# The Effect of Perfluorooctane Sulfonate, Exposure Time, and Chemical Mixtures on Methanogenic Community Structure and Function

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**ABSTRACT:** A plethora of organic micropollutant mixtures are found in untreated municipal wastewater. Anaerobic digesters receive large loadings of hydrophobic micropollutants that sorb to wastewater biosolids. Despite micropollutants being pervasive as mixtures, little research is available to explain the impact that mixtures of compounds, as well as exposure time, have on microbial communities in anaerobic digesters. Perfluorooctane sulfonate (PFOS) was added to anaerobic enrichment cultures in both short-term (14 days) and long-term (140 days) studies to determine the impact of exposure time. Additionally, triclosan was added during the experiments to investigate the impact of mixtures on community structure and function. PFOS did not alter methane production in short-term studies, but in long-term studies, methane production increased, consistent with our hypothesis that PFOS may act as a metabolic uncoupler. The impact of triclosan on methane production was exacerbated when PFOS was already present in the anaerobic enrichment cultures. Triclosan also had greater impacts on microbial community structures in the bottles that had been exposed to PFOS long-term. These results demonstrate that both chemical mixtures and exposure time are important parameters to address when trying to define the impacts of micropollutants on anaerobic microbial communities.

**KEYWORDS:** micropollutants, triclosan, perfluorooctane sulfonate, anaerobic digestion, emerging contaminants, biogas

## Introduction

Perfluorooctane sulfonate (PFOS) is an organic surfactant that is ubiquitous in the environment and has been found in the tissues of many animals, including bald eagles, polar bears, and marine animals.[1,2] PFOS has been found at nanogram per liter levels in surface waters[3,4] and was recently listed under the Stockholm Convention on Persistent Organic Pollutants.[5] Wastewater treatment plants receive PFOS directly with influent sewage, and it also forms as other fluorinated surfactants degrade.[6] In residual wastewater biosolids in the USA, PFOS is typically detected at concentrations between 10 and 100 µg (PFOS)/kg (dry solids), with levels up to 2,610 µg/kg; in Switzerland, concentrations as high as 600 µg/kg have been detected.[7–11]

The impacts of PFOS in biological systems are mixed. PFOS at very high concentrations, 500 mg/L, caused no detectable impacts on hydrogenotrophic methanogens in granular sludge when the exposure time was limited to 24 hours.[12] Interestingly, an alcohol alkoxylate surfactant present at 200 mg/L inhibited methanogenic activity after 9 days, but no inhibition was seen for the first 2 days of the experiment.[13]

Therefore, exposure time could be an important factor in assessing PFOS impacts and associated risk. In eukaryotes, PFOS has been linked to detrimental effects, causing mitochondrial cell membranes to leak at 5 mg/L.[14] At 30 mg/L, PFOS both increased and decreased the impacts of chlorinated compounds on algal cells,[15] suggesting that the impact of PFOS as a constituent of mixtures should also be further studied.

Because of the potential importance of exposure time and mixture effects, the objective of this research was to more fully explore the short-term and long-term impacts of PFOS, alone and in mixtures, on methanogenic communities. Methanogenic communities were selected as the focus of this research because PFOS is found in biosolids along with other micropollutants, such as triclosan.[7,16,17] Experiments were performed with methanogenic communities amended with environmentally relevant levels of PFOS, and in some cases, the ubiquitous antimicrobial triclosan. It was hypothesized that PFOS would have minimal impacts on communities alone, but would augment the impacts of triclosan. It was also hypothesized that effects would only be observed after PFOS exposure was

long enough to allow penetration through flocs and into cells. This work furthers our understanding of the impacts of this priority organic pollutant and sheds light on how testing conditions (short-term vs. long-term) impact risk evaluation.

