Biodegradation of PFOA in Microbial Electrolysis Cells by *Acidimicrobiaceae* sp. strain A6

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ABSTRACT

Acidimicrobiaceae sp. strain A6 (A6), is an anaerobic autotrophic bacterium capable of oxidizing ammonium (NH_4^+) while reducing ferric iron and is also able to defluorinate PFAS under these growth conditions. A6 is exoelectrogenic and can grow in microbial electrolysis cells (MECs) by using the anode as the electron acceptor in lieu of ferric iron. Therefore, cultures of A6 amended with perfluorooctanoic acid (PFOA) were incubated in MECs to investigate its ability to defluorinate PFAS in such reactors. Results show a significant decrease in PFOA concentration after 18 days of operation, while producing current and removing NH_4^+. The buildup of fluoride and shorter chain perfluorinated products was detected only in MECs with applied potential, active A6, and amended with PFOA, confirming the biodegradation of PFOA in these systems. This work sets the stage for further studies on the application of A6-based per- and polyfluorinated alkyl substances (PFAS) bioremediation in microbial electrochemical systems for water treatment.

Key Words: Microbial electrolysis cells, PFAS, PFOA, ammonium, *Acidimicrobiaceae* sp. strain A6, defluorination, dehalogenation, degradation.

Research Impact Statement: PFAS can be defluorinated in microbial electrochemical systems by Acidimicrobiaceae sp. strain A6 with NH_4^+ as electron donor.

1 Introduction

PFAS (per- and polyfluoroalkyl substances) are man-made chemicals that are widely used in consumer products such as non-sticking pans, paper coatings and firefighting foams. According to the National Institute of Environmental Health Sciences (NIEHS) (2021), there are over 7,500 different compounds that can be identified as PFAS, and the number is growing. Due to the ubiquitous usage and the recalcitrant characteristics of PFAS, their presence in the environment (Ahrens, 2011; Brusseau et al., 2020) and even in almost all of Americans' blood samples tested have been reported (CDC, 2012). Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are the two most common PFAS found in the environment (Brusseau *et al.*, 2020; Muir and Miaz, 2021) and pose a major health concern (ATSDR, 2021). The contamination of PFAS has been a concern for years, with its adverse health effects such as interfering with some hormones, affecting the immune system, and increasing the risk of cancer (Post et al., 2017; Saikat et al., 2013). Recently, the USEPA established a health advisory of 70 ng/L for PFOA and PFOS combined (USEPA, 2017). The state of New Jersey, for example, has an even more stringent advisory level of 13 ng/L (Health, 2020).

The C-F bond is the strongest covalent bond in organic chemistry (485 kJ/mol) (Natarajan et al., 2005), thus, it is extremely hard to breakdown perfluoroalkyl chemicals under environmental conditions via biodegradation. It has been reported that at least one hydrogen atom in the alkyl

chain was required to initiate the microbial cleavage of fluorinated alkyl compounds (Prevedouros et al., 2006). For example, research by Harding-Marjanovic et al. (2015) has documented the aerobic biotransformation of fluorotelomer thioether amido sulfonate (FtTAoS) over a 40-day incubation although the responsible organisms were not investigated. Another study indicated that *Mycobacterium vaccae* JOB5, *Pseudomonas oleovorans*, *Pseudomonas butanovora*, and *Pseudomonas fluorescens* DSM 8341 can degrade 6:2 fluorotelomer alcohol (6:2 FTOH) under aerobic conditions (Kim et al., 2014). Polyfluorinated telomeric structures were reported to be biodegradable under both aerobic and anaerobic conditions (Liu and Avendano, 2013). Unlike the above-mentioned polyfluorinated compounds, per-fluorinated compounds do not have hydrogen atoms on the alkyl chain thus making their biodegradation much more challenging, and evidence of their biodegradation is very limited. (Kwon et al., 2014) that incubations with *Pseudomonas aerug*inosa over 48 h could decompose 67% of PFOS. Yi et al. (2016) showed that incubations with *Pseudomonas parafulva* over 96 h resulted in a 32% decrease of PFOA. Chetverikov et al. (2017) showed that *Pseudomonas plecoglossicida* can use PFOS as a carbon source, decreasing the quantity of PFOS and releasing free fluoride ions during the incubation. However, these studies lack rigorous evidence of PFOS and PFOA biodegradation, since as mentioned in a comment on Kwon et al.'s work, identification of degradation products and careful analytical and microbiological methods are needed to ascertain that biodegradation of these compounds is occurring. In addition, there have been no reports on the biodegradation of PFOA nor PFOS by microorganisms via reductive defluorination until the recent findings by Huang and Jaffé (Huang and Jaffé, 2019), who found that the novel *Acidimicrobiaceae* sp. strain A6 (A6), an autotroph responsible for ammonium oxidation under iron reducing conditions, referred to as the Feammox process, can reductively defluorinate PFOA and PFOS under anaerobic conditions, and that

removal of up to 60% was achieved over 100-day incubations under anaerobic conditions. In follow-up studies they also reported defluorination of Perfluorohexane sulfonate (PFHxS) by A6 (Jaffé et al., 2021) and of PFOA in biosolids augmented with A6 and ferrihydrite (Huang et al., 2022).

A6 incubations using regular Feammox medium containing NH_4^+ and Fe(III) showed successful PFAS defluorination, and the presence of Fe(III) was necessary. However, the Feammox process requires large amounts of Fe(III) in a stoichiometric ratio of 6:1 of Fe(III):NH_4^+ ($3Fe_2O_3 \bullet 0.5H_2O + 10H^+ + NH_4^+ \rightarrow 6Fe^{2+} + 8.5H_2O + NO_2^-$) (Clement et al., 2005; Huang and Jaffé, 2015; 2018), thus, such a Fe(III) requirement would be a limitation for further applications, especially in biological reactors operated for long time periods or continuously. Previous studies on the bacterium A6 (Ruiz-Uriguen et al., 2018) and its bioelectrochemical characterization (Ruiz-Uriguen et al., 2019) have shown that it is an electrode-reducing bacterium (ERB) and can use NH_4^+ as the electron donor in microbial electrolysis cells (MECs) while reducing the electrode. We hypothesize that using MECs for PFAS defluorination with bacterium A6 will overcome the limitations of Fe(III) demand for maintaining active A6 cultures in biological reactors. Therefore, the objective of this study is to show that A6 is capable of PFOA degradation in MECs without the addition of solid Fe(III) phases.

