

FINAL REPORT

Biodegradation of Per- and Polyfluoroalkyl Substances (PFASs)
via Superoxide-Hyper-Producing Bacteria

SERDP Project ER20-1430

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Pedro J.J. Alvarez
Jordin Metz
Hassan Javed
Jacques Mathieu

Rice University

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EXECUTIVE SUMMARY

Objectives

PFASs are a class of over 4,700 synthetic compounds that are used in an array of industrial processes, consumer products, and in firefighting foam. This has resulted in their widespread distribution throughout the environment, with thousands of known or suspected release areas at Department of Defense facilities, and many more suspected releases at municipal and commercial sites. While research into the health and environmental effects of PFASs is still being explored, evidence of adverse health effects and widespread PFAS exposure is mounting. The Environmental Protection Agency (EPA) has established health advisory levels of 70 parts per trillion (ppt) for two of these compounds combined (PFOA and PFOS), and many state agencies have lower PFAS regulatory levels, reflecting their perceived toxicity and bioaccumulation potential. The objective of this limited-scope proposal is to evaluate a novel method to remediate PFAS, based on bacterial strains that are capable of producing superoxide at high rates and assessing their capacity to degrade model PFASs. Specifically, this project explored the potential of superoxide (including that generated by bacterial hyper-producers of extracellular superoxide) to degrade PFAS. Specific tasks included to:

1. Determine the potential for superoxide to degrade and defluorinate PFAS compounds, such as PFOA.
2. Identify and isolate bacteria with high superoxide production activity and explore factors to enhance their superoxide production.
3. Evaluate the ability of these superoxide hyper-producing bacteria to degrade PFAS.

In recent years, various Advanced Oxidation Processes (AOPs) have been explored for PFOA degradation. Yet, factors influencing their efficacy and degradation mechanism are not fully understood. Here, we resolve ambiguity in the literature regarding the role of superoxide in PFOA degradation (e.g., by nucleophilic attack) by considering three pure superoxide-producing systems: KO_2 in dimethyl sulfoxide (DMSO), xanthine oxidase with hypoxanthine, and WO_x/ZrO_2 catalyst with H_2O_2 . The use of these simple, ideal systems eliminates potential confounding effects to isolate the role of superoxide. We confirmed superoxide production in all three systems by electron paramagnetic resonance (EPR) spectroscopy and by precipitation of nitroblue tetrazolium (NBT), a common superoxide probe. Positive control experiments showed that the produced superoxide degrades ~48% bisphenol A (BPA) within one day, corroborating that superoxide was sufficiently stable and available for reaction in the test systems. However, no PFOA degradation was observed, which was corroborated by the absence of released fluoride and degradation by-products in all three systems. Therefore, other reaction pathways should be explored for PFOA degradation. Nevertheless, consistent with our driving hypothesis, superoxide can be generated extracellularly by bacterial hyper-producers, and this ROS can contribute to the degradation and natural attenuation of other commonly-co-occurring priority pollutants, such as trichloroethylene (TCE).

Technical Approach

Superoxide has been reported to degrade and defluorinate PFOA, and some prior publications suggest that it may play a critical role in the degradation of other PFASs. Furthermore, it was also recently discovered that many heterotrophic bacteria produce extracellular superoxide, with production rates spanning several orders of magnitude between different species. Moreover, superoxide production can be enhanced by the presence of cofactors (e.g., up to 100-fold using NADH), and its reactivity can be increased by certain solid matrices (with high surface area), redox mediators, or low polarity solvents. Therefore, we hypothesized that bacteria capable of producing high concentrations of superoxide can be used as a basis for PFAS bioremediation strategies. Accordingly, we first assessed PFAS degradation under various conditions in a low complexity, controlled system using chemically- and enzymatically-generated superoxide. We then planned to screen heterotrophic bacteria to identify the highest superoxide-producing strains (hyper-producers), and assess their ability to degrade PFASs (PFOA, PFOS, 8:2 FTOH) under conditions identified as maximally enhancing superoxide production and reactivity. Finally, PFAS degradation by superoxide hyper-producers would be assessed using PFAS-spiked aquifer material.

Results

For Objective 1, we conducted a series of experiments to assess whether superoxide (produced from various sources) could directly transform PFOA. These efforts are reported in an *Environmental Science & Technology Letters* paper,¹ with the conclusion that superoxide alone is not sufficient to degrade or defluorinate PFOA. Further tests were conducted to assess this potential remediation technology, and we found that superoxide was able to degrade other organic compounds (e.g., BPA, TCE). Overall, superoxide alone does not appear suitable for developing standalone remediation strategies for PFOA, but could be useful for other commonly co-occurring priority pollutants.

For Objective 2, we developed a superoxide production assay that could be used to easily screen for the presence of this capability in bacteria. We first acquired a model superoxide producer, *Pseudomonas putida* strain GB-1, to use for method development and as a benchmark. We then developed an assay based on the observation that microbial superoxide production is involved in manganese oxidation. Oxidized manganese (Mn(III+)) is very reactive, and can be quantified using the colorimetric indicator Leucoberbelin blue I. After successfully demonstrating this assay using GB-1, we tested various environmental samples and have identified some superoxide-producing bacteria. Preliminary tests have demonstrated that superoxide-producing bacteria can be easily and routinely isolated from environmental sources, which highlights the potential for developing bioremediation strategies based on the use of indigenous superoxide hyperproducers.

For Objective 3, since we showed that superoxide alone could not degrade PFOA, we also evaluated several other microbial enzymes that had been reported in the literature to degrade PFOA or PFOS. We tested a laccase-mediator system and a peroxidase-mediator system under various optimized conditions. Additionally, we tested the xanthine oxidase-hypoxanthine system in conjunction with MnCl₂ to promote manganese oxide formation. However, none of these systems demonstrated a significant removal of either PFOA or PFOS after one week, reflecting a common

problem with some PFAS degradation papers: the lack of reliable reproducibility. We are interested in continuing this research to the mechanism of superoxide TCE degradation by biogenic superoxide, which could be exploited to enhance natural attenuation or to develop permeable reactive barriers. We are also interested in testing superoxide-producing isolates for their capacity to degrade other common organic pollutants such as BPA or TCE via extracellular superoxide production.

Benefits

This work resolved a controversy in the literature regarding the role of superoxide in degrading PFOA, which will help advise and guide the remediation community in developing more effective treatment approaches that do not rely on production of such ROS. These results were published in a rigorous academic journal, *Environmental Science & Technology Letters*, highlighting the relevance of these results.

Additionally, the identification and characterization of superoxide hyper-producing bacteria will facilitate the development of both *in situ* and *ex situ* bioremediation strategies for other priority groundwater pollutants (e.g., TCE), which could lead to significantly reduced treatment costs. This research also increases our understanding of potential natural attenuation routes (via extracellular reactive species) and how biostimulation might be used to enhance their efficacy and increase biodegradation rates. This strategy should be evaluated for the remediation of a wide range of recalcitrant contaminants. Furthermore, the knowledge that superoxide and hydroxyl radicals² cannot directly degrade PFOA advises the remediation community to explore other strategies besides reactive oxygen species for degradation.

NOMENCLATURE

PFASs	Per- and polyfluoroalkyl substances
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PFH _x S	Perfluorohexanesulphonic acid
8:2 FTOH	8:2 fluorotelomer alcohol
6:2 FTOH	6:2 fluorotelomer alcohol
BPA	Bisphenol A
ROS	Reactive oxygen species
TCE	Trichloroethylene
LC-MS	Liquid chromatography–mass spectrometry
UV-Vis spectroscopy	Ultraviolet-Visible spectroscopy
IC	Ion chromatography
CHP	Catalyzed H ₂ O ₂ propagation
ISCO	<i>in situ</i> chemical oxidation
MFR	Modified Fenton's reagent
DI water	Deionized water

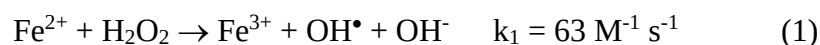
INTRODUCTION

PFASs are a class of over 4,700 synthetic compounds that are used in an array of industrial processes, consumer products, and in firefighting foam. This has resulted in their widespread distribution throughout the environment, with thousands of known or suspected release areas at Department of Defense facilities, and many more suspected releases at municipal and commercial sites.³ While research into the health and environmental effects of PFASs is still being explored, evidence of adverse health effects and widespread PFAS exposure is mounting. The Environmental Protection Agency (EPA) has established health advisory levels of 70 parts per trillion (ppt) for two of these compounds combined (PFOA and PFOS), and many state agencies have lower PFAS regulatory levels, reflecting their perceived toxicity and bioaccumulation potential.⁴

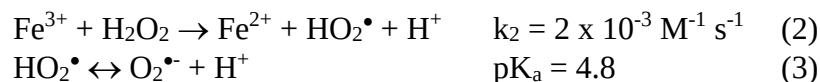
Developing effective remediation strategies for PFASs has numerous challenges related to their chemical diversity, uncertain behavior in different environments and conditions, and evolving regulatory landscape. Most regulated PFASs tend to absorb poorly and are relatively mobile in soil and groundwater due to their ionic nature, which can promote complex interactions with aquifer matrices (soil, minerals) and accumulation at phase interfaces (air-water, oil-water)³. This can present challenges for conventional remediation technologies that rely on either absorption or mobilization. Furthermore, the low volatility of PFASs renders air stripping and sparging ineffective. Presently, hydraulic containment coupled with commercial absorbents appears to be the standard remedial response, with a destructive treatment applied after contaminant concentration⁵. However, the efficacy of absorbents varies with PFAS functional groups and chain length^{6,7}, and the capital and operational expenses associated with absorbents and other pump-and-treat approaches are substantial.

In situ remediation technologies are desirable for PFAS remediation due to their generally lower costs and disturbance relative to *ex situ* technologies. However, the abundance of high-strength C–F bonds (116 kcal/mol) in PFAS make them extremely recalcitrant to degradation⁸. Reductive dehalogenation may seem to be a likely mechanism for PFAS degradation, but neither chemical reduction (using zero-valent iron, vitamin B12, or sodium dithionite) or anaerobic biodegradation has been shown to be effective^{9,10}. Currently, the only commercially-available option is *in situ* chemical oxidation (ISCO), which is not utilized for PFAS removal on a widespread basis¹¹. While persulfate has been used to successfully degrade many PFAS under lab conditions, its efficacy is significantly reduced by the presence of aquifer materials and other radical scavengers, and it is also incapable of degrading PFOS or PFHxS¹². Furthermore, persulfate-mediated PFAS degradation requires a high temperature and acidic environment¹³, which is difficult and expensive to create for large plumes.

Catalyzed H₂O₂ propagation (CHP; modified Fenton's reagent (MFR)) is a well-established and effective remediation approach for the *in situ* chemical oxidation (ISCO) of contaminants in soil and groundwater¹⁴, and has been successfully applied at the field scale for the treatment of a wide variety of contaminants including polynuclear aromatic hydrocarbons (PAHs), chlorinated solvents and non-chlorinated alkanes and alkenes¹⁵. In the Fenton's process, H₂O₂ is decomposed by Fe(II) generating hydroxyl radical (OH•):



The hydroxyl radical generated in the process can oxidize many organic pollutants, however, it does not react with many PFASs, including PFOA and PFOS^{16,17}. Thus, modification of Fenton's chemistry is needed for CHP of PFASs¹⁸. High concentrations of H₂O₂ and initiators such as soluble Fe(III), iron chelates, or minerals, can be employed to produce a variety of oxidants in addition to hydroxyl radical such as perhydroxyl radical (HO₂•), superoxide radical anion (O₂^{•-}), and hydroperoxide anion (HO₂⁻) as represented below:



Among these oxidants, superoxide and hydroperoxide produced in CHP reactions have been proposed to degrade PFAS, as described by Mitchell et al¹⁷. For instance, PFOA removal of up to 89% was reported within 150 min when exposed to initial H₂O₂ concentration of 1 M at pH 3.5, while superoxide generated with 2 M H₂O₂ catalyzed by birnessite at neutral pH removed 59% of PFOA, and 68% PFOA removal was obtained in Fe(III)-EDTA systems with 2 M H₂O₂¹⁷. Additionally, in the presence of hydroperoxide as the sole reactant in 2 M H₂O₂ solutions at pH 12.8, 80% of PFOA was removed. In these basic experiments, the percent fluoride released was in stoichiometric agreement to percent PFOA degraded, which suggests degradation of PFOA with no metabolite formation. However, implementing MFR/CHP *in situ* faces several challenges. The high H₂O₂ concentrations typically used for ISCO can result in rapid decomposition kinetics not optimal for sustained free radical generation; higher destruction of various contaminants is commonly reported in bench-scale studies using lower H₂O₂ dosing rates¹². Moreover, H₂O₂ rapidly decomposes in the presence of catalase-positive bacteria near injection wells¹⁸. Furthermore, the effectiveness of reactive oxygen species (such as superoxide) in degrading PFOA is unclear. While various studies have implied that superoxide plays a role in degrading PFOA,^{19–21} others argue that it is ineffective in attacking PFOA.^{22,23} It is important, therefore, to resolve this controversy; the results of this report describe this in detail.

