



PFAS biodegradation by *Labrys portucalensis* F11: Evidence of chain shortening and identification of metabolites of PFOS, 6:2 FTS, and 5:3 FTCA

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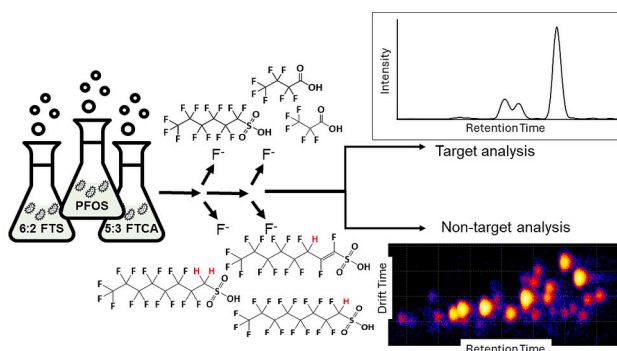
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HIGHLIGHTS

- Bacterial defluorination and chain shortening of PFOS, 6:2 FTS, 5:3 FTCA occurred
- PFOS removal of up to 96 % was observed after a 194-day incubation period
- After 100 day-incubation, up to 58 % of 5:3 FTCA and 21 % of 6:2 FTS were removed
- Several defluorinated PFOS metabolites were detected during biodegradation

GRAPHICAL ABSTRACT



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ABSTRACT

The biodegradation of three per- and polyfluoroalkyl substances (PFAS), namely perfluorooctane sulfonic acid (PFOS), 6:2-fluorotelomer sulfonic acid (6:2 FTS), and 5:3-fluorotelomer carboxylic acid (5:3 FTCA), were evaluated using *Labrys portucalensis* F11, an aerobic bacteria known to defluorinate fluorine-containing compounds. Cultures of *L. portucalensis* F11 were grown in minimal salts media and treated with 10,000 µg/L of individual PFAS as the sole carbon source in separate flasks. In PFOS-spiked media, several metabolites were detected, including perfluoroheptane sulfonic acid (PFHpS), perfluorohexane sulfonic acid (PFHxS), perfluorohexanoic acid (PFHxA), perfluoropentanoic acid (PFPeA), perfluorobutanoic acid (PFBA), and perfluoropropanoic acid (PFPrA). After 194-day incubation three de-fluorinated metabolites were identified: PFOS-F ($m/z = 480.940$), PFOS-2F ($m/z = 462.980$), and unsaturated PFOS-3F ($m/z = 442.943$). During the

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biodegradation of 5:3 FTCA, the following metabolites were observed: PFHxA, PFPeA, PFBA, PFPrA, and two fluorotelomer unsaturated carboxylic acids (5:3 FTUCA and 7:2 FTUCA). The biodegradation of 6:2 FTS was slower, with only 21 % decrease in concentration observed after 100 days, and subsequent formation of 4:2 FTS. On the contrary, 90 % of PFOS and 58 % of 5:3 FTCA were degraded after 100 days. These results indicate that *L. portucalensis* F11 can be potentially used for PFAS biodegradation in contaminated environments.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of highly fluorinated synthetic organic chemicals that have been extensively used in industrial, commercial, and domestic applications for over several decades now due to their surfactant-like, water-repellant, and oleophobic properties (Buck et al., 2011). The carbon-fluorine bonds characteristic of PFAS exhibit very high bond dissociation energies: around 485 kJ/mol, making PFAS generally resistant to degradation by oxidation, thermal treatment, and biological mechanisms; hence PFAS has been dubbed as “forever chemicals” (Brunn et al., 2023). PFAS can bioaccumulate in humans and animals following repetitive exposure (Taniyasu et al., 2003), leading to adverse health outcomes. These effects include thyroid disorders, immunotoxicity, neurotoxicity, and association with various types of cancer (Grandjean, 2018; Grandjean et al., 2020; Lewis et al., 2015; Vieira et al., 2013; Rios-Bonilla et al., 2024). The stability, vast applications, and improper waste disposal have made PFAS ubiquitous environmental pollutants (Barzen-Hanson et al., 2017; Prevedouros et al., 2006; Taniyasu et al., 2003) and requires costly remediation efforts (Cordner et al., 2021).

Strategies to enhance the biodegradation of PFAS are of great interest, particularly through bioaugmentation using microorganisms to degrade pollutants (Muter, 2023; Nzila et al., 2016). Bioaugmentation has been successfully implemented in soil bioremediation (Bokade et al., 2023; Dagliya et al., 2022), wastewater treatment (Chen et al., 2022; Dutta et al., 2022), and air biofiltration (Muter, 2023). However, there is limited information on the microbial degradation of fluorinated compounds, especially PFAS. Identifying microorganisms capable of degrading PFAS, evaluating their efficiency, and characterizing biodegradation products are critically needed because of the lack of cost-effective remediation technologies capable of removing PFAS in contaminated environments.

Recent studies have shown that microbial communities can defluorinate certain PFAS, suggesting that natural microbial defluorination exists (Yu et al., 2020). For instance, *Acidimicrobium* sp. strain A6 (A6) was found to reduce perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) in enriched cultures under anaerobic conditions (Huang and Jaffe, 2019). However, isolating the specific microorganisms responsible for PFAS biodegradation from mixed cultures can be challenging, especially if they are present at low abundance, making them difficult to identify and enrich. A recent study reported defluorination of α , β -unsaturated PFAS by *Acetobacterium* spp. (Yu et al., 2024), but only measured the increase in fluoride ion concentrations in the media without characterizing the metabolites formed during biodegradation of PFAS. Various *Pseudomonas* spp. have been shown to degrade some PFAS under aerobic conditions. For instance, *Pseudomonas parafulva* YAB1 resulted in 48 % reduction in PFOA over a 96-day incubation, but no biodegradation products nor fluoride ions were measured (Yi et al., 2016). Another study showed that *Pseudomonas aeruginosa* strain HJ4 isolated from the sludge of a municipal wastewater treatment plant decreased the concentration of PFOS by up to 67 % in 48 h (Kwon et al., 2014). However, no fluoride production was observed, and no major biodegradation products were uncovered, leaving one to suspect that the decrease in PFOS could be mostly due to adsorption rather than biodegradation. Another study demonstrated *Pseudomonas plecoglossicida* 2.4-D degraded PFOS by 75 %, increasing fluoride ions in the growth media, but also did not specify the degradation products formed (Chetverikov et al., 2017). Additional

information on bacterial degradation of PFAS under aerobic conditions from literature are listed in Table 1. Taking into account the aforementioned evidence, there is a need to identify microbial strains that have the ability to degrade PFAS and to characterize degradation products formed to assess their potential for PFAS bioremediation in highly contaminated sites.