Although the concentrations used in Huang and Jaffé (2019) were much higher than what is commonly observed in PFAS contaminated samples, at these concentrations (PFOA and PFOS up to 100 mg/L) they are not toxic to A6. Furthermore, at these concentrations the relative amount of PFOA lost to sorption is negligible for the Feammox incubations set up as described in Huang and Jaffé (2019) and conducted in this work. Finally, working at these high PFOA concentrations makes it easier to detect degradation intermediates. Hence, based on our previous experience, for

the proof-of-concept work conducted here, we used the same high PFOA concentrations used previously. Although not in the dissolved phase, very high PFOS concentrations, 460 mg/kg for sites in Australia and 373 mg/kg for US military installations, have been reported for soils by Brusseau et al. (2020).

2 Materials and Methods

2.1 Microbial electrolysis cell configuration

MECs (Figure 1) were constructed and run in parallel (Call and Logan, 2011; Ruiz-Uriguen *et al.*, 2019), using a stainless steel mesh (1.5 cm × 1.0 cm) as the cathode, and a graphite plate (1.5 cm × 1.0 cm × 0.32 cm) as the anode since it is chemically stable (Logan et al., 2006). MECs were set up in 10 mL serum bottles with rubber stoppers and aluminum seals. The headspace of each MEC was purged with an 80% N_2, 20% CO_2 gas mixture, and autoclaved. MECs were connected in parallel to a programmable power supply (model 3645A; Circuit Specialists Inc.) with a constant external applied voltage (V_{app}) set at 0.7 V(Ruiz-Uriguen *et al.*, 2019). Voltage was recorded hourly with a multimeter (model 2750; Keithley Instruments Inc.) across a 10 Ω resistor placed between the lead connecting the anode and the positive terminal of the power supply. All MEC reactors were placed on a mixing plate at 240 rpm. Current (I) was calculated using Ohm's law (I = V/R), where V is voltage and R the resistance. Data are reported as volumetric current density (I_d = A/m^3) which was obtained by dividing current by the volume of the culture (8 mL).

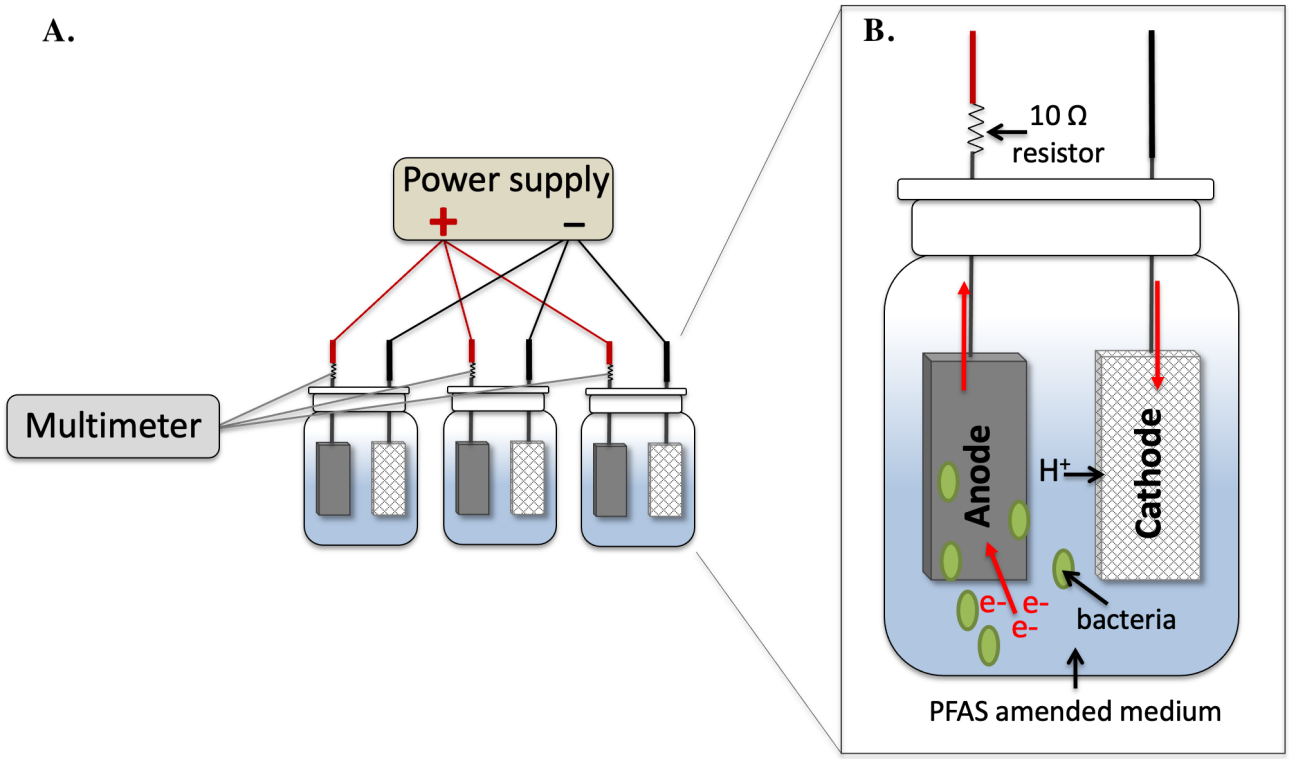


Figure 1. Schematic of (A) MECs multiplex setup, in which multiple MEC reactors can be connected to a single power supply and a single multimeter for voltage measurement. (B) Schematic of a single MEC inoculated with bacterial culture in a PFAS amended medium.

2.2 Acidimicrobiaceae sp. strain A6 cultures

The A6 enrichment culture was obtained from a Feammox laboratory-scale continuous-flow membrane reactor (Huang and Jaffé, 2015). This culture was maintained and incubated under anaerobic condition with inorganic Fe(III)-NH_4^+ enrichment medium (Huang and Jaffé, 2018). The A6 culture was obtained, as described in detail by Huang and Jaffé (2018) from the enrichment culture by streaking it on agar (18 g/l) iFeN medium plates with fresh ferrihydrite overlaid and incubating anaerobically at room temperature. Selected colonies were plated and incubated again, which was repeated three times, and then sub-cultured in vials containing a liquid iFeN medium

with about 10 mmol/l ferrihydrite and 2 mmol/l NH_4Cl, at a pH of 4.5. This procedure resulted in a highly enriched A6 culture. The cultures used for this study were then acclimated to 100 ppm PFOA for 250 days. These cultures were used to inoculate the MEC reactors.

