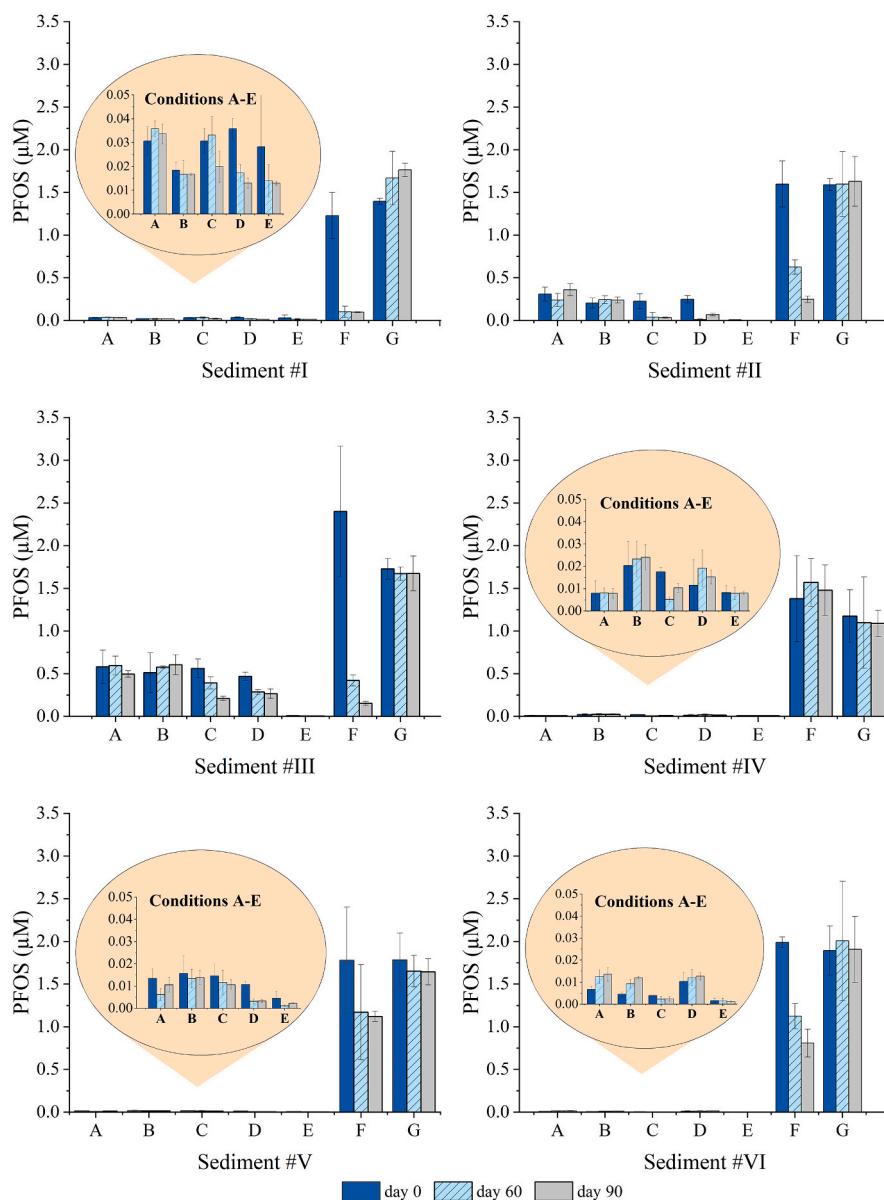


Fig. 3. Concentrations of PFOS during incubation with different sediment slurries. (A = DI; B = medium + NH_4^+ ; C = medium + Fe(III); D = medium + NH_4^+ + Fe(III); E = medium + NH_4^+ + Fe(III) + 2.4 μM PFOA; F = medium + NH_4^+ + Fe(III) + 2.0 μM PFOS; G = autoclaved control, spiked with 2.0 μM PFOS. Sediments V and VI are non-PFAS impacted sediments near to sediments II and III, respectively.

and could account, at least in part, for some of the missing fluorine).

