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Aqueous Film Forming Foam and Associated Perfluoroalkyl Substances Inhibit Methane Production and Co-contaminant Degradation in an Anaerobic Microbial Community

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METHODS

Microbial Culture

The feed sludge (thickened waste activated and primary sludge) was obtained from the Empire Wastewater Treatment Plant (Farmington, MN) and collected in a plastic carboy. Performance of source culture is shown in Figures SI.1 and SI.2.

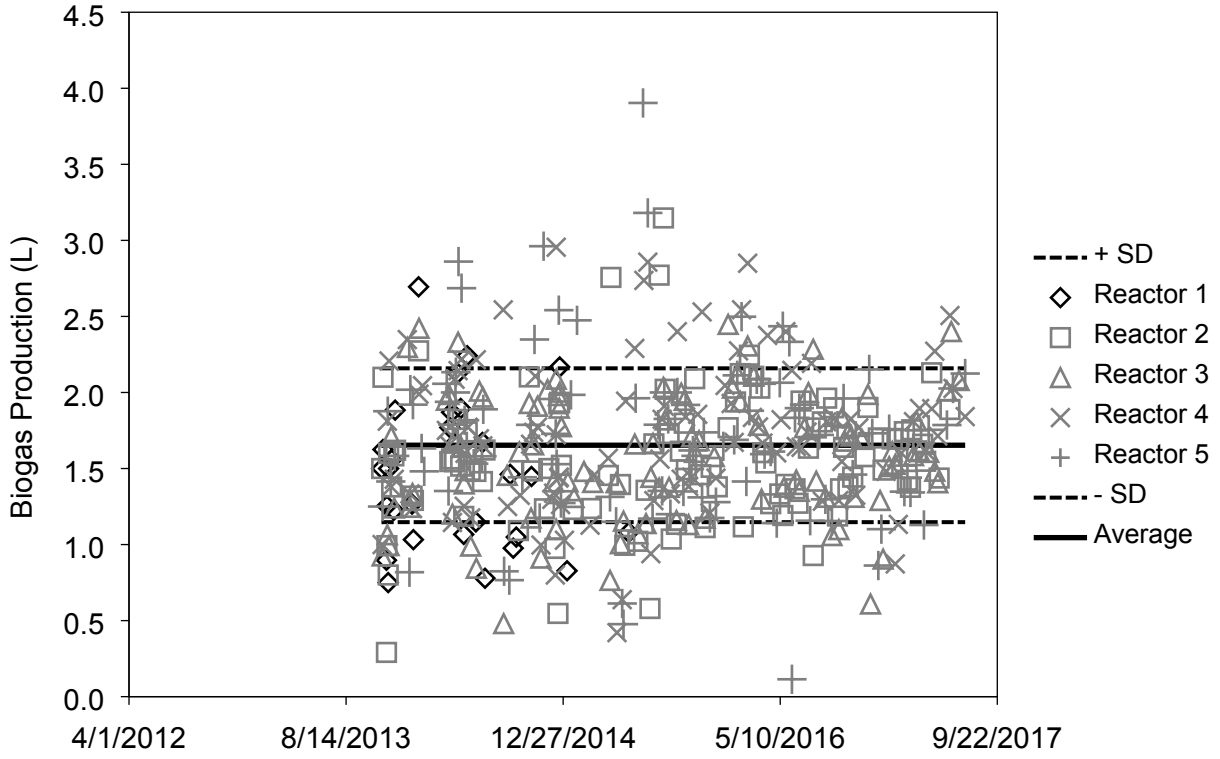

Figure SI.1. Biogas production from source reactors. All reactors are identical.