2.3 Experimental setup

Perfluorobutanesulfonic acid (PFBS, $C_4HF_9O_3S$) at 100 mg/L and perfluorooctanoic acid (PFOA, $C_8HF_{15}O_2$) at 100 mg/L were tested in a preliminary experiment in the presence and absence of the A6 culture to demonstrate A6's ability to degrade PFBS and PFOA in MECs. Vials were set up in triplicate. The preliminary MECs sets followed the exact experimental setup described throughout this section, except for the addition of resazurin (1 mg/L) which is an indicator of anaerobic conditions and was added only in the preliminary experiment to confirm the anaerobic conditions of the MEC reactors setup. Following the preliminary experiments, PFOA was selected for further detailed experiments since PFOA showed less detrimental effect on A6 incubations as compared to PFOS (Huang and Jaffé, 2019), and the longer chain of PFOA, as compared to PFBS, allows the detection of multiple shorter carbon chain intermediates. Table 1 shows the different incubation conditions for the treatment and control groups. No voltage was applied to the positive control group where ferrihydrite was added as the electron acceptor. This control group is essentially the same as a regular A6 incubations that were run in previous studies (Huang and Jaffé, 2018; 2019). Treatment group and control groups 1 to 4 were inoculated with the A6 highly enriched culture and the A6 enrichment culture in parallel, and each group had three replicates. Therefore, 33 individual vials were set up for the PFOA experiment in total.

Table 1. Experimental setup. All groups were set up for both MECs inoculated with A6 highly enriched culture and A6 enrichment culture, except for control 4 to which no A6 culture was added.

	PFOA	A6	NH_4^+	Fe(III)
Treatment	+	+	+	—
Control 1	—	+	+	—
Control 2	+	dead cells	+	—
Control 3	+	+	—	—
Control 4	+	—	+	—
Positive control *	+	+	+	+

* No voltage was applied to the positive control.

The Feammox medium consisted of 3.8 mM NH_4Cl, 0.6 mM $(NH_4)_2SO_4$, 0.24 mM $NaHCO_3$, 0.71 mM $KHCO_3$, 0.066 mM KH_2PO_4, 0.41 mM $MgSO_4 \cdot 7H_2O$, 0.41 mM $CaCl_2$, 0.15mM AQDS (9,10-Anthraquinone-2,6-disulfonic acid disodium salt), 1 ml/L vitamin supplement (ATCC® MD-VS), and trace element solution as described by (Sawayama, 2006). AQDS was added as the mediator since it can facilitate electron transfer to the anode from NH_4^+ oxidation and enhance both the amount of NH_4^+ oxidized and current in MECs (Ruiz-Uriguen *et al.*, 2019). In previous experiments with MECs (Ruiz-Uriguen *et al.*, 2019), NH_4^+ oxidation did not increase significantly for AQDS concentrations > 0.15 mM, hence this concentration was selected for the experiments conducted here. Nominal PFOA concentration in the medium was 100 mg/L for the treatment group as well as control groups 2-5. Neither NH_4Cl nor $(NH_4)_2SO_4$ was added the no ammonium control group (control group 3). The preliminary MECs sets amended with PFBS or PFOA contained resazurin (1 mg/L) as an indicator of anaerobic conditions. The pH

of the medium was initially set to 5 - 5.5 because the Feammox process works best at acidic conditions with pH below 6.3 (Huang and Jaffé, 2015; 2018).

The MECs were operated for 18 days before taken apart. A previous study (Ruiz-Uriguen *et al.*, 2019) shows that MEC reactors setup under the required shaking conditions results in the deterioration of the connections, therefore, operating periods beyond 3 weeks results in noisy readings generated by the connections between leads and electrodes becoming loose due to the movement of the whole reactor on the mixing plate. Additionally, due to the nature of the MEC reactors multiplex setup, it is not feasible to set up enough MECs to be destructively sampled over time to have a time course. Therefore, aliquots of initial and final samples were taken, which were filtered through 0.2 μm syringe filters and kept in 4°C until further analysis. Aliquots of unfiltered initial and final samples were frozen in -20°C until DNA extraction for A6 and total bacterial quantification.

2.4 Chemical analysis

NH_4^+ and F^- concentrations were analyzed via ion chromatography (IC). Filtered liquid samples were acidified using 6 N HCl to a final pH ~ 2, to prevent Fe^{2+} from oxidizing to Fe^{3+} and avoid Fe(III) fluoride complexes interfering with the IC measurements. Analyses were run on a Dionex™ Ion Chromatograph ICS3000, with a CS-16 column, CS-16 guard column, and a CERS 500 (4 mm) suppressor for cations; and with an AS-18 column with a KOH solution (26 mM) as the eluent at a flow rate of 0.8 mL/min, and suppressor current set at 85 mA, for anion analysis. Dionex™ Retention Time Standards for all the anion analyses were used. For F^- measurements, this method has a reported detection limit of 0.003 mg/L (IonPac AS18 Anion-Exchange Column; Dionex, 2011). In the second set of experiments, F^- was analyzed using a perfectION™

179 combination electrode, this method has a reported detection limit of 0.02 mg/L (Mettler-Toledo,
180 2011). Measured F^- values for the enrichment culture were for each method, whereas for the highly
181 enriched A6 culture samples there was an interference using the IC method, hence for that
182 experiments F^- results reported are from the measurements with the perfectION™ combination
183 electrode.

184 PFOA and its degradation intermediates were analyzed by the Guangdong Institute of
185 Microbiology (China) via ultraperformance liquid chromatography-tandem mass spectrometry
186 (UPLC-MS-MS: Agilent 1290-6430A), following well-established protocols (Shoemaker et al.,
187 2008; Yamamoto et al., 2014). A Waters Acquity UPLC™ C_{18} Column (50 mm × 2.1 mm, 1.7
188 μm) was used with two mobile phases (mobile phase A: water containing 2 mmol/L ammonium
189 acetate; mobile phase B: methanol containing 2 mmol/L ammonium acetate) under a gradient
190 elution program, as described by Huang and Jaffé (2019). The standard curve was in the range of
191 0.5 – 100 μg/L and all filtered samples were diluted to their appropriate concentrations. A blank
192 sample was included in the measurements to ensure that the target compounds were not detected
193 in their absence. Some of the samples were measured for a second time to verify the PFOA
194 amounts, where the method was slightly different (Supporting Information).

195 *2.5 Microbiological analysis*

196 Total genomic DNA was extracted from 1 ml of unfiltered samples using FastDNA™ SPIN
197 Kit for Soil (MP Biomedicals) following the manufacturer's manual, with an additional first step
198 in which 1ml of the unfiltered sample was centrifuged for 10 minutes at 14,000 relative centrifugal
199 force (RCF), then 500 μl of supernatant was removed, and the pellet was resuspended in the
200 remaining liquid. DNA concentrations were calculated based on the 1ml sample. Quantitative
201 polymerase chain reaction (qPCR) was conducted using an Applied Biosystems StepOnePlus™

Real-Time PCR system for total bacterial numbers and A6 quantification. Primer set 1055F/1392R (1055F: 5'-ATGGCTGTCGTCAGCT-3'; 1392R: 5'-ACGGGGCGGTGTGTAC-3) (Harms et al., 2003) was used for total bacteria quantification, and primer set acm_v1F/acm_v1R (acm_v1F: 5'-GGCGGCGTGCTTAACACAT-3'; acm_v1R: 5'- GAGCCCGTCCCAGAGTGATA-3') (this study) was used for A6 quantification. Each qPCR mixture (20 µl) was composed of 10 µl of SYBR Premix Ex Taq II 2X (TaKaRa, Japan), 0.8 µl of each forward and reverse primer at 10 µM, and the DNA template. Thermal cycling was initiated with 30 s at 95°C, followed by 40 cycles, each cycle consisted of 5 s at 94°C, 30 s at 56°C, and 30 s at 70°C. Each qPCR assay was run in triplicate for each sample and included negative controls and a standard curve; the latter consisted of serial dilutions of known numbers of copies of DNA.

