

FINAL REPORT

Treatment Train for In Situ Mineralization of PFOS using Heat- Activated Persulfate Oxidation (HAPO)

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List of Acronyms

AFFF	Aqueous film forming foam
AmPr-FHxSA	n-dimethylammoniopropyl perfluorohexane sulfonamide
CBZ	Carbamazepine
EPR	Electron paramagnetic resonance spectroscopy
FHxSA	Perfluorohexane sulfonamide
HBT	1-hydroxybenzotriazole
HPLC	High performance liquid chromatography
HAPO	Heat-activated persulfate oxidation
ISCO	In situ chemical oxidation
LC	Liquid chromatography
LMS	Laccase-mediator system
MS	Mass spectrometry
PFAA	Perfluoroalkyl acids
PFAS	Per- and polyfluoroalkyl substances
PFCA	Perfluoroalkyl carboxylic acid
PFHxA	Perfluorohexanoate
PFHxS	Perfluorohexane sulfonate
PFSA	Perfluoroalkyl sulfonic acid
PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
SERDP	U.S. Department of Defense's Strategic Environmental Research & Development Program
TvL	<i>Trametes versicolor</i> laccase

Keywords

PFAS, AFFF, bioremediation, laccase, laccase-mediator system, enzyme, treatment train, sorption, EPR

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Abstract

Introduction & Objectives

This project was in response to the ERSON-20-C1 objective of “improving understanding of biodegradation processes that could lead to PFAS biological treatment as part of a treatment train.” Our aim was to develop a novel proof-of-concept in situ treatment train to mineralize PFOS, by first transforming PFOS with a laccase-mediator system (LMS), an enzyme-based treatment method, and then subjecting transformation products to heat-activated persulfate oxidation (HAPO), a chemical treatment method. We considered that a dual-pronged treatment approach could address the challenges of treating complex mixtures of PFAS at aqueous-film forming (AFFF)-impacted DoD sites; namely, the initial bio-transformation step of perfluorooctane sulfonic acid (PFOS), which is notoriously difficult to treat in situ.

Our objectives were to 1) Identify laccase-mediator systems that could quickly transform PFOS; 2) Quantify and characterize the transformation products formed during laccase-mediator reactions; and 3) Evaluate the mineralization of transformation products by HAPO. In addressing these objectives, we sought to validate and optimize a treatment train approach for complex AFFF-contaminated matrices.

Technical Approach

The breadth of PFAS and non-fluorinated organics in AFFF as well as the co-contaminants present at DoD sites is further compounded by accumulation of perfluoroalkyl acids from precursor transformation. A single, isolated treatment unit is not a viable approach for remediation of these sites. However, a treatment train that takes advantage of the field-readiness and non-specificity of HAPO in combination with the novel enzyme-based LMS treatment approach, could make PFOS amenable to mineralization.

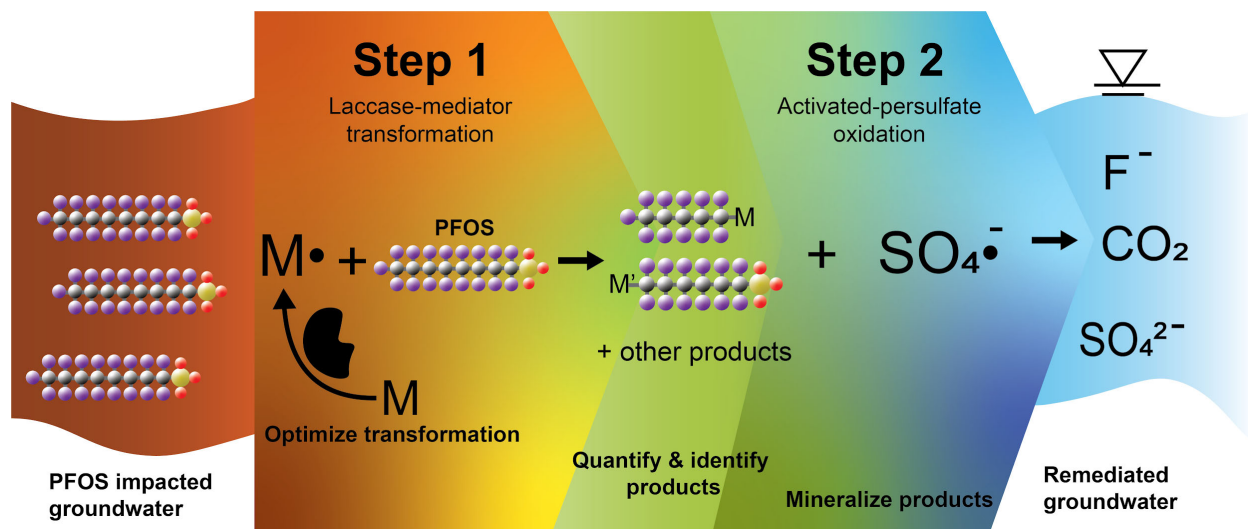
We viewed the LMS strategy as a tunable and optimizable treatment method due to the multiple components of the treatment system; namely, the choice of enzyme, the choice of mediator, and the choice of buffer. In the initial phase of our proposed research, we addressed the ‘optimization’ of the LMS for PFOS transformation, screening a variety of enzymes, mediator compounds, and buffers. We planned to evaluate the transformation rates and transformation products of PFOS subjected to these various conditions, and then to evaluate the subsequent treatment step, HAPO. We were unable to successfully or reliably show PFOS transformation in our screening studies. This led us to revise our approach and undertake a mechanistic investigation of the LMS, evaluating each of the oxidative reactions in the system. Our mechanistic investigation led to important insights about the nature of PFOS ‘loss’ in enzyme-based treatment systems.

Results

Screening studies of enzymes, mediators, and buffers for PFOS transformation did not indicate reactivity of either the laccase enzyme or the radical mediator compound towards PFOS. Upon validating the reactivity of the LMS towards a proxy compound, as well as validating enzyme activity and generation of the radical mediator, we concluded that the LMS was not able to reliably transform PFOS. We did find, however, that PFOS could be removed from solution when subjected to the enzyme-mediator treatment through a sorption-based mechanism.

Benefits

While unable to accomplish our initial objective of PFOS mineralization in a dual-step in situ treatment train approach, our findings suggested alternative removal mechanisms for enzyme-based treatment of PFOS. While not necessarily a feasible strategy at a large scale, the affinity of PFOS for proteins may be utilized for bio-inspired, selective materials for PFOS removal in complex contaminated matrices.



Executive Summary

Introduction

Biological treatment has been used for over a century for treatment of wastewater, and more recently for treatment of persistent organic pollutants, which have emerged as a consequence of industrialization and urbanization.^{1,2} Per- and polyfluoroalkyl substances (PFAS) are among the most recalcitrant pollutants, earning the colloquial title “forever chemicals” due to their long half-lives and resistance to biological, thermal, and chemical degradation. While typical removal and destruction technologies for PFAS are chemically and energetically intensive processes such as advanced oxidation/reduction processes, adsorption technology, and incineration³ the lower energy and chemical requirements of biological treatment strategies are an enticing, yet elusive, alternative for PFAS transformation.

Enzymatic biotransformation reactions are promising for the transformation of persistent organic pollutants, even PFAS, because of their rapid kinetics and the diversity of enzymes available for use. Horseradish peroxidase (HRP) and fungal laccase enzymes have been reported for biotransformation of perfluorooctanoic acid (PFOA).⁴⁻⁷ Laccase enzymes are of particular interest because unlike HRP, which requires hydrogen peroxide as a terminal electron acceptor, they utilize molecular oxygen, catalyzing the reduction of O₂ to H₂O.^{8,9} Laccase mediator systems (LMSs) further expand the utility of these enzymes— the addition of small molecular weight “mediator” compounds can facilitate electron transfer to organic substrates, which may not be oxidizable by laccase itself.¹⁰ LMSs have been investigated for the degradation of pesticides and pharmaceuticals and decolorization of dyes.^{11,12}

Laccase enzymes are also prevalent in the environment, and can be found in plants, insects, fungi, and bacteria,¹⁰ which could be beneficial for in situ treatment of organic compounds such as PFAS. Laccases are used to catalyze lignin biodegradation via one electron abstraction from phenolic hydroxyl groups.⁹ However, the oxidative power of these enzymes is limited by relatively low redox potentials (~0.2-0.8 V/NHE).¹¹ The addition of small molecule mediators, most commonly bearing nitroxyl or phenolic moieties, can facilitate electron transfer from target substrate to laccase, expanding the scope of laccase-catalyzed reactions to larger substrates with higher redox potentials.¹⁰ This multi-step oxidation cycle, in which laccase oxidizes a chemical mediator, the chemical mediator oxidizes a target substrate, and molecular oxygen is reduced to water, is referred to as the laccase-mediator system (LMS).

While initial results indicating application of an LMS to transform PFOA and its sulfonic acid analog, perfluorooctane sulfonic acid (PFOS) were promising, the transformation products reported were tentative and the reaction kinetics were inefficient.^{5,13} Additionally, the apparent requirement for copper in the reaction system^{6,13} poses some concern for in situ remediation applications. Therefore, we sought to improve the scope, efficacy, and mechanistic understanding of LMSs for PFAS treatment by investigating the following: what other laccase enzymes could catalyze the reaction? What other mediators? Could other metals be used to replace copper? Could the reaction rate be improved?

Our proposed project consisted of two steps: 1) Assess rates of PFAS transformation that can be achieved by a mediator radical system activated by laccase enzymes; and, 2) Assess the potential of using HAPO to mineralize the products of the laccase-mediator system. We began our investigation with a wide screening of laccase-mediator combinations for PFOS degradation, utilizing the previously published reaction conditions.¹³ However, after seeing no signs of PFOS

removal, we altered the scope of our investigation to probe the individual oxidation steps of the LMS reaction. Primarily, we focused on characterizing enzyme activity under a variety of conditions and the degradation of a proxy compound as a means of evaluating the general efficacy of the LMS. This has led to important insights regarding sorptive removal processes that may appear as artifacts of degradation in enzyme-based treatment systems.

Objectives

Our original objectives focused on assessment of rates of PFSA transformation achievable by LMSs, and assessment of the applicability of HAPO to mineralized products of LMS reactions. To that end, we proposed the following objectives:

1. We will identify laccase-mediator systems that quickly transform PFOS. The PFOS transformation rates and the effect of different laccase and mediators on reaction rates will be tested at reported optimal pH and temperature of the laccase enzyme. This analysis will identify the combination of laccase-mediator systems that are most efficient at converting PFOS.
2. We will then quantify and characterize the transformation products formed during laccase mediator reactions. To assess the formation of transformation products we will use the total oxidizable precursor (TOP) to help us characterize their nature (perfluoroalkyl chain length, etc.). We will also monitor the fluoride release during the laccase-mediator reactions. These analyses will provide insight into the extent and rate of defluorination and transformation by the laccase-mediator systems.
3. We will evaluate the mineralization of transformation products by HAPO. HAPO will be performed on the transformation products generated from the laccase-mediator reactions. A mass balance will be performed to reveal the efficiency of this integrated treatment and to gain insight into the steps and conditions affecting complete PFOS mineralization.

Technical Approach

Complex PFAS contamination at military sites impacted by AFFF.

PFAS are a class of chemicals that contain fully-fluorinated carbons. These short, stable C-F bonds provide PFAS with desirable traits for various industrial and consumer applications, including AFFFs.¹⁴ AFFFs have been used in the United States since the 1960s to extinguish fires at DoD sites¹⁵ and contain a wide range of PFAS, including perfluoroalkyl acids (PFAAs), such as PFCAs and PFSAs, as well as polyfluoroalkyl substances.¹⁶ These compounds were manufactured either via electrochemical fluorination (ECF) or telomerization. The transformation of polyfluoroalkyl substances can lead to the formation of more persistent PFAAs. For example, perfluoroalkane sulfonyl and sulfonamido compounds made by ECF in 3M AFFFs can be transformed to PFSAs.¹⁷ AFFFs made by other manufacturers, such as Ansul and National Foam, contain fluorotelomers that can be biotransformed to PFCAs in the environment.^{18–20} The polyfluoroalkyl substances in AFFFs can also be transformed to other polyfluoroalkyl

substances that have not been well characterized. As more sophisticated analytical methods have been applied, new classes of these compounds have been discovered in AFFF-impacted groundwaters, further complicating PFAS remediation.²¹

Heat-activated persulfate oxidation (HAPO) is a promising in situ technology for mineralization of most PFAS but inadequate for remediating PFSAs.

Activated persulfate is a relatively recent oxidation technique that has been applied in situ for various remediation projects around the country. Persulfate can be activated in a number of ways, including by heat (at least 35°C), alkaline conditions, and metals (e.g., iron).²² Under acidic conditions (i.e., pH<3), sulfate radicals mineralize PFCAs from AFFFs in both water and water-slurry samples.^{23,24} These radicals also oxidize polyfluorinated compounds through sequential shortening of the perfluoroalkyl chain. PFCAs and polyfluoroalkyl substances, including some compounds that can be transformed into PFSAs by microbes, are mineralized to CO₂ and fluoride. PFSAs, however, are not transformed by either persulfate or sulfate radicals. Because HAPO can mineralize a wide range of PFAS and prevent the future formation of PFOS, research targeting field applications has been intensively investigated in our lab through previous collaboration with the project ESTCP ER 201325.

Considering the full breadth of PFAS and non-fluorinated organics in AFFF as well as the co contaminants present at DoD sites, no single isolated treatment unit can fully remediate these sites. Therefore, a treatment train that takes advantage of the field-readiness and non-specificity of HAPO with the specificity of laccase-mediated biotransformation to make PFOS amenable to mineralization could show great technological promise.

The laccase-mediator reaction is a promising method to biotransform PFSAs to products that are amenable to mineralization by HAPO.

A fungal laccase (from *Pleurotus ostreatus*) in a system containing 1-hydroxybenzotriazole (HBT) as a mediator and 10 mM Cu²⁺ was shown to catalyze the transformation of PFOS to polyfluoroalkyl products with shorter perfluoroalkyl chains.¹³ HBT nitroxyl radicals generated from laccase oxidation can directly attack the C-C bond of the perfluoroalkyl chain of PFOS and generate shorter-chain perfluoroalkyl radicals. The perfluoroalkyl radicals can further react with either HBT radicals or other propagated organic radicals. The resulting products include polyfluoroalkyl substances without the sulfonate moiety or products that contain the sulfonate moiety linked on one side with fluorinated carbon atoms and non fluorinated hydrocarbons on the other.¹³

The use of this laccase-mediator transformation is limited as a standalone treatment of PFAS because of its potential to generate toxic aromatic and N-heterocyclic products in addition to the fluorinated compounds. However, in sequence with HAPO, any toxic organic products, together with both types of fluorinated products can be further oxidized by sulfate radicals in a subsequent HAPO step. Although promising to overcome the inert nature of the sulfonate group attached to the perfluoroalkyl chain tail, this enzymatic reaction is currently unfit for in situ engineering applications because of slow reaction rates, low conversion efficiency, and the short lifespan of the enzyme activity. In this proposed research, we aim to identify and test efficient laccase-mediator systems that will be compatible with subsequent HAPO treatment to develop a non-toxic treatment train, mineralizing PFOS in AFFF used by DoD.