A number of studies have explored PFAS biodegradation with mixed results. Though fluorinated compounds are rare in nature, some plants and bacteria synthesize monofluorinated compounds, such as nucleocidin, fluoroacetate, and 4-fluorothreonine²⁴. Exposure to synthetic mono- and difluorinated compounds is rather widespread due to their agricultural use as pesticides, herbicides, and insecticides, and as a result of the increased production of fluorinated drugs. Thus, several *Pseudomonas* strains and other bacteria possess fluoroacetate dehalogenases and other enzymes that can biodegrade naturally-occurring fluorinated compounds²⁴. However, PFASs are much more highly fluorinated, and the enzymes involved in their biodegradation have not been well documented yet. Several PFASs can be partially biodegraded to PFAAs under aerobic conditions, but complete biodegradation of PFAAs has not been demonstrated. The aerobic biodegradation of fluorotelomer alcohols (FTOHs) such as 8:2 and 6:2 FTOH have been extensively researched^{25–27}, and a number of studies have also reported their anaerobic biodegradation^{28,29}. Anaerobic degradation of 8:2 FTOH in digester sludge led to the accumulation of 8:2 FTUCA and PFOA²⁸. During aerobic biodegradation, 8:2 FTOH is transformed to 8:2 FTCA and is then defluorinated to 8:2 FTUCA. Further transformation to PFOA is expected to proceed via a “one-carbon removal” pathway. Despite the high number of fluorine atoms, 8:2 FTOH half-life ranges from < 2 to 30 days in laboratory studies^{26,27,30}, with a PFOA molar yield of 0.5% in a pure bacterial culture³¹ to 40% in one aerobic soil²⁷. This suggests 8:2 FTOH could be a very significant PFOA precursor in some environments.

6:2 FTOH biodegradation pathways are similar to 8:2 FTOH, but differ in the types and yields of terminal products. 6:2 FTOH showed a greater defluorination potential in aerobic soil, which was attributed to its smaller size and higher water solubility, which is believed to have resulted in increased bioavailability²⁵. During biotransformation by two *Pseudomonas* strains, 4:2 FTOH demonstrated a higher defluorination potential than either 6:2 FTOH or 8:2 FTOH³². Fungal biotransformation of 6:2 FTOH has also been explored³³. *Phanerochaete chrysosporium* transformed 6:2 FTOH to perfluorocarboxylic acids (PFCAs), polyfluorocarboxylic acids, and transient intermediates within 28 days³⁴. Fungi tested yielded more 5:3 acid (up to 51 mol % of initial 6:2 FTOH) relative to other metabolites. Production of lower amounts of PFCAs and more transformation of 6:2 FTOH toward 5:3 acid and other polyfluoroalkyl substances is desirable.

While there is a significant body of literature regarding the biodegradation of PFAS precursors, few reports exist for PFAA biodegradation. A strain of *Pseudomonas aeruginosa* was reported to aerobically degrade 67% of PFOS (600 mg L⁻¹) after 12 h, with PFBS and PFHxS detected as minor products³⁵. However, the methodology used raised some concern, and no corroborating data has been forthcoming³⁶. An earlier report demonstrated biodegradation of numerous fluorinated sulfonates, but not PFOS³⁷. Two studies of anaerobic biodegradation using either anaerobic sludge³⁸ or cattle rumen fluid also provided inconclusive results³⁹. Recently, *Acidimicrobium* sp. Strain A6 was shown to remove up to 60% of PFOA and PFOS (0.1 mg/L) over 100 days, while keeping a constant fluorine balance.⁴⁰ More promising results were obtained using enzymatic systems. A 68% reduction in PFOA was reported in 6 h using horseradish peroxidase with H₂O₂ and 4-methoxyphenol as co-substrates⁴¹, though negligible defluorination was observed. Enzyme-catalyzed oxidative humification reactions (ECOHR) using laccase achieved approximately 50% transformation of PFOA over 157 days using 1-hydroxybenzotriazole as a co-substrate, with 28.2% defluorination reported⁴². In a separate study using laccase and soybean meal as a natural mediator, a 24% removal of PFOA was achieved after 36 days in an aqueous system, while 40% degradation was observed after 140 days in a soil slurry⁴³. Another study reported 59% degradation of PFOS over 162 days using a laccase-mediator system with 1-hydroxybenzotriazole⁴⁴. Interestingly, a longer time was needed for PFOA degradation in the laccase system despite treating a PFOA concentration 1000-fold lower than in the horseradish peroxidase study, and using a substantially greater amount of enzyme. Furthermore, laccase required repeated amendments of enzyme and mediator (every 6 days). Nevertheless, these studies provide proof-of-concept that bacterial enzymes can be harnessed for the biodegradation of PFAAs in the presence of mediators.

Overall, there are some promising results from the biodegradation studies conducted to date, but most, if not all, face challenges that are likely to hinder their prospects for *in situ* use. For example, as many PFAS plumes are extremely dilute, there is a high probability that the enzymes needed for biodegradation would not be induced by the contaminant. Furthermore, little to nothing is known regarding PFAS uptake, which could be a significant barrier to PFAS biodegradation. Fungi appear useful for the biodegradation of fluorotelomer alcohols, but so far not PFAAs. Additionally, fungi are not typically useful for subsurface remediation as they are rarely found at depths greater than a few feet. Enzymatic treatment circumvents the need to induce genes or uptake contaminants, and may potentially be used to degrade PFAAs, but the costs associated with treating large plumes would likely be prohibitive. Thus, more broadly applicable and cost-effective bioremediation strategies are desirable.

APPROACH

Bioaugmentation has proven to be a successful remediation approach for a variety of contaminants^{45–47}, but is also susceptible to failure for several reasons. The main factors include a loss of viability during inoculation due to acute stresses⁴⁸, gradual cell death due to nutrient limitations or contaminant toxicity^{49,50}, competition by the indigenous microbial population^{51,52}, predation due to protozoan overgrowth⁵³, low soil water holding capacity, and extreme pH or temperature⁵⁴. Regardless of the cause, the overarching theme of bioaugmentation failures is that there is a lack of survival and growth of the introduced strain or consortium. Strain selection is therefore considered an extremely critical factor for successful bioaugmentation. Unfortunately, despite rapidly increasing interest in PFAS biodegradation over the past decade, no microbial strain suitable for *in situ* PFAS bioremediation has been identified. This is likely due to the extreme xenobiotic nature and wide variety of these relatively large molecules, which limits cellular uptake and metabolism. To develop an effective bioremediation strategy for PFASs, we sought to design an approach that avoids many of the pitfalls observed when implementing bioaugmentation for the remediation of other contaminants. Specifically, we postulate that the probability of using bioremediation to successfully biodegrade complex PFAS mixtures can be enhanced by:

- Avoiding the need for contaminant uptake by using an extracellular degradation pathway.
- Using a non-selective degradation mechanism that could react with many different PFASs.
- Focusing on pathways that are widespread in nature, thus enhancing the likelihood of isolating a variety of candidate bioaugmentation strains.

Recently, it was discovered that **the capacity to produce extracellular superoxide is widespread amongst heterotrophic bacteria** (27/30 tested produced superoxide)^{55,56}. The isolates tested were all environmentally common species, and included members of diverse phyla from a wide variety of aquatic and terrestrial environments, including lakes, soils, hydrothermal vents, marine sediments, estuaries, the subsurface, and the deep (> 1 km) ocean. Superoxide production between the tested isolates (bacteria and microalgae) ranged over almost four orders of magnitude ($0.1 - 10^3$ attomols superoxide $\text{cell}^{-1} \text{hr}^{-1}$). Assuming 10^6 cells/mL, production in a typical environment would range between $0.1 \text{ nmol} - 1 \text{ } \mu\text{mol}$ superoxide $\text{L}^{-1} \text{hr}^{-1}$. This is supported by a recent study that measured superoxide production rates in pond aphotic zones (assuming $\sim 10^5$ bacteria/mL) to be $30 - 70 \text{ nM h}^{-1}$.⁵⁷ Thus, given the results achieved for PFOA degradation by Mitchell et al. using a MFR/CHP system¹⁸, the question arises as to whether such biogenic superoxide production contributes to natural attenuation of PFASs, or could be harnessed to enhance PFAS removal *in situ*. Several recent discoveries support the proposed approach:

1. Superoxide has been shown to abiotically degrade and defluorinate PFOA¹⁸.
2. Superoxide reactivity can be significantly enhanced by the presence of solid matrices or solvents of lower polarity than water.⁵⁸
3. Extracellular superoxide production is widespread amongst a broad diversity of heterotrophic bacteria^{55,56}.
4. Bacterial extracellular superoxide production can be significantly enhanced by amendment with NADH⁵⁹.

These findings suggest it may be possible to utilize superoxide-hyper-producing bacteria in PFAS bioaugmentation strategies, and to develop biostimulatory approaches to enhance indigenous (or bioaugmented) superoxide hyper-production. Biogenic, extracellular superoxide production occurs via the activity of either multi-copper oxidases or animal heme peroxidases loosely attached to bacterial cell surfaces⁵⁹⁻⁶³, and is involved in the formation of manganese oxides^{56,64}. Numerous bacteria and fungi are known to degrade high molecular weight compounds (e.g., humic substances) via the production of reactive manganese oxides^{65,66}, and recent studies have demonstrated that soil manganese content is one of the biggest determinants of litter decomposition rates⁶⁷⁻⁶⁹. Additionally, manganese oxides may protect microorganisms from certain reactive oxygen species. Thus, the capacity to produce extracellular superoxide, and consequently manganese oxides, is a common trait amongst a broad diversity of bacteria. As our proposed strategy relies on bioaugmentation with superoxide-producing bacteria, such diversity is beneficial as it facilitates strain selection for different environments and enhances the likelihood of successful bioaugmentation.

Though superoxide is relatively unreactive in deionized water, its reactivity is greatly increased by the presence of solid matrices such as manganese oxides (e.g., birnessite), as well as low polarity solvents⁵⁸. For example, birnessite is highly effective at enhancing superoxide reactivity towards both carbon tetrachloride and perchloroethylene⁷⁰. For solid matrices, superoxide reactivity increases with increasing surface area. The finding that superoxide reactivity can be enhanced by solids and lower polarity solvents to a similar degree as H_2O_2 is important because the concentrations of H_2O_2 used to promote PFOA degradation in MFR/CHP reactions (1 – 2 M) are toxic to bacteria, which precludes its use as part of a broader bioremediation strategy. H_2O_2 is also difficult to apply *in situ* due to rapid decomposition by catalase-producing microbes and some mineral surfaces. Furthermore, H_2O_2 can inhibit the formation of manganese oxides⁶⁴.

While the primary focus of this limited scope proposal is direct transformation of PFASs by superoxide, manganese oxides can react with many organic pollutants directly, or indirectly via redox mediators, and have been described as having laccase-like activity⁷¹. It has also been shown that biogenically-produced manganese oxides have enhanced activity towards some contaminants under natural conditions relative to chemically-produced manganese oxides by virtue of their greater surface areas⁶⁴, and that degradation rates are further increased (10- to 15-fold) in the presence of Mn-oxidizing bacteria (i.e., superoxide-producing bacteria), which are able to reoxidize Mn(II)^{72,73}. Little is known about potential interactions between PFASs and the various manganese oxides, although both laccase and horseradish peroxidase have been demonstrated to degrade PFOA in the presence of redox mediators^{41,43}. Interestingly, the use of mediators facilitates the oxidation of compounds with redox potentials higher than the enzyme. For example, laccases (which can have redox potentials up to ~800 mV) cannot directly oxidize nonphenolic lignin subunits (redox potentials up to ~1500 mV). However, the addition of a mediator enables oxidation of such nonphenolic subunits through different mechanisms, such as electron transfer or radical hydrogen atom transfer⁷⁴. Manganese oxides have redox potentials similar to that of laccases, and have been used in conjunction with mediators to enhance contaminant removal via oxidative coupling reactions^{75,76}. Laccase is also capable of indirectly producing superoxide via autooxidation of its reaction products, which may explain its capacity to defluorinate PFOA and PFOS⁷⁷. Therefore, extracellular superoxide production has the potential to promote PFAS

degradation both directly through nucleophilic attack, and indirectly through the production of reactive intermediates.