To monitor the changes in the microbial communities during the experiment with both, the A6 highly enriched and the enrichment cultures, sequencing of 16S rRNA genes was conducted for initial and final subsamples of the treatment groups. The V4-V5 region was amplified using primer-set 515F-806R (Caporaso et al., 2012) and PCR reactions were carried out with a Veriti 96 well Thermal cycler (Applied Biosystems). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA), and the library quality was checked with a Qubit@ 2.0 Fluorometer (Thermo Scientific) before sequenced on an Illumina MiSeq platform. 250 bp paired-end reads were generated, and Operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off using the Uparse software (Uparse v7.0.100, http://drive5.com/uparse/). The taxonomy of each sequence was analyzed with Mother (version v.1.30.1) compared to Silva SSUrRNA database using a confidence threshold of 0.8-1.

223 *2.5 Statistical analysis*

224 The Student *t*-test was used to determine if there were statistical differences between
225 treatment and control groups.

226 ***3 Results and discussion***

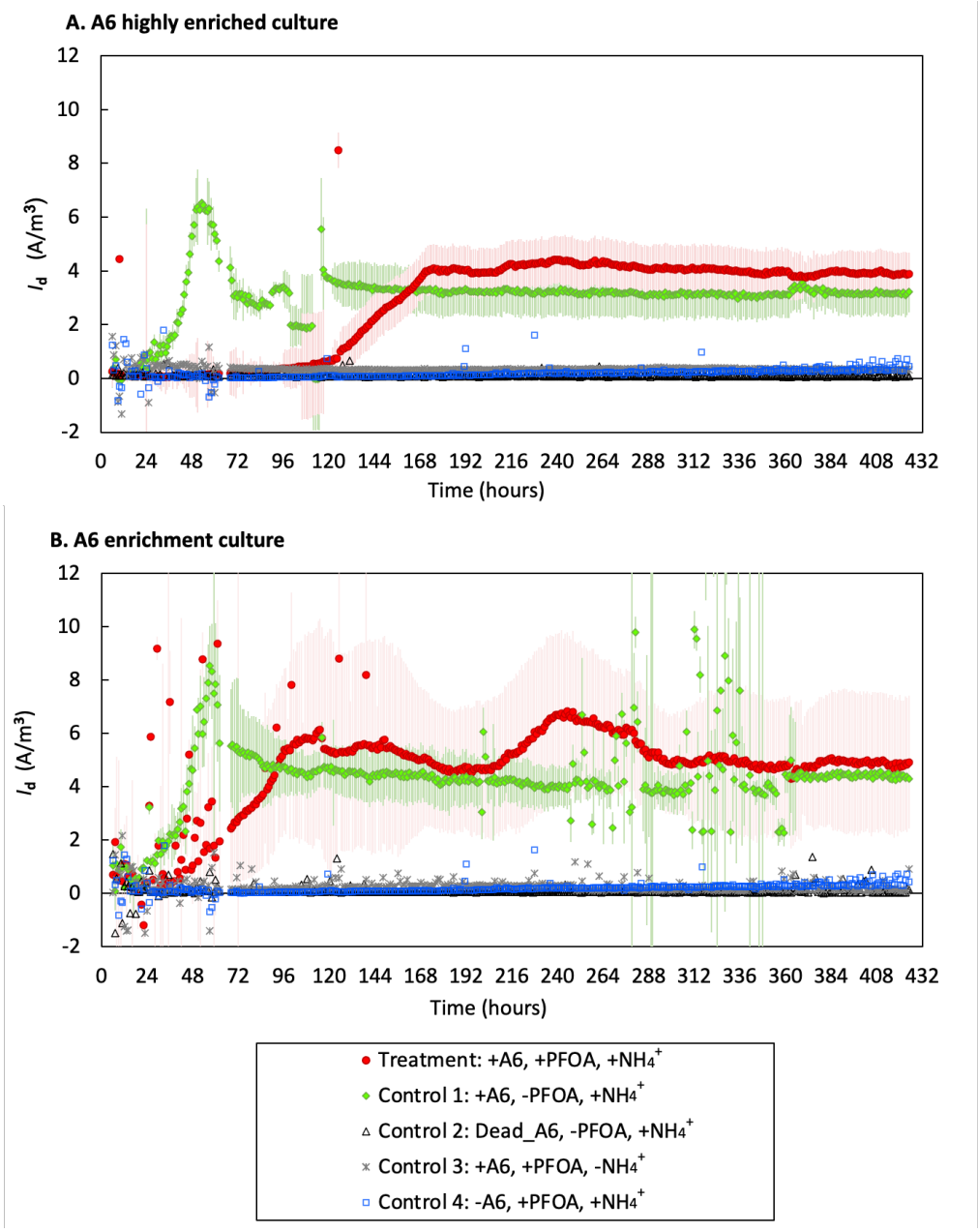
227 *3.1 Activity of Acidimicrobiaceae* sp. *strain A6 in the presence of PFOA*

228 As shown in Figure 2, only the groups with live A6 bacterium and NH_4^+ (treatment and
229 control group 1) had distinctive current production during the incubations, regardless of the
230 absence or presence of PFOA in the medium. This means PFOA at ppm concentration levels did
231 not have a detrimental effect on A6's electrochemistry activity in the MEC reactors. Similar results
232 were shown in the preliminary experiments, where current production (Figure S1) was observed
233 in the MECs inoculated with the A6 culture in the presence of PFBS (100 mg/L) or PFOA (100
234 mg/L).

235 Different MEC replicates showed different levels of current production for the same group,
236 and also different lag times before the onset for the current production, which has been observed
237 in previous MEC experiments (Escapa et al., 2015; Ruiz-Uriguen *et al.*, 2019), which was clearly
238 observed in the treatment groups, particularly in the one inoculated with the A6 enrichment culture.
239 This explains why MECs with A6 enrichment inoculum had more variability in the current density,
240 and thus, the larger error bars (Figure 1B), as each replica had a different lag times time and time
241 for the current density peak as shown in Figure S3.