3.3. Changes in the microbial community in the sediments during the incubations

Microbial analyses and community analyses were conducted to determine if: (i) *Acidimicrobium* sp. Strain A6 is present in these sediments, and whether stimulation with NH_4^+ and Fe(III) leads to its growth, and (ii) the presence of PFAS may impact the microbial community structure, before and after the incubations. Analysis of the microbial community structure were conducted on incubations of Sediments #I, #II, #III, #V, and #VI, with and without the addition of PFOA or PFOS.

In all incubations without the addition of PFOA/PFOS, except for Sediment #IV, the proportion of A6 remained between 0.3 % and 1 % (Table S5), showing little change before and after the incubations. In incubations with Sediments #I and #II, when supplemented with NH_4^+ and Fe(III), the A6 numbers increased during the incubation period by

approximately 5.7 times and 7.0 times, respectively (Fig. S1); however, the overall proportion of A6 did not increase substantially. After 90 days of incubation, the proportion of A6 in sediments with PFAS addition was higher compared to that in sediments without PFAS addition (Fig. S3, Table S5).

Comparing the alpha-diversity of the microbial communities between the AFFF-impacted sediments (Sediment #I, #II, and #III) and the non-AFFF-impacted sediments (Sediment #V and #VI), reveals that the alpha-diversity (Chao1 and Shannon index) is relatively higher in the non-impacted sediments (Table S4). Similarly, after 90 days of incubation, there was a slight decrease in the alpha-diversity of the microbial communities of Sediment #I and #II augmented with PFAS compared to their initial state (day 0) and the samples incubated without PFAS addition. In contrast, the addition of PFAS to AFFF-impacted sediments decreased the microbial diversity over time, whereas the addition of PFAS to the non-AFFF-impacted sediments (Sediments #V and #VI) resulted in minimal changes in the alpha-diversity. Notably, after 90 days of incubation with PFOA, Sediment

#V showed a slight increase in both the Chao1 and Shannon indices (Table S4).

The microbial community composition exhibits distinct differences between AFFF-impacted sediments (Sediment #I, #II, and #III), which have been exposed to long-term PFAS contamination, and the non-impacted sediments (Sediment #V and #VI) (Fig. 4, and S4). Notably, the microbial compositions of the AFFF-impacted sediments from different locations were more similar to each other than the AFFF-impacted vs. non-impacted sediments at the same location (Fig. 4 and S4). The PCoA analyses reveal significant variations in microbial communities during incubations with different treatments. There are significantly different ($p < 0.05$) in the microbial communities between samples with PFAS amended and those without PFAS amendment. Furthermore, in AFFF-impacted sediments (Sediments #I, #II, and #III), the microbial community composition on day 90 showed greater divergence compared to day 0 and day 30 (Fig. 5). *Burkholderiaceae* emerged as the most abundant family across all sediment samples, with *Paraburkholderia* and *Cupriavidus* being significantly more prevalent in AFFF-impacted sediments. Furthermore, families such as *Chthoniobacteraceae*, *Sphingomonadaceae*, *Alicyclobacillaceae*, and *Ktedonobacteraceae* were also more abundant in AFFF-impacted samples, whereas *Nitrosomonadaceae*, *Comamonadaceae*, and *Anaeromyxobacteraceae* were more prevalent in the non-impacted sediments (Fig. 3). The organisms mentioned above, that were higher in the AFFF-impacted sediments, increased further in the incubations with sediments augmented with PFOA or PFOS (Fig. S3).