This study investigates the ability of *Labrys portucalensis* F11, an aerobic bacterial strain from the Xanthobacteraceae family, to biodegrade three types of PFAS (listed in Fig. 1). Isolated from Estarreja, Portugal, *L. portucalensis* F11 has demonstrated metabolic versatility, effectively degrading fluorobenzene (Carvalho et al., 2008), 1,2-, 1,3-, and 1,4-difluorobenzenes (Moreira et al., 2012a), and fluorinated pharmaceuticals such as ofloxacin, norfloxacin, and ciprofloxacin (Amorim et al., 2014). Additionally, this bacterial strain can degrade fluoxetine, releasing fluoride from the perfluorinated methyl group ($-CF_3$) (Moreira et al., 2014). Therefore, the current study investigated the ability of *L. portucalensis* F11 to biodegrade 8-carbon chain PFAS with different headgroups and degree of fluorination on the carbon chain: 6:2-fluorotelomer sulfonic acid (6:2 FTS), 5:3-fluorotelomer carboxylic acid (5:3 FTCA), and PFOS, with structures shown in Fig. 1.

PFOS was chosen as the main test compound due to its high environmental abundance, frequent detection in biological samples, bioaccumulative nature, and known toxicity (Buck et al., 2011; Brunn et al., 2023). The study also included 6:2 FTS and 5:3 FTCA to compare the biodegradability of polyfluorinated versus perfluorinated substances, and the impact of different head groups (sulfonate vs. carboxylate). Further, there have been reports indicating that the presence of carbon without fluorine in PFAS makes them more biodegradable (Harding-Marjanovic et al., 2015; Zhang et al., 2016). Biodegradation was evaluated by measuring the decrease in PFAS concentrations and identifying biotransformation products using liquid chromatography with tandem mass spectrometry (LC-MS/MS) and ion mobility separation coupled with high-resolution mass spectrometry (IMS-HRMS). The results indicate that *L. portucalensis* F11 can defluorinate and shorten the chains of both poly- and perfluorinated PFAS, including those with sulfonated and carboxylated headgroups, highlighting the potential of this bacterial strain for bioremediation applications in PFAS-contaminated sites.

2. Materials and methods

2.1. Chemicals and reagents

Minimal salts medium (MM) was prepared with analytical grade chemicals (Sigma–Aldrich Saint Louis, MO) according to (Moreira et al., 2012b) without modification. PFOS and 6:2 FTS, were purchased from Sigma Aldrich (Saint Louis, MO) while 5:3 FTCA was purchased from SynQuest laboratories, Inc. (Alachua, FL). Other details can be found in Supporting Information (SI).

2.2. Microorganism

The bacterial strain *L. portucalensis* F11 (GenBank/EMBL/DBJ accession number AY362040; DSMZ accession number DSM 17916) was isolated from a sediment sample collected from an industrially contaminated site in Northern Portugal (Carvalho et al., 2008). This microorganism was selected for the biodegradation of PFAS due to its demonstrated ability to degrade fluorinated pharmaceuticals and fluorobenzenes (Amorim et al., 2014; Carvalho et al., 2008; Moreira et al.,

2012a; Moreira et al., 2014). The microorganism was routinely cultivated on nutrient agar (NA) plates incubated for 2 days at 30 °C to prepare the inoculum for the biodegradation experiments.

2.3. Biodegradation experiments

Biodegradation of PFOS, 6:2 FTS, and 5:3 FTCA by strain *L. portucalensis* F11 was carried out under aerobic conditions in 250 mL sealed flasks containing 50 mL of MM supplemented with 10,000 µg/L of PFOS, 6:2 FTS, and 5:3 FTCA, separately. Cells of *L. portucalensis* F11 were inoculated at an optical density (OD) of about 0.05 at 600 nm, and bacterial growth was monitored by measuring OD spectrophotometrically (Helios Gamma, Unicam Instruments, UK).

All the cultures were incubated at 30 °C on a rotary shaker (130 rpm). Experiments were performed in duplicate under sterile conditions. Abiotic control assays consisted of sealed flasks containing MM supplemented with 10,000 µg/L of PFOS, 6:2 FTS, and 5:3 FTCA separately, without bacterial inoculation. A control for cell growth was established with three flasks with the same concentration of methanol without PFOS, 6:2 FTS, and 5:3 FTCA addition. Samples were sacrificed for analysis at 0, 48, 100, and 194 days to assess microbial growth and PFOS, 6:2 FTS, and 5:3 FTCA biodegradation. The purity of the cultures was evaluated through regular plating on NA plates. Abiotic control experiments were conducted for PFOS and 5:3 FTCA incubating for 48-days. These experiments were performed under the same conditions as described above but without the presence of bacteria.

2.4. Analytical methods

2.4.1. Fluoride analysis

For each flask sacrificed for analysis, biomass was removed from culture samples by centrifugation at 14,000 rpm for 10 min at 4 °C. The concentration of fluoride ions in the supernatant was monitored weekly using an ion-selective electrode (model Orion 96-09, Thermo Electron Corporation, Beverly, MA), as previously described by (Moreira et al., 2014).

2.4.2. Solid phase extraction

Each of the culture supernatant (30 mL) was extracted and pre-concentrated through solid phase extraction (SPE) using Oasis® HLB and Resprep® MCX cartridges in tandem (HLB-WAX SPE), as described in our earlier works (Guardian et al., 2020; Halwatura and Aga, 2023). Detailed process can be found in the SI. Extraction recovery percentages, the method limits of detection (LOD) and limits of quantification (LOQ) can be found in our previous works (Guardian et al., 2020; Halwatura

and Aga, 2023).

2.4.3. Target analysis by liquid chromatography - tandem mass spectrometry (LC-MS/MS)

Target analysis for 40 PFAS (listed in Table S1) was carried out using the LC-MS/MS method described in our previous publication (Camdzic et al., 2023), and concentrations were determined using isotope dilution with ¹³C-labeled PFAS analogues (MPFAC – 24 ES). Detailed instrument parameters can be found in the SI. To prevent potential contamination, materials containing polytetrafluoroethylene (PTFE) were excluded. Each run incorporated blank injections following each PFAS sample injection, along with extraction blanks and quality control check standards, including a 50 µg/L PFAS standard mix. Throughout the runs, the areas of the analyte peaks, chromatographic retention times, and background contaminations were closely monitored.