242 The control groups with dead A6 bacterium (control group 2), with A6 but without NH_4^+
243 as the electron donor (control group 3), and without the inoculation of A6 (control group 4) showed
244 negligible current production. This confirms that the current was produced by microbial processes
245 carried out by A6, and that NH_4^+ was needed as the electron donor. After 250 hours of operation,

246 one of the thee replicas with A6 enrichment culture corresponding to control group 3 (+A6,
247 +PFOA, -NH_4^+), resulted in connection problems; therefore, those data points were not included
248 in the mean after 250 hours, and thus only the two remaining replicas' data were used.



249

250 **Figure 2**. Current density (I_d) measured in the MECs under different conditions inoculated with
251 the highly enriched A6 culture (A) and the A6 enrichment culture (B). Marks show the mean,
252 and the error bars represent the standard deviation (n=3).

253 The PFOA-amended MECs inoculated with highly enriched A6 and enrichment A6
254 cultures (Treatments: +A6, +PFOA, +$NH4^+$) showed longer lag time prior to current production,
255 compared to the no PFOA MECs (Control 1: +A6, -PFOA, +$NH4^+$). Current densities were
256 comparable for the two groups and presented no statistical difference (*t*-test, $p > 0.05$ for highly
257 enriched A6 culture, and $p > 0.1$ for enriched culture; however, the PFOA-amended treatments
258 show a slightly higher mean I_d than the no PFOA amended MECs (control 1). This is due to the
259 current production variability between the three treatment replicas (Figure S3), an occurrence
260 previously reported in MECs (Escapa *et al.*, 2015; Ruiz-Uriguen *et al.*, 2019). In this case, one of
261 the replicas has a higher I_d compared to the other two, thus increasing the mean I_d, and adding to
262 the large standard deviations. Therefore, the mean values are not sufficient to conclude that A6
263 MECs amended with PFOA produce higher current than those without PFOA, and given the
264 variability between running identical MECs, to fully track differences requires much higher
265 number of replicas. Additionally, the amount of NH_4^+ removal in the PFOA-amended MECs and
266 the MECs without PFOA showed no statistically significant difference (*t*-test, $p > 0.05$) (Figure
267 3). Since the overall NH_4^+ removal in this study was much less compared to the preliminary
268 experiment (less than 1 mM *versus* several mM), it is possible that lower microbial activities of
269 A6 in this study masked any potential inhibitory effect of PFOA in the MECs. Studies on toxicity
270 of PFOA on bacteria showed a large range of half growth inhibition concentrations from 10.6 ±
271 1.0 mg/L for *Escherichia coli* (Liu et al., 2016) to 72.2 mg/L for *Anabaena* CPB4337 and 524
272 mg/L for *Vibrio fischeri* (Rosal et al., 2010). Since one of the important toxicity effects by PFOA
273 is membrane disruption (Liu *et al.*, 2016), A6, as a gram-positive strain, may have different
274 responses to this chemical as compared to the above-mentioned gram-negative bacteria. Feammox
275 incubations of A6 with PFOA showed no discernable difference in the amount of NH_4^+ oxidized

and electron balance of Fe(II) + F^- production when comparing incubations without PFOA and with 100 mg/l of PFOA (Huang and Jaffé, 2019). A significant decrease in NH_4^+ oxidized and Fe(II) produced was observed when the PFOA concentration was 200 mg/l. More detailed studies are needed to understand the possible adverse effect of different PFAS on A6 bacterium.

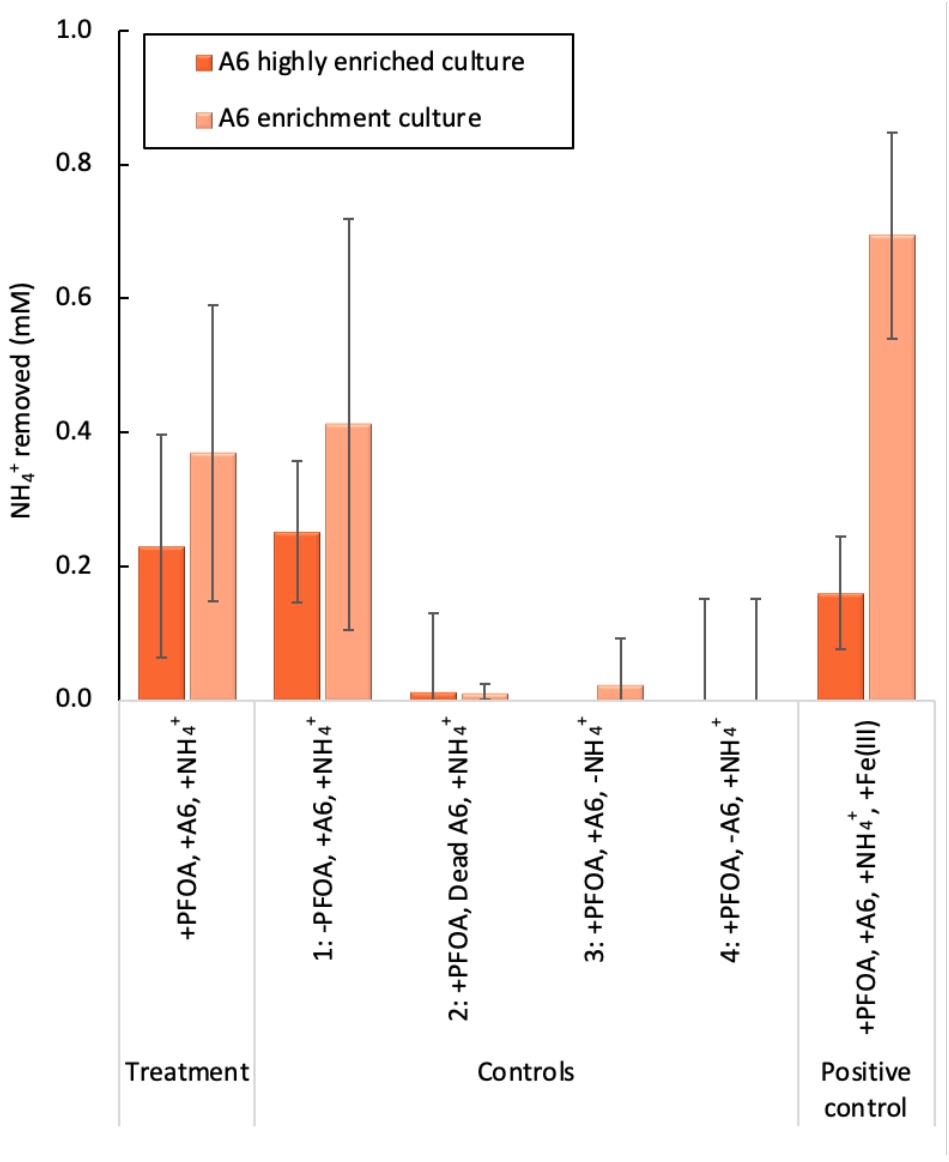


Figure 3. Amount of NH_4^+ removed, over 18 days of operation, of MECs inoculated with A6 highly enriched culture and A6 enrichment culture, with and without PFOA. Bars show the mean, and the error bars the standard deviation (n=3).