3.4. Distribution and expression of functional genes related to PFAS level in sediments

Functional gene quantification was performed to assess whether the distribution and activity of specific functional genes correlates with PFAS contamination in the soil and/or the biostimulation of these sediments. The genes of interest that are potentially related to PFAS and their possible degradation include dehalogenase genes and F^- ion

transporter genes. Dehalogenases are microbial enzymes that catalyze the cleavage of a carbon-halogen bonds in halogenated organic compounds, playing a crucial role in the degradation and dehalogenation of organic pollutants in the environment, especially under anaerobic conditions. Previous work has shown that the genome of *Acidimicrobium* sp. strain A6 contains dehalogenase genes, including a putative reductive dehalogenase, *rdhA*, and that in incubations with this bacterium in the presence of different PFAS a strong correlation between F^- produced and the expression of the *rdhA* gene is observed (Jaffé et al., 2021, 2024). Furthermore, experiments where this gene was knocked out did not result in PFAS defluorination by *Acidimicrobium* sp. strain A6 (Jaffé et al., 2021, 2024). Based on these results, it was postulated that the *rdhA* gene in *Acidimicrobium* sp. strain A6, or more accurately, its related enzyme, plays a crucial role in the defluorination of some PFAS, such as PFOA and PFOS. Hence, the *rdhA* gene number and its expression were tracked during the incubation experiments described in these bio-stimulation experiments. In addition to this *rdhA* gene, it is likely that many other dehalogenase genes are present in these sediment samples and may play a role in the degradation of specific PFAS. Based on existing research on dehalogenase genes commonly found in environmental soil samples, we used both broad-range and A6-specific primers (as detailed in Table S2) to screen, detect, and quantify various dehalogenase genes using qPCR in the sediment samples collected for this study. Genes showing significant presence include 2-haloacid dehalogenase, haloalkane dehalogenase, haloacetate dehalogenase, and the conserved *rdhA* in *Dehalococcoides*. These genes were prioritized for detailed functional studies due to their significant presence in the AFFF-impacted sediments. Since F^- is toxic to microorganisms, in environments that have high levels of dissolved F^- , or when bacteria defluorinate fluorinated organics, including PFAS, bacteria employ a specialized channel protein known as the putative F^- ion transporter *crcB* to cope with the F^- toxicity (Wackett, 2021, 2024). Primers targeting *crcB* genes, based on the genome of A6, have been designed and evaluated using Primer-BLAST (Jaffé et al., 2024).