Diverse laccase-mediator systems are promising for optimizing PFOS transformation.

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper enzymes that catalyze the reduction of molecular oxygen to water by one-electron oxidation of substrates such as substituted phenolic compounds.^{25,26} They are widely produced by plants, fungi, insects, and bacteria and are able to oxidize a wide range of aromatic and non-aromatic compounds directly. Laccases can also indirectly oxidize chemicals with bulky structures or high redox potentials with the aid of mediators.²⁷ Mediators are a group of low-molecular-weight laccase substrates, which can be easily oxidized by laccases to form highly potent intermediates, such as free radicals. The radicals can subsequently react with target compounds that cannot directly react with the enzymes. A recent study demonstrated the feasibility of using a laccase-mediator system to transform about 60% of 1 μ M of PFOS to a suite of shorter chain-length polyfluoroalkyl substances over a 162-day experiment.¹³ The conversion was slow but encouraging, and only the laccase from one fungus, *P. ostreatus*, was tested and only at room temperature. Given that a broad variety of both bacteria and fungi generate laccases with a wide range of activities and temperature optima, it is likely that a far more efficient, rapid and robust laccase-mediator system can be developed.

Thus far, more than 100 laccases have been isolated and characterized to various extents while numerous synthetic and natural mediators have been identified.²⁷ Because significant differences exist in biochemical properties of laccases and the reactions of these laccases with various mediators, selecting the appropriate laccase-mediator combination is crucial for efficient transformation of the target compound.^{27,28} Currently, except for the laccase from *P. ostreatus* and HBT, the PFOS transformation efficiency with other laccase-mediator systems remains unknown. As the efficiency of a laccase-mediator system is determined by both the enzyme stability, the kinetic parameters of the enzyme, and temperature, the conditions that can affect these parameters are crucial to PFSA transformation processes but have not yet been investigated. For example, given that HAPO requires heating of the groundwater to activate the persulfate, and given that enzyme activity tends to roughly double with every 10 °C increase, it is likely that the conditions that favor in situ HAPO (45-50 °C) will also favor enhanced laccase activity. To fill these knowledge gaps, this research will focus on identifying the laccase-mediator combinations that exhibit efficient transformation rates of PFOS under the conditions that favor application of HAPO.

Results & Discussion

Initial screening experiments of laccase enzymes and mediators did not indicate substantial transformation of PFOS. We screened selected nitroxyl mediator compounds in parallel with two high redox laccase species. Specifically, we tested *Agaricus bisporus* and white rot laccase over a 28-day period. Reactors were dosed weekly with enzyme (1 U/mL) and mediator (20 μ M). No significant PFOS degradation was observed (Figure ES1).

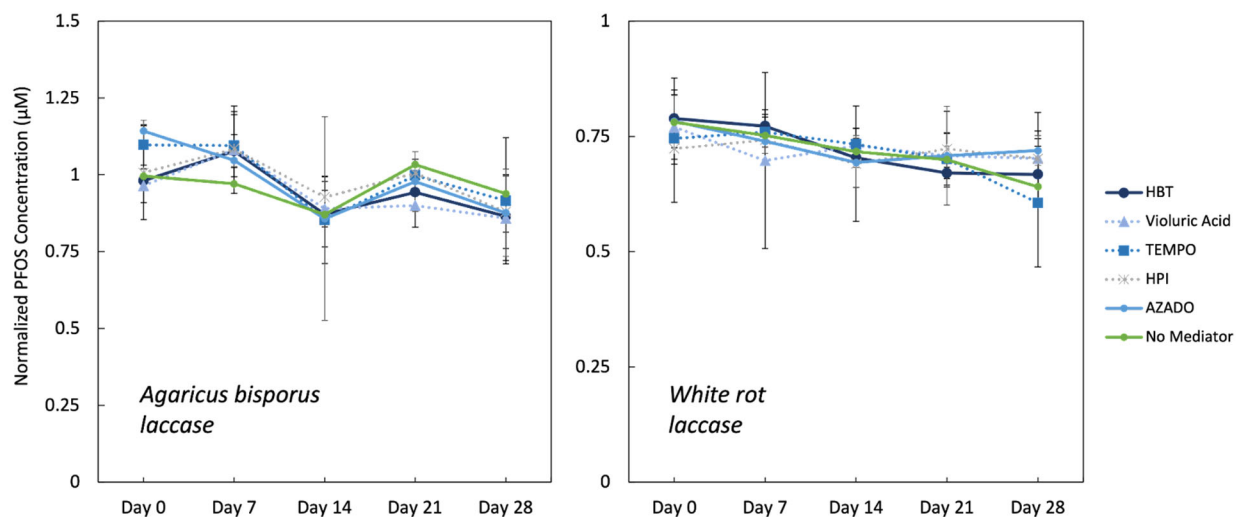


Figure ES1. Screening of laccase-mediator combinations for reactivity towards PFOS. PFOS concentrations do not appear to decrease significantly compared to the no mediator control over the 28 day period. Concentrations were normalized to a control (no laccase, no mediator) to account for possible evaporation. Laccase and mediator were added weekly at the time of sampling.

Upon confirming enzyme activity and the enzyme-mediator reaction, we considered slow reaction kinetics might prevent observable transformation of PFOA and PFOS. Thus, we lowered the concentrations to 0.1 μM PFOA and 0.1 μM PFOS in a 10 mM Cu_2SO_4 buffer. The reactors were treated with 1 U/mL *Trametes versicolor* and 1 mM HBT; doses of enzyme and mediator were added twice daily, for four days. Monitoring the concentration in the reactors revealed an apparent decrease in both PFOA and PFOS concentration after the first two doses of enzyme and mediator were added (within the first 24 hours), then the concentration appears to level off (Figure ES2). At the end of the experiment, the reactors were extracted with basic MeOH. Upon analyzing the total mass contained in the reactors at the beginning and end of the experiment, and comparing it to the mass determined from the ‘extract’ concentration, both PFOA and PFOS were fully recovered. The recovery of PFOS and PFOA suggests that the initial decrease in concentration is likely due to physical processes in the system (e.g. sorption).

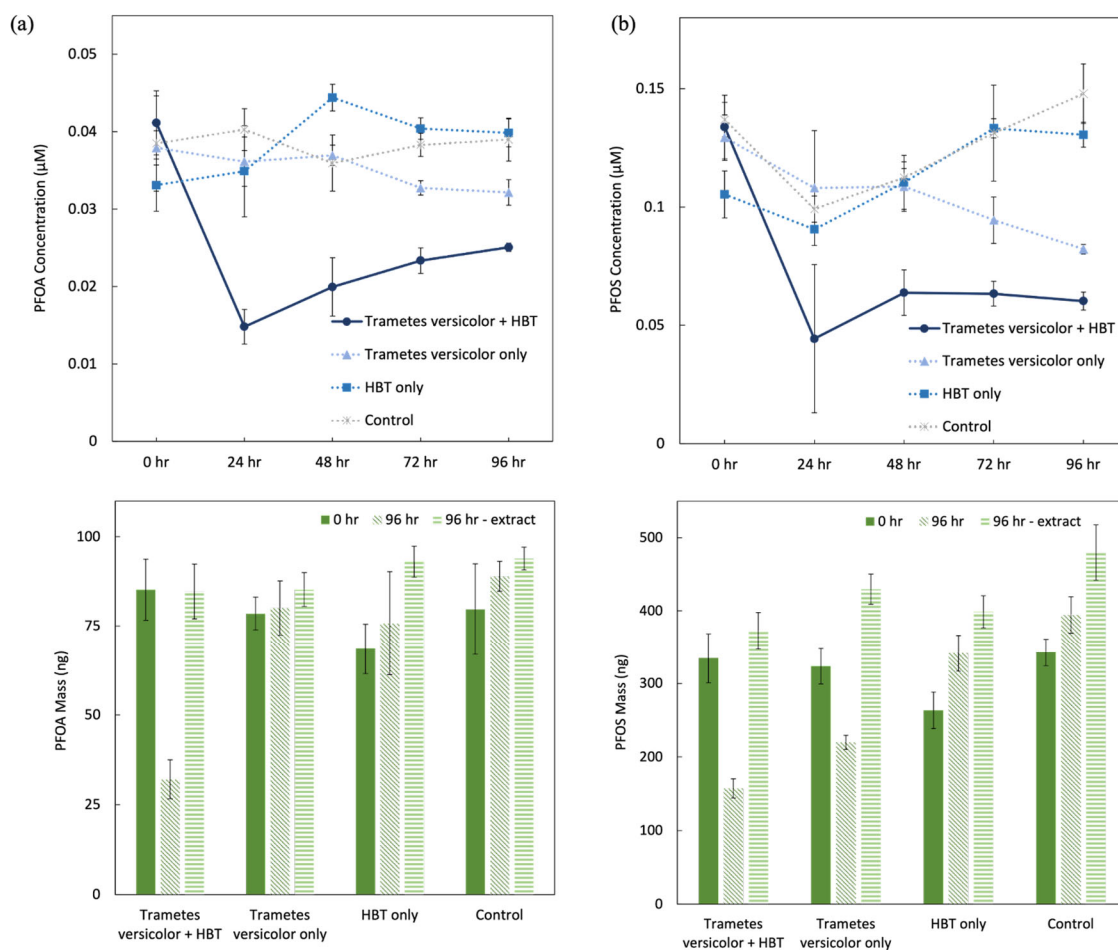
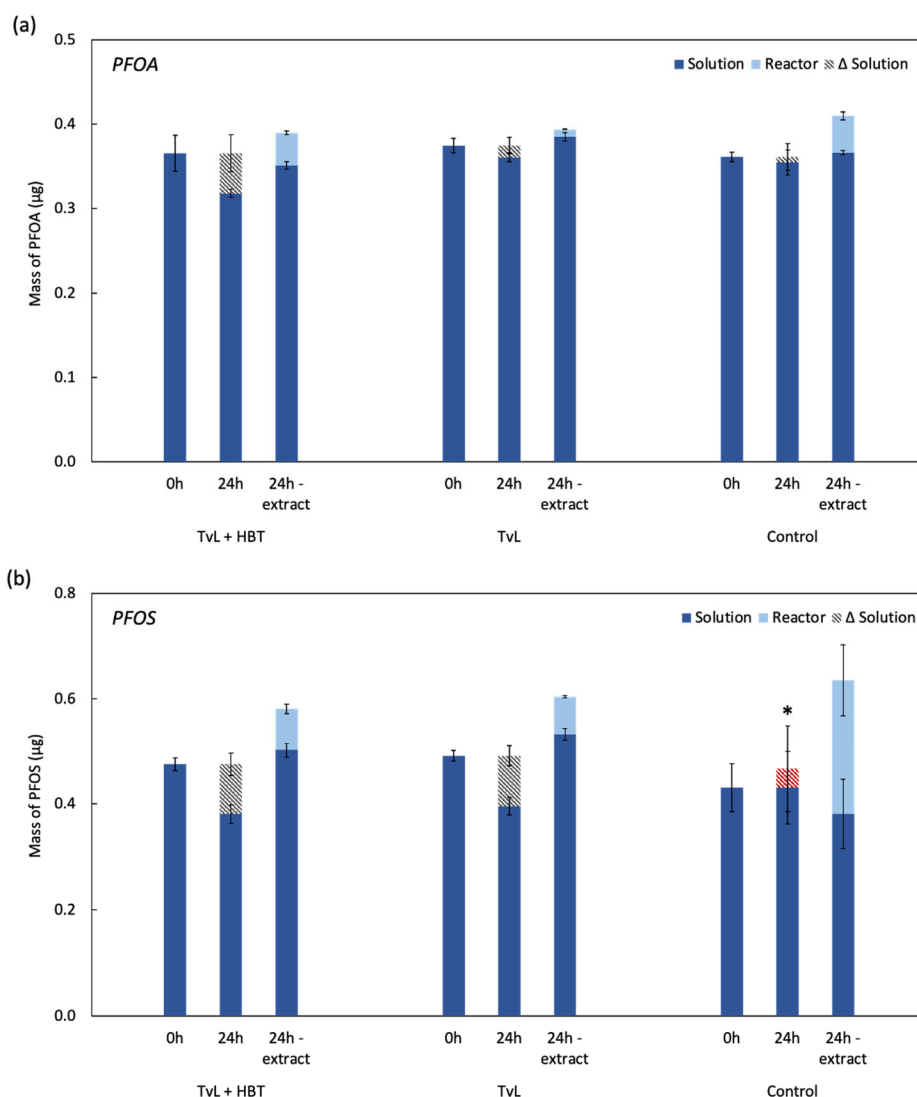


Figure ES2. Reactors were amended with TvL and HBT twice daily. a) Concentration of PFOA and b) Concentration of PFOS monitored via removal of an aqueous aliquot from the reactors; c) Mass of PFOA and d) Mass of PFOS in reactors calculated from detected concentration and total solution volume at the beginning of the experiment, at the 96 hour time point, and in the extracted solution volume at the 96 hour time point. Averages and error bars are representative of triplicate reactors.

Sorptive removal was further assessed and sorption processes due to enzyme sorption and reactor sorption were differentiated. We conducted an experiment in which enzyme-containing solutions and reactors were extracted separately with basic methanol. The results of this experiment are summarized in Figure ES3. The difference in mass detected in the 0h and 24h subsample (Δ Solution) can be reasonably attributed to enzyme sorption. For PFOA, a $13 \pm 6\%$ mass loss was observed in solution treated with TvL/HBT, while substantial mass loss was not observed in the TvL treated or control reactors. For PFOS, a $20 \pm 5\%$ mass loss was observed in solution treated with TvL/HBT, and a $19 \pm 4\%$ mass loss was observed in solution treated with TvL only.

Figure ES3. Reactors were treated with a single dose of 2 U/mL (60 mg) TvL and 2 mM HBT. Detected mass of a) PFOA and b) PFOS was measured from a solution aliquot immediately after reactor preparation (0h) and after 24 hours (24h) of incubation; Δ Solution is calculated from the mass detected in the aliquot between 0-24 hours (i.e., the mass attributed to protein sorption). At



24 hours, the protein-buffer solution and the reactor were extracted separately with basic MeOH. Error bars are the standard deviation of triplicate reactors.

Implications for Future Research & Benefits

While attractive as a bio-based treatment method for PFAS remediation, laccase-mediator systems appear to be unsuccessful at reliably degrading the perfluorinated compounds PFOA and PFOS. Previous studies had shown promising transformation results of PFOA and PFOS in a treatment system with the high redox laccase species, *Pleurotus ostreatus* and the nitroxyl mediator HBT.^{5,13} We were unable to reproduce transformation with multiple other enzymes and mediators tested, namely, *Trametes versicolor* and HBT, despite the multiple literature reports of this system for contaminant degradation and our own success at replicating the *Trametes versicolor* and HBT system for treatment of another prevalent contaminant, carbamazepine.