## Methods

**Long-term PFOS exposure tests.** This experiment was designed to test the hypothesis that long-term exposure of methanogenic communities to PFOS inhibits methane production, shifts microbial community structure, and renders communities more susceptible to the effects of triclosan. Experiments were performed for 140 days at environmentally relevant low (60 μg/kg) and high (800 μg/kg) PFOS concentrations.[7]

**Methanogenic source cultures.** Three-liter bench-scale anaerobic digesters were used to provide innocula for the smaller anaerobic serum bottles. These 3-L bench-scale digesters were seeded with biosolids from a full-scale anaerobic digester that treated cow manure (Haubenschild Farms).[18] The 3-L cultures were fed a synthetic feed that included (percent chemical oxygen demand (COD) in parenthesis): ethanol (40.5%), potassium acetate (10.9%), sodium acetate (9.4%), propionic acid (11.4%), butyric acid (8.5%), valeric acid (2.8%), 2-propanol (4%), and methanol (1.6%)[18] in minimal media.[19] The organic loading rate was 0.18 g COD/L/day and the hydraulic retention time (HRT) was 60 days.

**Anaerobic serum bottle cultures.** Anaerobic enrichment cultures were constructed from 160-mL serum bottles with 50 mL of active volume. Serum bottles were initially filled with 20 mL of biomass (12 g volatile solids (VS)/L) from the 3-L source cultures and 30 mL of minimal media.[19] Once a week 7 mL of the culture contents were removed and 7 mL of minimal media were added along with 0.2 mL of feed to provide organic loading rates of 0.05 g COD/L/day as acetate, 0.06 g COD/L/day as glucose, and 0.09 g COD/L/day as methanol; the average organic loading rate was 0.20 g COD/L/day. The mean HRT was 50 days. Nitrogen gas was used to purge the culture headspace, which were plugged with Teflon-lined septa and crimp-capped with aluminum seals. Cultures were incubated resting upright in the dark at 37°C. Periodically, a 1.5-mL aliquot of biomass was sampled from each culture for DNA extraction followed by microbial analysis.

Six sets of serum bottle cultures were operated in triplicate (Table 1). Two sets of cultures, called PFOS (60), were exposed to PFOS at approximately 60 μg/kg; two sets of cultures, called PFOS (800), were exposed to PFOS at 800 μg/kg; and two sets of control cultures were not exposed to PFOS.

PFOS (Sigma Aldrich, 97%) was weighed gravimetrically and dissolved in methanol. This stock solution was further diluted with methanol to achieve desired concentrations. The PFOS–methanol solutions were added to cultures with a gas tight syringe, and the control cultures received methanol containing no PFOS. The mass of PFOS in the culture at any given time was calculated from Equation 1, and the concentration

**Table 1.** Descriptions of experimental cultures for long-term exposure tests.

| SET | NAME | PFOS |
|-----|------|------|
| 1 | Control | No |
| 2 | Perturbed | No |
| 3 | PFOS (60) | Yes |
| 4 | PFOS (60) + perturbation | Yes |
| 5 | PFOS (800) | Yes |
| 6 | PFOS (800) + perturbation | Yes |

was determined from normalizing the mass of PFOS in the culture to total solids. The calculated concentrations of PFOS are shown in Figure 1.

$$C_t = C_0 e^{t/\tau} \qquad (1)$$

where $t$ is the time since PFOS feed, $C_t$ is concentration of PFOS in enrichment culture at time $t$, $C_0$ is initial PFOS concentration following feed, and $\tau = $ HRT.

One set of control cultures, one set of PFOS (60) cultures, and one set of PFOS (800) cultures were perturbed over the course of the experiment (Table 2) to study the effect of PFOS addition and stress on community structure and function. Perturbations included triclosan amendment. Triclosan (Irgasan, Sigma Aldrich, ≥97% [high-performance liquid chromatography]) was quantified gravimetrically and then dissolved in a methanol solution. The methanol solution was fed into the serum bottles with a gas tight syringe; again, control cultures received methanol containing no triclosan.