Using qPCR analysis, these genes were detected and quantified in

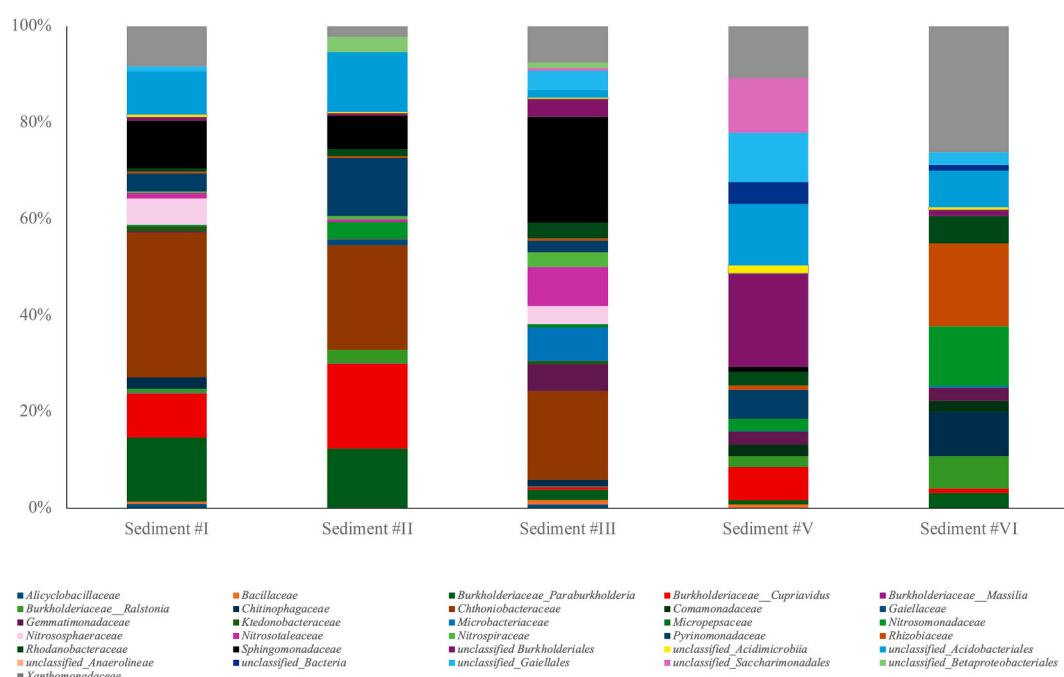


Fig. 4. Microbial community abundances at the family level of AFFF-impacted sediments (Sediment #I, Sediment #II and Sediment #III) and non-impacted sediments (Sediment #V and Sediment #VI). Only families with a relative abundance >0.5 % in at least 10 % of samples are represented. Detailed information at the genus level for the *Burkholderiaceae* family is provided. Specific information for the top 100 families is shown in Table S4.

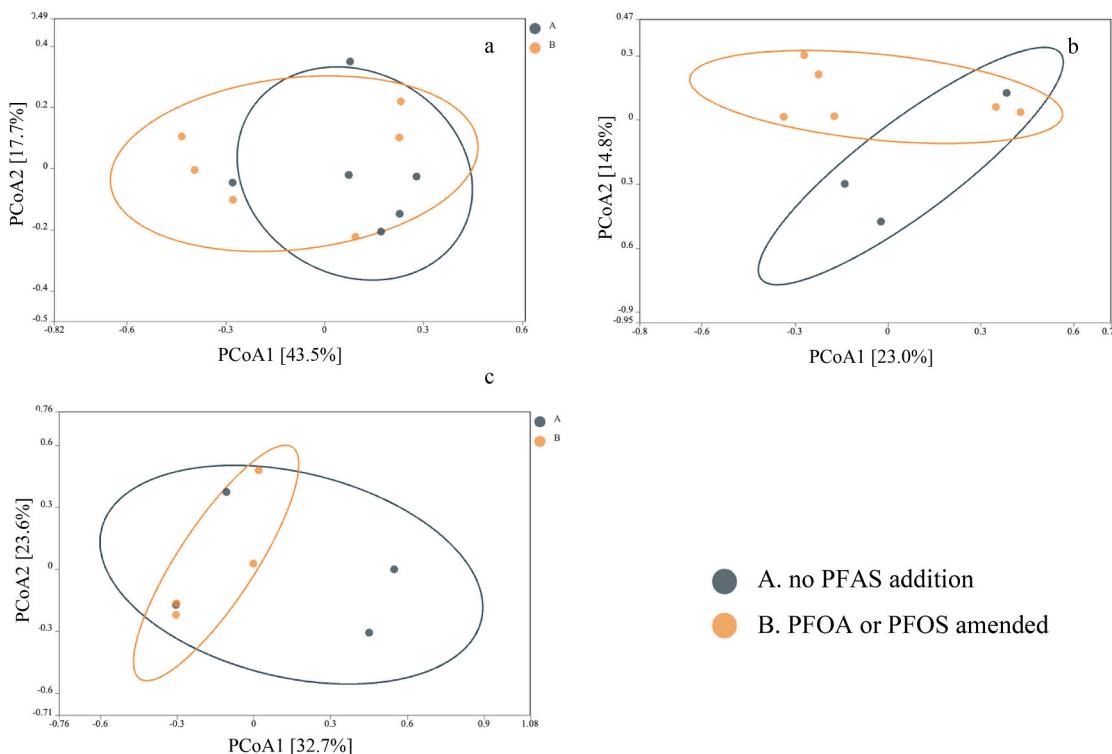


Fig. 5. Principal coordinate analysis (PCoA) ordination based on Bray-Curtis dissimilarity matrix showing significantly different ($p < 0.05$) microbial composition for Sediment #I, II and III on (a) day 0 and day 30, (b) day 90, as well as for Sediment #V and VI on (c) day 0 and day 90. Each point represents a sediment sample, color-coded according to different treatments applied, A. no PFAS addition; B. PFOA or PFOS.