Contaminant biodegradation via extracellular enzymes is widely known and utilized for remediation, but few studies have explored the importance of extracellular reactive oxygen species for *in situ* remediation. Perhaps one reason for this is the bulk of the literature on extracellular biodegradation is focused on fungi, which are generally unsuitable for use in subsurface applications beyond a few feet. The use of Fe(III)-reducing facultative anaerobes has been suggested as a way to sustain Fenton reactions *in situ*, for the biodegradation of 1,4-dioxane⁷⁸. However, the standard Fenton reaction is not effective for degrading most PFAS and the system requires an alternating aerobic and anaerobic environment, which may be difficult to implement *in situ*. Nevertheless, the recent discovery that heterotrophic bacteria produce extracellular superoxide (with a wide range of rates) presents new opportunities. Indeed, the basic premise of this project is supported by a recent study describing the biodegradation of tetrabromobisphenol A (80% reduction over 10 days) by extracellular reactive oxygen species (including superoxide) generated by a marine *Pseudoalteromonas* species⁷⁹. Similarly, we hypothesize that PFAS biodegradation will be substantially accelerated by increasing the concentration of superoxide-hyper-producing strains (e.g., bioaugmentation) or modulating environmental conditions to enhance either superoxide production or reactivity. Furthermore, a major problem with ISCO is that the oxidants react and are depleted prior to reaching the targeted contaminants. By bioaugmenting a site with bacteria that continually produce low levels of superoxide, this concern is at least partially mitigated. Recent studies have also demonstrated that many PFASs reversibly partition to bacterial membranes^{80,81}. Thus, it might be possible to use superoxide hyper-producing bacteria *in situ* for both sequestration and biodegradation of a wide variety of PFASs. And by utilizing pathways that generate reactive species such as superoxide, issues with enzyme substrate specificity are also avoided. This is important for PFAS biodegradation considering the diversity and abundance of compounds that could be encountered within a plume.

OBJECTIVES AND HYPOTHESES

- 1. Determine potential for superoxide-producing systems to degrade and defluorinate model PFAS compounds in the presence and absence of stabilizing surfaces (e.g., aquifer material), and derive pertinent rate laws.**

Hypothesis: Superoxide produced in a range of favorable, well-defined systems will react with model PFAS compounds, resulting in their degradation and defluorination in the presence of a solid matrix (e.g., glass beads, manganese-bearing birnessite, aquifer material) or low-polarity solvent (e.g. DMSO) that enhances superoxide stability and reactivity.

- 2. Identify or isolate bacteria with extracellular superoxide hyper-production, and discern factors enhancing superoxide production.**

Hypothesis: O_2^- production rates of at least $10 \text{ amol cell}^{-1} \text{ h}^{-1}$ will be common amongst heterotrophic bacteria, and rates up to $100 \text{ amol cell}^{-1} \text{ h}^{-1}$ will be identified in some bacteria through screening. Bacterial superoxide production will be enhanced at least 10-fold by

increasing Mn(II) and/or NAD(P)H concentrations. Mn(II) and NAD(P)H amendment will increase gene expression for superoxide-producing enzymes and enzyme activity, respectively.

3. Evaluate (extracellular) PFAS biodegradation potential of superoxide-hyper-producing bacteria.

Hypothesis: Cultures of heterotrophic bacteria capable of producing $> 10 \text{ amol superoxide cell}^{-1} \text{ h}^{-1}$ will promote the degradation and defluorination of model PFAS compounds in the presence of solvents or matrices that increase superoxide stability and reactivity. Degradation rates will also be enhanced by the addition of substrates that increase the concentration of pertinent bacteria, enzyme activity and/or gene expression.

MATERIALS AND METHODS

OBJECTIVE 1

Chemicals and Reagents. Perfluorooctanoic acid (95% purity), perfluoroheptanoic acid (99%), perfluorohexanoic acid ($\geq 97\%$), perfluoropentanoic acid (97%), perfluorobutanoic acid (98%), perfluoropropanoic acid (97%) were purchased from Sigma Aldrich. The catalyst material, a zirconia-supported tungsten oxide (“ WO_x/ZrO_2 ”, 20 wt% WO_3 content) was obtained from MEI Chemicals. H_2O_2 (30% w/v) and dimethyl sulfoxide (DMSO) (ACS grade, $\geq 99.9\%$) were obtained from Fisher Scientific. KO_2 powder, 18-crown-6 ($\geq 99.0\%$), fluoride standard (TraceCERT®, 1000 mg/L in water), nitroblue tetrazolium, bisphenol A (BPA) ($> 99\%$), were obtained from MilliporeSigma. Deionized water ($18 \text{ M}\Omega \text{ cm}^{-1}$) was used for all experiments.

Potassium Superoxide Experiments. KO_2 powder was added to DMSO, followed by 18-crown-6 to facilitate dissolution, at a ratio of KO_2 :18-crown-6 of 1:2.5, with 1 mM and 30 mM KO_2 concentrations. This was stirred for at least 15 min. A 20 mL capped glass vial contained the 10 mL solution with 10 mg/L PFOA or 20 mg/L BPA in DMSO. Control reactions without KO_2 were also set up. The reactions stirred for 24 h. The reactions were quenched with water at a ratio of a 10:1 of water: DMSO.

Xanthine Oxidase Enzyme Experiments. Two sets of 5-mL reactions were performed in 15-mL polypropylene tubes containing 0.5 mM hypoxanthine, 0.25 units/mL xanthine oxidase and 0.5 mg/L PFOA or 20 mg/L BPA in a phosphate buffer solution (pH 7.4). One set of reactions was amended with MnO_2 (1000 mg/L) to stabilize superoxide. Control reactions containing no xanthine oxidase were also setup. All reactions were run at 37°C for 3-days (PFOA) or 1-day (BPA) under constant agitation. Samples from reactions containing MnO_2 were filtered using $0.2 \text{ }\mu\text{m}$ polypropylene filter prior to analysis.

MnO_2 Synthesis. MnO_2 was prepared as previously reported.^{18,82} Briefly, 2 M hydrochloric acid was added dropwise to boiling 1 M potassium permanganate solution under vigorous stirring. MnO_2 precipitates were separated, washed with deionized water and ethanol, and dried at 60°C overnight.

WO_x/ZrO₂ Catalyst Experiments. In a 20 mL capped glass vial, 10 mL solution containing 50 mg/L PFOA or 20 mg/L BPA, 0.1 M H₂O₂ and 2,000 mg/L WO_x/ZrO₂ was prepared. Control reactions without 0.1 M H₂O₂ were also set up. For PFOA, additional control reactions to assess fluoride recovery were prepared, containing 50 mg/L PFOA, 2,000 mg/L WO_x/ZrO₂ and 5 mg/L spiked fluoride. All reactions were stirred for 2-days (PFOA) or 1-day (BPA). PFOA (and BPA) was extracted from the catalyst by raising the pH of the solutions to pH 12 by adding NaOH (1 M), and stirring them overnight. Aliquots (5 mL) were withdrawn from the solution, filtered through 0.2-μm polypropylene filters and adjusted to neutral pH using 0.5-M H₂SO₄ prior to analysis.

Analytical methods. PFOA was analyzed with a Shimadzu IT-ToF LC-MS equipped with a C-18 column (Ascentis, 2.7μm, 1.0 mm × 150mm). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The flow rate was 0.15 mL/min. The gradient ran from 30%B to 95%B. All sample pH values were adjusted to ~7 before analysis. This HPLC-TOF-MS system was used to detect both PFOA and its degradation by-products. Two scan events were set up where the first scan was used to detect PFOA based on the nominal mass of the molecular ion with 3 Da wide mass window for PFOA quantification. The second scan event was used to scan the samples for degradation by-products in the 100-500 m/z range. Standard PFOA degradation byproducts, C3 – C7 shorter chain perfluorinated carboxylic acids, were used to confirm the accuracy of the second scan, which was capable of detecting the by-products down to 25 ppb. A calibration curve was created for each of the standard degradation byproducts.

Blanks and standard samples were injected periodically to monitor the performance and stability of HPLC-TOF-MS instrument. No leaching of PFOA in-between the injections was observed and the periodically run standard samples confirmed that the system was stable and only experienced ~8% drift.

BPA was analyzed using a HPLC (LC20AT, Shimadzu) equipped with a C-18 column (Shimadzu, 5 μm, 4.6 mm × 50 mm) and an UV–Vis photodiode array detector (SPD-M20A, Shimadzu). The mobile phase (1 mL/min) was 60% acetonitrile and 40% water.

A Dionex Aquion chromatography system with an anion-exchange column (Dionex, RFICTM IonPacTM AS23 column, 4 x 250 mm) and a conductivity detector (Dionex, DS6 heated conductivity cell) was used for fluoride (F⁻) analysis. The mobile phase (1 mL/min) was sodium carbonate (4.5 mM) and sodium bicarbonate (0.8 mM) in DI water.

Nitroblue Tetrazolium (NBT) (a classical biochemical assay for superoxide detection)² was used as a spectroscopic probe for superoxide measurement. NBT (350 mg/L) was added to the KO₂, XO, and WO_x/ZrO₂ systems. H₂SO₄ was added to the KO₂ solution to lower the pH to ~7. NBT forms a formazan product after reaction with superoxide, which is insoluble in water. Therefore, for enzymatic and catalytic systems, the formazan product was dissolved by adding DMSO to the reaction mixture (DMSO:H₂O of 3:1). Samples were filtered through a 0.2 μm polypropylene filter and measured using a UV-Vis spectrometer.

Electron Paramagnetic Resonance (EPR) experiments were conducted with a Bruker EMX spectrometer. The KO₂ reaction was measured using freeze-trapping while the WO_x/ZrO₂ catalyst

and XO/HX enzymatic systems were measured using BMPO as a superoxide spin-trapping agent. Spin-trap experiments were conducted by adding 1000 mg/L BMPO to the catalyst system (2000 mg/L $\text{WO}_x/\text{ZrO}_2 + 0.1 \text{ M H}_2\text{O}_2$) and the enzymatic system (0.5 mM hypoxanthine, 0.25 units/mL xanthine oxidase in phosphate buffer, pH 7.4) and were mixed for 10 min. BMPO forms a stable radical adduct upon reaction with the short-lived superoxide radical. Room temperature EPR measurements were conducted at a frequency of 9.32 GHz, with 100 kHz modulation frequency, 0.2 - 1 G modulation amplitude, 20 mW power, and 163 ms time constant. Different parameters were used for catalyst and enzymatic systems to optimize signal size. The catalyst system gave a strong superoxide signal and was measured using 0.2 to 0.5G modulation amplitude in a single scan, whereas the enzymatic system was measured using 1G modulation amplitude over eight accumulated scans due to a weaker signal. The superoxide in KO_2/DMSO stock (30 mM KO_2 , 75 mM 18-crown-6) was measured by freeze trapping a 1/10 dilution of KO_2/DMSO stock in 50 mM NaOH, placed in an EtOH/dry ice bath, and then transferred into liquid nitrogen. Freeze trapping experiments were performed at 115 - 120 K, with a frequency of 9.29 GHz, 100 kHz modulation frequency, 2 G modulation amplitude, 1 mW power, and 163 ms time constant. KO_2 measurement at room temperature did not yield a spectrum.

All experiments were conducted in triplicate and the statistical significance was determined by Student's t-test ($p \leq 0.05$).

PFAS Analysis. Several standard methods have been published for the determination of PFASs in different matrices: EPA method 537 for drinking water⁸³; ASTM D7979 for water, influent/effluent and waste water and slurries⁸⁴; and ASTM D7968 for soil⁸⁵. For this work, we characterized the degradation of model PFASs by superoxide in aqueous systems (buffers, synthetic groundwater, and/or bacterial growth media), with and without solids (aquifer material, birnessite, glass beads). For screening purposes, we quantified PFAS concentrations in-house using ASTM D7979 and D7968. To validate our results, end-point samples were sent to Eurofins Lancaster Environmental Lab, which is DoD QSM 5.1-accredited.

For screening purposes, we found that high PFAS concentrations (100 ppb – 1 ppm) can be accurately determined using LC-MS. External calibration curves have been built for PFOA in water phase and sludge, with $R^2 = 0.999$ and 0.998 respectively, using an Agilent 1200 liquid chromatography system with a Bruker MicroToF mass spectrometer. This system is managed and maintained by the Rice Shared Equipment Authority, which offers access to over 100 advanced research instruments. Blanks are run every 3 - 4 samples to ensure column cleanliness over the sample sequence. Standards are analyzed every new day of use to construct a calibration curve. Interferences are mainly the chemicals and salts from the original medium, resulting in different retention time between standard samples and experimental samples, which should be eliminated by preparing them in the same media.

In order to identify metabolites, or achieve a lower detection limit (low ppb or ppt concentrations), an LC-MS/MS system is necessary. Our lab has successfully quantified PFOA and analyzed metabolites after advanced oxidative treatments using LC-MS/MS. The documented detection limits for PFOA and PFOS were 1.7 and 2.2 ng/L respectively, based on the standard. Two LC-MS/MS systems are managed by the Rice Shared Equipment Authority: a Shimadzu IT-ToF LC-MS System and a Thermo LTQ-Orbitrap.