2.4.4. Non-target analysis using ultra performance liquid chromatography with ion mobility separation coupled to a time-of-flight mass spectrometer (UPLC/IMS-QToF/MS)

To determine the formation of PFAS metabolites that were not included in the targeted PFAS analysis, a non-target analysis approach was performed using a Waters™ Acquity Ultra Performance Liquid Chromatograph with a SELECT SERIES™ cyclic ion mobility quadrupole-time-of-flight mass spectrometer (UPLC/IMS-QToF/MS) adopting a procedure described by (Organtini et al., 2023). A 5.0-µL sample was injected into the Atlantis™ Premier BEH™ C18 AX analytical column (100 × 2.1 mm × 1.7 µm) obtained from Waters Technology Corporation (Milford, MA). Gradient chromatography was performed using a flow rate of 0.30 mL/min with 2 mM ammonium acetate in Nanopure™ water (mobile phase A) and 0.1 % ammonium hydroxide in methanol (mobile phase B). Details of the UPLC/IMS-QToF/MS mobile phase conditions and MS parameters are described in SI, Table S2, and Table S3.

3. Results and discussion

High starting concentrations of PFAS that exceed typical levels found in environments were utilized for several critical reasons. Firstly, higher concentrations enable us to observe changes in the substance's concentration and detection of metabolites formed without pre-concentration. Secondly, elevated concentrations help the microbial community adapt to the substance, reducing the initial lag phase during which minimal degradation occurs (Özel Duygan et al., 2021). While the spiking PFAS concentration used in this study is not typical in the natural environment, PFAS concentrations can reach up to hundreds of mg/

Table 1

Microbial transformation of perfluoroalkyl sulfonic acids and polyfluoroalkyl sulfonic acids under aerobic conditions from literature.

PFAS	Microbial strain	Incubation time (days)	% of removal of starting PFAS	Transformation products	Reference
Perfluoroalkyl sulfonic acids					
PFHxS	<i>Pseudomonas</i> strains PS27 and PDMF10	7	~30–40 % reduction of bioaccumulation	–	(Presentato et al., 2020)
PFOS	<i>Pseudomonas aeruginosa</i> HJ4	48	67	PFHxS, PFBS	(Kwon et al., 2014) (Chetverikov et al., 2017)
	<i>Pseudomonas plecoglossicida</i> 2.4-D	6	In liquid media- ~100	PFHpA, F ⁻	
	<i>Pseudomonas plecoglossicida</i> 2.4-D	~180	In soil- 75	–	
Polyfluoroalkyl sulfonic acids					
6:2 FTS	<i>Gordonia</i> sp. NB4-1Y	7	99.9	6:2 FTCA (C6F13CH2CO2H), 6:2 FTUCA (C4F11CF=CHCO2H), 5:3 FTCA, 4:3 FTCA, PFHxA, PFPeA, PFBA	(Shaw et al., 2019)
	<i>Rhodococcus jostii</i> RHA1	~3	99	6:2 FTUCA, α-OH 5:3 FTCA, PFHpA, 6:2 FTUSA, (C6F13CH=CHSO3H)	(Yang et al., 2022)
	Enrich with <i>Dietzia aurantiaca</i> J3	7	~100	6:2 FTCA, 6:2 FTUCA, 5:3 FTCA, PFHxA, PFPeA, F ⁻	(Mendez et al., 2022)

L levels in manufacturing wastes (Prevedouros et al., 2006) and in spent granulated activated carbon (DiStefano et al., 2022).

3.1. Evidence of defluorination and chain-shortening of PFAS

A significant increase in the concentration of F^- (114 $\mu\text{g/L}$: 2 % of defluorination) was observed for PFOS after 48 days of incubation, which increased further in the last sample collected at 194 days (219 $\mu\text{g/L}$: 3 % of defluorination). Removal of up to 96 % of the initial PFOS was also observed after 194-day incubation, indicating biodegradation of PFOS by *L. portucalensis* F11. The increase in fluoride ion concentration and a corresponding decrease in PFOS over the period of incubation is shown in Fig. 2A.

Defluorination of 6:2 FTS and 5:3 FTCA by *L. portucalensis* F11 were also observed. Analysis of the culture media spiked with 6:2 FTS collected after 100-day incubation revealed increase in F^- concentration (76 $\mu\text{g/L}$: 1 % of defluorination) and a corresponding decrease in 6:2 FTS concentration (Table S6); the 48-day sample did not have detectable F^- and was therefore not analyzed by LC-MS/MS. Similarly, decrease in 5:3 FTCA over the period of 100 days was accompanied by increase in fluoride ions, with concentration reaching 86 $\mu\text{g/L}$ (1 % of defluorination) (Fig. 2C) at the end of the incubation period. Both concentrations of 6:2 FTS and 5:3 FTCA decreased in the culture media by 21 % and 58 % respectively, at around 100 days of incubation (Fig. 6).

The cell growth pattern was very similar in all the tested conditions, in the presence or absence (growth control) of PFAS, with the maximum OD_{600} of 0.233 ± 0.017 observed at 48 days. There was no evidence that the degradation of PFAS contributed to the growth of the bacteria; however, at the concentrations of PFOS, 6:2 FTS, and 5:3 FTCA used in the experiments, the amount of carbon could be too low to support bacterial growth. On the other hand, there was also no evidence of the toxic effect of the tested PFAS on *L. portucalensis* F11 cells, as the presence of these compounds did not affect growth.

No fluoride release was observed in the abiotic control, indicating that chemical or photolytic degradation of PFAS did not occur in the absence of *L. portucalensis* F11 cells. The abiotic controls for PFOS and 5:3 FTCA showed only around 0.2 % decrease in the corresponding PFAS in the culture media, indicating that the loss of spiked PFAS during the experimental procedure is negligible and there is no significant adsorption to the glassware. Data obtained from the biomass (Table S4) also showed that no significant amount of PFAS accumulated in the bacterial cells.

3.2. Biodegradation of PFOS and formation of metabolites

The spiked PFOS used as sole carbon source by *L. portucalensis* F11 was biodegraded into shorter chain metabolites, which were detected across the sampling points at 48, 100 and 194 days. Target analysis of the 48-day culture media revealed the formation of perfluoroheptane sulfonic acid (PFHpS), perfluorohexane sulfonic acid (PFHxS), perfluorohexanoic acid (PFHxA), perfluoropentanoic acid (PFPeA), perfluorobutanoic acid (PFBA), and perfluoropropanoic acid (PFPrA) (Fig. 2B). The metabolites detected in the target analysis matched the retention times of the isotopically labeled standards, as well as the expected MS/MS fragmentation pattern and ion ratios of the PFAS standards. Similar transformation products were detected for the repeated 6:2 FTS and 5:3 FTCA spiked samples for 194-day incubation (Table S5).