AFFF-impacted sediments (Sediment #I, #II and #III) and non-impacted sediments (Sediment #V and #VI). The results show that the abundance of total dehalogenase genes (including 2-haloacid dehalogenase, haloalkane dehalogenase, haloacetate dehalogenase, reductive dehalogenase) was significantly higher in the AFFF-impacted sediments as compared to the non-impacted sediment samples (Fig. 6). Specifically, when targeting the reductive dehalogenase (*rdhA*) genes (including conserved *rdhA* in *Dehalococcoides* and *Acidimicrobiaceae rdhA* genes), the number of these genes in AFFF-contaminated samples were about two orders of magnitude higher than in the non-contaminated sediments (Fig. 6). Interestingly, Sediment #I, which harbored the highest abundance of *Acidimicrobiaceae rdhA* genes (Fig. 6), also demonstrated the greatest PFOA and PFOS removal during incubations following stimulation and the addition of these PFAAs (Figs. 2 and 3). The abundance of the F⁻ ion transporter *crcB* showed no significant difference between AFFF-impacted sediments (Sediment #II and #III) and non-impacted sediment samples (Sediment #V and #VI) (Fig. 6).

To determine the impact of higher PFAS concentrations on the abundance of these genes, analyses were also performed on incubations with AFFF-impacted sediments (Sediment #II and #III) and non-impacted sediment samples (Sediment #V and #VI) amended with PFOA and PFOS. Quantification of RNA using RT-qPCR revealed that the expression of selected dehalogenase genes (including 2-haloacid dehalogenase, conserved *rdhA*, and *Acidimicrobiaceae rdhA* genes) increased significantly ($p < 0.05$) during incubations with the addition of PFOA and PFOS, as compared to no PFAS addition in both AFFF-impacted and non-impacted sediments. The expression of *crcB* was significantly higher ($p < 0.05$) in PFAS-amended samples as compared to the non-amended samples (Fig. S5). The higher expression of *crcB* might be attributed to the higher F⁻ levels present in these incubations because of the defluorination of these PFAS.

4. Discussion

4.1. Stimulation of the A6/Feammox reaction and PFAS degradation in AFFF impacted sediment incubations

Anaerobic batch incubations were conducted with iron-rich sediments from different AFFF-impacted sediments. Results show that Sediment #I, #II, and #III, which have pH values around 6–6.5, foster an environment suitable for the Feammox reaction (Fig. 1). Incubations of these sediment samples demonstrated that A6 growth can be stimulated with added Fe(III), but the most significant growth occurs when both Fe(III) and NH₄⁺ are supplied (Fig. S1). The impact of added Fe(III) is greater for sediments with lower background Fe(III) levels, such as Sediment #III, where little to no NH₄⁺ oxidation occurs unless Fe(III) is added. In contrast, the addition of Fe(III) has a negligible effect on NH₄⁺ oxidation in Sediment #I, which has the highest Fe(III) levels. The effect of Fe(III) addition on NH₄⁺ oxidation in Sediment #IV was minor due to the higher pH, which is not conducive to the growth of A6 and the Feammox process. In incubations with active Feammox reactions and A6 growth, particularly those stimulated via the addition of Fe(III) and NH₄⁺, there was enhanced degradation of PFAS. In these incubations, the degradation of PFOS was higher than that of PFOA. After 90 days of incubation with PFAS amended, in Sediments #I, #II, and #III, there was an 84.6 % to 93.7 % decrease of PFOS, while the decrease of PFOA was 43.7 % to 87.9 % (Figs. 2 and 3, Fig. S6, shows the same results comparing changes in the different constituents between sediments and treatments).