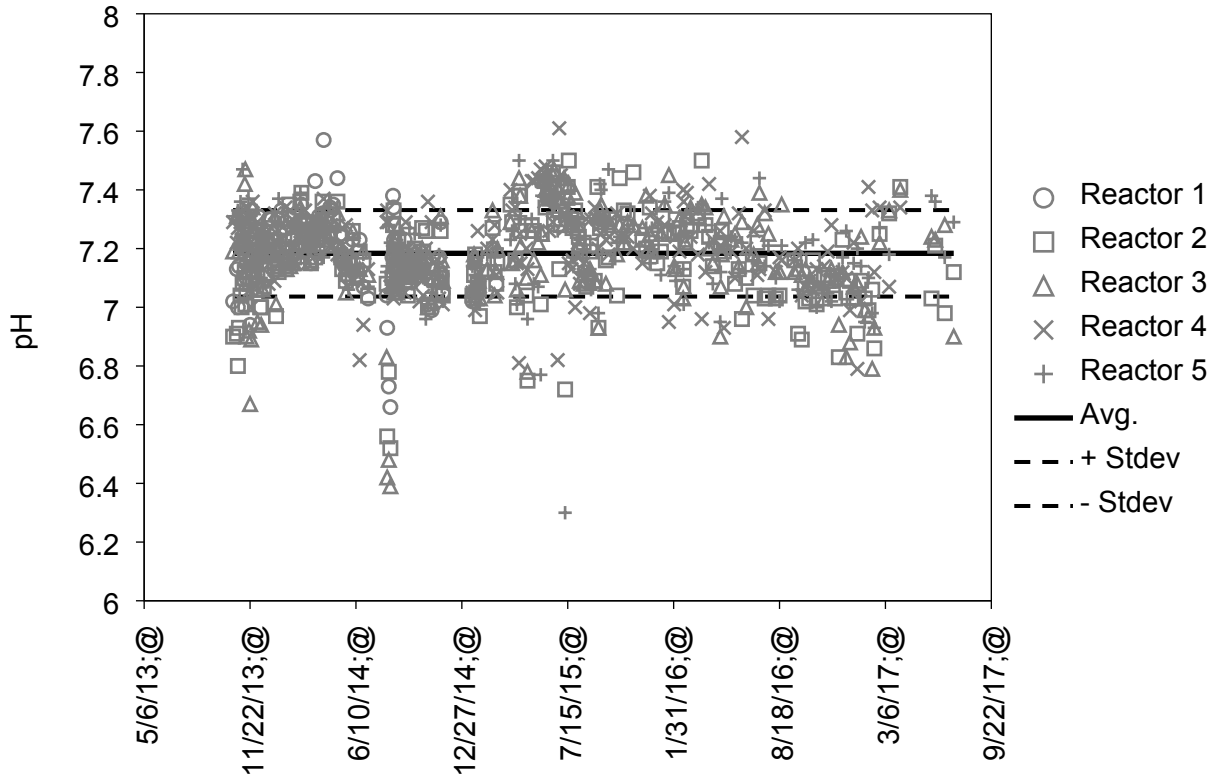

Figure SI.2. pH in source reactors. All reactors are identical.

Microbial Analysis

Illumina Sequencing. Samples were taken for DNA analysis in 1 ml aliquots, pelleted, and frozen immediately at -20ºC until DNA was extracted. The Fast DNA extraction kit (MP Bio) was used according to the manufacturer's instructions. Extracted DNA was stored at -20ºC until analysis. Illumina sequencing of the 16S rRNA gene was completed by the University of Minnesota Genomics Center (UMGC). The V4-V6 region of the 16S rRNA gene was amplified with primers F- GTGCCAGCMGCCGCGGTAA and R- GACRRCCATGCANCACCT and sequenced using Illumina MiSeq paired end sequencing (2x300). Initial quality filtering of the data was completed with the Gopher pipeline available on the Minnesota Supercomputing Institute (MSI) and is available for public use (Gopher pipelines by John Garbe; accessed 7/8/19,

https://bitbucket.org/jgarbe/gopher-pipelines/src/default/). Trimmomatic was used for primer removal and quality filtering, trimming the reads when the average read quality dropped below 25 over a 4 base pair window. The forward and reverse reads were concatenated. The samples were then rarified to the sample with the least number of quality filtered reads. Denovo OTU picking was completed with QIIME using the Uclust algorithm and 97% similarity. Taxonomy was assigned with QIIME using the SILVA database. Whole community data and data from which the *Archaea* were excluded (*i.e.*, *Bacteria* only) were both analyzed; results were very similar and therefore suggested that the *Archaea* were not adequately represented with the Illumina primers selected. Therefore, only the community data for *Bacteria* were considered in our analysis. Beta diversity analysis was completed in R with the Phyloseq package. The beta diversity between the samples was calculated using Bray Curtis. R was then used to complete a hierarchal clustering of the samples.