**Short-term PFOS exposure tests.** This experiment was designed to test the hypothesis that the flocculant nature of
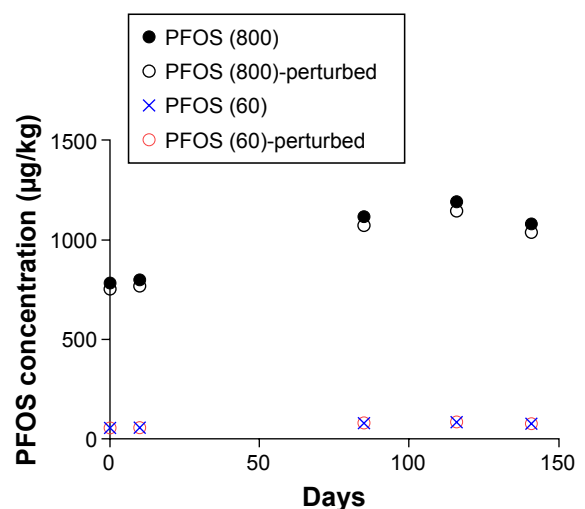


**Figure 1.** Calculated concentrations of PFOS during long-term PFOS exposure tests. Data points represent average concentration in triplicate serum bottles. Error bars represent standard deviation ($n = 3$), but are too small to be seen.

**Table 2.** Perturbations imposed on control-perturbed, PFOS (60)-perturbed, and PFOS (800)-perturbed cultures.

| DAY | PERTURBATION NAME | PERTURBATION DETAILS |
|-----|-------------------|----------------------|
| 3 | Mixing | 125 r.p.m., 1 week |
| 10 | Ammonia | $NH_4Cl$ (1 g/L as $NH_4$) |
| 20 | Cold shock | 4°C, 72 h |
| 115 | Triclosan | 20 mg/kg |
| 121 | Triclosan | 60 mg/kg |
| 131 | Triclosan | 225 mg/kg |

the biomass was protective and short-term exposure to PFOS, even at high concentrations, would not impact methane production or alter the impacts of triclosan in a PFOS mixture. PFOS concentrations were set to 5× environmental levels in this short-term experiment to test a worst-case environmental scenario. If short-term effects were not observed at these concentrations then it would be presumed that current environmental levels do not have short-term effects. Anaerobic serum bottles (160 mL) were initially set up and fed as described for the long-term exposure tests. After 7 days, the bottles were re-fed acetate and glucose. The bottles were amended with micropollutants on Day 0. Micropollutants were weighed gravimetrically and dissolved in methanol. The micropollutant solutions were added with a gas tight syringe. The concentrations of micropollutants in the cultures were calculated from the mass of micropollutant added and divided by the total solids present (Table 3). The experiment lasted for 14 days.

**DNA extraction and automated ribosomal intergenic spacer analysis (ARISA).** To extract DNA, biomass samples (1.5 mL) were centrifuged (13,200 *g*) for 1.5 minutes, the supernatant was discarded, and pellets were frozen at −20°C until extraction. Lysis buffer (MP Biomedicals) was added to pellets, and cells were lysed with three freeze–thaw cycles followed by incubation at 70°C for 90 minutes. The DNA was extracted using the FastDNA Spin Kit (MP Biomedicals), and the extracts were kept at −20°C until use.[18] Community fingerprints were assessed by ARISA following the procedure described previously.[18] Briefly, primers ITSF and ITSReub were used to amplify the intergenic spacer region of Bacteria.[20] The phosphoramidite dye HEX was used to label the forward primers. The total volume of the polymerase chain reaction (PCR)