3.2 Decrease of PFOA in the A6-inoculated MECs

The designed PFOA concentration in the MEC medium with PFOA was 100 mg/L. However, because of the hydrophobicity and its surface-active characteristic, dispensing a stock solution of PFOA and sampling from individual vials may need extremely careful handling. Substantial PFOA adsorption on glassware, filter membrane and centrifuge tubes has been reported (Lath et al., 2019), which could contribute largely to the variability in the initial concentration of the various MECs in this study. However, PFOA measured on day 0 in the study by Huang and Jaffé (2019) was very close to what was added to the vials, suggesting that PFOA sorption is negligible under those experimental conditions. In the current study, a master mix was made for each treatment or control condition, and then dispensed in three individual vials to form triplicates. The PFOA solution used to make the master mix was at 1000 mg/L concentration, although sampling and to make dilute solutions did not result in replicable concentrations. Thus, although the aim was to prepare initial concentration of 100 mg/L PFOA, once the MECs were set up and sampled, the highest initial concentration of PFOA measurement was 47.1 mg/L. Therefore, PFOA concentrations measured for day 0 were used as the initial values to calculate PFOA decrease.

The performance of individual MECs showed differences among the replicates, such as current production time trajectories, peak current levels, and NH_4^+ removal even in the absence of PFAS, something that has been previously reported (Escapa *et al.*, 2015; Ruiz-Uriguen *et al.*, 2019). Hence, a high variance in PFOA decrease, intermediates production from PFOA degradation, and fluoride production were also expected in this study due to the characteristics of the MEC experiments. Here, the mean and standard deviation of the PFOA decrease with statistical significance were presented and compared to demonstrate that PFOA removal occurred in MECs inoculated with the active A6 highly enriched and A6 enrichment cultures. As shown in Figure

4, PFOA decrease was seen in both treatment groups with PFOA, NH_4^+, and the inoculum with either of the two A6, while the control group 2 with dead A6 cells did not show PFOA removal, nor did control group 3 (+PFOA, +A6, -NH_4^+) and 4 (+PFOA, -A6, +NH_4^+) show statistically significant changes. Control group 3 shows large error bars because some PFOA removal was detected in some MECs, most likely due to NH_4^+ carry-over from A6 cultures and from the release of NH_4^+ from other organisms during biomass turnover. A6 bacteria could have taken advantage of the available NH_4^+, thus resulting in some PFOA degradation, particularly in MECs inoculated with A6 enrichment culture.

Statistical analysis showed significant difference in PFOA decrease from initial samples to final samples for the treatment groups (*t*-test, $p < 0.01$ for the A6 inoculum, and $p < 0.1$ for the A6 enrichment inoculum), but no statistically significant difference for the control groups. For the positive control group incubated with Fe(III) as the electron acceptor, and no applied voltage, PFOA decreased 77.6 ± 16.2 % with the A6 highly enriched inoculum, and 48.1 ± 6.6 % with the A6 enrichment inoculum, respectively, which is comparable to the PFOA decrease in the active MECs.

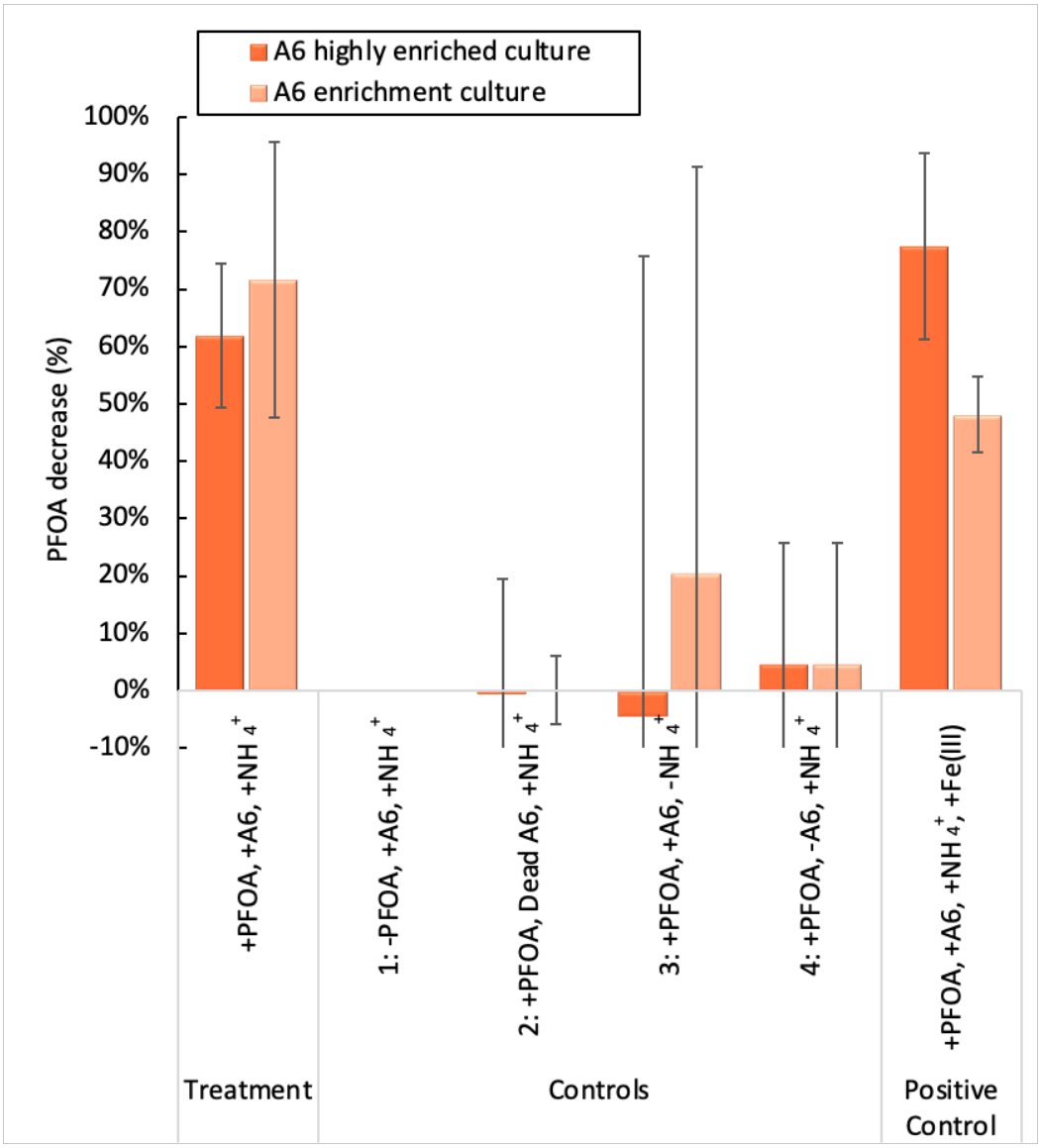


Figure 4. Decrease of PFOA from A6 inoculated MECs.