The production of perfluorocarboxylic acids (PFCAs) indicates that during the biodegradation of PFOS, the sulfonic acid head group is converted to a carboxylic acid group, as indicated in the proposed biotransformation pathway in Fig. 3. Based on a previous study (Cook et al., 1998), desulfonative enzymes in aerobic bacteria are generally regulated by induction when the sulfonate acts as a carbon and energy source, or by a global sulfur scavenging network when the sulfonate is utilized as a sulfur source. Further, the formation of carboxylated metabolites may have been promoted by the enzymatic activities of the bacteria as discussed by Yang et al. (2022). Another study suggested that the increased concentration of acetate in the culture media accompanies the biodegradation of PFOS (Huang and Jaffe, 2019), which may have promoted the formation of carboxylated metabolites. In proposing degradation pathways based on the metabolites identified in this study, we conducted a comparison with other PFAS-degrading bacteria and analyzed various degradation pathways to strengthen our findings. Table 1 summarizes the microbial transformation of perfluoroalkyl sulfonic acids, polyfluoroalkyl carboxylic acids, and polyfluoroalkyl sulfonic acids under aerobic conditions, as reported in the literature. These literature findings are consistent with our results and proposed degradation pathways.

The detection of shorter-chain PFAS in the culture media, along with the increased formation of fluoride ions, clearly indicate that *L. portucalensis* F11 has the ability to degrade PFOS. However, we hypothesize that the concentrations of the detected shorter-chain PFAS shown in Fig. 2B are underestimated because there may be other metabolites that have escaped detection by LC-MS/MS, including those that are volatile. The volatility of carboxylated PFAS increases with a decrease in chain length, resulting in loss to the atmosphere at the culture temperatures used (Mancinelli et al., 2023). In fact, the data from the second set of experiments (Table S5) showed the formation of shorter-chain PFAS, trifluoroacetic acid (TFA) and PFPrA, supporting our hypothesis. Unlike the first set, the samples from this second set of experiments were shipped and analyzed immediately, and were not stored for a prolonged period of time.

3.3. Identification of additional by-products of PFOS by non-target analysis

Non-target analysis using HRMS combined with IMS was used to identify other metabolites that were not included in the targeted analysis. Adding IMS gives the possibility to resolve species of interest from coeluting matrix interferences and separate structural isomers based on their collision cross section (CCS) (Izquierdo-Sandoval et al., 2022). Measured drift times (DT) in the IMS cell can be used to calculate CCS values that can serve as an additional parameter for identifying unknown metabolites. Based on the detected amounts of targeted PFAS metabolites, the remaining PFOS concentration, and the released F^- in the culture media, much of the fluorine content of the spiked PFAS remained unaccounted for.

While many peaks were detected with non-targeted analysis, a few major peaks of interest were selected for identification. Previous studies have demonstrated that compounds containing halogens such as fluorine occupy a unique space in the plots of CCS vs m/z values because halogenated compounds have smaller CCS values than compounds without halogen with the same m/z (Dodds et al., 2020; Foster et al., 2022; MacNeil et al., 2022; Mullin et al., 2020).

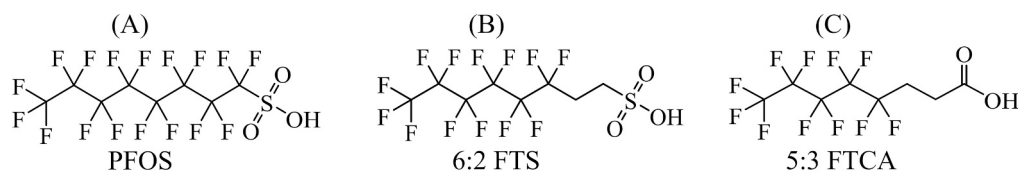


Fig. 1. Structures of A) perfluorooctane sulfonic acid (PFOS), B) 6:2-fluorotelomer sulfonic acid (6:2 FTS), and C) 5:3-fluorotelomer carboxylic acid (5:3 FTCA).

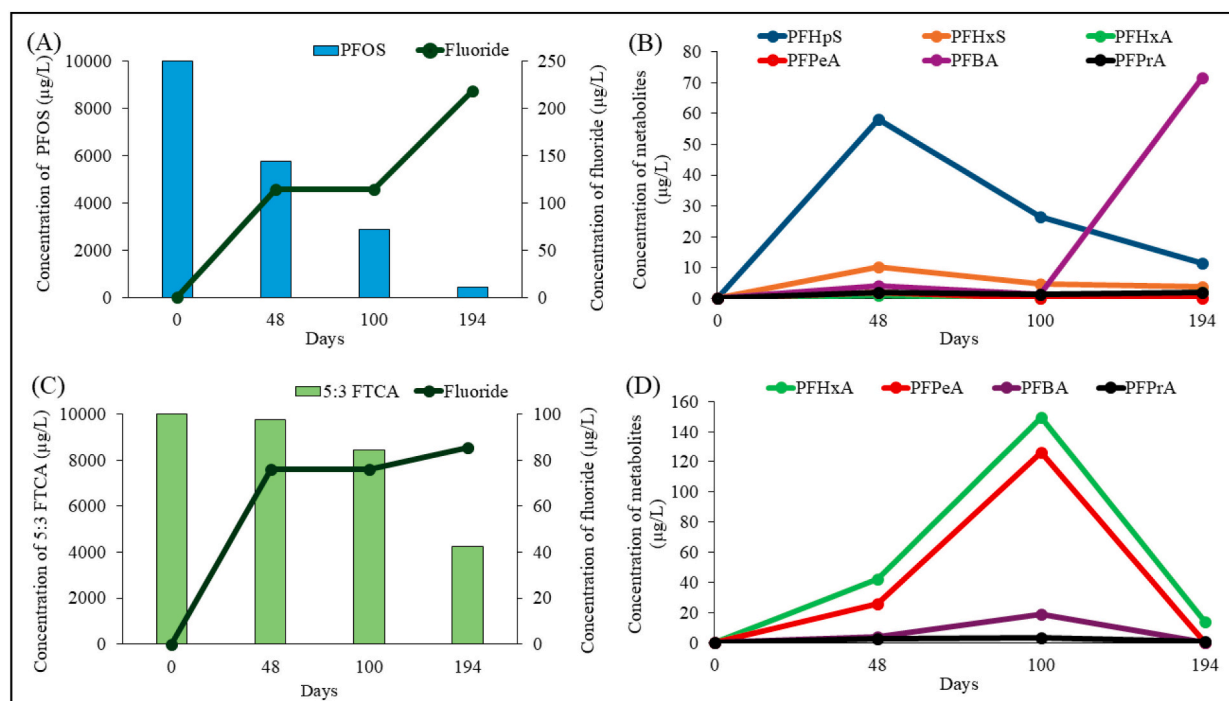


Fig. 2. Plot showing the decrease in PFOS and the corresponding increase in fluoride ions (A), the formation of shorter-chain PFAS metabolites (PFHpS, PFHxS, PFHxA, PFPeA, PFBA and PFPrA) from PFOS biodegradation (B) plot showing the decrease in 5:3 FTCA and the corresponding increase in fluoride ions (C), the formation of shorter-chain PFAS metabolites (PFHxA, PFPeA, PFBA and PFPrA) from 5:3 FTCA biodegradation (D) detected at 0, 48, 100, and 194 days of incubation.