A thorough investigation of the proposed, multi-step oxidation mechanism, including confirming the enzyme activity and activity retention, the enzyme-mediator reaction (i.e., radical

generation), and the reactivity of the radical mediator towards a proxy substrate, carbamazepine, led us to conclude that the oxidative mechanism and reaction conditions were effective. However, lack of substantial removal of the target perfluorinated substrates (PFOA, PFOS), indicated that the system was not effective at oxidizing these species. This was not altogether surprising, given the recalcitrant nature of the compounds, and the extremely stable C–F bonds required to break for transformation. Additionally, laccase enzymes on their own typically react directly with substrates containing phenol or aniline functional groups (i.e., native to lignin), while nitroxyl-type mediators typically react directly to oxidize alcohols,²⁹ both of which have substantially lower bond strengths and redox potentials than the perfluorinated compounds.

While we did not observe transformation of PFOA and PFOS, close examination of the treatment reactors did reveal a removal artifact that we attributed to protein sorption. Analysis of the substrate mass contained in the protein-buffer solutions, and residual substrate mass on the reactor container, revealed that sorption to both enzyme and the reactor container contributed to apparent concentration decreases in the reaction system. These concentration decreases could appear as a removal artifact of 20-30%; however, extraction of the reactors resulted in full recovery of the substrates at the end of treatment times. Sorptive removal of PFAAs is a previously reported phenomenon, and can be attributed to the hydrophobicity of the PFAAs as well as specific interactions between PFAA functional groups (i.e., carboxylate) and protein side chains.

This project set out to learn if laccase-mediator systems could be applied as a robust, bio-based treatment strategy for PFAA transformation. In particular, we sought to combine enzyme based treatment with well studied chemical oxidation mechanisms (e.g., H₂O₂), to achieve complete mineralization of PFAAs in situ. We considered that the multiple components of the laccase-mediator system (enzyme, mediator, buffer, metals) lend themselves to a highly tunable system, which we thought could be optimized for kinetics and treatment efficiency. Ultimately, we found that laccase-mediator systems could not reliably transform PFAAs, but rather, that laccase enzymes remove PFAAs via adsorption. Enzyme adsorption technologies may not be feasible on a scale necessary to treat PFAAs, however, further investigation of the enzyme-PFAA binding modes and interactions may provide inspiration to novel sorptive materials capable of selective PFAA removal from complex contaminant matrices.

Objectives

This project was proposed in response to the ERSON-20-C1 objective to “improve understanding of biodegradation processes that could lead to per-, polyfluoroalkyl substance (PFAS) biological treatment as part of a treatment train”. Our aim was to: (1) elucidate the biochemical mechanisms governing the efficiency and reaction rates of perfluoroalkyl sulfonate (PFSA) transformation by laccase-mediator systems and (2) identify engineering operational parameters that ensure successful integration of the laccase-mediator system with heat-activated persulfate oxidation (HAPO) in a three-stage treatment train, i.e., HAPO-laccase transformation-HAPO (HAPO-lac-HAPO) to create a novel in situ treatment for mineralization of PFASs at aqueous film-forming foam (AFFF)-impacted United States Department of Defense (DoD) sites.

Key questions guiding our proposed research were:

1. Which laccase-mediator systems will lead to high efficiency PFSA transformation under conditions compatible to post-HAPO treatment? How do elevated temperatures (30-50°C) and acidic pH conditions (from HAPO) affect laccase stability and PFOS transformation? What are the key factors affecting the PFSA transformation efficiency?
2. What strategies can be used to direct the laccase-mediator reactions towards complete PFSA transformation?
3. What are the key configurations and operational conditions of integrated laccase-mediator and HAPO treatment for in situ remediation?

We aimed to optimize highly efficient laccase-mediator systems to integrate as a treatment step between two HAPO treatments as a means to develop a non-toxic in situ treatment train for mineralizing PFAS contaminants at AFFF-impacted DoD sites (Figure 1). To achieve this overall goal, we had three specific research objectives:

1. Identify laccase-mediator systems that efficiently convert PFASs to polyfluoroalkyl substances that can subsequently be mineralized by HAPO. The PFOS transformation rates, effect of mediator concentration on reaction rates and enzyme activities will be tested at elevated temperatures and low pH conditions typical of those produced by HAPO. We will also investigate the effects of aquifer solids and minerals (e.g., iron oxides) on PFSA transformation.
2. Investigate the reaction conditions and treatment strategies that maximize the rates and efficiency of PFSA transformation. Inhibition factors affecting complete PFOS transformation will be identified and strategies to overcome these factors will be compared. Here we aim to identify a reaction time, enzyme-mediator ratio, and amendment regime that will lead to complete PFSA transformation.
3. Develop a treatment train combining the enzymatic transformation of PFASs and HAPO to mineralize PFCAs, PFASs, and polyfluoroalkyl substances (Figure 1). A

treatment train will be designed based upon three consecutive processes: HAPO, laccase transformation, and repeated HAPO (HAPO-lac-HAPO). The first HAPO step will mineralize PFCAs and polyfluoroalkyl substances, leaving PFSA's untreated. Then, the lac step will transform the residual PFSA's into a suite of polyfluoroalkyl substances that are likely to be more amenable to HAPO treatment. Finally, the second HAPO step completes the treatment train by mineralizing the remaining polyfluoroalkyl substance transformation products and any co-contaminants or byproducts. We will first investigate the effects of inorganic compounds generated in a post-HAPO environment on the PFAS transformation efficiency in the lac step and identify treatment strategies that ameliorate the identified inhibitions. We will also perform experiments to understand the effects of remaining reaction constituents (e.g., transformed products as well as leftover mediators, metals, and enzymes) on subsequent HAPO treatment. At the last phase of this task, we will use real groundwater samples to optimize this HAPO-lac-HAPO treatment train and to identify engineering parameters for successful in situ PFAS mineralization.

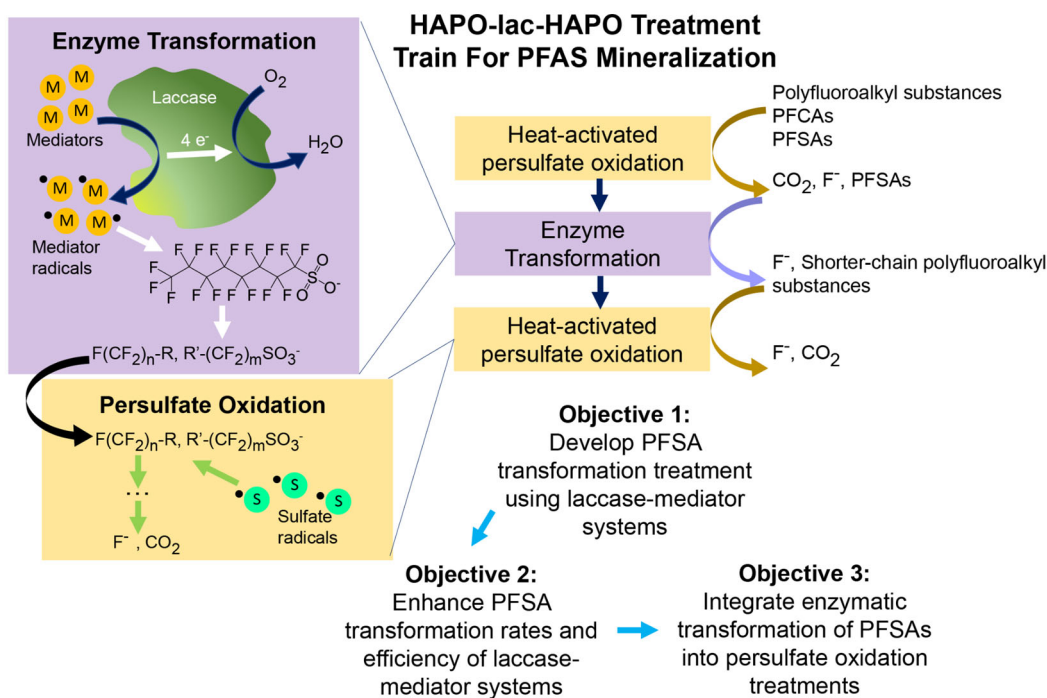


Figure 1. Summary of the proposed research.

Consistent with the SERDP mission to develop and apply innovative environmental technologies to reduce DoD's current and future environmental liabilities, we sought to develop mineralization treatment methods for PFASs to achieve site closure.

Background

PFAS are a class of chemicals that contain fully-fluorinated carbons. These short, stable C-F bonds provide PFAS with highly desirable traits for various industrial and consumer applications, including aqueous film-forming foams (AFFFs).³⁰ AFFFs have been used in the United States since the 1960s to extinguish fires at DoD sites¹⁵ and contain a wide range of PFASs, including perfluoroalkyl acids (PFAAs), such as perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs), as well as polyfluoroalkyl substance.¹⁶ PFAS are manufactured either via electrochemical fluorination (ECF) or telomerization processes. The transformation of polyfluoroalkyl substances can lead to the formation of more persistent PFAAs. For example, perfluoroalkane sulfonyl/sulfonamido compounds found in ECF made formulations, such as 3M AFFFs, can be transformed to PFSAs.¹⁷ AFFFs made by manufacturers such as Ansul and National Foam, contain fluorotelomers (i.e., polyfluoroalkyl substances made by the telomerization process) that can be biotransformed to PFCAs in the environment.^{18–20} The polyfluoroalkyl substances in AFFFs can also be transformed to other polyfluoroalkyl substances that have not been well characterized. As more sophisticated analytical methods have been applied, new classes of these compounds have been discovered in AFFF-impacted groundwaters, further complicating PFAS assessment and remediation at contaminated sites.³¹

Activated persulfate is a relatively recent oxidation technique that has been applied in situ for various remediation projects around the country. Persulfate can be activated in a number of ways, including by heat (at least 35°C), alkaline conditions, and metals (e.g., iron).²² Under acidic conditions (i.e., pH<3), sulfate radicals mineralize PFCAs from AFFFs in both water and water-slurry samples.^{23,24} These radicals also oxidize polyfluorinated compounds through sequential shortening of the perfluoroalkyl chain. PFCAs and polyfluoroalkyl substances, including some compounds that can be transformed into PFSAs by microbes, are mineralized to CO₂ and fluoride. PFSAs, however, are not transformed by either persulfate or sulfate radicals. Because HAPO can mineralize a wide range of PFAS and prevent the future formation of PFOS, research targeting field applications has been intensively investigated in our lab through previous collaboration with the project ESTCP ER 201325. A treatment train that can take advantage of the current field technology development with HAPO to achieve complete mineralization of all PFASs during in situ remediation would be highly valuable.

A fungal laccase (from *Pleurotus ostreatus*) in a system containing 1-hydroxybenzotriazole (HBT) as a mediator and 10 mM Cu²⁺ was shown to catalyze the transformation of PFOS to polyfluoroalkyl products with shorter perfluoroalkyl chains.¹³ Although unlikely to be a stand-alone treatment of PFSAs due to the generation of possibly toxic aromatic products and N-heterocycles, a laccase-mediator system represents a promising method for transforming PFSAs to compounds amenable to subsequent HAPO treatment. HBT nitroxyl radicals generated via laccase oxidation were proposed to attack the C-C bond of the perfluoroalkyl chain of PFOS and generate shorter-chain perfluoroalkyl radicals. The perfluoroalkyl radicals were suggested to further react with either HBT radicals or other propagated organic radicals. The resulting products include polyfluoroalkyl substances without the sulfonate moiety or products that contain the sulfonate moiety linked on one side with fluorinated carbon atoms and non-fluorinated hydrocarbons on the other.¹³ Both types of products can be further oxidized by sulfate radicals in a subsequent HAPO

step. Although promising to overcome the inert nature of the sulfonate group attached to the perfluoroalkyl chain tail, the enzymatic reaction is currently unfit for in situ engineering applications because of slow reaction rates, low conversion efficiency, the short lifespan of enzyme activities, and the potential generation of toxic end products. Furthermore, the mechanism of the oxidation reaction between the nitroxyl radical mediators and PFOS is not well documented. In this proposed research, we aim to select and optimize highly efficient laccase-mediator systems to integrate between two HAPO steps to develop a non-toxic in situ treatment train, mineralizing the three most abundant types of PFASs in AFFF formulations.

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper enzymes that catalyze the reduction of molecular oxygen to water by one-electron oxidation of substrates such as substituted phenolic compounds.^{26,32} They are widely produced by plants, fungi, insects, and bacteria and are able to oxidize a wide range of aromatic and non-aromatic compounds directly. Laccases can also indirectly oxidize chemicals with bulky structures or high redox potentials with the aid of mediators.²⁷ Mediators are a group of low-molecular-weight laccase substrates, which can be easily oxidized by laccases to form highly potent intermediates, such as free radicals. The radicals can subsequently react with target compounds that cannot be directly oxidized by the enzymes. A recent study has demonstrated the feasibility of using a laccase-mediator system to transform about 60% of 1 μ M of PFOS to a suite of shorter chain-length polyfluoroalkyl substances over a 162-day experiment.¹³ The conversion was slow but encouraging, and only the laccase from the fungus *P. ostreatus* was tested. This particular fungal strain is no longer sold commercially, thus other high redox fungal laccases were of interest to expand the utility of the laccase-mediator system.

In fact, more than 100 laccases have been isolated and characterized to various extents while numerous synthetic and natural mediators have been identified²⁷. Because significant differences exist in biochemical properties of laccases and the reactions of laccases with different types of mediators, selecting the appropriate laccase-mediator combination is crucial for the efficient transformation of the target compound.^{27,28} Currently, except for the laccase from *P. ostreatus* and the mediator HBT, the PFOS transformation efficiency of other laccase-mediator systems remains largely untested. Additionally, whether the laccase-mediator systems can be used under the in situ HAPO conditions is also unknown. As the efficiency of a laccase-mediator system is affected by both the enzyme stability and the kinetic parameters of the enzyme, the conditions that can affect these parameters are also crucial to PFSA transformation processes but have not been systematically investigated. To fill these knowledge gaps, this research will focus on identifying the laccase-mediator combinations that exhibit enhanced transformation rates of PFASs. Upon identifying successful laccase-mediator systems, the reaction will be optimized with respect to treatment regimen timeline and quantity of enzyme and mediator. Evaluation of the reaction in combination with HAPO treatment will act as a proof of concept for field-based application of the new treatment train.

Materials & Methods

Chemicals

Chemicals used in the experiments were of reagent grade quality or higher, and were purchased from the following suppliers: MilliporeSigma, Fisher Scientific, Spectrum, and VWR. Commercial enzymes were purchased from MilliporeSigma (*Trametes versicolor*, *Agaricus bisporus*, *Aspergillus sp.*) and from Creative Enzymes (Native laccase, white rot fungi). Chemicals and enzymes were used without further purification. Mass-labeled internal standard compounds were purchased from Wellington Laboratories. For preparation of aqueous buffers for the reactors, Milli-Q water was used. For preparation of mediator stocks for reactor amendments, HPLC-grade acetonitrile (MeCN) was used unless noted otherwise. Reactors were prepared using 50 mL polypropylene (PP) Corning centrifuge tubes, or 20 mL glass scintillation vials.