While 8:2 FTOH is an important precursor for PFASs⁸⁶, it is not in the analyte list of ASTM standards, so methods for sample collection, preparation and analysis are based on recently published literature. In order to assess the degradative capacity of superoxide-producing bacterial strains, 8:2 FTOH was spiked into the bacterial culture medium. Glass serum bottles or polypropylene tubes are used, capped, and sealed with sealing film to avoid the loss of semi-volatile 8:2 FTOH²⁸. Triplicate containers and biotic/abiotic controls ensure the validity and accuracy of results. After centrifugation, supernatants are cleaned up by solid-phase extraction (SPE) and dispersive carbon sorbent, and loaded into LC-MS or LC-MS/MS for the analysis of 8:2 FTOH and its metabolites. Methanol extraction is performed on the pellet, and the extract will be purified by dispersive carbon sorbent as well prior to analysis.

Quality Assurance/Quality Control

General. The responsibility for quality assurance and quality control (QA/QC) lies primarily with the principal investigators and research scientists. The primary components of the lab's QA/QC plan involve trained analysts, reliable instrumentation, appropriate use of analytical methodologies, and external validation of data. Training of analysts is generally on a one-on-one basis, and is carried out by Prof. Alvarez, postdoctoral researchers, and doctoral students. The instrumentation in these labs is all relatively new and of high quality. Appropriate use of analytical methodologies is ensured by constant literature review and contact with other researchers in the field. Prof. Alvarez oversees instrument maintenance and provides assistance in selection of analytical techniques. Quality assurance for research carried out using the Rice University's Core facilities will be carried out in consultation with the staff of those facilities. As part of the safety program, Rice University requires regular thorough inspections by the Building's Safety Officer and Rice's Environmental Health & Safety Department (EHSD). Protective equipment and waste disposal procedures follow Rice's EHSD's policy, which is based on both Federal safety guidelines and the Texas Department of Health. Worker safety in the laboratory is a primary concern of the Environmental Engineering program.

Chemicals and Instrumentation. A Milli-Q water system is available in the laboratory to provide high quality deionized water when necessary. Reagents and solvents used are of ACS reagent grade quality or better. When appropriate, published EPA analytical methodologies are used. Logbooks are maintained for each instrument, including maintenance records. A detailed QA/QC plan, based on EPA guidelines, can be provided upon request.

Sample Handling. Samples requiring refrigeration are stored at 4°C in refrigerators that are monitored weekly for temperature fluctuations. Samples requiring freezing are stored at -20°C or -80°C. Samples for microbial analyses are handled with extreme care using sterilized tools in a laminar flow hood to prevent contamination. Standard safety procedures are followed, and all samples containing bacteria are autoclaved twice before disposal.

Analytical Methods. For each analyte of interest, calibration standards at a minimum of five concentrations by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with deionized water were used. One standard was at a concentration near, but above, the method detection limit. The other standards will correspond to the range of concentrations found in laboratory samples with appropriate dilutions made to conform to detector range and linearity. Samples, blanks and standards were analyzed using identical techniques to compensate

for extraction efficiency. Standard curves were generated daily before each analysis batch and responses will be calculated using a linear least-squares fit technique. Continuing calibration verification were performed after every 10 injections using a midrange standard and deemed acceptable if the percent difference is less than 15% from the expected value.

Precision, Accuracy, and Completeness. The reproducibility of quantification techniques are determined periodically by comparison of triplicate independent of standard samples. For analytical methods, acceptable levels of precision are < 10% relative standard deviation (RSD). Biomass estimates, such as absorbance or plate counts, are inherently more variable and target RSD for these measures is < 20%. The RSD is calculated as $RSD = (s/y_m) * 100\%$, where RSD = relative standard deviation, s = standard deviation, and y_m = mean of replicate analyses. The standard deviation, s , is defined as $s = \text{SQRT}(\text{sum of } I = 1 \text{ to } n (y_i - y_m)^2 / (n-1))$, where S = standard deviation, y = measured value of the i th replicate, y_m = mean of replicate measurements, and n = number of replicates.

The accuracy of analysis is determined by spiking triplicate samples with approved standard solutions used for instrument calibration. This process will be repeated periodically to ensure data quality in different experimental phases of the research. For measurements where matrix spikes are used, accuracy is defined as the percent recovery (R). $\%R = 100\% - (s-u)/c_{sa}$, where s = measured concentration in spike aliquot, u = measured concentration in unspiked aliquot, and c_{sa} = actual concentration of spike added. For measurements where standard reference materials are used instead or in addition to a matrix spike, the accuracy is expressed as $R\% = 100\% * c_m/c_{srn}$, where c_m = measured concentration of standard reference material (SRM), and c_{srn} = actual concentration of SRM. Duplicate or triplicate analyses were performed (except for those parameters measured real time) and reported mean $\pm$ the standard deviation with a 90% degree of confidence.

Data Reduction and Reporting. All data is contained in a centralized notebook. A large portion of the data was also maintained electronically in the form of spreadsheets and will be backed up weekly and stored in various locations. Computer records are backed-up weekly using portable backup devices (e.g., flash drives) with copies stored at the laboratory and at another location. All statistical analyses are performed using standard methodologies. Unless specifically reported otherwise, all hypothesis testing (comparison of means and variances, comparison of least-squares estimates for model coefficients, determining zero and non-zero coefficients, etc.) is done using a significance level of 5%. Values from triplicate samples will be averaged. Sample tracking, beginning with a transfer of data from log books to a summary form indicating sample status, ensures the timely analysis of each sample and the completeness of the data set. Soon after collection, data is arranged in tabular and graphic formats. These data are reviewed to evaluate a) consistency and quality of the data set, b) remedial actions required in the sampling or analytical procedures, and c) the possible need for modification of the experimental program. Records of all raw analytical data, instrument maintenance, sample records and laboratory notebooks will be maintained for future reference. The quality of all measurement data generated and processed is assessed for precision, accuracy, completeness, and comparability. Most analysis involved in this project can be performed using EPA-recommended procedures. Multiple samples from replicate tests are analyzed to establish the precision. The accuracy of the data is established using EPA-

improved procedures. The data are also compared to literature data to determine the accuracy and representativeness of the data.

OBJECTIVE 2

Bacterial strains and isolation. *Pseudomonas putida* GB-1, a model manganese-oxidizing and superoxide-producing bacteria, was kindly provided by Dr. Bradley Tebo (UCSD). Soil bacteria were isolated by suspending 1 g soil in 10 mL phosphate-buffered saline (PBS) and gently shaking for 10 mins. Ten-fold serial dilutions of the soil suspension were prepared and 100 μ L was streaked onto the surface of Leptothrix (Lept) plates (1.5% agar). Lept medium was prepared by autoclaving 1 L deionized water containing 0.48 mM CaCl_2 , 0.83 mM MgSO_4 , and 0.5 g yeast extract. 15 g agar was added when medium was prepared for plates. After autoclaving, the following filter sterilized solutions were added: 5 mL 1 M glucose (5 mM final concentration), 500 μ L 1 g/mL casamino acid solution (0.5 g/L final concentration), 10 mL 1 M HEPES buffer (pH 7.5, 10 mM final concentration), 1 mL trace elements solution, 0.1 mL 1 M MnCl_2 (100 μ M final concentration), and 0.37 mL freshly prepared 10 mM FeCl_3 (3.7 μ M final concentration). Lept trace elements included 10 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 44 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 13 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ per 1 L deionized water. Streaked plates were maintained at room temperature for at least three days and up to two weeks.

Manganese oxidation and superoxide production assays. Biogenic, extracellular superoxide production is mediated by either multi-copper oxidases or animal heme peroxidases loosely attached to bacterial cell surfaces, and is involved in the formation of manganese oxides. Thus, superoxide production can be indirectly observed as brown manganese oxide precipitate that forms on bacterial colonies when grown on low-nutrient media containing Mn(II). To verify manganese oxidation, a solution of 0.04% Leucoberberlin Blue I (LBB) in 45 mM acetic acid was spotted directly onto bacterial colonies, and the development of a bright blue color was used to indicate the presence of Mn(III/IV+) oxides. Bacterial colonies that tested positive by LBB spot assay were then streaked purified on Lept media, and single colonies were transferred into nutrient broth (NB), grown overnight, and preserved as 10% glycerol stocks. To quantify manganese oxidation, 0.1 mL culture samples were added to 0.5 mL of 0.04% LBB and the mixture was incubated for 10 mins in the dark at room temperature. The reaction was filtered using a 0.22 μ m PES syringe filter, and the absorbance was measured at 620 nm. Standard curves were prepared with KMnO_4 (with 40 μ M KMnO_4 being equivalent to 100 μ M MnO_2) and blanks were prepared using Lept media without bacteria.

Bacterial identification. Isolates were identified using their 16S rRNA gene sequences. Individual colonies were subjected to colony PCR (cPCR) after being streak purified three times. A pipet tip was used to transfer a small portion of each colony into a 25 μ L PCR reaction containing 12.5 μ L Kapa HiFi Ready Mix, 2 μ L 16S primer mix (27F and 1492R universal bacterial primers), and 10.5 μ L molecular grade H_2O . The thermocycler program was as follows: 3 mins at 95°C followed by 30 cycles of a 30 sec denaturation step at 95°C, a 15 sec 53°C annealing step, and a 30 sec 72°C extension step. A final extension step was performed for 5 mins at 72°C. PCR products were then separated by gel electrophoresis using a 0.7% agarose gel containing SYBR Safe stain. PCR amplicons of the appropriate size were then subjected to gel extraction using an Omega Bio-tek E.Z.N.A. Gel Extraction Kit according to the manufacturer's instructions. Sanger sequencing was

then performed on each fragment (Genewiz, New Jersey), and DNA sequences were analyzed using the NCBI BLAST algorithm (nr database).

OBJECTIVE 3

Potassium Superoxide Experiments with TCE. A similar procedure to that used above for PFAS was utilized for the TCE experiments, although working in gas-tight conditions. KO_2 powder was added to DMSO, followed by 18-crown-6 to facilitate dissolution, at a ratio of KO_2 :18-crown-6 of 1:2.5, with a stock solution of 200 mM. This was covered in parafilm and stirred for at least 15 min. A 20 mL serum vial was filled with DMSO and a stir bar and sealed with a PTFE/butyl septum and a 20 mm aluminum crimp seal. TCE was injected using a gas-tight syringe and the vials were allowed to equilibrate for at least 30 minutes. Then, the stock solution of KO_2 was injected through the septa into the vials to yield a range of KO_2 concentrations from 25 μM to 30 mM. These solutions were stirred at room temperature for one day at 600 rpm. After this time, an aliquot of solution was removed and quenched with DI water at a ratio of 10:1 water: DMSO, and the solution was used for IC analysis of chloride. The IC analysis instrument and procedure was the same as the one described above.

WO_x/ZrO_2 Catalyst Experiments. A similar procedure to that used above for PFAS was utilized for the TCE experiments, although working in gas-tight conditions. A 70 mL serum vial was filled (without headspace) with DI water, 2,000 mg/L WO_x/ZrO_2 , and a stir bar, and sealed with a PTFE/butyl septum and a 20 mm aluminum crimp seal. Neat TCE was injected using a gas-tight syringe for a final concentration of 200 $\mu\text{g/L}$ and the vials were allowed to equilibrate for at least 60 minutes. Then stock 30% H_2O_2 was injected for a final concentration of 0.1 M, and the solutions were allowed to stir at room temperature for one day at 600 rpm. Control reactions without WO_x/ZrO_2 of H_2O_2 were also set up.

After the one-day reaction time, solutions were transferred to 40 mL vials and sent for external analysis by Eurofins Lancaster Laboratories Env, LLC using EPA Method 8260C on a GC-MS instrument using purge-and-trap.

Enzyme assays. Enzymes tested for their capacity to degrade PFOA or PFOS included xanthine oxidase (XO), horseradish peroxidase (HRP), and laccase (LAC). All enzymes were purchased from Sigma-Aldrich. Enzyme reactions were optimized in a 100 μL volume in 96-well plates containing phosphate buffer (pH 7), and were monitored over 12 hours. XO reactions were optimized by varying the concentrations of XO (0 to 0.15 U/mL) and hypoxanthine (0 to 100 μM) and assessing reduction of WST-1 (100 μM) at 440 nm. HRP reactions (0 – 0.18 U/mL) were optimized by monitoring ABTS (100 μM) oxidation at 405 nm at various H_2O_2 concentrations (0 – 1 mM). Laccase reactions (0 – 10 U/mL) were optimized based on the oxidation of 1-HBT (100 μM) while monitoring at 408 nm. Mn(II) oxidation was assessed in 10 mL volumes using optimized XO conditions without WST-1 and with 100 μM MnCl_2 . Samples were taken after 24 hours and analyzed by LBB assay, as described above. PFAS degradation was assessed using optimized enzyme conditions in 10 mL volumes with the addition of 0.5 μM PFOA and 0.5 μM PFOS. All reactions were maintained at room temperature in the dark with gentle shaking (120 rpm). XO reactions contained MnCl_2 , while HRP reactions using 4-methoxyphenol as the mediator instead of ABTS. A second set of laccase/1-HBT reactions was prepared containing 0.2% Tween 20. All enzyme reactions containing PFAS were respiked with both enzyme and the substrate or

mediator every day for five days. Reactions were allowed to incubate for an additional two days before being sent for external PFAS analysis (Eurofins Lancaster Labs).