As a starting point, the PFOS sample from the 194-day incubation was screened because it exhibited the highest number of metabolites detected by targeted LC-MS/MS analysis. Interestingly, non-targeted analysis identified three new additional metabolites of PFOS (Fig. 3). Each detected metabolite was assigned a corresponding confidence level based on the experimental m/z ratio, fragmentation pattern, retention time, mass defect, and isotopic pattern, as described in a previous publication (Charbonnet et al., 2022). The first metabolite identified (PFOS-F) had the formula of $C_8H_2F_{16}O_3S$, indicating the substitution of one fluorine with a hydrogen atom. The observed m/z value for the [M-H]⁻ adduct for PFOS-F ($m/z = 480.940$) matched the theoretical m/z within 5 ppm error. Fragment ions detected in the high collision energy spectrum of this compound matched the theoretical fragmentation of the proposed structure (Fig. S1). The PFOS-F detection was therefore assigned a confidence level of 3a because the location of the hydrogen substitution remains ambiguous.

The second metabolite (PFOS-2F) was tentatively identified with the formula of $C_8H_3F_{15}O_3S$ where two fluorine atoms in PFOS were substituted with two hydrogen atoms. The observed m/z for PFOS-2F ($m/z = 462.980$) matched the theoretical m/z value within 5 ppm error, and the MS/MS fragmentation matched the proposed structure (Fig. S2). Consequently, the PFOS-2F detection was assigned a confidence level of 3a because the locations of the hydrogen substitutions also remain ambiguous.

Lastly, a third metabolite (PFAS-3F) was tentatively identified with the formula of $C_8H_2F_{14}O_3S$; an unsaturated PFOS resulting from defluorination and dehydrogenation with a measured mass ($m/z = 442.943$) that matched the theoretical m/z value within 5 ppm error (Fig. S3). While more than 3 diagnostic fragment ions were detected, the confidence level was also assigned as 3a because the locations of defluorination and unsaturation in the molecule remain ambiguous.

Many PFAS, including PFOS, exist as branched and linear isomeric mixtures in natural environments due to their manufacturing process (Benskin et al., 2010; Dodds et al., 2020). The PFOS standard used as the carbon source for *L. portucalensis* F11 was a mixture of these linear and branched isomers which are partially separated chromatographically

(Fig. 4A). Ion mobility spectrometry provides an extra dimension of separation and allows the separation of the branched PFOS isomers. Coupling LC separation with 6 passes in the cyclic IMS device (IMS resolution $\sim 159 \Omega/\Delta\Omega$) revealed over 11 isomers of PFOS (Fig. 4A).

The defluorination processes and the subsequent formation of double bonds can occur in any of the isomers of PFOS, which results in metabolites that consist of numerous structural isomers with different CCS values; these isomers may be separated based on their CCS values using UPLC/IMS-QToF/MS. The use of IMS with 3 passes in the cyclic device (IMS resolution $\sim 112 \Omega/\Delta\Omega$) to characterize the defluorinated metabolites of PFOS revealed the presence of at least 9 isomers, for PFOS-F (Fig. 4B), with possibly more at lower intensities. For PFOS-3F analysis using 3 passes in the cyclic device (IMS resolution $\sim 112 \Omega/\Delta\Omega$), at least 13 isomers were observed (Fig. 4C). The formation of isomers during metabolism likely results from the bacterial strain's ability to remove fluoride from any carbon, and the potential to form the double bond at any place along the carbon backbone of PFOS. Results from these analyses suggest that while chain shortening is an important degradation pathway, transformation into defluorinated by-products of the same chain length might be as equally important during the biodegradation of PFOS. It is notable that only 3 % of fluoride ions were detected in the media, even after a 90 % decrease in PFOS was observed. While the identified degradation products suggest chain-shortening and dehalogenation as important biodegradation pathways it was difficult to quantify how much of the parent PFAS were actually converted to dehalogenated PFAS because quantification of metabolites was not possible without standards. Similarly, mass balance based on quantification of fluoride ions released was not achieved using the current set-up, hence it is not possible to accurately determine what the main degradation pathway is and is outside the scope of this study.

3.4. Degradation of sulfonated (6:2 FTS) and carboxylated (5:3 FTCA) fluorotelomers

Additional studies were performed to assess the ability of *L. portucalensis* F11 to degrade other PFAS and to determine the