Enzyme Activity Assay

Assays were performed in clear, flat-bottom 96-well plates; plate wells can hold a volume of up to 400 μL , and a total volume of 200 μL was used in the assay reactions. Buffer solutions were prepared with reagent grade compounds. ABTS and DMP were purchased from MilliporeSigma. A UV-Visible spectrophotometer (BioTek Epoch 2 Microplate Reader) equipped with a plate reader in combination with Gen5 Version 3.02 processing software was used to calculate the change in absorbance of the samples. Absorbance was measured via continuous readings every 60 seconds, over the course of 0-10 minutes. In the case that absorbance hit a maximum within the 10-minute measurement, only the absorbance readings in the linear region were used to determine activity. For the ABTS assay, absorbance was measured at the wavelength maxima of $\lambda = 420 \text{ nm}$ ³³; for the DMP assay, absorbance was measured at the wavelength maxima of $\lambda = 468 \text{ nm}$.³⁴

A colorimetric UV-spectrophotometric assay was used to determine the relative enzyme activity among batches and species of commercial laccase enzymes. The assay measures the oxidative conversion of 2,6-dimethoxyphenol (DMP) to the corresponding 3,5,3',5'-tetramethoxydiphenylquinone.³⁴ The absorbance spectra of the quinone is measured at a wavelength maxima of 468 nm ($\epsilon = 49,600 \text{ M}^{-1}\text{cm}^{-1}$) in a citrate-phosphate buffer (pH 3.8) prepared from a 0.1 M solution of citric acid and a 0.2 M solution of Na_2HPO_4 . Upon assay preparation, buffer solution was added to the plate followed by the laccase containing solution. DMP or ABTS was then quickly added before placing the plate in a Shimadzu UV-vis reader for absorbance measurements. The laccase containing solution was diluted appropriately (1000 - 10,000x) in order to achieve an absorbance in the range of 0.1-1 AU for best accuracy. In the majority of experiments, the target enzyme activity was 0.5-2 U/mL; in order to accurately measure enzyme activity of these experiment solutions, a total dilution of approximately 1000x was required to obtain an absorbance reading in the appropriate range. Absorbance was measured continuously over 10 minutes. Solutions were assayed in triplicate from which the average absorbance measurements were calculated. Absorbance data was related to enzyme activity using the Beer-Lambert law,

$$A = \epsilon bC$$

where ε = *molar extinction coefficient*, b = *path length*, and C = *concentration*.

Absorbance data was related to enzyme activity using the Beer-Lambert law, $A = \varepsilon bC$ where ε = *molar extinction coefficient*, b = *path length*, and C = *concentration*. For ABTS, $\varepsilon = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$ for a wavelength maxima of 420 nm; for DMP, $\varepsilon = 49,600 \text{ M}^{-1} \text{ cm}^{-1}$ for a wavelength maxima of 468 nm. Path length was calibrated to 1 cm from a matrix blank using Gen5 Version 3.02 processing software. The change in concentration of substrate per minute ($\mu\text{mol}/\text{minute}$) was used to determine the enzyme activity, adjusted for dilution. The following equation was used,

$$\text{Activity (U/mL)} = \frac{\Delta A}{\varepsilon} \times 10^6 \times \frac{1}{D}$$

with a factor of 10^6 to convert from mol of product to μmol of product, and a factor of $1/D$ to correct for total dilution from the original enzyme containing solution.

Batch Transformation Experiments

Reactors were prepared in triplicate, unless noted otherwise. Reactor solutions consisted of the target substrate in a selected buffer (specified throughout Results & Discussion), to which selected mediator and enzyme concentrations were added. Reactors were prepared either in Corning 50-mL PP centrifuge tubes, with up to 10-mL solution volume, or 20-mL glass scintillation vials, with up to 5-mL solution volume, to ensure oxygenated headspace. Solutions were prepared in the following order: 1) Buffer was added to the tube/vial (e.g., sodium malonate); 2) A small volume of concentrated substrate stock was added to the buffer (e.g., 100 μL of aqueous PFOS, PFOA, or carbamazepine); 3) A small volume of concentrated mediator stock was added to the buffer containing substrate, i.e., the reactor solution (e.g., 50-100 μL of 1-HBT in acetonitrile); 4) Laccase enzyme was added to the reactor solution, either from a concentrated stock in buffer or as a solid powder. In the case that a concentrated laccase stock was used, it was prepared in the same buffer solution used in the reactors. For control reactors (i.e., those without mediator, or without enzyme), equivalent volumes of acetonitrile and/or buffer were added to the reactors such that volume changes throughout the course of the experiment were as consistent as possible for all reactors.

Protocols established in Luo et al. (2015, 2018)^{5,13} called for ‘dosing’ of enzyme and mediator every 6 days; that is, reactors were dosed with 1 U/mL of freshly prepared laccase solution and 20 μM of HBT (mediator) in acetonitrile. The purpose of this protocol was to maintain enzyme activity and continue stimulating the reaction. We followed a similar dosing protocol in our experiments, amending reactors ranging from two-times daily to once a week, depending on the experimental set-up (specified throughout Results & Discussion). The quantity and frequency of enzyme and mediator doses was one of the variables evaluated in this study, and the specifics of the dosing schedule varied for individual experiments. We evaluated target enzyme activity in the range of 0.5-2 U/mL. We evaluated mediator quantities in the range of 20 μM to 5 mM.

The reactors were sampled to monitor concentration changes for the target substrates on a predetermined sampling schedule. Reactors were sampled as frequently as once per day, up to once a week, depending on the length of the experiment as well as the mediator/enzyme

amendment schedule. At the sampling time, all reactors were opened to promote aeration. Reactors were kept open for a minimum of 10 minutes to ensure oxygen was replenished in the solution. Sample aliquots were removed from reactors and immediately diluted into methanol (MeOH) or basic methanol (2% ammonium hydroxide) to terminate the reaction. A minimum of 100 μ L aqueous sample was removed for dilution. For the majority of the experiments, the aqueous solution volume at the end of the experiment was weighed and the equivalent amount of MeOH was added directly to the reactor (i.e., 2x dilution, 5-mL of MeOH added to 5-mL aqueous reaction solution), to ensure complete 'extraction' from the reactor in the case that substrate had sorbed to the container walls. Additionally, internal standards (MPFOS, MPFOA) will be compared for their adherence to the reactor container walls. In order to evaluate this, the standard will be spiked into the reactor prior to MeOH extraction, and detected concentration of the standard will be compared before and after the MeOH extraction step. Substrate concentrations from the subsample were compared to those taken directly from the reactor (after dilution with MeOH) at the final time point to determine if sorption processes were a significant factor in any observed concentration variation.

Analytical Methods

Samples for LC-MS/MS analysis were prepared in 2 mL screw cap amber vials purchased from Agilent Technologies. LC-MS grade methanol and LC-MS grade water were used for sample preparation and dilution. In the case that samples were pre-diluted prior to addition to amber vials, dilutions were in 1.5-2 mL PP centrifuge tubes using methanol to ensure maximum solubilization of the analytes. Samples were vortexed between any subsequent dilutions to ensure solubilization. For copper containing samples, samples were diluted in basic methanol (2% ammonium hydroxide in methanol) in order to precipitate the copper. These samples were then centrifuged (15,000 g, 25°C, 10 minutes) to separate out a copper pellet and avoid copper interference in the LC-MS/MS analysis.

Mass-labeled internal standard compounds were purchased from Wellington Laboratories. The mass-labeled compounds arrive in glass ampules ranging from 1.0-50 μ g/L, and are diluted to make stock solutions for preparation of internal standard solution and standard working solutions for the preparation of a standard curve. PFAS are quantified by LC-MS/MS equipped with electrospray ionization in negative mode (Triple Quad 6460A, Agilent Technologies) multiple reaction monitoring (MRM).

To ensure accuracy and precision in measurements obtained from LC-MS/MS analysis, the method included quantification of a known sample supplied by the EPA, method blanks, and standard checks throughout the sample run. For example, the EPA sample of known PFOA, PFOS concentration was analyzed at the beginning of each LC-MS/MS run, as well as throughout the duration of the run, approximately every 30 samples. Along with running method blanks approximately every 10 samples, this allowed determination of any changes in concentration of target analytes due to instrumentation or analyte carry over. Selected standard curve samples were also checked throughout the run. A method blank sample (50/50 MeOH:H₂O) was run at the beginning of an LC-MS run and approximately every 10 samples throughout the analytical run. An acceptable level of detection for any analytes in the method blank was expected to be <50% of the lowest calibration standard. A laboratory control sample prepared from an EPA standard solution was run at the beginning and end of each sample run, with the acceptable level of each analyte being between 80-120% of the nominal value. If the

EPA sample is quantified as $\geq 20\%$ or $\leq 20\%$ of the known value, the samples will be re-run. A calibration curve was run and analyzed at the beginning of each run, and acceptable with an $R^2 \geq 0.99$. For all analytes, the $S/N \geq 10$ were considered significant peaks for quantification.

Table 1. PFAS compounds analyzed via LC-MS/MS.

Compound	Internal Standard	Precursor Ion	Product Ion	Fragmentor Voltage (V)	Collision Energy (V)	Polarity
MPFBA	-	217	172	50	5	Neg
MPFPeA	-	266	222 221	60	2	Neg
MPFHxA	-	315	270	60	5	Neg
MPFOA	-	417	372	70	2	Neg
MPFNA	-	468	423	70	5	Neg
MPFHxS	-	403	103	150	40	Neg
MPFOS	-	503	80	190	60	Neg
PFBA	MPFBA	213	169	50	2	Neg
PFPeA	MPFPeA	263	219 68.9	60 92	2 8	Neg
PFHxA	MPFHxA	313	269 119	80	2 15	Neg
PFHpA	MPFOA	363	319 169	80	2	Neg
PFOA	MPFOA	413	369 169	80	3 14	Neg
PFNA	MPFNA	463	419 219	80	2 15	Neg
PFBS	MPFHxS	299	99 80	120	30 70	Neg
PFHxS	MPFHxS	399	99 80	125	50 80	Neg
PFOS	MPFOS	499	99 80	122	50 80	Neg
FHxSA	MPFOS	398	78	125	36	Neg

FOSA	MPFOS	498	78	125	36	Neg
6-2 FtS	MPFOS	427	407 80	140	25 35	Neg
AmPr-FHxSA	MPFOS	485	85	135	30	Pos

EPR Spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy studies were performed in the CalEPR center in the University of California, Davis. X-band (9.4 GHz) continuous-wave (CW) EPR spectra were recorded on a Bruker Biospin EleXsys E500 spectrometer with a super high Q resonator (ER4122SHQE), an ESR900 liquid helium cryostat with a temperature controller (Oxford Instruments ITC503) and gas flow meter. CW EPR spectra were recorded under slow-passage, non-saturating conditions. Spectral simulations were performed in Matlab 2018a with Easyspin 5.2.35 toolkit.³⁵

The EPR sample of BTNO (benzotriazole-N-oxyl) radical in CH₃CN was prepared by mixing 100 μ L 20 mM Cerium (IV) ammonium nitrate in CH₃CN and 100 μ L 20 mM HBT in CH₃CN. The spectrum was recorded at room temperature, using the following spectrometer settings: conversion time = 120ms, modulation frequency = 100 kHz, modulation amplitude = 0.025 mT, and simulated using previously reported parameters.²⁹ EPR samples of HBT radicals generated by laccase enzymes were prepared by incubating aqueous solutions containing 1 mM HBT, 12 mg/mL *Trametes versicolor* laccase, 10 μ M CuSO₄, and 1 mM of different substrates for varying periods. The spectra were recorded at 50 K using the following spectrometer settings: conversion time = 120ms, modulation frequency = 100 kHz, modulation amplitude = 0.5 mT.

Results & Discussion

Overview

Our initial aims in this project assumed successful identification of multiple laccase-mediator type reactions for PFOS transformation, and a focus on determining transformation products, pathways, and kinetics in order to optimize the reaction and integrate it with chemical treatment (e.g., H₂O₂) for complete contaminant mineralization. However, in the initial phases of our investigation, we were not able to reliably detect removal of PFOS, thus, we altered the scope of our investigation to mechanistically evaluate the laccase-mediator system in an attempt to better understand the scope and utility of the oxidation reaction. This included evaluating enzyme activity, evaluating the enzyme-mediator reaction, and evaluating the enzyme-mediator reaction towards not only the target perfluorinated substrates (e.g., PFOA and PFOS), but also towards a proxy substrate (e.g., carbamazepine) (Figure 2). This step-wise investigation led us to interesting findings related to artifacts of PFOA and PFOS removal that we attributed to sorptive processes in the reaction system.

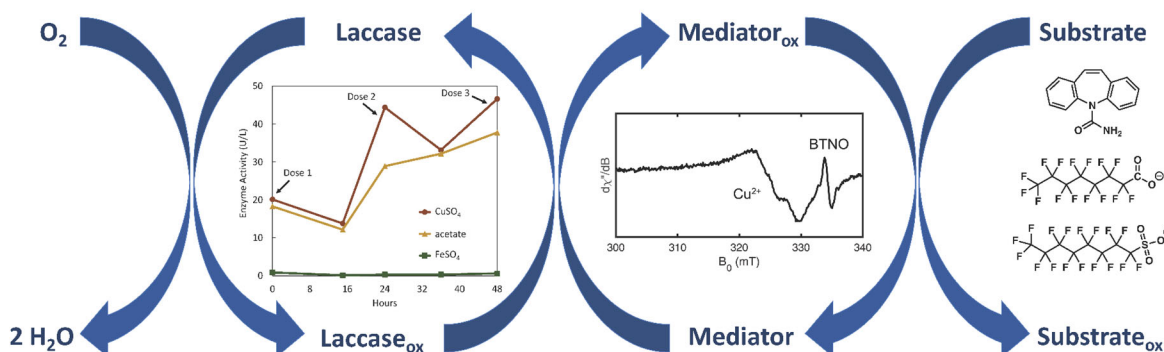


Figure 2. An overview of the strategy employed to investigate the multi-step laccase-mediator oxidation mechanism towards the target perfluorinated substrates, PFOA and PFOS, and the proxy substrate employed, carbamazepine (CBZ).