RESULTS AND DISCUSSION

OBJECTIVE 1

Three Superoxide-Producing Systems

Superoxide was claimed to degrade PFOA in a CHP system¹⁸, and various other studies have implied that superoxide plays a role in degrading PFOA.^{19–21} In theory, superoxide could degrade PFOA via two reaction pathways (Figure 1e): (a) nucleophilic attack of the C-F bond, as postulated for degradation of CCL₄ by superoxide;^{70,87} or (b) step-wise PFOA degradation via a superoxide-mediated decarboxylation. However, other studies argue that superoxide is ineffective in attacking PFOA.^{22,23} Therefore, it is important to resolve this controversy and complete the first objective of this proposal to show if and how superoxide degrades PFOA.

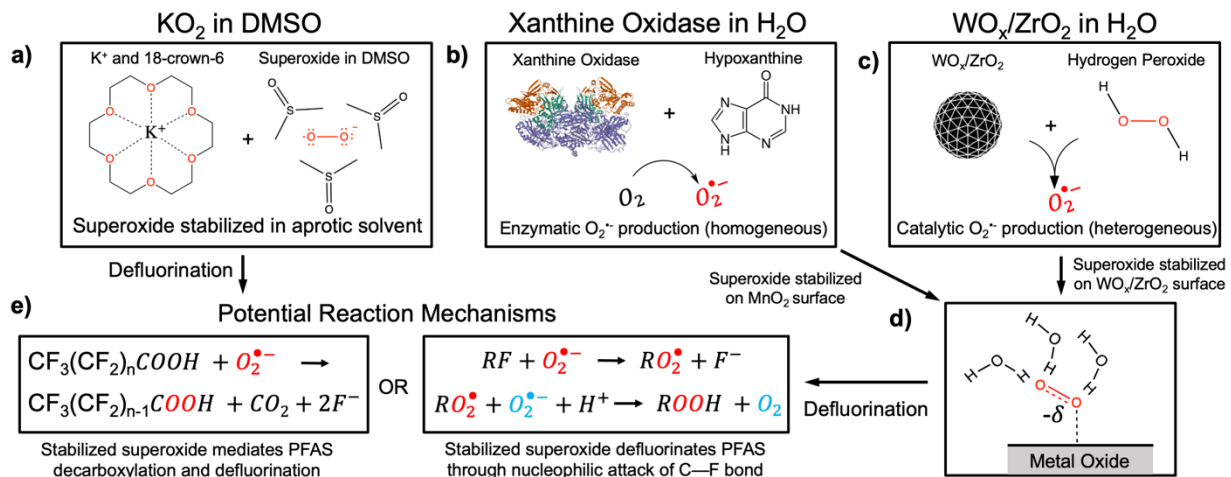


Figure 1. Hypothetical PFAS defluorination approaches using superoxide-stabilizing systems. Superoxide is strongly solvated by protic solvents (e.g. water), in the absence of a stabilizer, limiting its reactivity and facilitating rapid disproportionation. We hypothesize that superoxide-stabilizing systems, both homogeneous and heterogeneous, could enhance superoxide reactivity. A) KO₂ produces stabilized superoxide radicals in the aprotic solvent DMSO in the presence of 18-crown-6, which facilitates dissolution. B) Xanthine Oxidase reacts with hypoxanthine to generate superoxide from dissolved oxygen in the aqueous solution. Adding MnO₂ to the solution stabilizes the radical on the metal oxide surface. C) The WO_x/ZrO₂ catalyst reacts with H₂O₂ to form superoxide heterogeneously in aqueous solution. D) Superoxide is hypothesized to be stabilized on the metal oxide surface (MnO₂ for the enzyme system, WO_x/ZrO₂ for the catalyst system) in aqueous solution. E) Stabilized superoxide may defluorinate PFOA via two mechanisms: attack of the carboxyl group, decarboxylating and releasing F⁻. or nucleophilic attack of the C—F bond, releasing F⁻ and resulting in an alkyl hydroperoxide moiety.

We isolated superoxide production from other ROS to advance mechanistic understanding of its reaction with PFOA. Superoxide undergoes nucleophilic reactions in aprotic solvent, but its reactivity in water is diminished by strong solvation and rapid disproportionation.^{17,88} However, superoxide can be stabilized on the surfaces of metal oxides (e.g. birnessite) and by the addition

of low-polarity solvents.^{58,70} Here, we considered three systems that produce stabilized superoxide (Figure 1): (1) KO₂ dissolved in aprotic solvent, which facilitates superoxide-mediated nucleophilic dehalogenation^{89,90} (Figure 1a); (2) Xanthine Oxidase (XO) and hypoxanthine (HX) enzymatic system, which is well-known to produce superoxide,⁹¹ amended with MnO₂ for surface stabilization of superoxide (Figure 1b); and (3) WO_x/ZrO₂ + H₂O₂, which was recently demonstrated to degrade 1,4-dioxane via stabilized superoxide produced by H₂O₂ decomposition (Figure 1c).⁹²

In particular, the xanthine oxidase/hypoxanthine system is a well-established biological means for generating O₂^{•-}. This was used to assess the ability of biologically-produced superoxide to degrade PFAS using hypoxanthine as a substrate (Figure 2)⁹³. One unit of xanthine oxidase converts 1 μmole of xanthine to uric acid per min at pH 7.5 at 25 °C, and about 50% of this activity is obtained with hypoxanthine. Using 1 mg/L xanthine oxidase (~7 U) would generate 3.5 μM superoxide/min with hypoxanthine as the substrate, which is approximately 15 – 20-fold slower than the theoretical rate achieved via MFR/CHP as previously reported¹⁸. Other amendments found to enhance superoxide reactivity (e.g., birnessite) were added to reactions. Batch reactions (10 mL) were prepared in polypropylene vials using model PFASs and precursors (e.g., PFOA, PFOS, 8:2 FTOH) in phosphate buffer (pH 7).

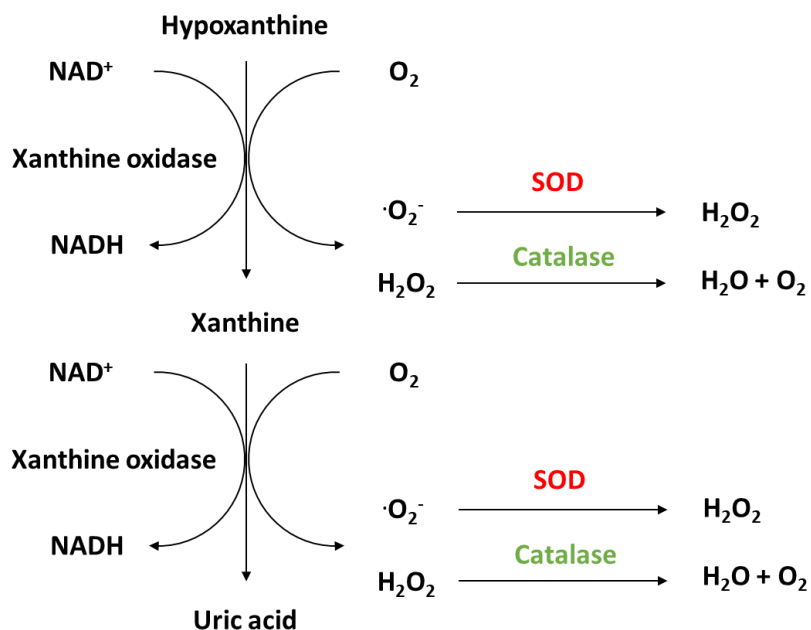


Figure 2. Generation of superoxide (O₂^{•-}) from hypoxanthine using xanthine oxidase. Superoxide dismutase (SOD) is used to verify PFAS degradation is due to superoxide. Catalase is added to verify PFAS degradation is not due to H₂O₂.

Verifying Superoxide Production in All Three Systems

We used nitroblue tetrazolium (NBT) precipitation and electron paramagnetic resonance (EPR) spectroscopy to corroborate superoxide generation in the three systems. NBT (350 mg/L) was added to all three superoxide-producing systems, as well as controls. The active reactions reduced

NBT to NBT-formazan in all three systems, creating a purple colored precipitate. This precipitate was not formed in the control reactions, leaving the solutions yellow in color. The precipitate was redissolved in a mixture of DMSO and water and its extinction peak was measured using UV-Vis spectroscopy, as shown by the 526-nm peak in Figure 3a. Additionally, EPR spectroscopy corroborated superoxide production. KO_2 produces high concentrations of stabilized superoxide in aprotic solvents such as DMSO. Here, the 30 mM KO_2 DMSO solution produced 358 μM superoxide at the time of measurement, which was determined by EPR using the freeze-trap method (Figure 3b).⁹⁴ Superoxide production in the remaining systems was confirmed by spin-trapping the radicals with BMPO, a common method to measure lower concentrations of superoxide at room temperature⁹⁵. Significantly less superoxide was trapped for the enzymatic system with a different and less common BMPO-superoxide adduct conformation (Figure 3c). This conformation is also characteristic of superoxide and has been previously reported by others.^{95–97} The WO_x/ZrO_2 catalyst system produced a spin-trapped BMPO radical (Figure 3d). The line shape obtained is characteristic of a BMPO-superoxide adduct, with all four peaks at approximately the same height.⁹⁸ This spectrum indicated that the BMPO-trapped radical was primarily superoxide.

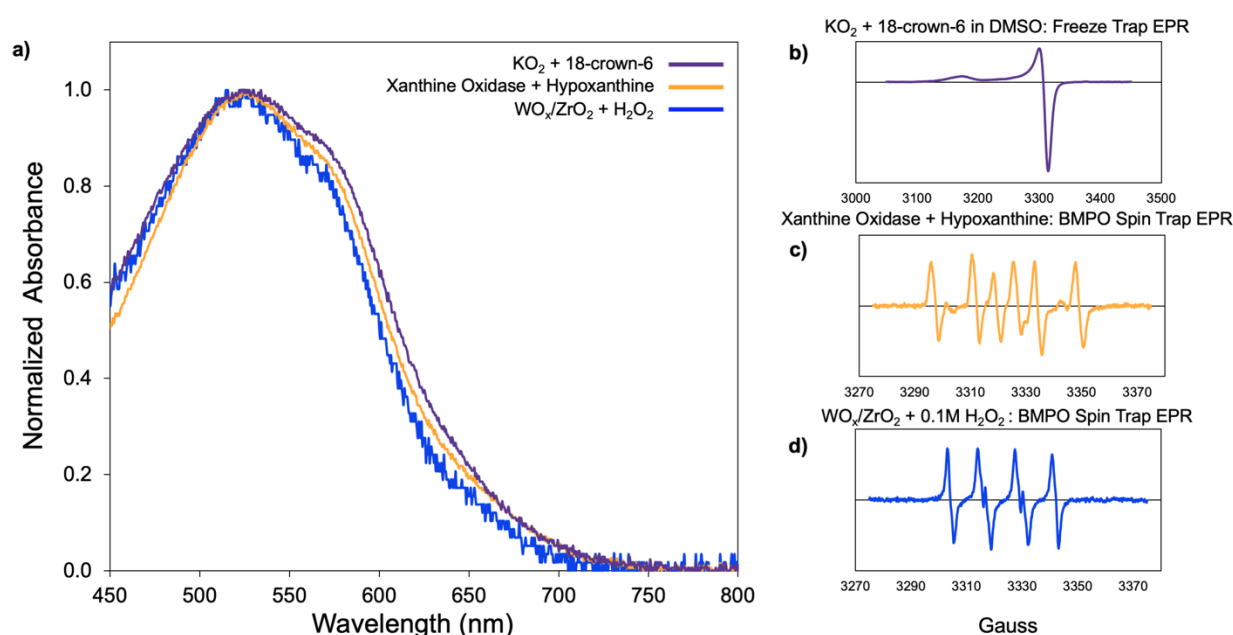


Figure 3. Superoxide production by the three tested systems. a) UV-Vis spectra of the reduced NBT product (NBT-formazan) after reacting with superoxide. Insoluble NBT-formazan precipitates were dissolved in DMSO/ H_2O solution. Spectra were baseline-corrected (by subtracting the spectrum of NBT solution without superoxide) and normalized to the highest absorption value. b) EPR spectrum (115 – 120K) of 30 mM KO_2 in DMSO with 75 mM 18-crown-6 stock diluted 1:10 in 50 mM NaOH after freeze trapping in liquid nitrogen. c) EPR spectrum of superoxide produced by xanthine oxidase and hypoxanthine after spin-trapping with BMPO. This is the less common BMPO-adduct conformation. d) EPR spectrum indicative of the superoxide produced by the WO_x/ZrO_2 catalyst with 0.1M H_2O_2 , after spin-trapping with BMPO.