Quantitative Polymerase Chain Reaction. To quantify methanogens, primers specific for the 16S rRNA gene of methanogens were used (630 F- GGATTAGATACCCSGGTAGT and 803R –GTTGARTCCAATTAAACCGCA)(1). This targeted an approximate 175 bp region of the 16S rRNA gene and was verified in other publications(1–3). Each qPCR reaction contained 1X SYBR green MasterMix (Bio-Rad Laboratories), 100 nM of each primer (IDT), 1 mg/L BSA, and 1 µl of purified DNA. All qPCR analysis was performed on a Biorad CFX Connect Real-Time System. The general qPCR cycle was an initial denaturation at 95°C for 1 min followed by 40 cycles of: 95°C for 15 s and 60°C for 1 min. A melt curve analysis was completed at the end of each run for quality control. A 10-fold dilution standard curve ranging from 10^9 to 10^0 was used to quantify the number of gene copies in each sample. Standards were purchased from IDT as gblocks based on the 16S rRNA gene sequence of *Methanobrevibacter*

smithii (Genbank accession no. U55234). The standard curve was linear from 10^9 to 10^1 and the limit of detection was determined to be 10^1. The qPCR efficiency was 106% and the standard curve had an R^2 of 0.996. No template controls were used as qPCR negative controls and nothing was detected from these samples. To determine whether differences in methanogen numbers between samples were significant, t-tests were performed with P-values corrected via the Tukey's correction.

Data Analysis

Toxicity. The rate of methane production was calculated for each bottle by linear regression of the data from the first three days of the experiment. Rates were then averaged among the triplicate bottles. Methane production during this time frame was consistently linear as depicted in Figure SI.3.

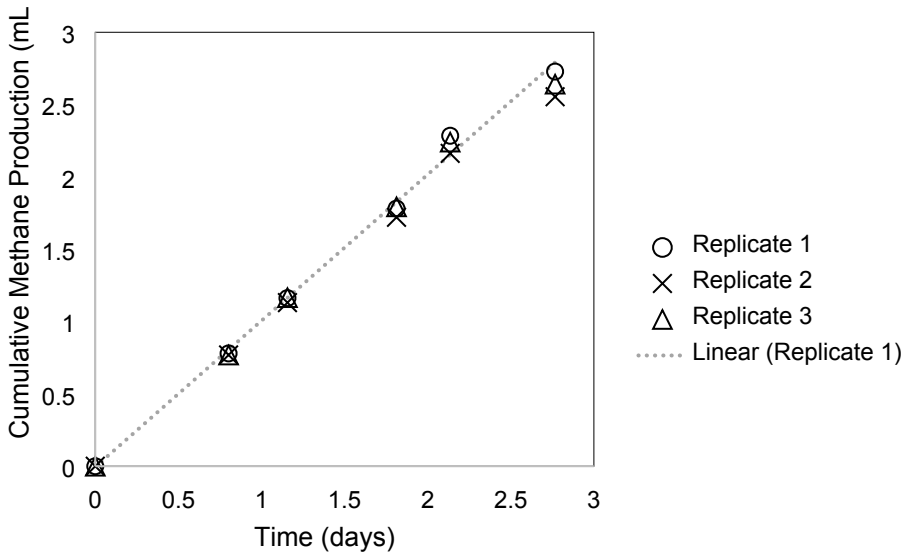

Figure SI.3. Linear regression of replicate control bottles during the first three days of an experiment.

RESULTS & DISCUSSION

Table SI.1 shows the *P*-values obtained during statistical comparisons (t-tests) of the rate of methane production in the PFAS-amended treatments compared to the no-PFAS control. Outliers were removed with Grubbs' correction with α=0.05. The Tukey correction was used to correct *P*-values for multiple comparisons. Some experiments were repeated; therefore, in some cases multiple *P*-values are presented.

Table SI.1. *P*-values corrected for multiple comparisons and obtained from statistical comparisons between the rate of methane production in PFAS-amended treatments and the no-PFAS control treatments (Figure SI.3). Some experiments were repeated, hence multiple *P*-values are presented.