Screening of Enzyme-Mediator Combinations

While previously published work reported *Pleurotus ostreatus* laccase enzyme in combination with HBT,^{5,13} this particular laccase enzyme is no longer commercially available. However, in the proposed mechanism, the key species for initiating oxidation of the target substrates (i.e. PFOA, PFOS) is the oxidized HBT radical (Luo et al. 2015, 2018). Therefore, the species of laccase should be interchangeable, as long as the enzyme has a high enough redox potential to oxidize HBT. With the aim of expanding the relevance of LMSs for PFAS degradation, we sought to evaluate other laccase species and other nitroxyl moiety bearing mediator compounds that we hypothesized could catalyze PFOS transformation. We selected common and commercially available laccases, *Agaricus bisporus* (Sigma), white rot species (Creative Enzymes), and *Trametes versicolor* (Sigma), all of which are considered to be ‘high-redox’ species capable of oxidizing typical mediator compounds.^{36,37} Based on previously

reported mediator species capable of oxidizing PFOA and PFOS.^{4,38} we chose to screen a variety of mediator compounds (Figure 3) in order to optimize the transformation reaction. Of particular interest were nitroxyl compounds similar in structure to the compounds HBT, violuric acid, and ABTS, which were previously shown to decrease PFOA and PFOS concentrations in soil slurries.³⁸ Additionally, PFOA degradation was observed without a mediator in laccase systems containing soil slurries as well as soybean meal, perhaps indicating that natural mediators present in the soybean meal could promote degradation.¹³ Recent reports of highly efficient alcohol oxidation reactions with 2-azaadamantane (AZADO)³⁹ also inspired us to evaluate the oxidation potential towards PFAS.

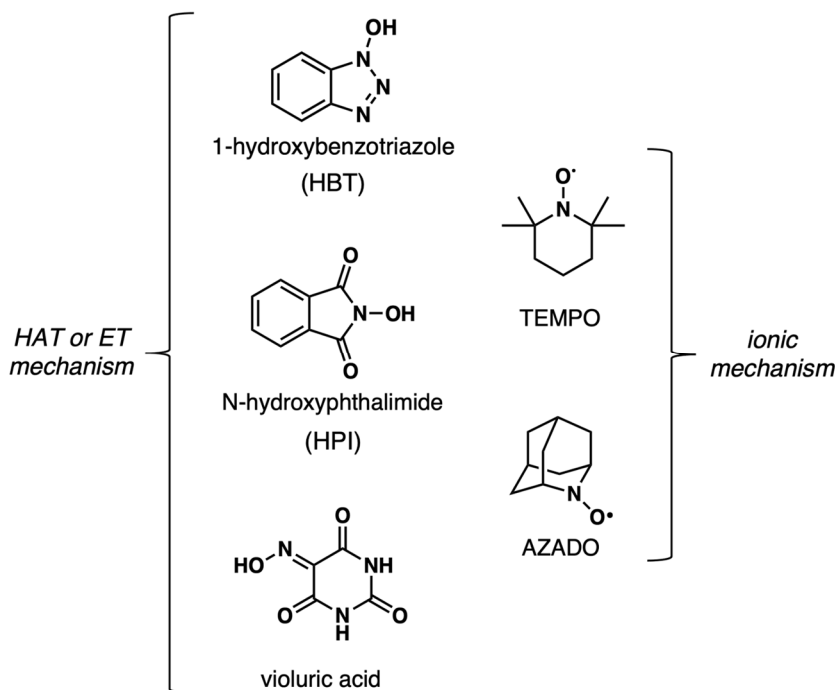


Figure 3. Nitroxyl (N-OH) mediator compounds selected for screening.

The nature of electron transfer mediated by the nitroxyl groups may differ, we wanted to evaluate the impact of the mechanism on degradation efficiency. Compounds such as HBT, violuric acid, and *N*-hydroxyphthalimide (HPI) generally perform oxidation through a hydrogen atom transfer (HAT) mechanism by abstracting a hydrogen atom from the substrate, but can also perform electron transfer (ET) in some cases.²⁹ Alternatively, compounds such as TEMPO and AZADO generally perform oxidation through an ionic mechanism, through which the radical forms a positive charge and the mediator initiates a typical nucleophilic-type reaction mechanism. Given that our primary target substrates, PFOS and PFOA, contain no hydrogen atoms at relevant pH ranges, we hypothesized that the reaction with HAT/ET type mediators should proceed via the ET route.

Additionally, in a previous study,¹³ it was shown that copper was essential to PFOS degradation in the LMS with HBT and *Pleurotus ostreatus*. It was hypothesized that the Cu²⁺ could induce a structural change in PFOS, ‘unlocking’ the helical CF₂ backbone of PFOS. While density functional theory (DFT) calculations reported in this study did not indicate a significant

change in the C–C bond strength in the backbone units, they showed a change in the dihedral bond angles in the chain. It was suggested that the decrease in the dihedral angles allowed for greater accessibility for attack by the oxidized HBT radical, facilitating the breaking of the CF₂ backbone. While it is interesting that copper may induce this change in the helical structure of PFOS, which has been similarly shown for other metal cations,⁴⁰ it is not immediately clear how a change in dihedral angle would facilitate the oxidative reaction. Based on the hypothesized function of Cu²⁺, we considered that other mediators could potentially decrease steric hindrance with the substrate, eliminating the requirement for copper and improving compatibility for *in situ* treatment.

Initial screening experiments with the selected enzymes were performed in a similar manner to those reported previously. Reactors containing PFAS mixtures in a 10 mM CuSO₄ buffer were ‘dosed’ with laccase enzyme (1 U/mL) and mediator (20 μM) every 7 days, and a solution aliquot was taken at each dosing time to measure PFAS concentrations. While the previous studies were reported over the course of 160 days, substantial transformation of PFOA and PFOS (~20%) was observed after just 20 days. Therefore, we expected to see some indication of transformation during the 20 day period, that could warrant a longer term evaluation of identified enzyme-mediator combinations. The white rot laccase in combination with TEMPO indicated minor losses in PFOS concentration over the course of 20 days, and more substantial losses of PFHxSaAm (Figure 4). A treatment with *Agaricus bisporus* enzyme and the violuric acid mediator indicated more substantial losses of PFOS and PFHxSaAm (Figure 5). The treatment with *Trametes versicolor* enzyme and the HBT and TEMPO mediators indicated similar losses for both mediators with regard to PFOS, and more substantial loss of PFHxSaAm with the TEMPO treatment (Figure 6 & Figure 7). However, there was no evidence of shorter chain PFCAs or PFSAAs on Day 20, thus leaving the question of potential transformation products unanswered.

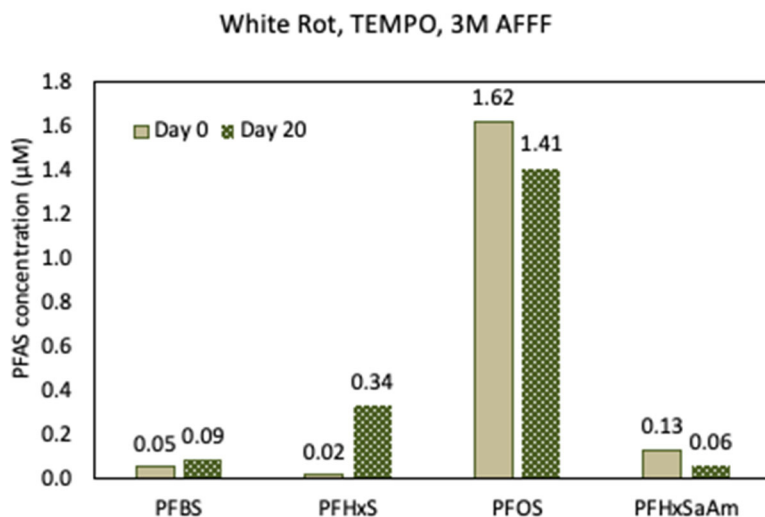


Figure 4. Treatment of a 3M AFFF solution containing PFBS, PFHxS, PFOS, and PFHxSaAm with white rot laccase (1 U/mL) and TEMPO (20 μM); 3 doses were added over the course of the 20 day period. Solution aliquots were removed and diluted in basic methanol for quantification by LC-MS/MS.

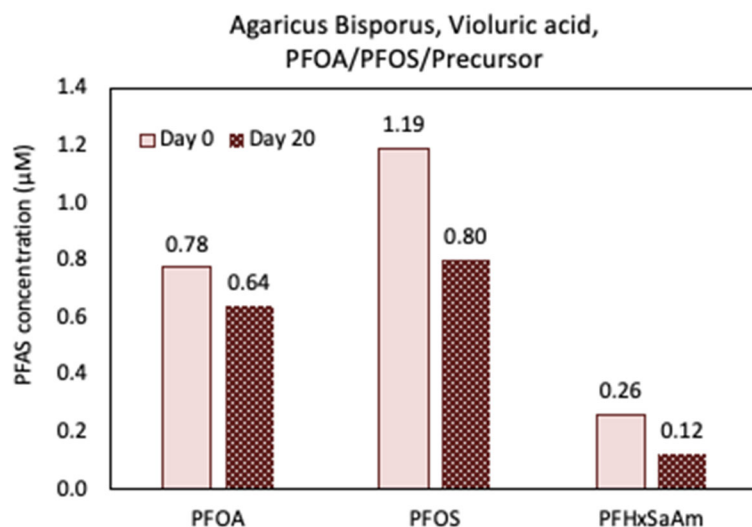


Figure 5. Treatment of a prepared PFAS solution containing PFOA, PFOS, and PFHxSaAm with *Agaricus bisporus* laccase (1 U/mL) and violuric acid (20 µM); 3 doses were added over the course of the 20 day period. Solution aliquots were removed and diluted in basic methanol for quantification by LC-MS/MS.

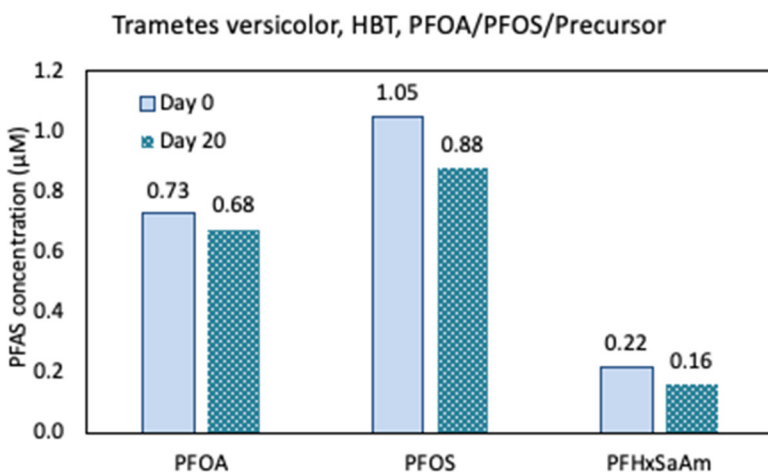


Figure 6. Treatment of a prepared PFAS solution containing PFOA, PFOS, and PFHxSaAm with *Trametes versicolor* laccase (1 U/mL) and HBT (20 µM); 3 doses were added over the course of the 20 day period. Solution aliquots were removed and diluted in basic methanol for quantification by LC-MS/MS.

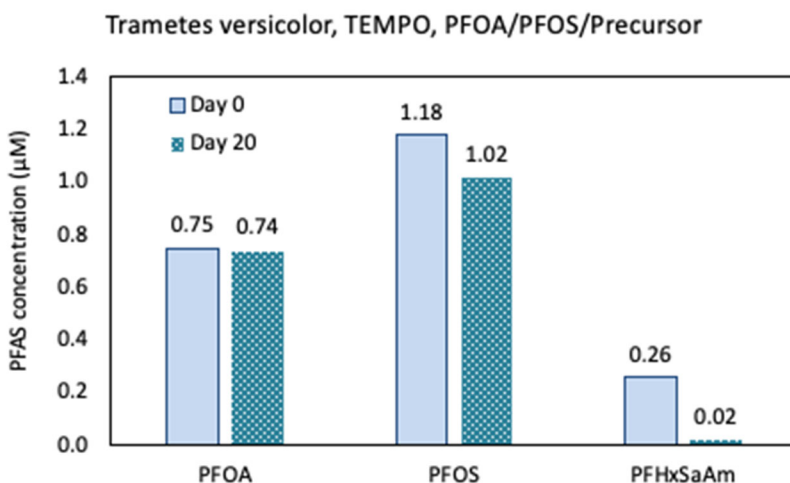


Figure 7. Treatment of a prepared PFAS solution containing PFOA, PFOS, and PFHxSaAm with *Trametes versicolor* laccase (1 U/mL) and TEMPO (20 µM); 3 doses were added over the course of the 20 day period. Solution aliquots were removed and diluted in basic methanol for quantification by LC-MS/MS.

We also conducted an experiment to screen all the selected mediator compounds in parallel and only a single target compound, PFOS. Specifically, we screened the *Agaricus bisporus* and white rot laccase, over a 28-day period. Reactors were dosed weekly with enzyme (1 U/mL) and mediator (20 µM). No significant PFOS degradation was observed (Figure 8). The data summarized below shows both the normalized concentrations (Figure 8), which were adjusted for evaporation, and the actual concentrations (Figure 9). To correct for evaporation, we compared the detected concentrations ($C_{\text{detected-Day } X}$) in the treatment reactors between sampling timepoints to the concentration in the control reactor ($C_{\text{control-Day } X}$), which contained no enzyme and no mediator. Specifically, $C_{\text{detected-Day } X}$ in each treatment reactor for Day 0, 7, 14, 21, 28 was divided by the quotient of the concentrations in the control on Day 0 and Day 0, 7, 14, 21, 28:

$$C_{\text{corrected-Day } X} = \frac{C_{\text{detected - Day } X}}{C_{\text{control - Day 0}} \div C_{\text{control - Day } X}}$$

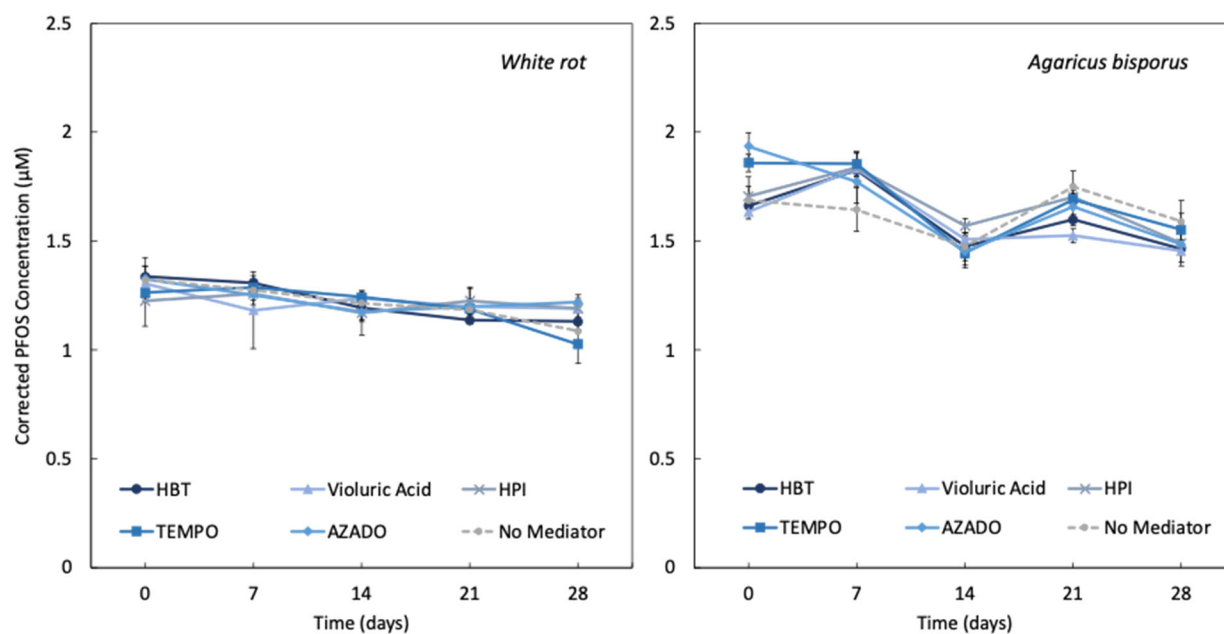


Figure 8. Screening of laccase-mediator combinations for reactivity towards PFOS. Laccase (1 U/mL) and mediator (20 µM) were added weekly. PFOS concentrations in the a) Native White Rot treated reactors and b) *Agaricus bisporus* treated reactors do not decrease significantly compared to the No Mediator control over the 28-day period ($p = 0.58$ White rot, $p = 0.24$ *Agaricus bisporus*). Error bars are the standard deviations of triplicate reactors.