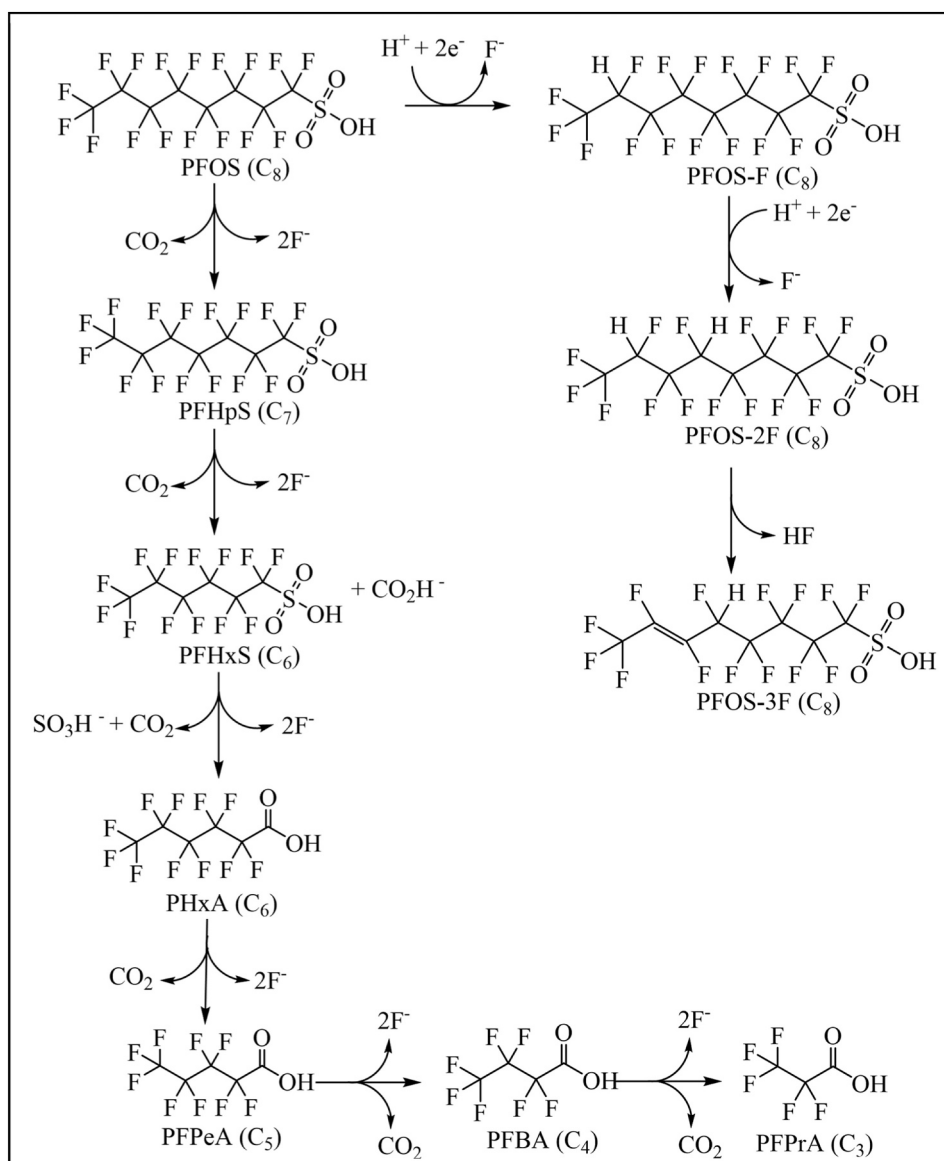


Fig. 3. Proposed biotransformation pathway for PFOS, showing the formation of detected metabolites, during the biodegradation by *L. portucalensis* F11. Defluorination, desulfonation, carboxylation, dehydrogenation, and chain-shortening of PFOS were observed. The number of carbons in the backbone chain is indicated inside the parenthesis to indicate the shortening of PFAS during biodegradation. PFOS-F, PFOS-2F, and PFOS-3F were identified at confidence level 3a, while the others were identified as level 1.

influence of headgroup and degree of fluorination. Hence, separate bacterial cultures were treated with 6:2 FTS and 5:3 FTCA, both of which are 8-carbon chain fluorotelomers, but with different headgroups. When fluoride ions were detectable in cultures, samples were harvested and analyzed by target LC-MS/MS. Hence, the initial sample (0-day incubation) and final sample (~100-day incubation) cultures treated with 6:2 FTS and 5:3 FTCA were analyzed by LC-MS/MS.

Removal of up to 21 % of the initial 6:2 FTS was observed in the final sample Fig. 6; 6:2 FTS concentrations in the spiked cultures decreased with time while the detected free fluoride in the culture media was observed above the limit of detection (Table S6). Over 100 days, the shorter chain fluorotelomer 4:2 FTS (Table S6) was detected as a metabolite as shown in Fig. 5, demonstrating that the spiked 6:2 FTS was degraded by the *L. portucalensis* F11. The metabolite matched the retention time, MS/MS fragmentation pattern, and ion ratio of the corresponding PFAS standard 4:2 FTS. The formation of short-chain metabolite by defluorination and decarboxylation is shown in Fig. 5.

Removal of up to 58 % of the initial 5:3 FTCA was observed in the

final sample Fig. 6; 5:3 FTCA concentrations in the spiked cultures decreased with time (Fig. 2C) while the detected free fluoride in the culture media increased. The 5:3 FTCA biodegradation products were shorter chain PFAS, including PFHxA, PFPeA, PFBA, and PFPrA, and were detected over 100 to 194-day incubation (Fig. 2D), demonstrating that the spiked 5:3 FTCA was degraded by the *L. portucalensis* F11 (Fig. 7). Similar transformation products with closer concentrations were detected for the repeated 6:2 FTS and 5:3 FTCA spiked samples for 194-day incubation (Table S5).

The metabolites detected in the targeted analysis matched the retention times of the isotopically labeled standards, MS/MS fragmentation pattern, and ion ratios with corresponding PFAS standards. The MS/MS fragmentation pattern and retention times of the metabolites also matched the corresponding standards. However, as noted above it is likely that the concentrations of the detected shorter-chain PFAS are underestimated because there may be other metabolites that could have escaped detection by target LC-MS/MS, including those that are volatile.