Treatment	*P*-value
Carboxylates	
PFBA (3C)	*>0.99, 0.91,*
PFOA (7C)	*>0.99*
PFNA (8C)	*0.30, <0.01*
Sulfonates	
PFBS (4C)	*0.72*
PFHxS (6C)	*>0.99, >0.99*
PFOS (8C)	*<0.01, <0.01, >0.99*

Figure SI.4. shows the degradation of 2,4-dichlorophenol (DCP) over time in no-PFAS control treatments and in treatments amended with 50 mg/L of various PFAS. The figure shows the average from triplicate microcosms; the error bars show the standard deviation of triplicates. This data was fit with the Gompertz model to determine the lag period prior to DCP degradation. Panels A and B show data obtained in experiments performed on two different dates.

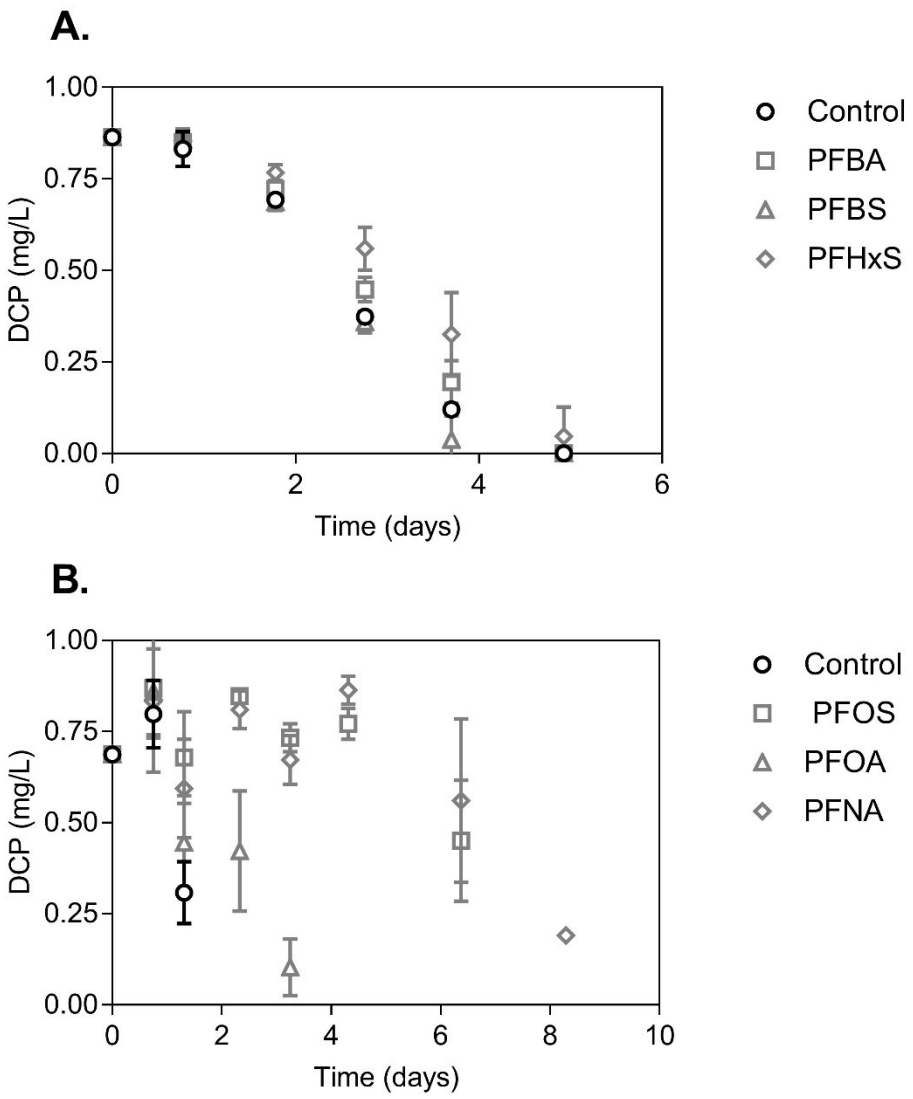

Figure SI.4. Degradation of 2,4-dichlorophenol (DCP) in the no-PFAS added control and in the presence of 50 mg/L of varying PFAS. Panel A. shows data from treatments amended with PFBA, PFBS, and PFHxS, and Panel B. shows data from treatments amended with PFOS, PFOA, and PFNA. The data on these two panels was obtained from experiments performed on different days.