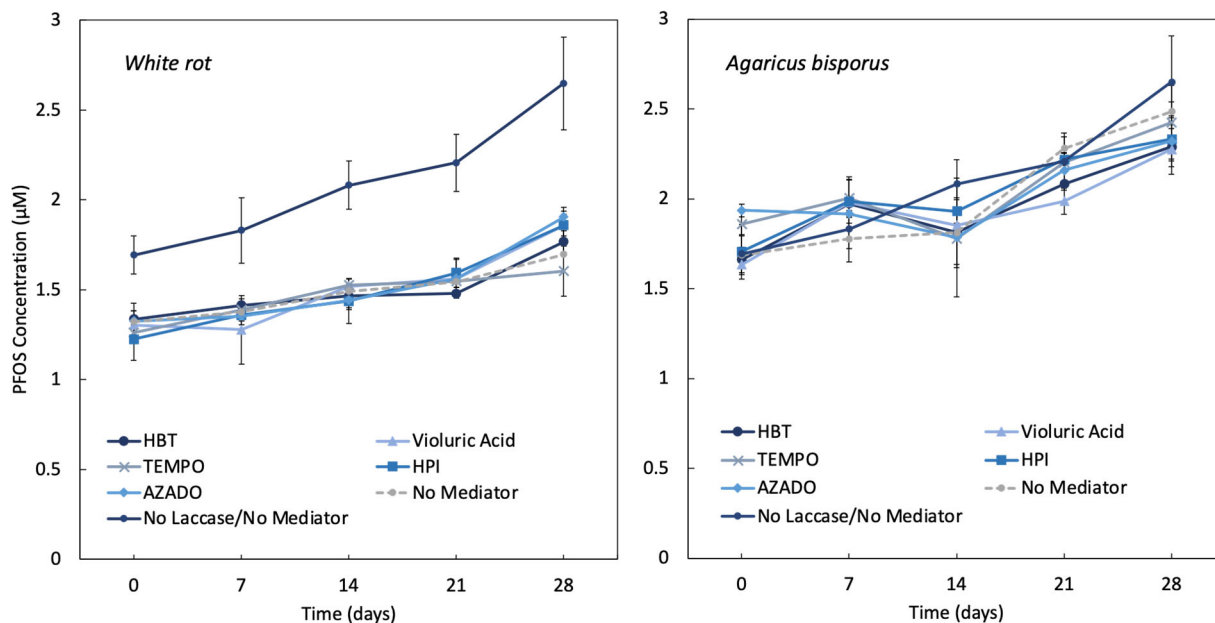


Figure 9. Measured concentrations observed in treated reactors. Concentrations appear to increase due to evaporation in the reactors over the 28-day period. Error bars are the standard deviations of triplicate reactors.

The apparent decrease in PFOS concentration across the 28 day reaction is similar among the treatment conditions and the enzyme-only control, suggesting that the laccase-mediator reaction is not initiating detectable PFOS degradation. Additionally, no detectable differences were observed between the mediator compounds screened. This suggested that the LMS was not effectively degrading PFOS among the conditions studied. Because the main difference in this system and the previously reported reaction was the enzyme itself, we wanted to further evaluate the enzyme activity.

Enzyme Activity Tests

Our initial hypothesis was that the specific laccase species (*Pleurotus ostreatus*) used in the previously reported studies was not critical to achieve transformation of the target substrates, PFOA and PFOS. Because the enzyme only is responsible for the initial oxidation step of the nitroxyl mediator, we considered that any laccase with a high enough redox potential to generate the nitroxyl radical species would be proficient in initiating the laccase-mediator reactivity towards PFOA or PFOS. The nitroxyl radical species generated can then react directly with PFOA or PFOS. Considering that the *Trametes versicolor* enzyme is one of the most well documented in terms of laccase-mediator treatment systems for water contaminants^{33,34,41}, we focused on evaluating the enzymatic activity of this particular enzyme for evaluating our treatment regimen. Generally, the enzyme activity can be extrapolated from the relationship between the change in absorbance and the concentration of enzyme (Figure 10); details of the extrapolation can be found in the Materials & Methods section.

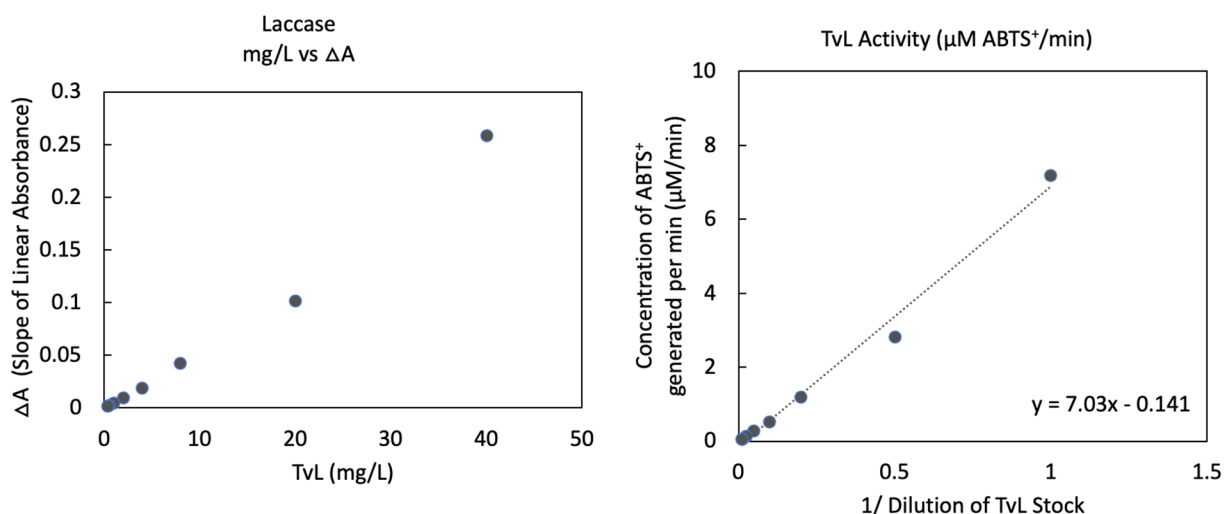


Figure 10. Determination of *Trametes versicolor* activity.

Multiple methods for a colorimetric UV-spectrophotometric assay have been reported for evaluating laccase activity. We chose to compare two assay methods, one of which used ABTS as a laccase oxidation substrate and the other of which used 2,6-dimethoxyphenol (DMP). We found that the assay utilizing ABTS indicated 100-fold higher activity than the assay utilizing DMP for *Trametes versicolor* activity.

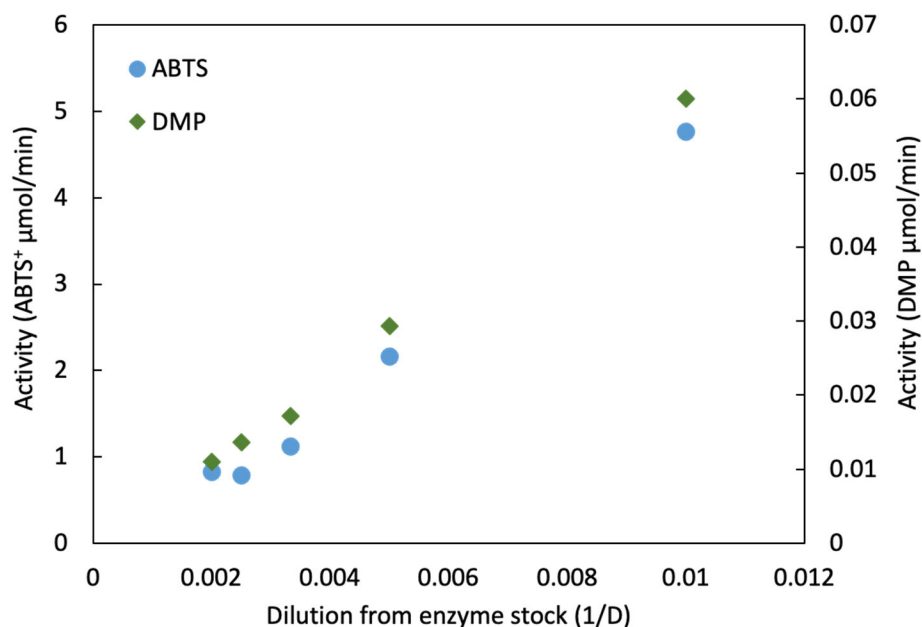


Figure 11. Comparison of *Trametes versicolor* activity using ABTS and DMP.

We compared the activity retention of *Trametes versicolor* in multiple buffer solutions, including a CuSO_4 solution and a sodium acetate buffer, which has been previously described in treatment systems with *Trametes versicolor* (cite) (Figure 12). We also compared a FeSO_4 solution, as an alternative to utilizing copper in the system. We found that enzyme activity was well retained in both the copper and acetate solutions, however, complete loss of enzyme activity was observed in the iron solution.

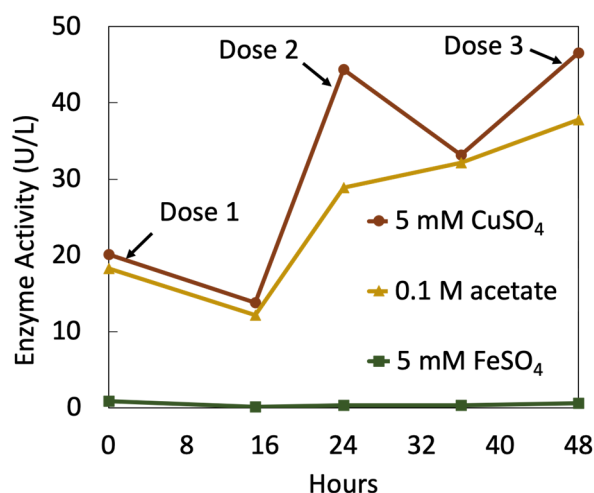


Figure 12. Monitoring *Trametes versicolor* activity in three different solutions indicated that enzyme activity was retained in 5 mM CuSO_4 solution and 0.1 M sodium acetate buffer; no enzyme activity was observed in 5 mM FeSO_4 solution.

Detection of nitroxyl radicals with electron paramagnetic resonance (EPR) spectroscopy

In addition to confirming enzyme activity by enzymatic assays, we wanted to confirm the reaction between the enzyme and the mediator was producing reactive radical species. The laccase enzyme should catalyze the conversion of HBT to the oxidized form BTNO (Figure 13). Some radical species are detectable by electron paramagnetic resonance (EPR) spectroscopy, if they have a long enough lifetime. EPR has been used to evaluate LMSs previously, however, these studies focused on lignin systems, rather than on organic contaminants.^{42,43}

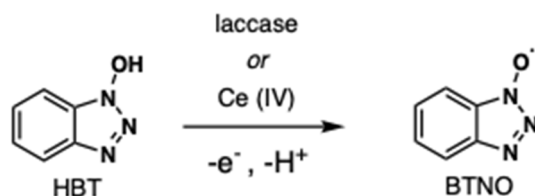


Figure 13. Oxidation of 1-hydroxybenzotriazole (HBT) to the radical species benzotriazole-N-oxyl (BTNO) by electron and proton transfer. The oxidation can be carried out by Ce (IV) ammonium nitrate in CH₃CN in an equimolar ratio, or by laccase in an aqueous buffer solution.

Following a previous report,²⁹ we were able to generate the BTNO radical in the CH₃CN solution by oxidizing HBT with Ce (IV). The room-temperature CW EPR spectrum of the resulting dark solution showed a hyperfine-split feature that can be simulated using previously reported ¹⁴N and ¹H hyperfine parameters (Figure 14).²⁹ Formation of the BTNO radical in aqueous solution was also observed by incubating TvL laccase and HBT at the presence of Cu²⁺. However, the hyperfine patterns were not resolved in the EPR spectrum of the frozen aqueous solution, which may have been due to hydrogen bonding effects between water and the radical species causing line broadening in the spectrum. Similar EPR signals for N-oxide radicals in aqueous solutions have been documented previously.⁴³

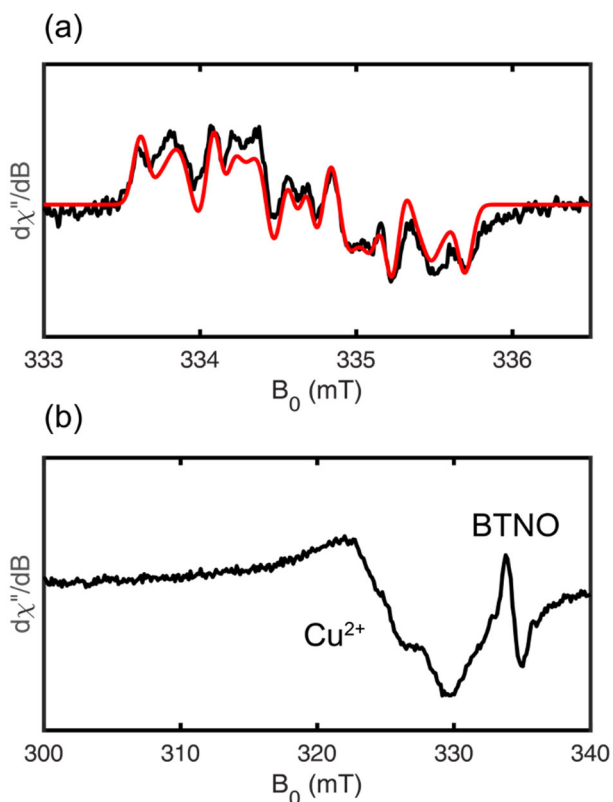


Figure 14. X-band CW EPR spectra. (Top) Room temperature EPR spectrum of BTNO radical generated in CH₃CN (black trace) and simulation (red trace). Simulation parameters: $g = 2.0069$, three ^1H with $a(^1\text{H}) = 1.0, 5.7$ and 12.8 MHz, three ^{14}N $a(^{14}\text{N}) = 1.6, 4.3$ and 13.3 MHz. (Bottom) EPR spectrum of BTNO radical generated in the laccase system. The feature on the left is due to Cu^{2+} EPR signal.