**Table 3.** Concentration of micropollutants in short-term exposure tests.

| NAME | CONCENTRATION |
|------|---------------|
| Control | NA |
| Triclosan | 230 mg/kg |
| PFOS | 14,000 µg/kg |
| Triclosan + PFOS | 230 mg/kg; 14,000 µg/kg |

mixture was 25 µL and consisted of 20 nmol deoxynucleoside triphosphates, 25 pmol forward and reverse primers, 1 × PCR buffer (Promega), 1.25 units of GoTaq DNA polymerase (Promega), and approximately 1 ng of genomic DNA. The PCR protocol consisted of 3 minutes of initial denaturation at 94°C, 35 cycles of 94°C for 45 seconds, 55°C for 1 minute, 72°C for 2 minutes, and a final extension of 7 minutes at 72°C. PCR products were separated by capillary electrophoresis, and peak areas were analyzed with PeakScanner Software version 1.0. Fragment lengths <156 bp were removed from subsequent analyses to eliminate primer dimers.[20] Fragments >1,000 bp were eliminated because the maximum size standard was 1,000 bp. Peaks had to account for at least 0.5% of the total area in a sample to be used for further analysis. The relative contribution of one operational taxonomic unit (OTU) normalized to the total community (area of one OTU divided by area of all OTUs) was averaged across replicates, and this average contribution was used for statistical analysis.[18,21,22]

**Analytical methods.** The biogas produced by each culture was quantified by measuring the displacement volume of a wetted glass syringe. Methane was quantified on a gas chromatograph equipped with a thermal conductivity detector as described previously.[23] The detection limit was 0.95 nmol of methane per microliter of sample injected.[18] Samples from the cultures were dried overnight at 105°C to determine the total solids content. VS were determined by quantifying the mass that volatilized upon heating the dried sample for 2 hours at 550°C. Total and volatile suspended solids (TSS and VSS) were quantified by passing a known volume of sample through a glass fiber filter (Millipore AP4004700) and drying the filter at 105°C (TSS) and then at 550°C (VSS).

**Statistical analysis.** Analysis of variance (ANOVA) and Student's *t*-tests were performed using Graphpad Prism v. 5.04 (Graphpad Software, Inc.). A *P*-value of ≤0.05 was considered significant. ARISA data were plotted and analyzed using nonmetric multidimensional scaling (nMDS). *R* was used for nMDS analysis using the *vegan* package. The distance between samples in nMDS plots correlates to dissimilarity between samples.[24]

## Results and Discussion

**Long-term PFOS exposure tests.** In serum bottle cultures that were exposed to 800 µg/kg PFOS and simultaneously perturbed, the community structure shifted and methane production increased 51% relative to control cultures (Figs. 2 and 3). This increase was statistically significant (ANOVA, *P* = 0.018). At these higher but still environmentally relevant concentrations,[7] PFOS could have been exerting subtle uncoupling impacts, as observed previously with fluorinated surfactants.[14] The mixing perturbation that occurred at Day 3 could have increased the mass transfer of PFOS into biomass flocs. The ARISA profiles revealed that PFOS had no impact on Bacteria community structure through the first 50 days, but by Day 115, the higher levels of PFOS had
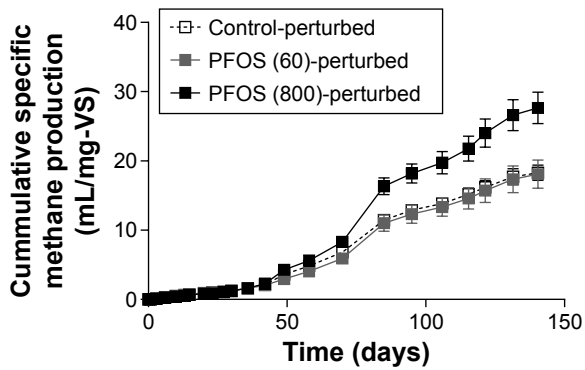
**Figure 2.** Impact of PFOS on methane production in perturbed cultures in long-term 140-day exposure experiment. Error bars represent standard error of the mean, $n = 3$. Data prior to Day 84 were normalized to total volatile solids; data after Day 84 were normalized to total volatile suspended solids.

shifted the community profile away from the control, and this shift persisted through Day 140, which was at the end of the experiment (Fig. 3).