Figure SI.5. shows the degradation of DCP over time in no-PFAS control treatments and in treatments amended with 5 mg/L of PFOS, PFOA, and PFNA. The figure shows the average of data from triplicate microcosms and the error bars show the standard deviation of those data. This data was fit with the Gompertz model to determine the lag period prior to DCP degradation. This experiment was performed on a different date than the experiments shown in Figure SI.4.

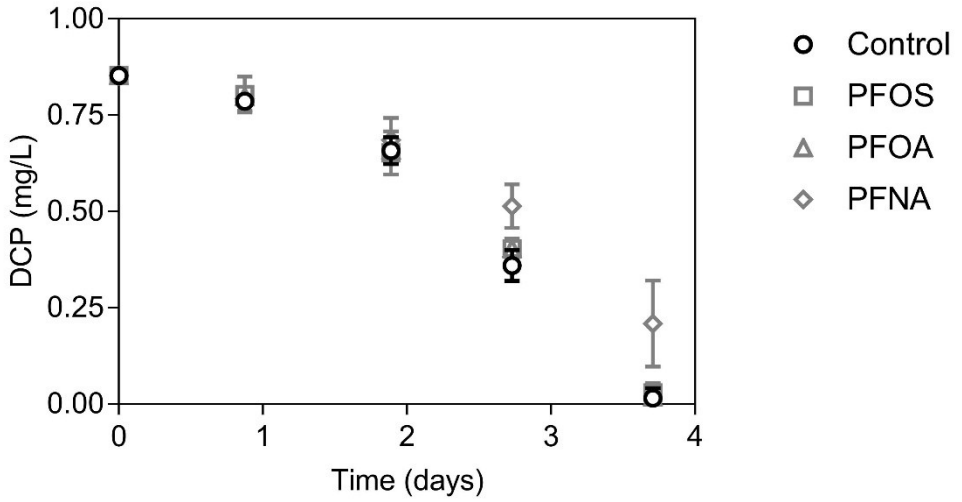

Figure SI.5. Degradation of DCP in no-PFAS added control treatments and in treatments amended with 5 mg/L of either PFOS, PFOA, or PFNA. The average of triplicate treatments are shown with error bars showing the standard deviation.

Figure SI.6. shows the clustering of the samples based on the beta diversity between samples, as determined by Bray Curtis. The Illumina analysis was performed on samples taken on Day 1 and Day 25 (the end of the experiment) of the DCP-degradation experiment. The initial samples clustered together and then shifted as a result of time and PFAS exposure. The AFFF samples clustered apart from the other samples. Two controls and one PFHxS+PFOS samples also clustered separately. The Kruskal-Wallis test was used to determine if any OTUs with a relative abundance above 0.5% were statistically different between the treatments; none were. The largest difference between the three samples that were most different from the others (two controls and one PFOS+PFHxS) was the high abundance of a *Clostridia* OTU and the lower abundance of an *Anaerolinea* OTU (Figure SI.6)