Superoxide Degrades BPA (Positive Control)

We first demonstrated (as positive control) the efficacy of superoxide to degrade bisphenol A (BPA), a known endocrine disruptor, as superoxide has been shown to degrade BPA.⁹⁹ We tested

the three superoxide-producing systems with BPA and analyzed the compound using a HPLC with an UV–Vis photodiode array detector. About 48% BPA (initially 20 mg/L) was degraded in the KO_2 system (30 mM KO_2 and 75 mM 18-crown-6 in DMSO) after one-day reaction (Figure 4a), but no degradation occurred in the absence of KO_2 . A similar BPA removal efficiency was observed in the WO_x/ZrO_2 catalytic system (2,000 mg/L catalyst + 0.1M H_2O_2) (Figure 4b). Partial BPA removal in the control containing only WO_x/ZrO_2 was likely due to adsorption on the catalyst surface. These results corroborate that superoxide was produced and was sufficiently stable to react, both in aprotic solvent (DMSO) and in deionized water with a metal oxide surface (WO_x/ZrO_2). BPA degradation in the enzymatic system could not be accurately quantified due to direct transformation of BPA by MnO_2 , which is known to react with benzylic alcohols (Figure 5).¹⁰⁰

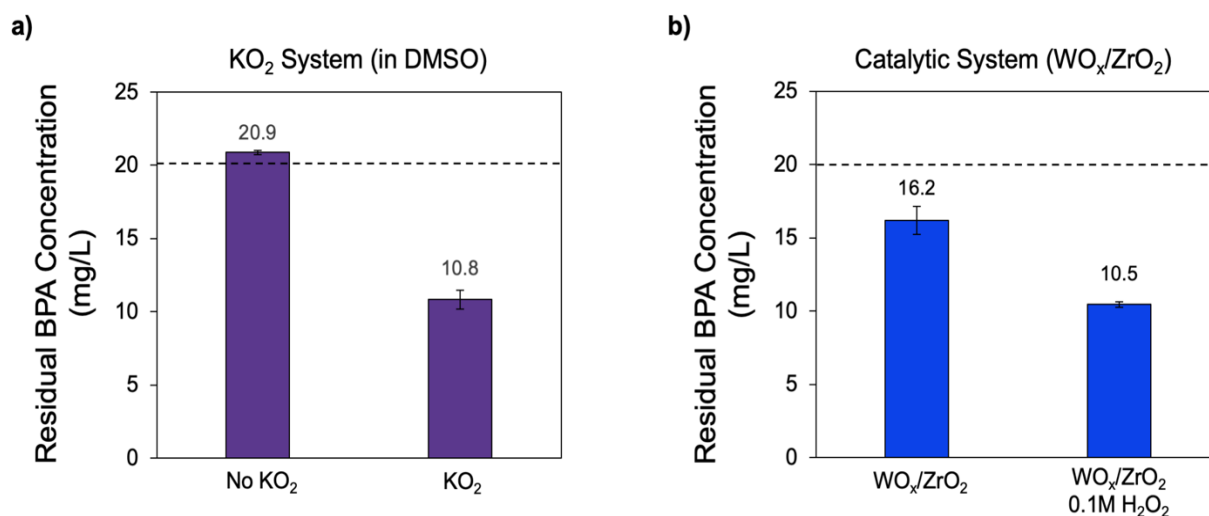


Figure 4. BPA degradation (positive control) in the KO_2 and catalytic systems. a) 48% BPA (20 mg/L initial) was degraded in the KO_2 system (30 mM KO_2 , 75 mM 18-crown-6) during a one-day reaction compared to the control (75 mM 18-crown-6). b) 48% BPA (20 mg/L initial) was removed by the WO_x/ZrO_2 system (2000 mg/L WO_x/ZrO_2 , 0.1M H_2O_2), while only 19% was removed in the control (2000 mg/L WO_x/ZrO_2) during a one-day reaction.

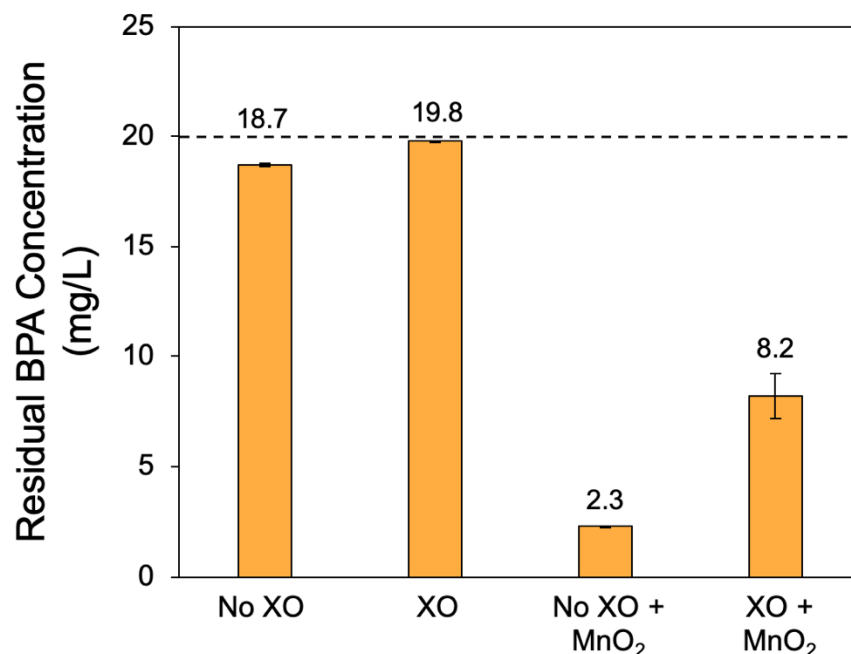


Figure 5: No BPA (20 mg/L) degradation was observed after a one-day reaction in the enzymatic system (0.25 units/mL xanthine oxidase (XO), 0.5 mM hypoxanthine, pH 7.4) compared to the control (0.5 mM hypoxanthine, pH 7.4). The effect of stabilizing superoxide on MnO₂ (1000 mg/L) surface for BPA degradation could not be evaluated due to direct transformation of BPA by MnO₂ which confounded the outcome of the experiment.

Superoxide Does Not Degrade PFOA

Finally, we investigated the degradation of PFOA by superoxide. Though numerous studies have explored PFOA degradation mechanisms in AOP and ARP,^{2,101–106} the role of superoxide remains unclear. Here, we used three well-defined superoxide-producing systems to isolate its efficacy to degrade PFOA at different initial concentrations (0.5 to 50 mg/L). In all cases, no PFOA degradation was observed (Figure 6), no degradation byproducts were detected (including C3 – C7 shorter-chain perfluoroalkyl acids, which are common PFOA degradation byproducts), and no significant fluoride release was detected compared to controls (Table 1). A wide range of KO₂ concentrations was used to ensure sufficient (but not excessive) superoxide concentrations and avoid significant self-dismutation.¹⁰⁷ However, no statistically significant difference between initial and final concentrations was found after accounting for ~8% LC-MS system variability.

The enzyme system produced relatively low levels of superoxide, so a smaller amount of PFOA, 0.5 mg/L, was used to ensure sufficient radical-contaminant interactions. Figure 6b shows the two sets of reactions. All reactions were run in a buffered pH 7.4 solution with 0.5 mM hypoxanthine, and those with enzyme had 0.25 units/mL xanthine oxidase. The second set was run with superoxide-stabilizing MnO₂, which adsorbed some PFOA.

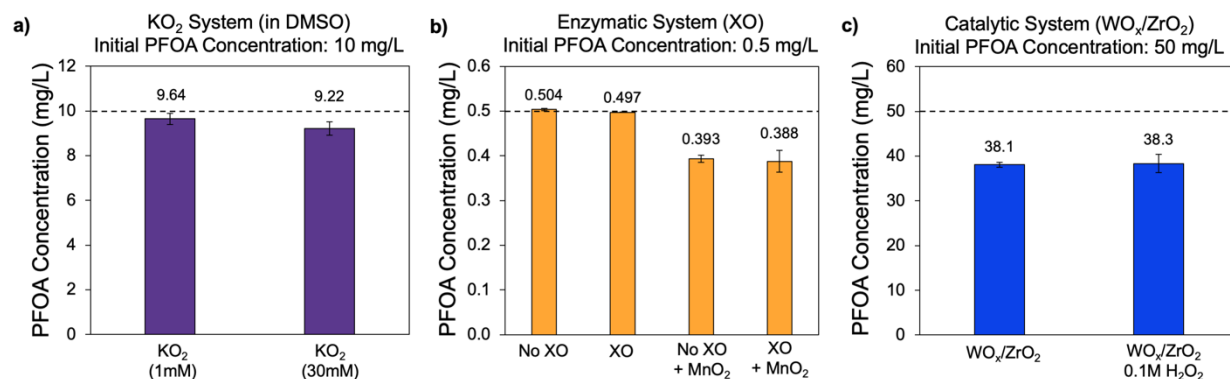


Figure 6. No significant PFOA degradation was detected in any of the reaction systems. a) No PFOA (10 mg/L) degradation was observed after a one-day reaction in KO_2 /18-crown-6/DMSO solution (1mM KO_2 and 30mM KO_2). This was corroborated by a lack of degradation byproducts and no detectable F^- release. b) No PFOA (0.5 mg/L) degradation was observed after a three-day reaction in the enzymatic system (0.25 units/mL xanthine oxidase (XO), 0.5 mM hypoxanthine, pH 7.4) compared to the control (0.5 mM hypoxanthine, pH 7.4). The addition of MnO_2 (1000 mg/L) as a superoxide stabilizing agent did not enhance PFOA degradation compared to the control. No PFOA degradation byproducts or F^- were detected. c) No PFOA (50 mg/L) degradation was observed after a two-day reaction in the catalytic system (2000 mg/L WO_x/ZrO_2 and 0.1M H_2O_2). No PFOA degradation byproducts or F^- were detected.

The catalyst system was tested using 50 mg/L PFOA, one hundred times higher than that of the enzymatic system. A high PFOA concentration was required to get significant recovery, to overcome adsorption to the catalyst surface and loss during the extraction process. A control study with just PFOA and water yielded 89% PFOA recovery (a loss of 5 mg/L) after extraction with NaOH (pH 12) and subsequent filtration. Figure 6c shows that the system with 2,000 mg/L WO_x/ZrO_2 catalyst and 0.1M H_2O_2 yielded no significant PFOA degradation compared to the control without H_2O_2 , which is required for superoxide production.

While some publications infer that superoxide is involved in PFOA degradation,^{18,19,21} these studies involved more complex systems with confounding factors that preclude isolating the role of a single ROS. We recognize that our data does not rule out the possibility of superoxide participating in PFOA degradation in some complex systems that activate PFOA (e.g., via heterogeneous catalysis) or promote concerted degradation via multiple ROS and reaction pathways. Nevertheless, our reductionist experiments favored high concentrations of stabilized superoxide that enhance degradation of dissolved contaminants, and we eliminated confounding factors to prove that superoxide alone plays no significant role in PFOA degradation. Previous studies in our lab took a similar reductionist approach to conclusively show that hydroxyl radical alone also cannot degrade PFOA.² These two major ROS species are unable to degrade and defluorinate PFOA on their own. This finding has important implications for the selection, design, and optimization of remediation strategies to degrade PFOA, and to avoid sub-optimal allocation of treatment resources.

Table 1: F⁻ recovery by IC and PFAS byproduct detection in LC-MS.

Reaction	F ⁻ conc (mg/L) [†]	PFOA byproducts detected C3 – C7 ^{††}
1 mM KO ₂ , 2.5 mM 18-crown-6, PFOA 10 mg/L	None detected	None detected
1 mM KO ₂ , 2.5 mM 18-crown-6, PFOA 100 mg/L	None detected	None detected
30 mM KO ₂ , 75 mM 18-crown-6, PFOA 10 mg/L	None detected	None detected
0.5 mM Hypoxanthine, 0.5 mg/L PFOA	None detected	None detected
0.25 units/mL Xanthine Oxidase, 0.5 mM Hypoxanthine, 0.5 mg/L PFOA	None detected	None detected
0.5 mM Hypoxanthine, 0.5 mg/L PFOA, 1000 mg/L MnO ₂	None detected	None detected
0.25 units/mL Xanthine Oxidase, 0.5 mM Hypoxanthine, 0.5 mg/L PFOA, 1000 mg/L MnO ₂	None detected	None detected
2000 mg/L WO _x /ZrO ₂ , 50 mg/L PFOA	0.357 ± 0.106 *	None detected
2000 mg/L WO _x /ZrO ₂ , 0.1 M H ₂ O ₂ , 50 mg/L PFOA	0.352 ± 0.059 *	None detected
2000 mg/L WO _x /ZrO ₂ , 50 mg/L PFOA, 5 mg/L spiked F ⁻	4.652 ± 0.124 **	None detected

[†] IC limit of detection for F⁻ < 0.01 mg/L

^{††} LC-MS limit of detection for PFOA and C3 – C7 byproducts < 0.01 mg/L

* No statistically significant difference (p > 0.05). The observed F⁻ likely leached from the catalyst.