We hypothesized that EPR may give some indication as to whether the HBT radical interacted with the target perfluorinated substrates. We evaluated the reaction under similar conditions used in the aqueous reactors monitored via LC-MS/MS (2 U/mL TvL, 1 mM HBT); we chose to use a lower copper concentration (10 μM rather than 10 mM) to avoid interference with the organic radical signal. The decrease in the radical signal within 20 minutes upon PFOA and PFOS addition could suggest an interaction between BTNO and the substrate; however, the signal increased to the initial level for both substrates after 120 minutes (Figure 15). While it is unclear why the addition of PFOS and PFOA causes the radical signal to decrease, a parallel experiment monitoring PFOA and PFOS concentration by LC-MS/MS confirmed that no removal was detected under these conditions (Figure 16).

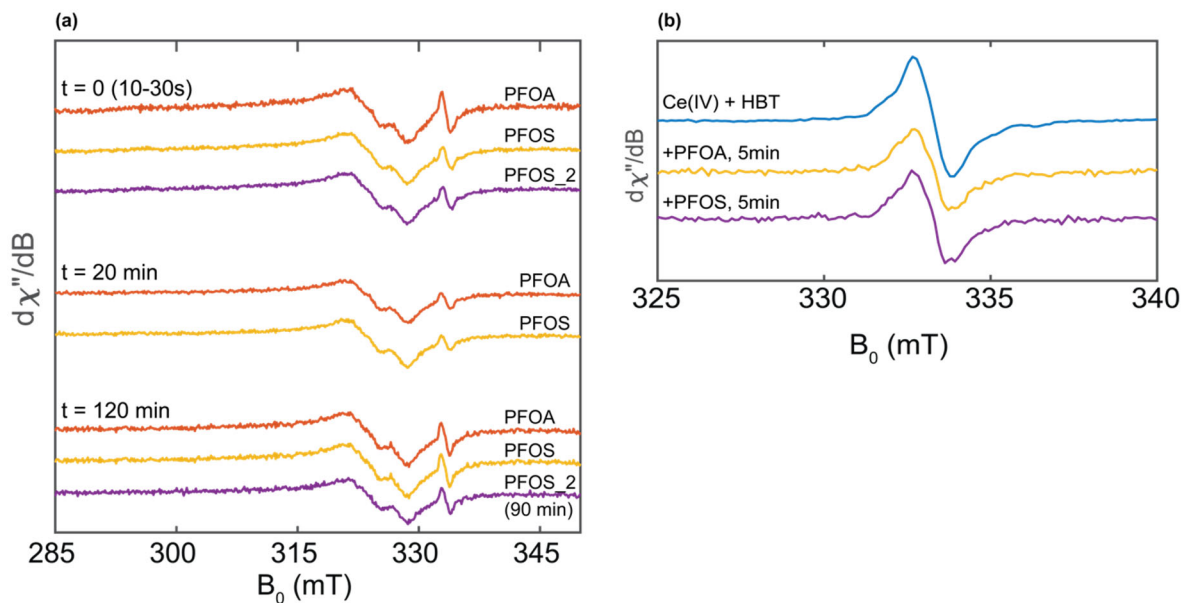


Figure 15. Detection of BTNO radical signal (335 mT) in the presence of PFOA, PFOS in a) a *Trametes versicolor* aqueous solution and b) in an acetonitrile solution with Ce(IV) catalyst.

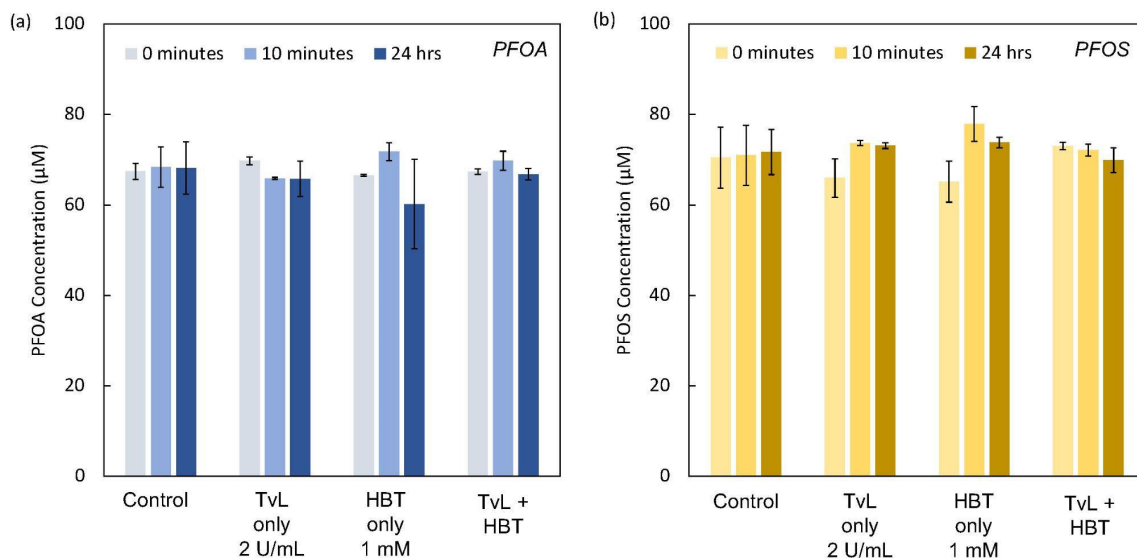


Figure 16. a) PFOA and b) PFOS concentrations monitored by LC-MS/MS in reaction conditions replicated from the EPR experiment. A reactor containing 2 U/mL TvL and 1 mM HBT in 10 μM CuSO_4 was incubated for ~ 45 minutes to allow for substantial radical generation; 200 μL aliquots were then taken and added to microcentrifuge tubes to which 50 μM PFOA, PFOS were added to directly. Reactions were quenched by addition of 200 μL MeOH directly to the microcentrifuge tubes and diluted for LC-MS/MS analysis. Error bars represent standard deviations of triplicate reactor conditions.

Reactivity towards a proxy compound

Upon observing no apparent degradation of PFOS or PFOA in the LMSs initially screened, we chose to evaluate the degradation of a proxy compound, carbamazepine (CBZ), in order to probe the reactivity of the system. The evaluation of enzyme activity and reactive radical generation left us to conclude that the enzyme-mediator oxidation reaction was occurring, and we wanted to confirm that the system as a whole (buffer, timing of enzyme-mediator doses) was conducive to contaminant transformation. Though non-fluorinated, CBZ is hydrophobic ($\log K_{ow} > 2$)⁴⁴ and generally requires a strong oxidizing agent (e.g., hydroxyl radical) for complete mineralization.⁴⁵ Multiple other researchers have investigated LMS treatment for CBZ as an environmentally benign approach that requires lower energy inputs and avoids accumulation of a waste stream.³³ It has been shown that *Trametes versicolor* laccase (TvL) and HBT can result in CBZ removal of up to 60%.⁴¹ Both *Trametes versicolor* and *Pleurotus ostreatus* are considered high redox potential species, with redox potentials of 800 mV (vs. NHE)⁴⁶ and 650-740 mV (vs. NHE),³⁷ respectively. With this assumption, submitting PFOS and PFOA to the reaction conditions capable of CBZ transformation, should result in oxidation of the perfluorinated substrates as well.

We initially tested *Trametes versicolor* in an attempt to replicate previous reports of this system for CBZ degradation.⁴¹ We evaluated both the effect of enzyme dose (Figure 17) and mediator concentration (Figure 18) on CBZ removal. We also evaluated two alternative enzymes, an additional high redox laccase enzyme, *Aspergillus* (770 mV vs. NHE,⁴⁷) (Figure 19). We chose to proceed evaluating the reaction with *Trametes versicolor* and HBT given that previous researchers had also reported the success of the HBT mediator strategy.^{5,13} In evaluating the effect of mediator dosage with *Trametes versicolor*, we observed that higher mediator doses (1 mM, 5 mM) may slightly improve the removal efficiency of CBZ ($C_0 = 20 \mu\text{M}$) compared to the ‘No Mediator’ control (Figure 18). To improve the removal efficiency, we increased the enzyme dose from 0.6 U/mL to 2 U/mL and decreased the initial concentration of CBZ 0.1 μM . These conditions resulted in approximately 35% removal of CBZ over the course of 120 hours (Figure 20). While we were satisfied with these results as an indication that the laccase-mediator reaction was occurring, it should be noted that we were not able to achieve the 60-95% removal levels reported previously.^{33,41}

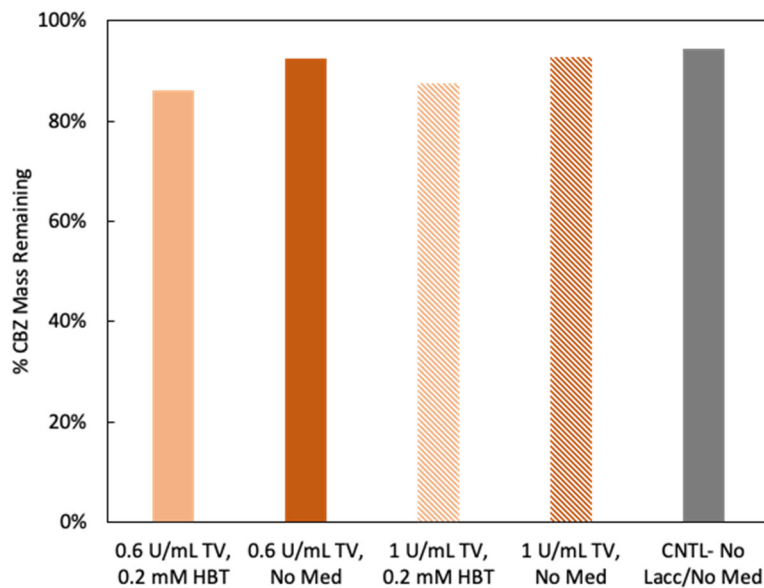


Figure 17. Comparison of 0.6 - 1 U/mL doses of *Trametes versicolor* (TV). Mass of CBZ measured pre-treatment and 48-hours after treatment; 4 doses of TV were added over the course of 48 hours. Reactors were contained in a shaker incubator at 30°C, 130 rpm. Values are averages of duplicate reactors.

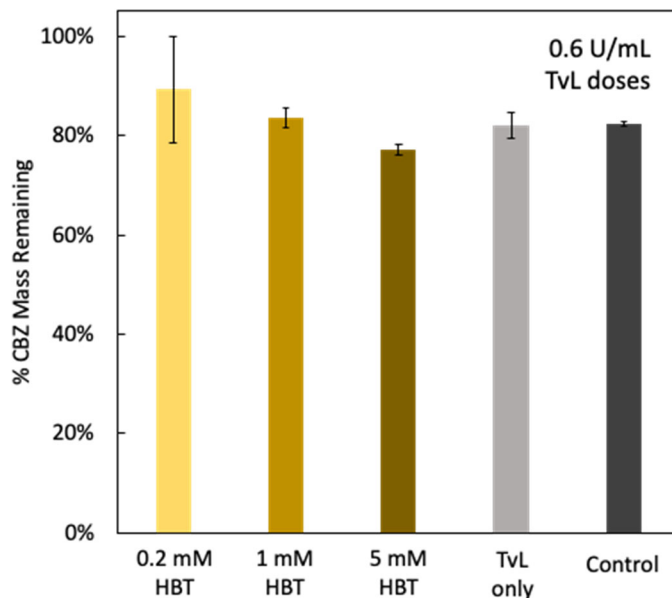


Figure 18. Comparison of 0.2 - 1 mM doses of HBT with *Trametes versicolor*. Mass of CBZ measured pre-treatment and 120-hours after treatment; 4 doses of TV were added over the course of 120 hours. Reactors were contained in a shaker incubator at 30°C, 130 rpm. Error bars are standard deviations for triplicate reactors.

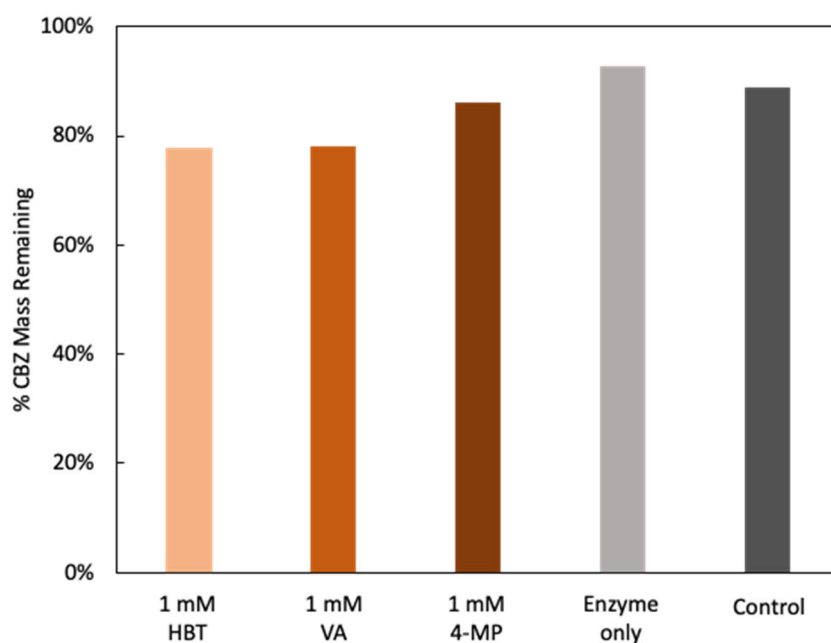


Figure 19. Comparison of HBT, violuric acid (VA), and 4-methoxyphenol mediators with *Aspergillus* laccase enzyme (2.5 U/mL). Mass of CBZ measured pre-treatment and 72-hours after treatment; 4 doses of *Asp.* and mediator were added over the course of 72 hours. Reactors were contained in a shaker incubator at 30°C, 130 rpm. Values are averages of duplicate reactors.