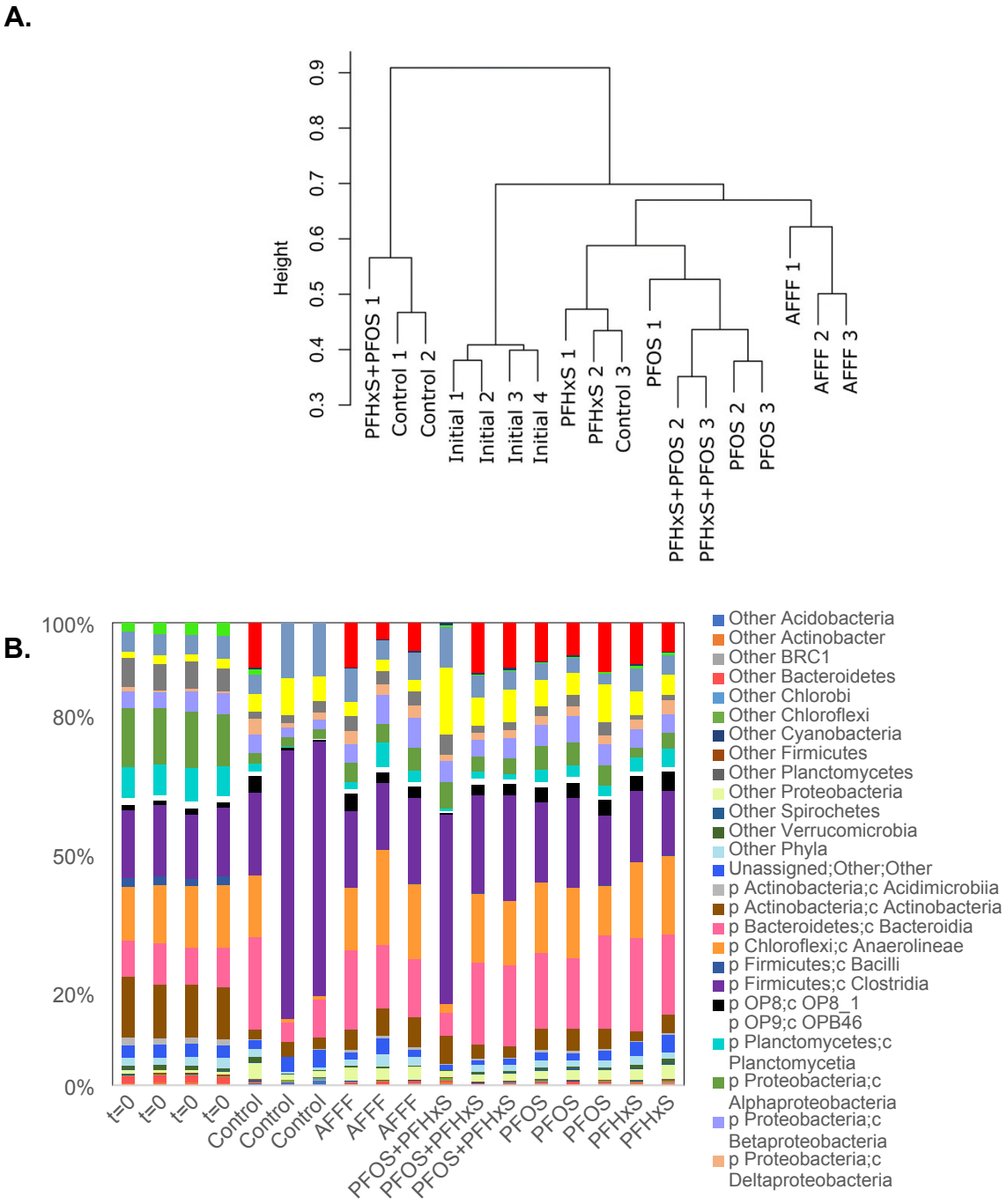

Figure SI.6. Microbial community analysis from the DCP-degradation experiments. Panel A shows hierarchical clustering of samples based on Bray Curtis beta diversity analysis. Panel B shows the relative abundance of the 16S rRNA gene sequences at the Class phylogenetic level for the initial and each of the treatments. Specific Classes that were present at less than 1% were lumped at the Phylum phylogenetic level. Phyla present at less than 1% were also lumped together.

Table SI.2. shows the chemical oxygen demand (COD) (mg/L) in no-PFAS control treatments, treatments amended with PFAS only or with DCP+PFAS. The time zero measurement was taken of the initial diluted digester culture prior to the addition of PFAS and/or DCP. The measurements taken on Day 3 were from the microcosms to which no DCP was added. The measurements taken on Day 25 were from the microcosms to which DCP was added. These data show the large COD added with the AFFF that was not present in the other treatments.

Table SI.2. Chemical oxygen demand (COD) (mg/L) in no-PFAS controls and in treatments amended with PFAS only or PFAS+DCP. The t=0 measurement was from the diluted digester culture. The t=3 measurements were taken from treatments that did not contain DCP. The t=25 measurements were taken from treatments amended with DCP.