Surfactants have been observed to produce subtle, broad-spectrum inhibitory effects on bacteria. Effects of the surfactant nonylphenol on bacteria are more widely studied than the effects of PFOS, and nonylphenol has been shown to inhibit *Escherichia coli*, *Staphylococcus aereus*, and nitrifiers.[25–27] Both compounds, however, have been linked to the increases in membrane permeability.[14,28] An increase in membrane permeability would diminish the proton motive force and force microorganisms to consume more substrate to maintain ATP levels; this could explain the increase in methane concentrations observed in these cultures. Alternatively, chemical uncouplers could also decrease microbial biomass as a result of less availability of ATP for anabolic activity.[29–31] No statistical difference was observed between the solids content in control
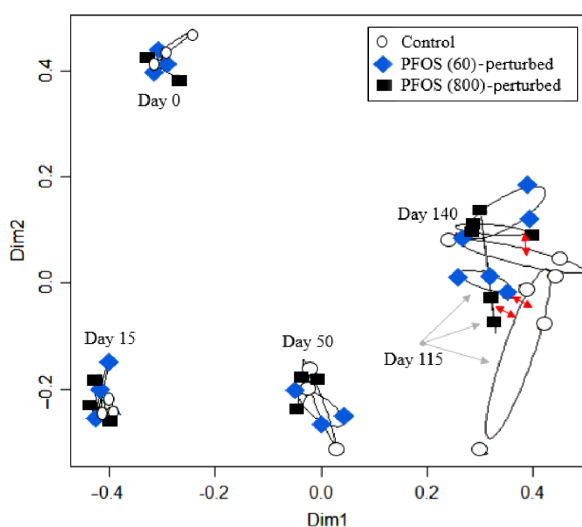


**Figure 3.** nMDS on Bacteria in perturbed cultures. Differences between communities at a given time point are highlighted by red arrows.

reactors or any of the PFOS-exposed reactors (two-way ANOVA, $P = 0.9101$ for the effect of treatment; Supplementary Fig. 1). These gravimetric solids measurements, however, may not be sensitive enough to accurately determine changes in biomass as a result of uncoupling. The increase in gas production implies that microbes increased their respiration rates to maintain ATP levels. Future work should be conducted with real-time measurement of ATP levels to further understand the impacts of PFOS on microbes.

Results from this study are different from those of Hollingsworth et al,[12] who found that PFOS had no impact on hydrogenotrophic methanogens. Other than exposure time, another difference between the two studies is that Hollingsworth et al fed only $H_2/CO_2$, and thus the impacts of PFOS on Bacteria and acetoclastic methanogens were not tested. Methanogenesis is a syntrophic, sequential process. It is feasible that PFOS uncouples Bacteria, causing faster turnover of methanogenic substrate followed by increased methane production. Alternatively, acetoclastic methanogens, which are thought to account for approximately two-thirds of methane production during anaerobic digestion,[32] may be impacted by PFOS. As opposed to the results of Hollingsworth and coworkers, the results presented herein demonstrate that, over exposure times of >50 days, PFOS can alter the function and structure of a methanogenic community at environmentally relevant levels if other perturbations, which are common in both engineered and natural systems, also occur.