The 5:3 FTCA final sample was used for non-target analysis as it

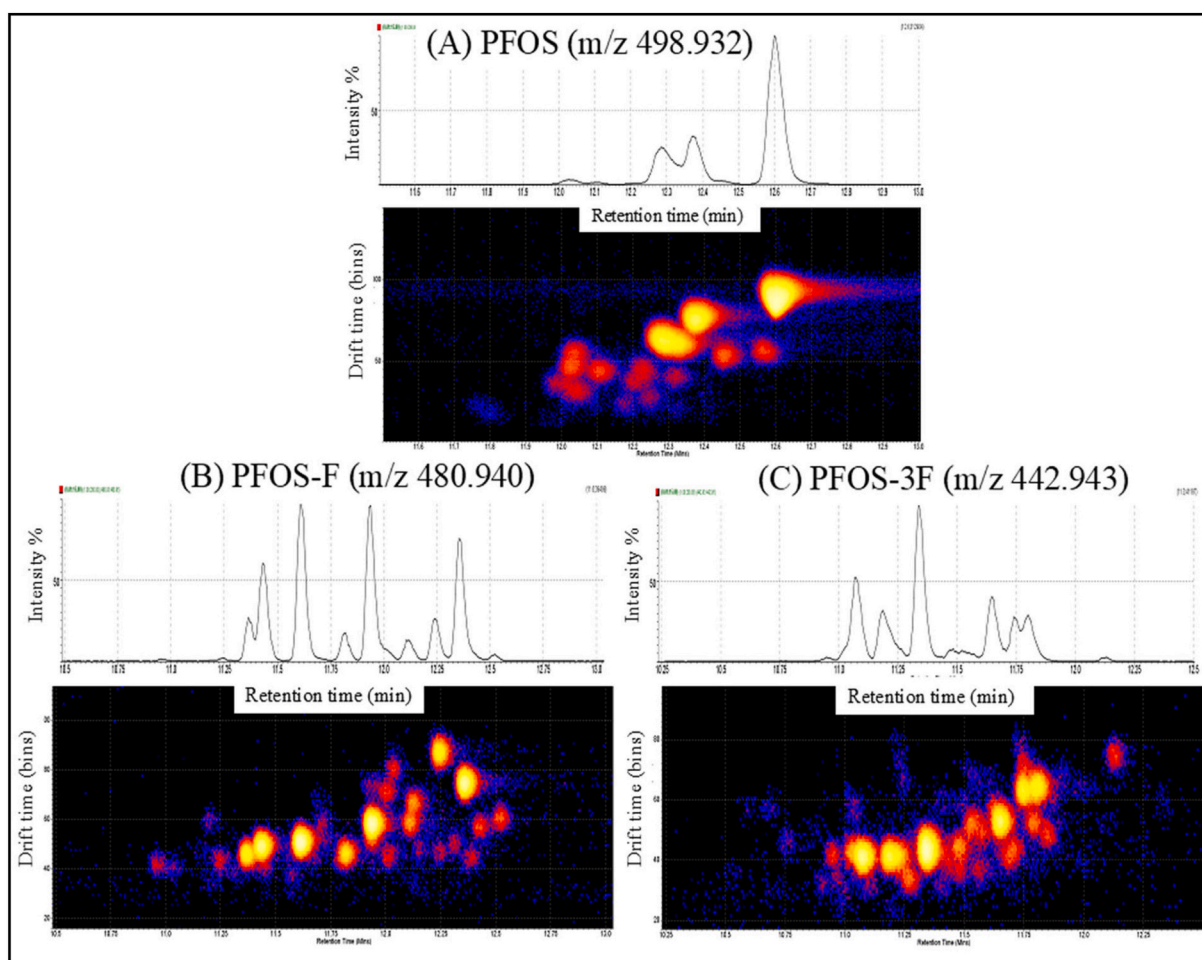


Fig. 4. Extracted ion chromatograms (top figure) and extracted ion mobilograms (bottom figures) exhibiting separation of (A) isomers of PFOS ($m/z = 498.932$), and isomers of defluorinated PFOS: (B) Isomers of PFOS-F ($m/z = 480.940$), and (C) Isomers of PFOS-3F ($m/z = 442.942$). Mobilograms are plots of drift times versus retention times for the selected m/z .

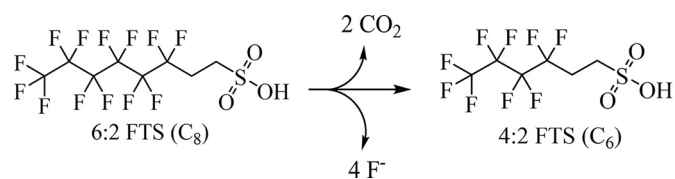


Fig. 5. Proposed biotransformation pathways of detected metabolites detected during the biodegradation of 6:2 FTS by *L. portucalensis* F11 using targeted analysis by Liquid chromatography - Tandem mass spectrometry (LC-MS/MS). All the compounds were identified as confidence level 1.

exhibited the highest number of metabolites detected during targeted LC-MS/MS analysis. Interestingly, the non-targeted analysis identified two metabolites of 5:3 FTCA in addition to those detected via targeted analysis (Fig. 7). The first putative metabolite was assigned with the formula $C_8H_3F_{11}O_2$ and a proposed structure of 5:3 fluorotelomer unsaturated carboxylic acid (5:3 FTUCA), resulting from dehydrogenation. The observed m/z value for the 5:3 FTUCA ($m/z = 338.990$) matched the theoretical m/z within 5 ppm, and the high collision energy fragmentation had diagnostic ions at 10.83-min (Fig. S4). The 5:3 FTUCA detection was therefore assigned a confidence level of 2b.

The second metabolite of 5:3 FTCA was one carbon shorter and assigned with a formula $C_7H_2F_{10}O_2$ ($m/z = 306.982$), this was tentatively identified as 5:2 fluorotelomer unsaturated carboxylic acid (5:2 FTUCA). The identification of this metabolite was assigned a confidence

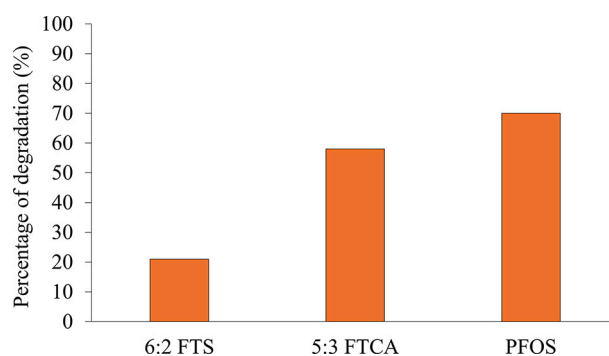


Fig. 6. Plot showing the percentage of degradation of 6:2 FTS, 5:3 FTCA, and PFOS in the final sample (100-day incubation) by *L. portucalensis* F11 bacterial strain.

level of 2b. The observed fragmentation pattern of 5:2 FTUCA in the high collision energy spectrum (Fig. S5) matched that of a reference standard for 6:2 FTUCA. Additionally, the observed CCS of the tentatively identified 5:2 FTUCA sits on the trendline created when plotting CCS vs m/z with the measured CCS values of 6:2 FTUCA and 8:2 FTUCA (Fig. S6). Subclasses of PFAS with the same headgroup have distinct mass/CCS trendlines (Dodds et al., 2020), and seeing that the value for the proposed identification of 5:2 FTUCA is on this trendline gives increased confidence in its identification. During the non-target analysis