	Day		
	0	**3**	**25**
Control	391.5	319.0	203.3
AFFF as 50 mg/L PFOS	391.5	2,681.3	1,339.6
PFOS + PFHxS	391.5	585.3	216.7
50 mg/L PFOS	391.5	583.3	226.0
8.8 mg/L PFHxS	391.5	321.3	207.3

Figure SI.7. shows the methane production over time in the DCP-degradation experiments. Data from no-PFAS control treatments and treatments amended with 50 mg/L PFOS, 8.8 mg/L PFHxS, PFOS (50 mg/L)+PFHxS (8.8 mg/L), and AFFF is shown. The figure shows the average of data from triplicate microcosms and the error bars show the standard deviation of those data. This data also shows the extra methane production that occurred in the AFFF-amended treatments. This rapid increase in methane production was observed after the initial COD present in the diluted digester culture appeared to be degraded (on about Day 25), presumably a result of the large COD amended to the microcosms as part of the AFFF.

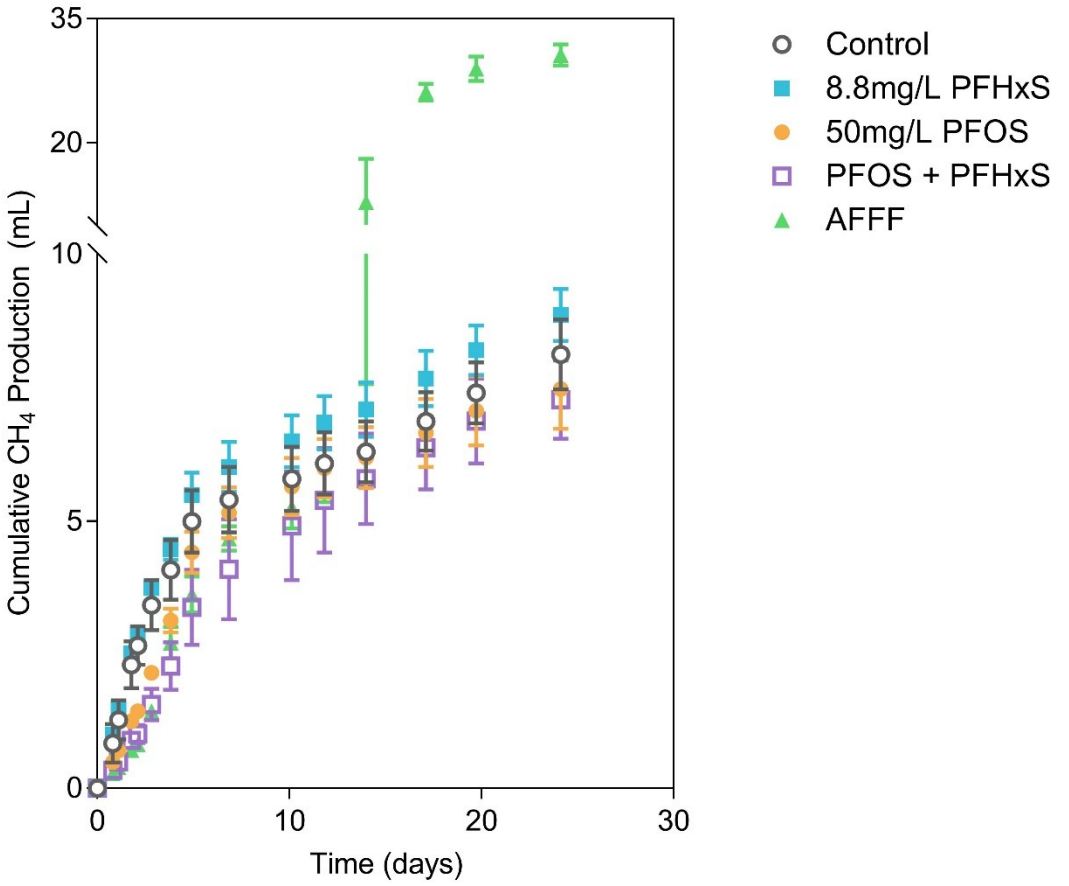

Figure SI.7. Methane production in treatments used to determine the effect of AFFF and its major PFAS constituents on the degradation of 2,4-dichlorophenol (DCP).

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