PFOS also exacerbated the impacts of triclosan on methane production and community structure. When communities were amended with PFOS, triclosan addition had greater impacts on the methane production rate relative to control communities to which no PFOS was added (Fig. 4, Table 4). In the PFOS (60) and PFOS (800) sets of cultures, triclosan amendment led to significantly higher methane production rates compared to just methanol amendment (Table 4). In the control set of cultures, triclosan amendment did not significantly increase methane production. Subsequently, methane production declined more rapidly in PFOS cultures amended with triclosan compared to PFOS cultures amended with only methanol. Again, no difference in the decline in methane production was observed between control cultures with triclosan relative to control cultures with just methanol added (Table 4). These results suggest that PFOS enhanced the potential uncoupling effects of triclosan.[33] In addition, PFOS may have increased the bioavailability of triclosan. PFOS is a surfactant, and surfactants have been shown to increase the bioavailability of organic compounds.[34] This may have resulted in PFOS impacting the microbes' ability to take up triclosan, resulting in increased toxicity over time. These results are especially important because PFOS and triclosan are both found in residual wastewater biosolids.[7,17] Nevertheless, because of the limited number of data points, more work is needed to corroborate these results. In addition to functional impacts, triclosan also had larger impacts on the community structure
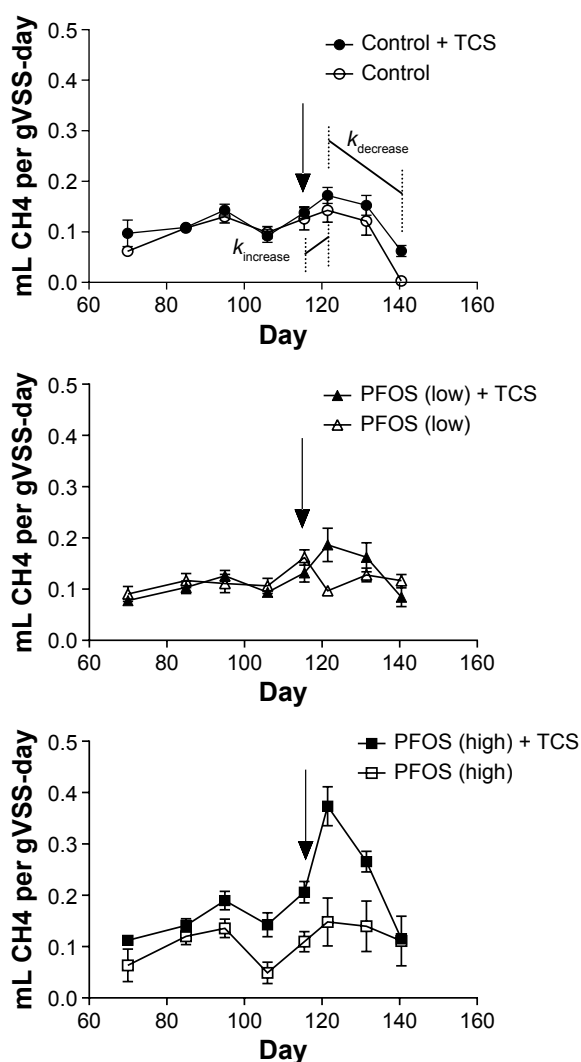
**Figure 4.** Impact of triclosan on methane production rate. Arrows denote time of triclosan addition. Error bars represent standard error of the mean, $n = 3$.

mixed with either triclosan or trichlorophenol, the inhibition of algae decreased, but when all three compounds were combined, inhibition increased.[35] Thus, evaluating toxicity by adding the effects of individual compounds could substantially underestimate or overestimate the toxicity of mixtures as seen in the environment. Indeed, the results presented herein demonstrated that following 115 days of exposure to PFOS at 0.02 mg/L (a relevant concentration in wastewater biosolids, equivalent to 800 µg/kg), the impacts of triclosan were more drastic, by generating faster methane production rates and causing larger shifts in community structure. PFOS directly impacted methanogenic communities in this study (Figs. 2 and 3), while also appearing to play a role in the impact of other micropollutants as part of a mixture.

**Short-term PFOS exposure tests.** While PFOS had functional and structural effects on methanogenic communities in long-term exposure experiments, no effects were observed in the short-term 14-day exposure experiments, even with much higher levels of PFOS added. PFOS did not alter methane production during the 14-day experiment (Fig. 6; ANOVA $P = 0.197$). Similarly, PFOS did not alter methane production during the first 14 days of the long-term 140-day exposure experiment (Supplementary Fig. 2; ANOVA $P = 0.1233$). Neither did PFOS augment the impacts of triclosan in these short-term experiments, again indicating that short-term impacts of PFOS are different than long-term impacts. These results indicate that more time is likely required for PFOS to transport into flocs or cells where it can have an impact on microorganisms. Similarly, an alcohol alkoxylate surfactant at 200 mg/L had no effect on methane production after 2 days, but after 9 days the compound was inhibitory.[13] Thus, in the 24-hour exposure study by Hollingsworth et al,[12] different results may have been observed had their experiments been longer term.

in those cultures also amended with PFOS, according to the nMDS analysis (Fig. 5).