3.3 Degradation products confirm the biodegradation by A6 in MECs

Since PFOA measurements might be affected by sample handling and loss to adsorption, detection of shorter carbon chain degradation intermediates from the final samples of each MEC, as well as the production of F^-, were used as an additional key evidence of PFOA degradation, in addition to the decrease in PFOA concentration. No shorter carbon chain perfluoroalkyl acids

329 (PFAAs) intermediates were detected in the initial samples, while four PFOA degradation
330 intermediates were detected in some of the final samples: perfluoroheptanoic acid (PFHpA,
331 $C_7HF_{13}O_2$), perfluorohexanoic acid (PFHxA, $C_6HF_{11}O_2$), perfluoropentanoic acid (PFPeA,
332 $C_5HF_9O_2$), and perfluorobutanoic acid (PFBA, $C_4HF_7O_2$). Although not all replicates in the
333 treatment and positive control groups showed all four measured intermediate species,
334 intermediates only appeared, as expected, in the treatments or positive control groups (Table 2),
335 where viable A6, NH_4^+, PFOA, and electron acceptors [either electrodes or Fe(III)] were available.
336 The production F^- and of acetate in the MEC incubations with PFBS (Fig. S2) shows that at least
337 some of shorter than eight carbon chain compounds can also be degraded on the MECs.
338
339 These results show that A6-mediated PFOA degradation can occur using either ferrihydrite
340 or electrodes as the primary electron acceptor, and that NH_4^+ as the electron donor is necessary for
341 this process, although the use of H_2 as electron donor was not examined here. Overall, higher
342 intermediate concentrations were observed in the reactors inoculated with the A6 enrichment
343 culture as compared to the ones inoculated with the A6 highly enriched culture, for all four
344 intermediate species both in MECs and regular Feammox incubations. This result is consistent
345 with the results from the previous study, where much lower intermediate concentrations were
346 observed in incubations inoculated with A6 than in the incubations with enriched A6 culture
347 (Huang and Jaffé, 2019).

348 Table 2. Concentrations of intermediates from A6-inoculated MECs. The treatment group
349 (+PFOA, +A6, +NH_4^+) and control groups had an applied voltage of 0.7 V, while the positive
350 control group [+PFOA, +A6, + NH_4^+, +Fe(III)] had no applied voltage and ferrihydrite [Fe(III)]
351 was provided as the electron acceptor. No intermediates were detected from other control groups.

Intermediate species		PFBA (nM)	PFPeA (nM)	PFHxA (nM)	PFHpA (nM)*
A6 highly enriched culture					
Treatment	+PFOA, +A6, +NH_4^+	N.D.**	N.D.	1.38 (2.4)***	75.5 (130.7)
Positive control	+PFOA, +A6, + NH_4^+, +Fe(III)	N.D.	3.7 (4.2)	10.0 (12.9)	1252.5 (1375.6)
A6 enrichment culture					
Treatment	+PFOA, +A6, + NH_4^+	162.0 (162.0)	71.7 (124.2)	1.63 (2.8)	1490.6 (2582.0)
Positive control	+PFOA, +A6, + NH_4^+, +Fe(III)	578.1 (578.0)	350 (596.4)	172.4 (299)	3234.6 (3235.0)

* Note that maximum initial PFOA measured in the experiment was 132.5 μM.

** N.D. indicates "not detected".

*** Data presented as average (standard deviation). Standard deviations are large because not detected measurements in replicates were calculated as zero values.

Results from recent incubations with PFOA and pure A6 cultures have shown the production of HPFOA, with the fluorine missing from the alpha carbon (Jaffé et al., 2021). Given that there are many organisms that can break a C-C bond of polyfluorinated alky substances, where not all H are substituted by a F (Liu et al., 2007; Liu et al., 2010; Wang et al., 2011), it was hypothesized by Huang and Jaffé (2019) that once A6 has partially defluorinated a PFAA heterotrophs are then capable of breaking the respective C-C bond, producing shorter chain PFAAs. Shorter carbon chain PFAAs like PFHxA and PFPeA were measured in the MECs and the positive controls during this experiment, with higher amounts of these shorter PFAAs having

364 been measured in the enrichment culture incubations, that have a much higher population of
365 heterotrophs as discussed below.

366 Based on the qPCR results of A6 gene and 16S gene counts from MEC samples (Figure
367 S4), all vials inoculated with the either of the A6 cultures contained other organisms. Data obtained
368 from 16s rRNA sequencing for total community composition analysis at the genus level (Table
369 S1) show that the A6 highly enriched inoculum was composed of 95% A6 at the beginning (t = 0)
370 and 90% at the end (t = 18 days) of the experiments. The A6 enrichment inoculum was composed
371 of 21% A6 at the beginning (t = 0) and 16% at the end (t = 18 days) of the experiments. At both
372 timepoints A6 was the most abundant organism of these microbial communities. In addition to A6,
373 the only other genus present in the A6 highly enriched (0.2% at t=0 and 0.7% at t=18 days) and
374 the A6 enrichement (5.7% at t=0 and 4.3% at t=18 days) cultures, that has been reported to be
375 capable to biodegrade PFOS, a type of PFAS, is *Pseudomonas* (Kwon *et al.*, 2014). However, such
376 biodegradation takes place under aerobic conditions, which is not the case for the MECs.
377 Additionally, the community composition analysis showed that in the A6 enrichment culture two
378 other potential ammonia oxidizers are present, *Sphingomonas* and *Nitrospiraceae*, which were
379 2.5% and 0.1% of the total bacterial abundance. They are not known as exoelectrogens, and there
380 is no research showing that they are involved in PFOA degradation. Both *Sphingomonas* and
381 *Nitrospiraceae* are considered to be aerobic bacteria, so under the anaerobic conditions at which
382 the MECs were operated, NH_4^+ oxidation, and thus I_d production, is conducted by A6.