When A6 was stimulated in these sediments and amended with PFOA/PFOS, a significantly greater decrease in the concentration of these PFAS was observed in the AFFF-impacted sediments (Sediments #I, #II, and #III) as compared to the non-impacted sediments (Sediments #V, and #VI), indicating that the microbial community of the AFFF-impacted sediments had experienced an acclimation/adaptation to PFAS. This might also explain why the degradation of PFOS was more

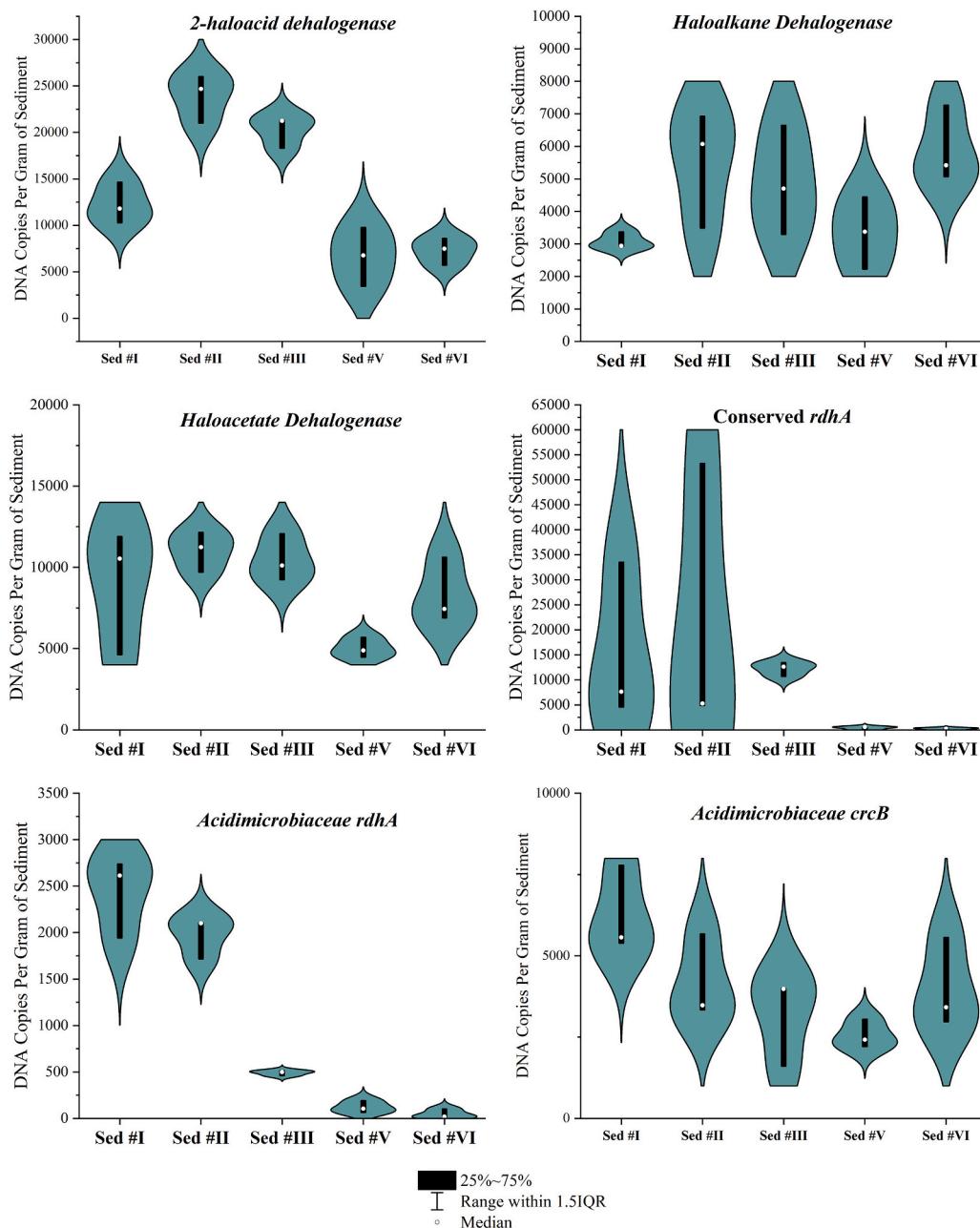


Fig. 6. qPCR results of selected dehalogenase genes and fluoride transporter genes from environmental samples. All measurements are shown in units DNA copy per gram sediments (Sediment # I, II and # III are AFFF-impacted sediments, Sediment # V and # VI are non-impacted sediments).

active in the AFFF-impacted sediments vs. that of PFOA, since PFOS was the most prevalent PFAA.