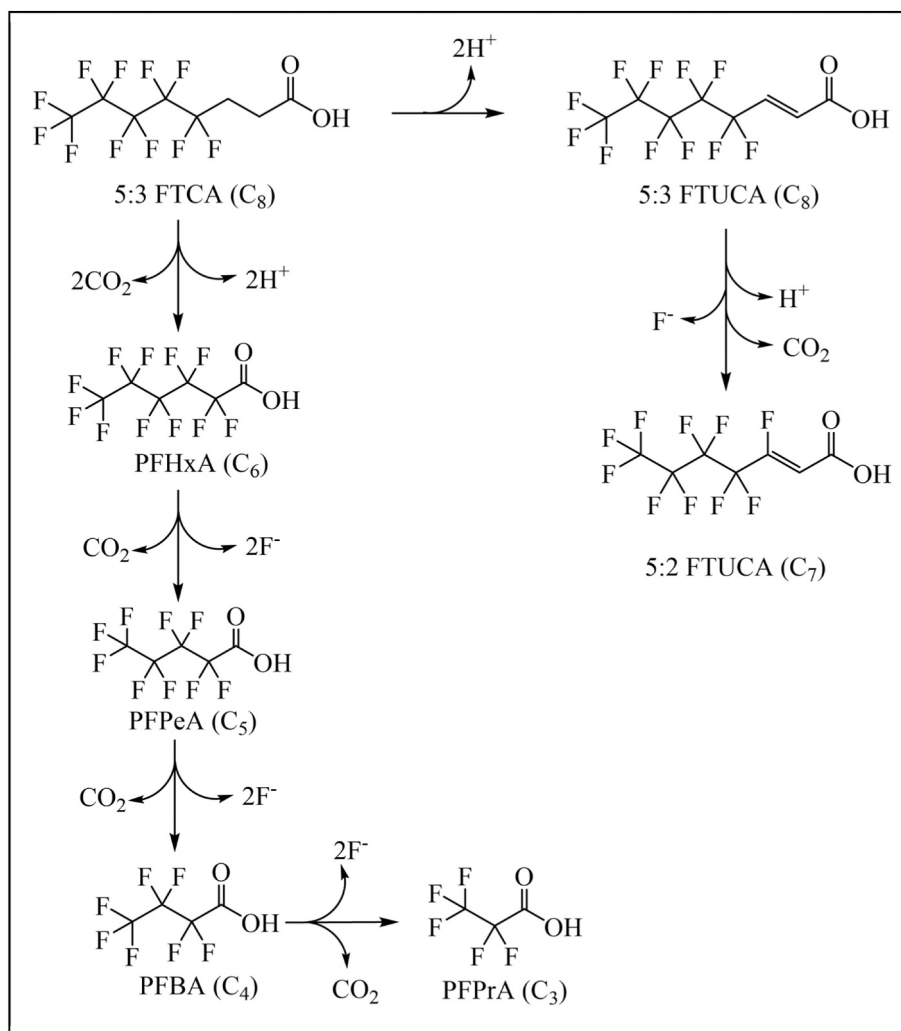


Fig. 7. Proposed biotransformation pathways of detected metabolites formed during the biodegradation of 5:3 FTCA by *L. portucalensis* F11 showing defluorination, hydrogenation, and decarboxylation. The 5:3 FTUCA and 5:2 FTUCA were identified with confidence level 2b while the others were identified with level 1.

of the 194-day incubation 5:3 FTCA sample, isomeric products as we demonstrated for PFOS (Fig. 4) were not identified.

In this study, the combined results from target and non-target analysis demonstrated the remarkable ability of *L. portucalensis* F11 to cleave the strong and stable C–F bonds present in PFOS, 6:2 FTS and 5:3 FTCA as shown in Fig. 3, Fig. 5, and Fig. 7 respectively. PFOS possesses a sulfonic acid head group, while 5:3 FTCA contains a carboxylic acid head group; despite the differences in head group chemistry, we observed the formation of biodegradation products, including shorter-chain PFAS, for the three tested compounds.

3.5. Environmental implications

The results of this study demonstrated that this bacterial strain may be a good candidate for bioremediation of PFAS-contaminated sites, such as in bioaugmentation strategies. *L. portucalensis* F11 may also be potentially used for seeding activated sludge in wastewater treatment plants, especially in the treatment of industrial wastes from manufacturing companies or in the semiconductor industries where PFAS are used in large quantities and end up in wastewater (Ober et al., 2022). As this aerobic bacterial strain was isolated from industrial waste-contaminated sites (Carvalho et al., 2008), it is well adapted to harsh environmental conditions extending its applicability in highly contaminated sites. The strain is able to grow at a temperature range of 16–37 °C and a pH range of 4.0–8.0 (Carvalho et al., 2008), which

confers a great potential for adaptation to various environmental conditions.

In this study, we showed the formation of several structural isomers resulting from the biodegradation of PFOS. These findings have several important implications because the federal regulations on the occurrence of PFAS in the environment are implemented only for the linear isomers, and toxicity studies do not consider the influence of the branched isomers. However, since PFAS bioaccumulation is facilitated by protein transporters (Forsthuber et al., 2020), it is likely that binding affinities and toxicity vary among isomers. It is also well known that structural isomers of organic chemicals significantly affect their biological activities. Therefore, the isomeric distribution of PFAS cannot be neglected as it may impact negative health effects, resulting in higher or lower potency compared with their linear counterparts. The findings in this study not only underscore the importance of fundamental scientific research but also create opportunities for engineering applications to carry out bioremediation of persistent halogenated compounds such as PFAS.

4. Conclusion

To date, *L. portucalensis* strain F11 is one of the few isolated microorganisms that has been reported to degrade PFAS successfully, as evidenced in the biodegradation of PFOS, 6:2 FTS, and 5:3 FTCA into shorter-chain compounds and production of fluoride ions in the

culture media. Notably, *L. portucalensis* F11 has the ability to degrade both per-fluorinated and poly-fluorinated compounds: up to 96 % of spiked PFOS after a 194-day incubation, and 58 % of spiked 5:3 FTCA and 21 % of spiked 6:2 FTS removed after 100 days incubation. Metabolites of PFOS degradation from 7-carbon to 3-carbon: PFHpS, PFHxS, PFHxA, PFPeA, PFBA, and PFPrA were detected in PFOS-spiked media. In 194-day incubation, three de-fluorinated metabolites were identified: PFOS-F ($m/z = 480.940$), PFOS-2F ($m/z = 462.980$), and unsaturated PFOS-3F ($m/z = 442.943$). During the degradation of 5:3 FTCA, several metabolites were formed: PFHxA, PFPeA, PFBA, PFPrA, 5:3 FTUCA, and 7:2 FTUCA. In 6:2 FTS-spiked media, 4:2 FTS was detected. While further experiments are needed to evaluate the ability of *L. portucalensis* F11 in degrading a wide range of “forever chemicals”, results from this study lay the foundation for optimizing analytical conditions to understand the mechanism of PFAS biodegradation by *L. portucalensis* F11, which in turn will provide critical insights that will inform the design of effective bioremediation strategies.

CRedit authorship contribution statement

Mindula K. Wijayahena: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Irina S. Moreira:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paula M.L. Castro:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Sarah Dowd:** Writing – review & editing, Methodology, Formal analysis. **Melissa I. Marciesky:** Writing – review & editing. **Carla Ng:** Writing – review & editing. **Diana S. Aga:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there are no financial conflicts of interest or personal relationships that could have influenced the research presented in this paper.

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Appendix A. Supplementary data

Additional chemical, experimental, and instrumental details, including additional mass spectra, repeated sample data. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.178348>.

Data availability

Data will be made available on request.

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