Interestingly, other studies have also shown that mixtures containing PFOS alter, both synergistically and antagonistically, the effects of other micropollutants. PFOS exposure at 30 mg/L for 72 hours decreased the inhibition of atrazine and diuron to algae.[15] In contrast, it increased the inhibitory effects of pentachlorophenol on algae.[15] When PFOS was

## Conclusion

In summary, exposure time, in addition to the presence of other stressors/perturbations, and whether co-contaminants are present, matter when assessing the risk and impacts of PFOS. Overall, this research demonstrated that PFOS has subtle but definitive impacts on methanogenic community function and structure. Exposure time appears to greatly influence the impacts of PFOS, as it does with other surfactants[13] and

**Table 4.** Average $k$-values from triplicate cultures for the increase and decrease in methane rates following triclosan amendment. $k$-values were calculated between Days 115 and 121 and between Days 121 and 140. $P$-values were determined by a Student's $t$-test between perturbed and unperturbed culture sets for each PFOS amendment level (control, 60, 800).

| | CONTROL PERT[a] | CONTROL | *P*-VALUE | PFOS (60) PERT | PFOS (60) | *P*-VALUE | PFOS (800) PERT | PFOS (800) | *P*-VALUE |
|---|---|---|---|---|---|---|---|---|---|
| $k_{increase}$ | 0.006 | 0.003 | 0.236 | 0.009 | −0.011 | **0.010** | 0.028 | 0.006 | **0.016** |
| $k_{decrease}$ | −0.006 | −0.007 | 0.442 | −0.005 | 0.001 | **0.028** | −0.014 | −0.002 | **0.003** |