These incubation experimental results indicate that in iron-rich, acidic, AFFF-impacted sediments where A6 is present, some defluorination of perfluoroalkyl substances such as PFOA and PFOS can be achieved through the stimulation of the Feammox reaction. Although based on previous incubation results, the defluorination of PFOA/PFOS might be attributed to the activity of A6, the potential presence of other PFAS-degrading microbes can't be ruled out.

4.2. Effects of PFAS and Feammox stimulation on the microbial community structure

Significant differences were observed in the microbial communities between AFFF-impacted and non-impacted sediments (Fig. 4 and S4). As the incubations progressed under different treatments, the divergence in

community structure between PFAS-amended and non-amended sediments became more substantial (Fig. 5). Higher proportions of the genera *Paraburkholderia* and *Sphingomonas* were observed in AFFF-impacted sediments, and these genera being further enriched in the PFAS-amended incubations (Fig. S3). During incubation with PFAS, the relative abundance of *Paraburkholderia* increased from 11 % to 24 % after 30 days, but decreased to 2 % in the non-PFAS samples, and dropped in all samples by day 90 (Fig. S3). The genome of *Paraburkholderia* includes genes for (S)-2-haloacid dehalogenase, an enzyme recently identified as involved in PFOA degradation (Harris et al., 2022). Similarly, *Sphingomonas* showed higher proportions in PFAS-amended samples at day 30 and day 90 compared to non-PFAS incubations as well as the initial samples (Fig. S3). Recent studies indicate that *Sphingomonas* are more prevalent in PFOS-contaminated soil (Qiao et al., 2018; Yin et al., 2023) and are potential degraders of 8:2 FTOH, based on their increased abundance after exposure to 8:2 FTOH (Dong et al.,

2023). After 90 days of incubation, sulfate-reducing bacteria such as *Desulfoviroga* and *Desulfurispora*, along with sulfur/sulfide-oxidizing bacteria *Sphingomonas*, exhibited significant growth in incubations augmented with PFOS. *Desulfoviroga* and *Desulfurispora* may potentially be involved in PFOS degradation (Lorah et al., 2024), and/or their growth may be stimulated by the production of sulfate resulting from the degradation of PFOS. The presence of these organisms has been observed in A6 enrichment cultures (Huang and Jaffé, 2019; Ruiz-Urgüen et al., 2021) to which only PFOA or PFOS were added, and it was hypothesized that as A6 partially defluorinates these PFAA's, these organisms can then degrade the partially defluorinated compounds further.