383 Obtaining and maintaining a pure A6 culture is very challenging, especially during
384 continuous subculturing, and although over 90% of the highly enriched culture was composed of
385 A6, other heterotrophs may have become viable in the A6 inoculated MECs and contributed to the
386 low concentration of short chain intermediate. Nonetheless, A6 is thought to be crucial for the first

387 defluorination step that allows other heterotrophs to further degrade the partially defluorinated
388 PFOA and produce degradation intermediates. (Huang and Jaffé, 2019). Much less PFHpA was
389 observed in the A6 enrichment culture inoculated MECs or incubations from this study (< 5 μM)
390 compared to the previous study (> 20 μM) (Huang and Jaffé, 2019), possibly because of the shorter
391 operation time used (18-day incubations in this study *versus* 100-day incubations in the previous
392 study) and the lower initial PFOA concentration in this study. The quantity of intermediates
393 produced in this study only accounted for 0.3 to 5.2 % of the initial PFOA measured in this
394 experiment (based on calculations for individual vials), which is small compared to the decrease
395 in PFOA concentration. Nonetheless, as expected, intermediates were only detected in the
396 treatment MECs and positive control cultures, and not in any replica of the control groups. As
397 mentioned above, results shown in Table 2 reveal that the concentrations of intermediates in the
398 A6 enrichment culture were much higher than in the highly enriched A6 culture, which is
399 consistent with our hypothesis that heterotrophs in the enrichment culture can break down the C-
400 C bond once A6 has partially defluorinated the PFAA.
401 The C7 intermediate had the higher concentration compared to the other shorter chain
402 intermediates, which is also consistent with the results from previous conventional Feammox
403 incubations. It indicates that PFOA is possibly degraded in a series of steps, where one carbon is
404 cleaved off at a time, and longer incubation times might have resulted in increasing concentrations
405 of the shorter carbon chain intermediates. Detailed studies are ongoing to better understand the
406 mechanism of PFOA degradation by A6.

407 **Table 3.** Production of F^- from the A6 culture and A6 enrichment culture incubations over 18 days
408 of incubation (average [standard deviation]). No F^- was detected in control groups except for the
409 positive control in which Fe(III) was added as the electron acceptor.

	F^- increase (μM)	A6 highly enriched culture	A6 enrichment culture
Treatment	+PFOA, +A6, $+NH_4^+$	21.69 (6.06)	14.80 (11.88)
Positive control	+PFOA, +A6, $+NH_4^+$, +Fe(III)	19.53 (6.80)	28.67 (18.27)

Due to the F^- loss at the pH of the experiment (Figure S6), a fluorine balance is not feasible for these experiments. Given that acetate, the production of which was detected in the MECs (Figure S2C), and other possible defluorinated compounds produced, are intermediates that are further consumed by heterotrophs present in the enrichment culture, and to a lesser degree in the A6 highly enriched culture, a rigorous carbon balance is not feasible unless labeled carbon is used. Hence, as discussed above, we rely here on the decrease in PFOA concentration, coupled with the production of F^- and fluorinated intermediates as indicators that these PFAS are being partially degraded over the 18-day MEC operation period. As mentioned above, the final pH in the second set of MECs was higher, resulting in significantly lower F^- recovery than the initial MEC experiments, for which the pH remained lower. Whereas the measured F^- concentration in the second set of MECs (Figure 6) accounts only for a small fraction of the F^- expected based on the amount of PFOA removed, concentrations of F^- produced in the first set of MECs (Figure S2B) are on the order of what one would expect for that amount of PFOA degraded.

Results from the preliminary MECs operated with PFBS and PFOA showed F^- production from the vials with live A6 cells, and no F^- increase was observed in the control group without PFAS addition (Figure 6). The amount of F^- produced, 0.14 and 0.28 mM from PFBS and PFOA respectively, is consistent with the amount of F^- ions produced during comparable incubation

periods of experiments run with A6 using ferrihydrite as the main electron acceptor (Huang and Jaffé, 2019). As expected, F^- production was only observed in the treatment group and positive control group, which confirmed defluorination of PFOA by active A6 cultures. However, it was at the μM level, which was orders of magnitude lower compared to the preliminary MEC experiment and the previous study (Huang and Jaffé, 2019), where the F^- production was in mM level. The reason for these results is attributed to a lower bacterial activity in comparison to the preliminary MECs, which is also reflected in the amount of NH_4^+ removed which was much lower (0.23 ± 0.17 mM for the A6 highly enriched inoculated MECs, and 0.37 ± 0.22 mM for enriched A6 inoculated MECs) than the amount removed in the preliminary set of the A6 cultured amended with PFOA (0.76 ± 0.85 mM). Moreover, the MECs medium has an initial pH of 5 – 5.5, and under natural conditions, as the Feammox reaction proceeds, the pH of the system increases. When MECs are inoculated with A6 cultures, there is some Fe carry-over (Figure S5). Fe(II) can react with NO_2^- formed during the Feammox reaction and be reoxidized to Fe(III) in the system. As the pH increases above 3, F^- reacts with Fe(III) to form hexafluoroferrates(III) (Chaudhuri and Islam, 1986), and the recovery of F^- at pH ≥ 6 is expected to be low in the presence of Fe(III) (Figure S6). The final pH from this study (pH 6.5 – 7.5) was higher than the preliminary experiment (final pH ≤ 5), which can account for the difference in F^- recovery. Nevertheless, the absence of F^- in control groups and the presence of F^- in treatment group and positive control group serves as evidence of PFAS degradation in addition to the production of the degradation intermediates.

4 Conclusions

The results from this study demonstrate that A6 can defluorinate PFOA in MECs using electrodes as the primary electron acceptor instead of Fe(III). The intermediates produced from the A6-inoculated MECs are consistent with the intermediate species produced in the regular

Feammox incubations inoculated with an A6 enrichment culture. Although for environmental applications one would not use a pure culture, in part because it is difficult to maintain a pure culture for extended time periods, especially in a continuous flow system, experiments with the highly enriched A6 culture show that A6 plays a key role in the defluorination of PFAS. By contrasting differences between the defluorination performance of the highly enriched and the enrichment culture it is possible to gain insights into what steps are carried out by A6 and what steps by other organisms present in the enrichment culture once the initial defluorination of a perfluorinated compound has been achieved by A6. Since in this study we were unable to obtain and maintain a pure A6 culture over the incubation time, further studies with a pure A6 culture should be designed and conducted to better understand the defluorination mechanisms once the maintenance of a pure A6 culture is achieved for longer periods of time.

Findings from this research contribute to the application of A6 and its metabolic processes, validating the concept of utilizing A6-based microbial electrochemical systems for PFAS treatment. Given the high ferric iron demand by A6 it would be a challenge to operate a biological reactor for the long term or continuously in which PFAS can be defluorinated by A6. The results presented here show that biological defluorination of PFAS by A6 can be achieved in the absence of an Fe(III) source in bioelectrochemical reactors.

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