**Note:** [a]Perturbed.

**Figure 6.** Impact of PFOS, triclosan, and PFOS–triclosan mixture on methane production during short-term 14-day exposure experiment. Error bars represent standard error of the mean, $n = 3$.
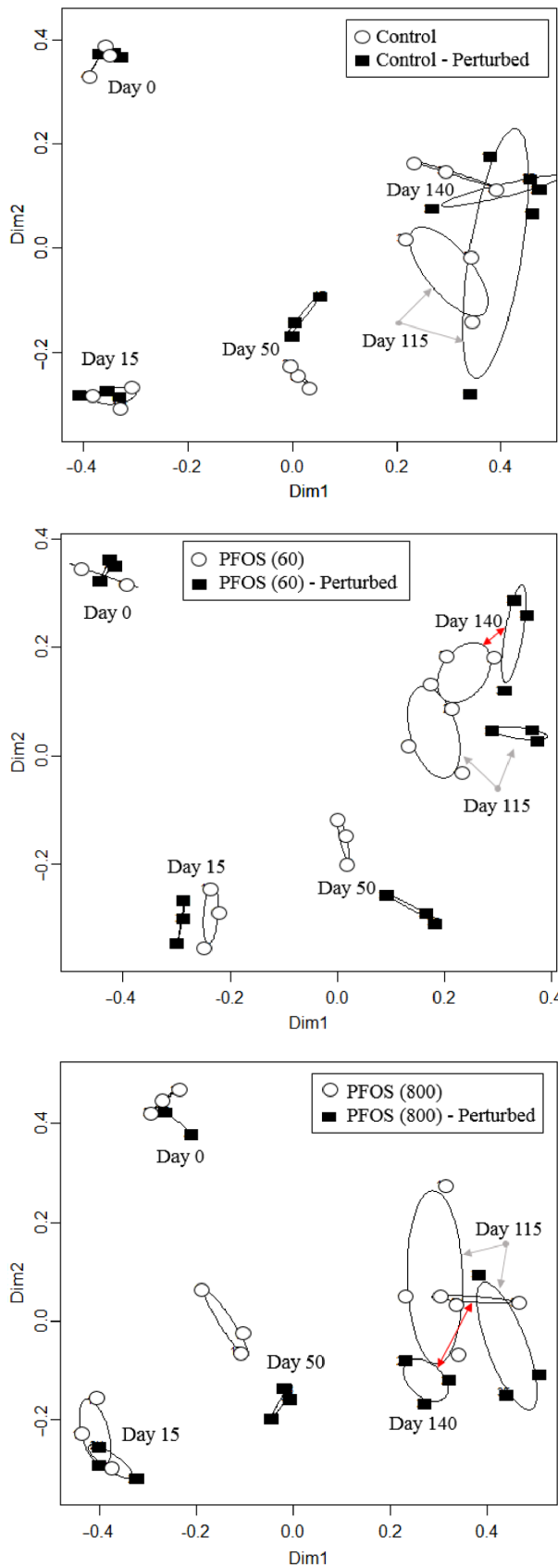
**Figure 5.** nMDS on Bacteria between perturbed and unperturbed cultures for control (top), low PFOS (60 µg/kg) (middle), and high PFOS (800 µg/kg) (bottom) cultures. Red arrows indicate dissimilarity between triplicate cultures at Day 140.
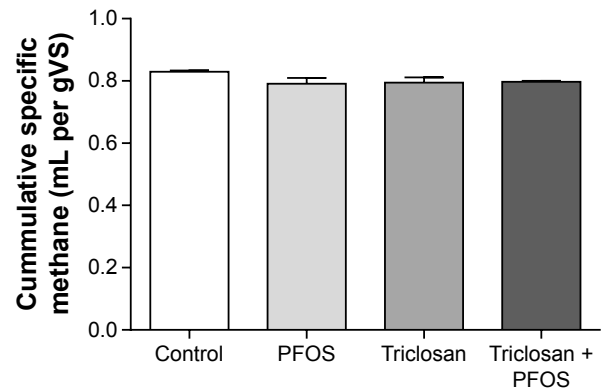
chlorinated chemicals.[36,37] Exposure time also influences the impacts of mixtures. When triclosan was added after 115 days of PFOS exposure, methane production and community structure were impacted, but function was not altered when triclosan and PFOS were added simultaneously in short-term (14 days) experiments. Other researchers have demonstrated that PFOS can alter the toxicity of mixtures in algal cultures.[15,35] Tests designed to evaluate environmental risk must therefore account for mixtures and incorporate an element of exposure time if they are to appropriately evaluate chemical risk. While it is impossible to test all chemicals in every possible mixture for prolonged periods of time, testing the effects of individual compounds on one particular microorganism, for example, *E. coli*, may do little to establish risk. Additionally, the duration of the test should reflect the predicted fate of a chemical and the time that a given chemical will reside in a particular environment. For example, effects from a hydrophobic chemical that will reside in sediments should be assessed after a much greater exposure time than those effects from a hydrophilic chemical that will degrade quickly in river water. Without a proper understanding of the long-term effects of micropollutants, alone and in mixtures, we could be stressing environments far beyond what has been predicted in laboratories or, if organisms are protected by flocs, far less.

## Acknowledgment

## Author Contributions

Conceived and designed the experiments: PJM, TML, and PJN. Analyzed the data: PJM, TML, and PJN. Wrote the first draft of the manuscript: PJM and PJN. Contributed to the writing of the manuscript: PJM, TML, and PJN. Agree with manuscript results and conclusions: PJM, TML, and PJN. Jointly developed the structure and arguments for the paper: PJM and PJN. Made critical revisions and approved

final version: PJM, TML, and PJN. All authors reviewed and approved of the final manuscript.

## Supplementary Materials

**Supplementary figure 1.** Total mass of volatile solids in serum bottles during long-term experiment.

**Supplementary figure 2.** Impact of PFOS on methane production in unperturbed cultures after 14 days during long-term experiment.

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