** Corresponds to 86% F⁻ recovery after accounting for background F⁻ in the WO_x/ZrO₂ system.

OBJECTIVE 2

Implementing a bacterially-mediated subsurface superoxide hyper-production strategy for PFAS remediation would require the use of heterotrophic bacteria. Though few stationary phase heterotrophic bacteria have been assessed for superoxide production, the highest reported average rate is 14.2 amol cell⁻¹ h⁻¹ for *Pseudomonas putida* GB-1.⁵⁵ While this is two orders of magnitude lower than that reported for some microalgae, these rates are comparable when normalized to cell surface area. Nevertheless, it is important to screen a broader range of bacteria and also to understand how these rates can be enhanced so that complete PFAS biodegradation and defluorination might be achieved. Understanding the physiological limits of superoxide production within different bacterial species, as well as modulating factors, is important for assessing their potential for various bioremediation strategies, including natural attenuation, biostimulation, and bioaugmentation. Moreover, advancing understanding of the molecular basis of superoxide production could lead to the development of biomarkers to assess this potential natural attenuation mechanism, and would facilitate development of synthetic biology strategies that enhance superoxide production.

Development of an assay for identifying and characterizing superoxide-producing bacteria.

Superoxide can be quantified using various methods, which can be broadly grouped as either electrochemical, UV/Vis spectroscopy, luminescent, or vibrational spectroscopy. UV/Vis and luminescence are most commonly used for analyses conducted within biological systems. For enzymatic assays, we utilized a well-established colorimetric assay based on the reduction of tetrazolium by superoxide to form the deep-blue diformazan product. However, this assay is not compatible with selectively assessing superoxide production in the presence of viable bacterial cells. Instead, as recent studies have demonstrated that microbially-produced superoxide is responsible for manganese oxidation, we utilized a colorimetric assay that relies on the reaction of manganese oxides (Mn(III+)) with LBB to form a deep-blue product. Thus, superoxide production can be indirectly monitored by oxidation of Mn(II) to form Mn(III+) oxides, which then react with LBB. The presence of Mn(III+) can be visualized by the rapid development of a deep blue color after spotting a 0.04% LBB solution directly onto bacterial colonies on agar plates, or by quantifying the increase in absorbance at 620 nm in cultures.

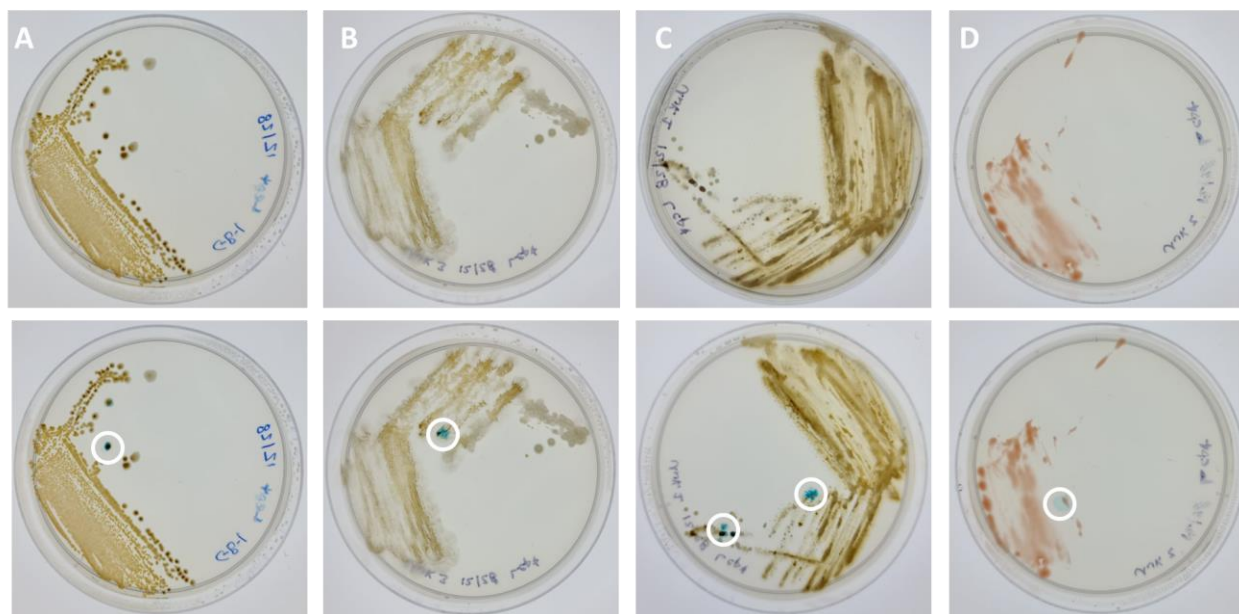


Figure 7. LBB spot assays of bacterial isolates. The top panel is the plate prior to the assay and the bottom panel is the same plate within ten minutes of spotting a 0.04% LBB solution onto one or two colonies (white circles). **A.** *Pseudomonas putida* GB-1; **B.** *Pseudomonas* sp. isolated from soil; **C.** *Pseudomonas* sp. isolated from soil; **D.** *Hymenobacter* sp. isolated from soil. The colorimetric change elicited by the *Hymenobacter* sp. isolate was less intense and took longer to develop relative to the *Pseudomonas* isolates.

Isolation and characterization of superoxide-producing bacteria from the environment

The model manganese-oxidizing bacteria *Pseudomonas putida* GB-1 was obtained and used as both a positive control and benchmark for assay development and subsequent studies. Surface soil containing organic litter was used for bacterial isolation as manganese cycling has been demonstrated to be a major factor in the degradation of high molecular weight organics. Putative manganese-oxidizing bacteria were identified by LBB spot tests and then streak purified.

Numerous, distinct manganese oxidizing bacteria were isolated from each sample, suggesting this metabolic capacity is ubiquitous in soil communities (Figure 7). 16S rRNA gene sequencing revealed two of the isolates demonstrating the highest activity were also *Pseudomonas* species. The bias towards *Pseudomonas* may be due to the media used for isolation (Lept). However, another isolate was obtained that produced pink colonies and did not display the brown coloration indicative of the presence of manganese oxides. However, this isolate also tested positive during the LBB assays and was retained for further characterization.

To quantify manganese oxidation, and indirectly superoxide production, single colonies of each isolate were transferred to Lept broth and grown to stationary phase. Each culture was then diluted to normalize OD₆₀₀ and equivalent volumes were diluted 5-fold using a 0.04% LBB solution. After a 10-minute incubation in the dark, cultures were filtered and the supernatant A₆₂₀ measured (Figure 8). There were no statistically significant differences between the three *Pseudomonas* species tested, with all oxidizing approximately 70% of the available Mn(II). The *Hymenobacter* isolate was only able to oxidize approximately half of the Mn(II) over the same period of time.

The ease with which we were able to isolate superoxide-producing bacteria from the environment is supported by prior research demonstrating the widespread prevalence of heterotrophic bacteria capable of extracellular superoxide production. This is relevant for the design of potential bioremediation strategies as the primary reason bioaugmentation fails is a lack of survival of exogenous strains. Isolation and amplification of native strains has been proposed for bioaugmentation, but it is often difficult if not impossible to find suitable strains at a specific contaminated site. However, the ubiquity of superoxide-producing bacteria supports the development of a more generalized remediation approach based on extracellular ROS production. Moreover, a biostimulation strategy designed to induce superoxide production amongst native bacteria might be envisioned and could be broadly implemented. Additionally, extracellular ROS could potentially play a role in natural attenuation, and should be further investigated.

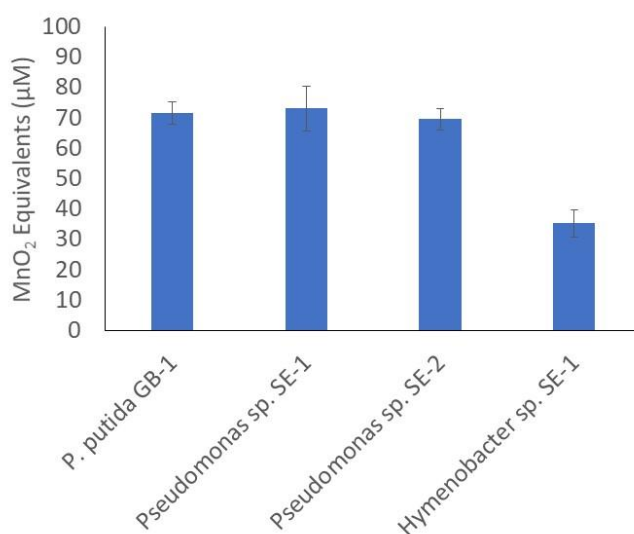


Figure 8. Manganese oxidizing activity of bacterial isolates. Standard curves were prepared using KMnO₄ and converted to MnO₂ equivalents.

OBJECTIVE 3

For Objective 3, since we proved that superoxide alone could not degrade PFAS, we evaluated several other microbial enzymes which had been previously reported in the literature. We tested a laccase-mediator system and a peroxidase-mediator system under various optimized conditions. Parameters optimized included enzyme, mediator, and substrate concentrations (e.g., lower oxidant dosing rates have been shown to be better for degradation of organics, to avoid auto-decomposition¹³). As an example, Figure 9 shows the optimization of a xanthine oxidase-hypoxanthine system. We calculated conditions that would extend reaction times over hours to almost a day versus other studies that used reactions lasting only minutes or even seconds. Longer reaction times were sought to limit the rapid decay kinetics seen when high oxidant concentrations are used, and which are not optimal for contaminant degradation. Additionally, longer reaction times would more likely represent microbial processes occurring in the subsurface.

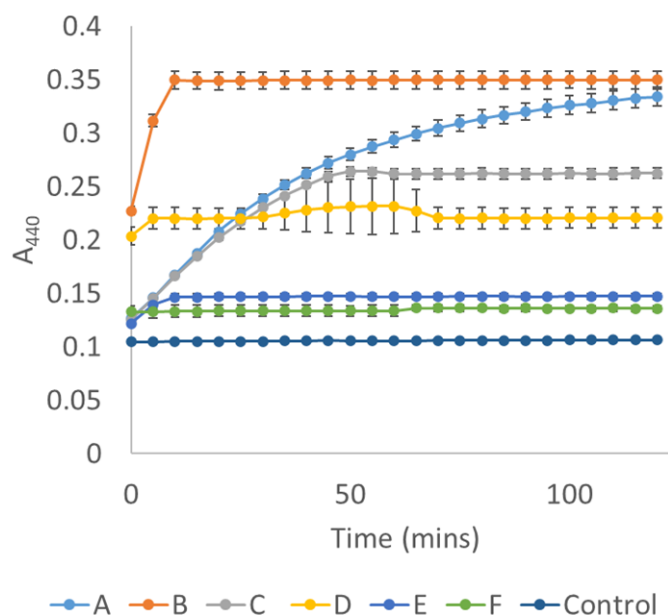


Figure 9. Enzymatic reactions (e.g., xanthine oxidase–hypoxanthine system) were optimized to simulate microbial processes. Conditions tested included (A) 0.0015 U XO/mL and 100 μ M hypoxanthine; (B) 0.015 U XO/mL and 100 μ M hypoxanthine; (C) 0.0015 U XO/mL and 50 μ M hypoxanthine; (D) 0.015 U XO/mL and 50 μ M hypoxanthine; (E) 0.0015 U XO/mL and 10 μ M hypoxanthine; (F) 0.015 U XO/mL and 10 μ M hypoxanthine; and (Control) 0.015 U XO/mL and 0 μ M hypoxanthine. All reactions were prepared in phosphate buffer (pH 7) and analyzed in triplicated.

Our HRP-4MP assay reaction conditions were modified from Colosi et al. (2009) as their mediator concentration was relatively low and they used a very high PFOA concentration⁴¹. Since they report degradation in 6 h, they did not re-spike with enzyme or mediator. However, we optimized our conditions to extend the reaction time and re-spiked both enzyme and mediator daily for 5 days. For the LAC-1HBT system, our conditions included 1/10 the laccase and 50X the mediator relative to that reported by Luo et al. (2015)^{42,44}, to extend the reaction. For laccase, 1 U degrades 1 μ mol substrate/min. Thus, the conditions used by Luo et al. would appear to have run to

completion within seconds, yet they only re-spiked every six days. We did not add Cu^{2+} in our reactions as they did for their PFOS study. They used a mineral salts medium for their PFOA study, which included copper and magnesium.

In summary, no PFOA or PFOS degradation was observed in different enzymatic systems after one week (Figure 10), suggesting the *need to reevaluate previous positive reports of enzymatic degradation*.