4.3. Quantification and expression of selected functional genes during the biostimulation experiments

The microbial response to PFAA contamination involves complex mechanisms, which may be influenced by the presence or absence of various functional genes. Although much remains unknown, two key steps are essential for the degradation of PFAAs (Wackett, 2021). First, microbes must possess enzymes capable of catalyzing the C–F bond cleavage. Since all incubations of this study were conducted under anaerobic conditions, dehalogenase genes were the primary focus for catalyzing the C–F bond cleavage. Previous studies have shown a strong correlation between reductive dehalogenase gene expression and fluoride production in *Acidimicrobium* sp. Strain A6 incubations with PFOA, PFOS, and PFHxS (Jaffé et al., 2021). Consistent with these findings, this study revealed that dehalogenase genes are more prevalent in AFFF-impacted sediments and highly expressed in PFAS-amended incubations (Fig. 6 and Fig. S5). Results presented in Fig. 6 show that the largest difference between the presence of the genes tracked here in the AFFF-impacted vs. the non-impacted sediments is for the conserved *rdhA* and the *Acidimicrobeaceae rdhA*. Of these two, the *Acidimicrobeaceae rdhA* copy numbers in the initial AFFF-impacted sediments, tracks closest to the amount of PFOA/PFOS removed during biostimulation (Fig. S5). This further suggests that these enzymes may facilitate the removal of fluorine atoms from the carbon backbone of PFAS, leading to their breakdown and subsequent biodegradation/biotransformation. Secondly, microbes must protect themselves against fluoride toxicity, potentially through fluoride-proton antiporters. The *crcB* gene, a fluoride transcription regulator, is critical for microbial survival in PFAS-contaminated environments (Stockbridge and Wackett, 2024). Although the distribution of *crcB* genes did not show significant differences between AFFF-impacted and non-impacted sediments (Fig. 6), microbes lacking the *crcB* gene decreased significantly in number in incubations amended with PFAS, likely due to fluoride toxicity. Genera such as *Pseudolabrys*, *Gemmimonadaceae*, *Candidatus Omnitrophus*, *Tumebacillus*, *Candidatus Nitrosotalea*, *Dehalococcoidia*, and *Gaiellales* showed substantial reductions in PFAS-amended incubations compared to non-amended incubations (Fig. 4 and Fig. S3). A comprehensive investigation was conducted using the NCBI and KEGG databases to examine the presence of the *crcB* gene in the specified bacterial genera. Upon searching the genomic data available in these databases, no annotated *crcB* genes were found within these genera, indicating that these bacteria do not possess known *crcB* genes. In the sediment incubations described above, the expression of the *Acidimicrobeaceae crcB* gene was significantly higher in PFAS-amended sediments (Fig. S5), where the defluorination of PFOA/PFOS resulted in a significant increase in the dissolved F⁻ concentration.

These findings highlight the potential to use selected functional genes, such as dehalogenase genes and perhaps fluoride transcription regulator genes, as possible indicators for PFAS degradation. Monitoring the presence and expression of these genes could provide valuable insights into the microbial mechanisms driving PFAS biotransformation and inform bioremediation strategies.

5. Conclusions

Results of the incubations conducted here indicate that *Acidimicrobium* sp. Strain A6 is present at some acidic, iron rich sites, where it can be stimulated via the addition of Fe(III) and NH₄⁺, which results in an enhanced PFAS defluorination. Interestingly, when *Acidimicrobium* sp. Strain A6 was stimulated in these sediments, and they were amended with PFOA/PFOS, significantly more decrease in the concentration of these PFAS, and most importantly a corresponding increase in F⁻ was observed, which provided strong evidence that the added PFAS were being defluorinated, especially considering that no significant differences in the PFOA/PFOS or F⁻ concentration over the incubation period were detected in any of the autoclaved incubations.

Even though there is some noise in the analyses of PFOA/PFOS and F⁻ in the sediments without the addition of PFOA/PFOS, results do indicate that there is little effect from the addition of just NH₄⁺ on PFOA/PFOS degradation and F⁻ production, but once either Fe(III) or Fe(III) and NH₄⁺ are added, there is an overall measurable effect on PFOA/PFOS removal and F⁻ production, indicating that Fe(III) is limiting their degradation process.

Although the experiments did not focus on the specific role of *Acidimicrobium* sp. Strain A6 in the defluorination of these PFAAs, results presented here indicate that in iron-rich, acidic, AFFF-impacted sediments, where this bacterium is present, defluorination of perfluoroalkyl substances such as PFOA and PFOS may be enhanced via its stimulation with an Fe(III) source and NH₄⁺. The overall conclusion of these findings is that there might be sites with the proper biogeochemical characteristics, where biostimulation might result in the degradation of PFAAs, opening potential avenues for novel bioremediation schemes for PFAS contaminated soils.

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CRediT authorship contribution statement

Shan Huang: Writing – original draft, Methodology, Investigation. **Chiara Smorada:** Writing – original draft, Investigation. **Charles E. Schaefer:** Methodology, Writing – review & editing. **Peter R. Jaffé:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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 was provided by US Department of Defense. Peter Jaffe has patent #“Biodegradation of Fluorochemicals,” U.S. Provisional Application No. 62/792,971, January 16, 2019, (with S. Huang). pending to Princeton University. 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.

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Data availability

Data will be made available on request.

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