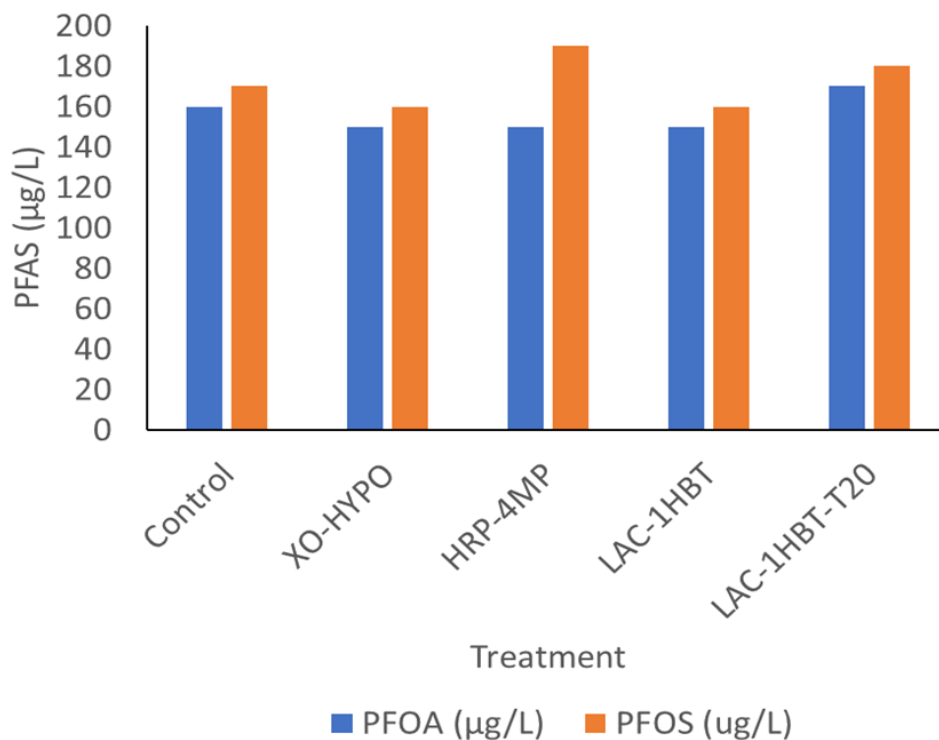


Figure 10. No PFOA or PFOS degradation observed in different enzymatic systems.

The ultimate goal of this project was to demonstrate and characterize PFAS degradation by superoxide, identify superoxide-hyper-producing bacteria, then evaluate the extracellular PFAS biodegradation of those bacteria. However, the results from Objective 1 demonstrated that superoxide alone, stabilized in a variety of aqueous and non-aqueous environments, is not able to degrade PFOA. This resulted in a publication to inform the remediation community of this result.¹ After analyzing these data, we turned to other relevant targets for SERDP and the Department of Defense where superoxide might be generated in situ and productively utilized. We obtained preliminary results that superoxide is effective at degrading trichloroethylene (TCE). The mechanism of degradation is currently underway and requires further investigation.

TCE degradation was analyzed in all three of the superoxide-producing systems identified in Task 1 to determine the viability of TCE degradation by superoxide. TCE was added directly to KO_2 with 18-crown-6 (in a 1: 2.5 molar ratio) in DMSO. Superoxide is stabilized in polar aprotic solvents, so this experiment was to test the effect of high concentrations of stabilized superoxide

derived from the potassium superoxide salt. These experiments were run in triplicate in 20 mL serum vials sealed with PTFE/butyl septa and a 20 mm aluminum crimp seal. TCE was injected through the septum into 20 mL DMSO solution for an initial concentration of 50 μM (6.6 mg/L). The reactions ran for 24 hours, stirring at 600 rpm. Then, aliquots of each solution were taken and quenched in DI water, diluted 10:1 DI: DMSO, then analyzed for chloride release by ion chromatography. A range of KO_2 concentrations were tested with the fixed amount of TCE. Figure 11 shows the IC results. For an initial concentration of 50 μM , the maximum Cl^- concentration for completely degraded TCE was 150 μM . If a single superoxide molecule were required to completely break a C—Cl bond, and if the added concentration of KO_2 accurately reflected the real amount of superoxide present in solution, then 150 μM of KO_2 should completely dechlorinate all 50 μM of TCE. This is not reflected in the data. However, if two superoxide molecules can effectively remove a single chlorine, then 300 μM of KO_2 should completely dechlorinate all 50 μM of TCE. This would reflect two electrons added per C—Cl bond broken, which has been previously reported for CCl_4 .⁸⁹ This is approximately what the data showed, as 300 μM of KO_2 produced 105 μM Cl^- . It is likely that not all of the added superoxide salt was available for reaction, leading to a lower amount of chloride release than predicted. Higher KO_2 concentrations, up to 100x more, yielded approximately 125 μM Cl^- . These data showed that superoxide stabilized in low polarity solvents can effectively dechlorinate TCE.

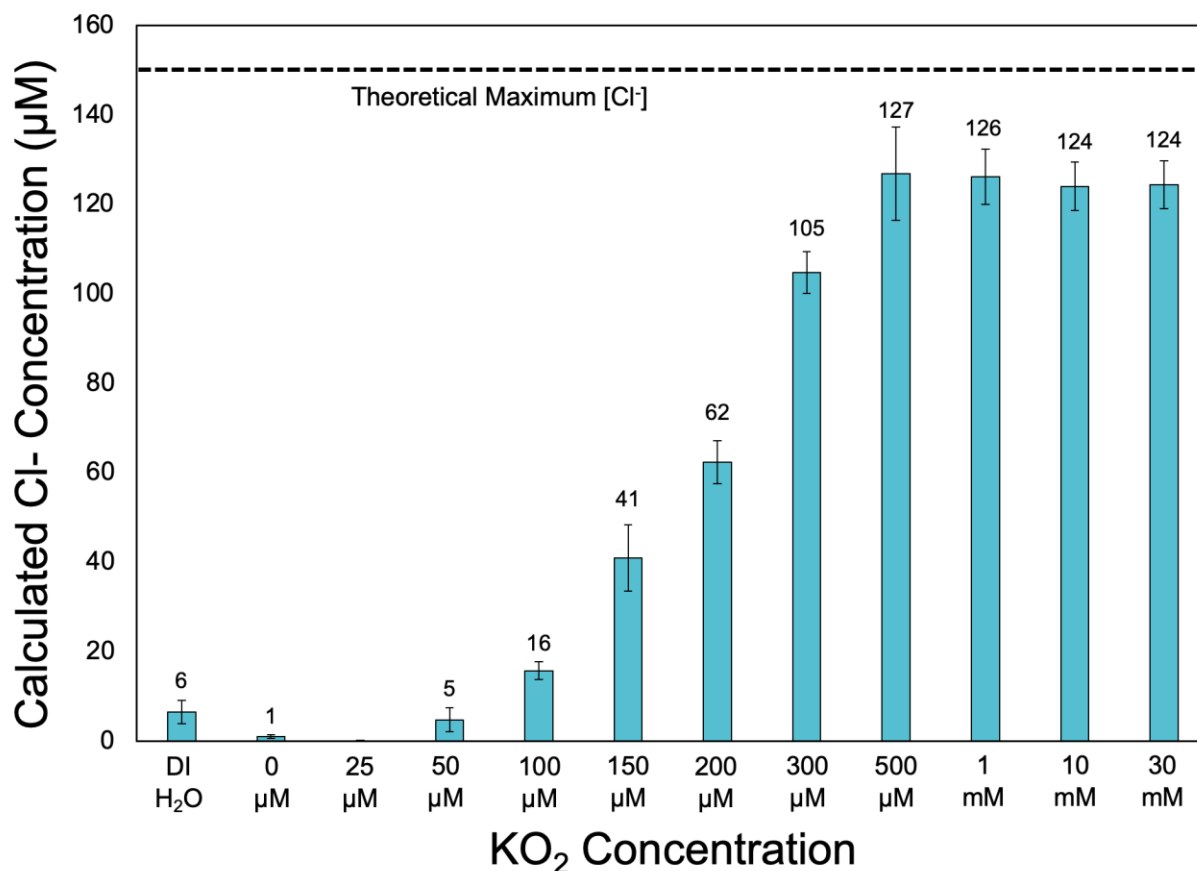


Figure 11. Chloride concentrations after reacting 50 μM TCE with increasing concentrations of KO_2 (and 18-crown-6, in a 1: 2.5 molar ratio) in DMSO. After running for one day, the reactions were quenched in DI water and analyzed for chloride release. The theoretical maximum chloride release was 150 μM .

These data show the potential of TCE degradation from superoxide in polar aprotic solvent, but that is not a realistic environmental system. Further experiments were conducted with TCE in the $\text{WO}_x/\text{ZrO}_2 + \text{H}_2\text{O}_2$ catalyst system (described in Objective 1) to determine the degradation potential of superoxide generated in aqueous systems and stabilized on a metal oxide surface. An environmentally relevant concentration of TCE ($\sim 200 \mu\text{g/L}$) was utilized in this experiment. Serum vials (70 mL) containing 2000 mg/L WO_x/ZrO_2 and/or 0.1 M H_2O_2 in deionized water were reacted with TCE for one day. TCE was analyzed by Eurofins Lancaster Environmental Lab, which is DoD QSM 5.1-accredited, and the results are shown in Figure 12. These data show $\sim 65\%$ TCE removal compared to controls. A wide range of other volatile organic compounds (VOCs) was screened and none were detected in this experiment relative to controls, indicating that degradation byproducts were no longer present after the 24 hour reaction. Experiments are ongoing to isolate the degradation mechanism. These data show that stabilized superoxide in aerobic, aqueous systems can degrade TCE at environmentally relevant concentrations.

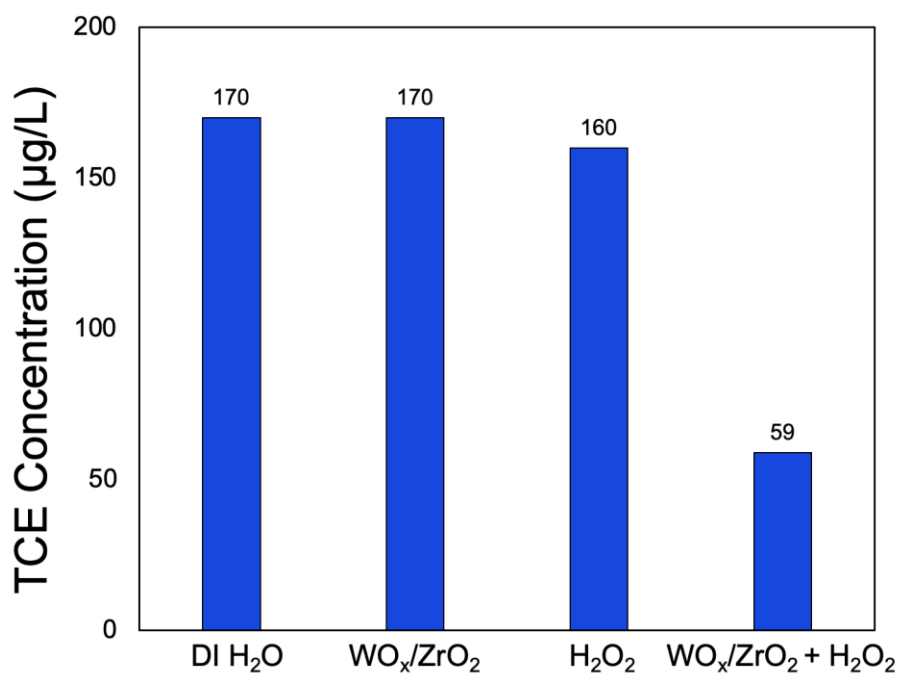


Figure 12. TCE concentrations in samples analyzed by Eurofins Lancaster Environmental Lab for the superoxide-producing catalytic system of 2000 mg/L WO_x/ZrO_2 with 0.1 M H_2O_2 . Treatment controls show negligible degradation, while the superoxide-producing reaction yielded significant TCE removal.

CONCLUSION

This project resulted in a publication¹ in *Environmental Science & Technology Letters* that resolved the controversy around superoxide's role in PFOA degradation. Briefly, we demonstrated that superoxide alone cannot degrade PFOA. These results are an important contribution to the PFAS remediation community in advising researchers against utilizing ineffective treatment

strategies. Additionally, we isolated superoxide hyper-producing bacteria and developed an assay to test their superoxide production capability. These bacteria were then analyzed to show that their superoxide production could degrade contaminants in water such as BPA. Finally, superoxide-producing systems were utilized to degrade TCE, a groundwater contaminant that is commonly found with PFAS plumes. These results suggest that superoxide hyper-producing bacteria could be utilized for cleanup of a variety of water pollutants, including those that co-occur with PFAS, allowing for a treatment train to effectively degrade a range of co-occurring contaminants in a contaminated site. Therefore, further research on characterizing these mechanisms and evaluating their implementation in permeable reactive barriers or natural attenuation strategies is recommended.

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