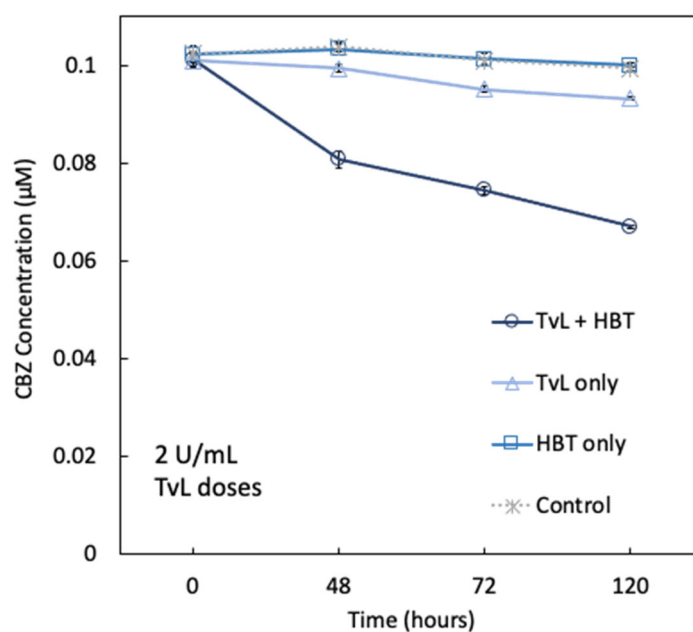


Figure 20. CBZ degradation with *Trametes versicolor* (2 U/mL) and HBT (1 mM) treatment over 120 hours. Error bars are standard deviations for triplicate reactors.

Replicating system for reactivity of TvL towards PFOA

Upon confirming the reactivity of the *Trametes versicolor* and HBT system towards a proxy compound, we evaluated the reactivity towards another previously reported LMS substrate, perfluorooctanoic acid (PFOA), the carboxylic acid analogue of PFOS. The degradation of PFOA was previously reported by an LMS system.^{4,5,38} As a typically easier to oxidize substrate than PFOS using other destructive technologies,²⁴ we believed that we might have greater success in reproducing PFOA removal and degradation. Additionally, reports of PFOA degradation in some iterations of the LMS studied included solids in the reaction matrix, and we hypothesized that the addition of solids may have impacted laccase reactivity.

To evaluate this hypothesis, we compared treatment of a solution spiked with 1 μ M PFOA in soil and soil-free reactors. The treatment conditions included an enzyme-only control, as well as an untreated control to account for potential sorption effects to the soil or the reactor containers. We chose to dose reactors more frequently over a shorter period of time— 6 doses over two weeks— based on the previously reported conditions³⁸ as well as the loss of enzyme activity we observed throughout the dosing regimen.

At each timepoint, triplicate reactors were sampled sacrificially by adding MeOH directly to the vial such that the volume was doubled; the solution was then allowed to equilibrate overnight before an aliquot was taken for LC-MS/MS analysis. As shown in Figure 21, neither the enzyme-mediator treatment nor the enzyme-only treatment resulted in removal of PFOA compared to the control. Additionally, no difference in removal was observed between the soil and soil-free conditions. However, the soil did impact the retention of enzyme activity (Figure 22). Between doses (approximately 48-hours), the enzyme activity decreased nearly to zero in the soil-free reactors dosed with laccase and mediator, while it decreased by roughly 60% in the soil-containing reactors. Similarly, in the reactors dosed with enzyme-only, the enzyme lost approximately 56% of its activity in the soil-free reactors, and only 35% in the soil-containing reactors. While the higher enzyme activity retention in the soil-containing reactors did not apparently improve PFOA removal, this observation did provide us with important insight for the dosing regimen. The near complete loss of activity within 48 hours in the soil-free reactors suggested that dosing frequency could be increased to minimize the amount of time that laccase was inactive during experiments.

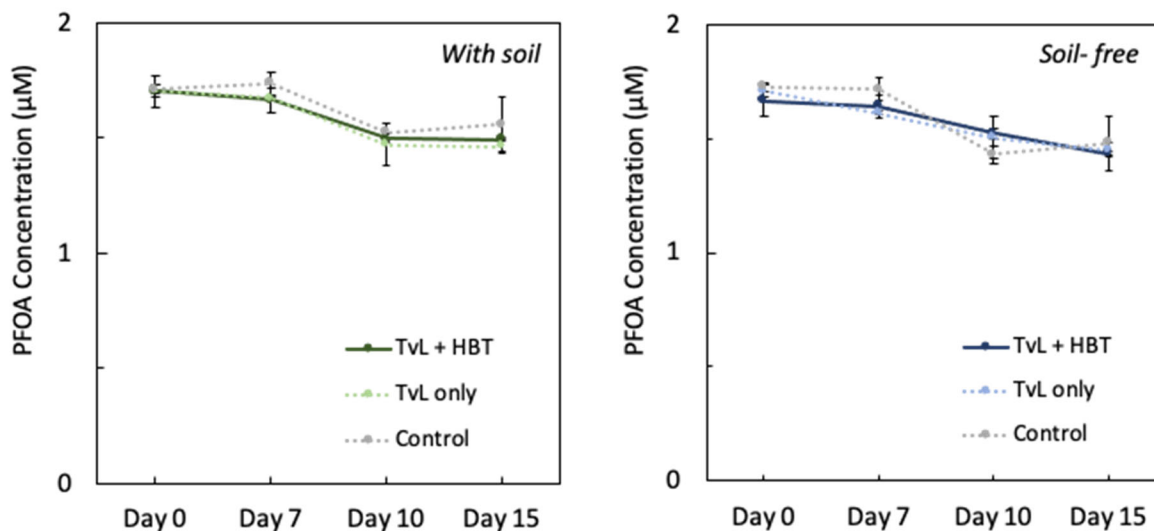


Figure 21. PFOA transformation does not proceed in soil or soil-free conditions over the course of 15 days compared to an untreated control; TvL (2 U/mL) and HBT (1 mM) were added 6x total every 2-3 days.

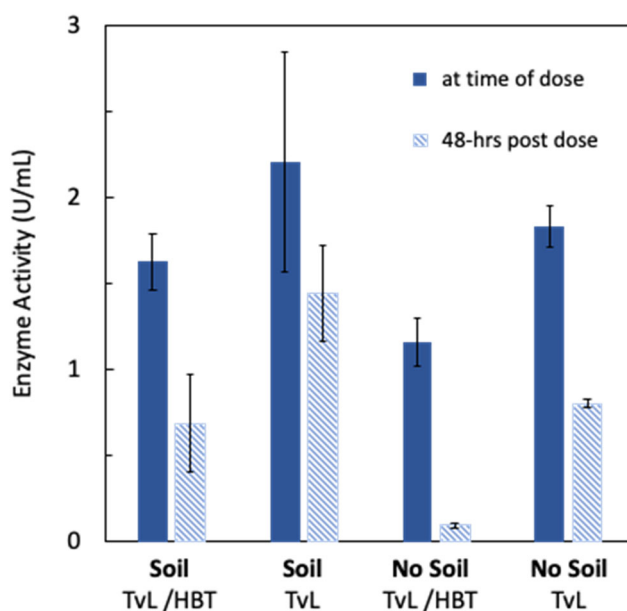


Figure 22. Enzyme activity comparison between soil and soil-free reactors. Enzyme activity is better retained in soil-containing reactors; addition of mediator decreases enzyme activity over the course of the experiment as would be expected by the enzyme-mediator oxidation reaction.

Sorptive removal with low concentration substrate

Considering that slow reaction kinetics might be preventing detectable transformation of PFOA or PFOS with the substrate concentrations initially tested (e.g., 1 μ M PFOA, 1 μ M PFOS), we lowered the concentrations to either 0.1 μ M PFOA or 0.1 μ M PFOS in a 10 mM Cu_2SO_4 buffer. The reactors were treated with 1 U/mL *Trametes versicolor* and 1 mM HBT; doses of enzyme and mediator were added twice daily, for four days. We increased the frequency of dosing because we observed that enzyme activity decreased nearly to zero over the course of 6-8 hours (Figure 23). Aliquots were removed daily to measure concentration.

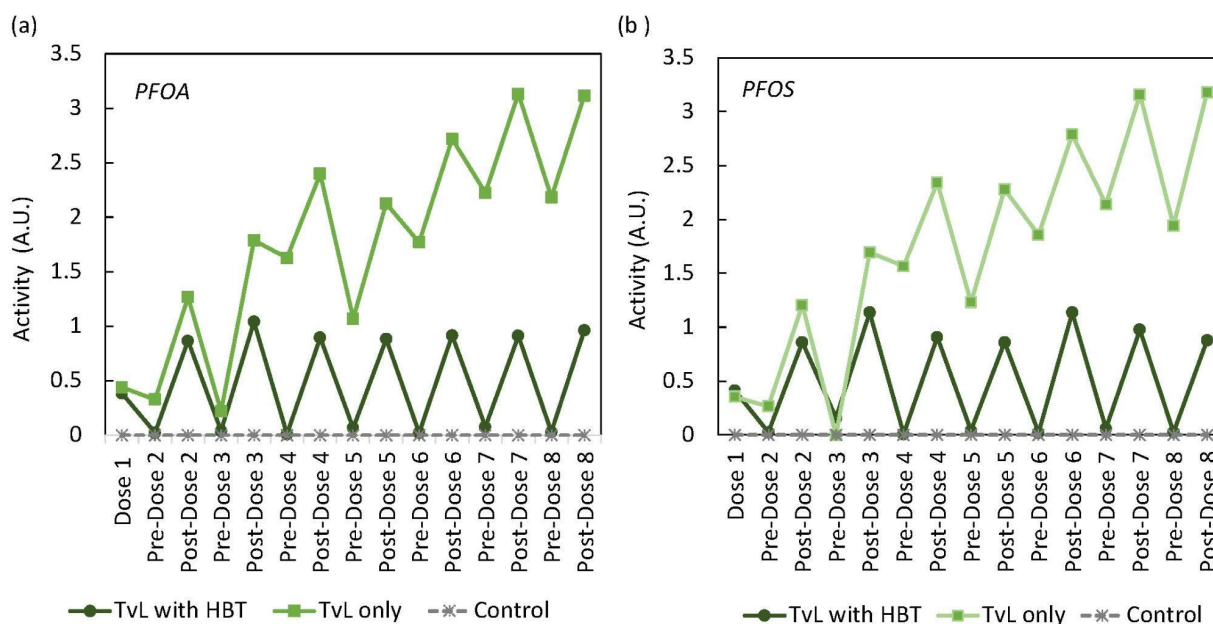


Figure 23. Enzyme activity in a) PFOA reactors and b) PFOS reactors monitored by the DMP assay over the course of the reaction; reactors treated with ~1 U/mL TvL and 1 mM HBT. Doses were added twice daily, with approximately 6-8 hours in between doses.

Monitoring the concentration in the reactors revealed an apparent decrease in both PFOA and PFOS concentration after the first two doses of enzyme and mediator were added (within the first 24 hours), then the concentration appears to level off (Figure 24). At the end of the experiment, the reactors were extracted with basic MeOH. Upon analyzing the total mass contained in the reactors at the beginning and end of the experiment, and comparing it to the mass determined from the ‘extract’ concentration, both PFOA and PFOS were fully recovered. The recovery of PFOS and PFOA suggests that the initial decrease in concentration is likely due to physical processes in the system (e.g. sorption). Both PFOA and PFOS have been shown to have a strong affinity for proteins, namely human serum albumin (HSA)^{48,49} and bovine serum albumin (BSA).^{50,51} Recently, one group of researchers even investigated proteins as adsorbents

for PFOA, including BSA, casein, egg white albumin, and lysozyme,⁵² and found that these proteins could achieve up to 93% removal depending on the aqueous conditions. The adsorption mechanism in proteins is understood to be primarily due to hydrophobic interactions, and specific protein binding sites have been reported for proteins of interest such as HSA.⁵³

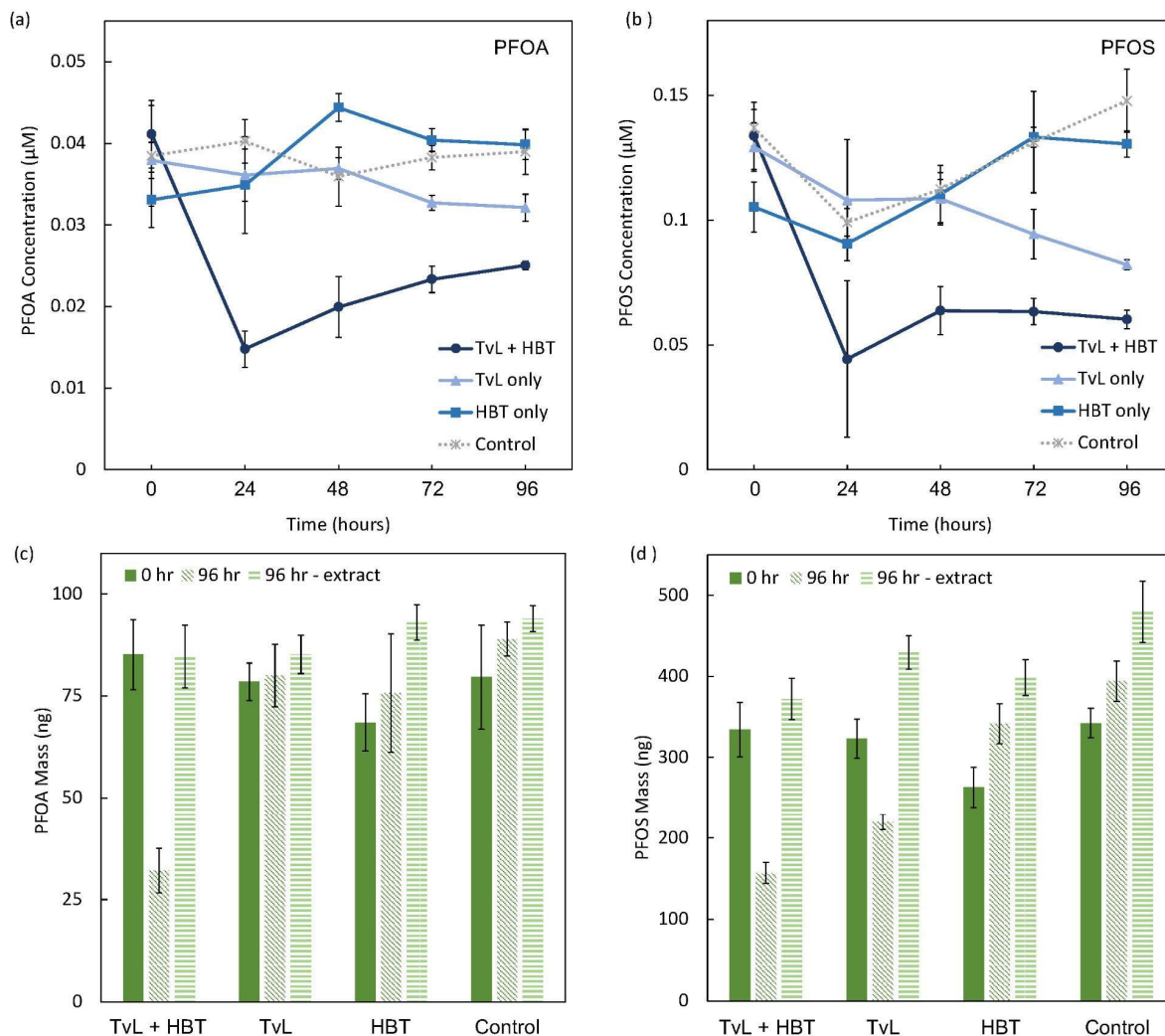


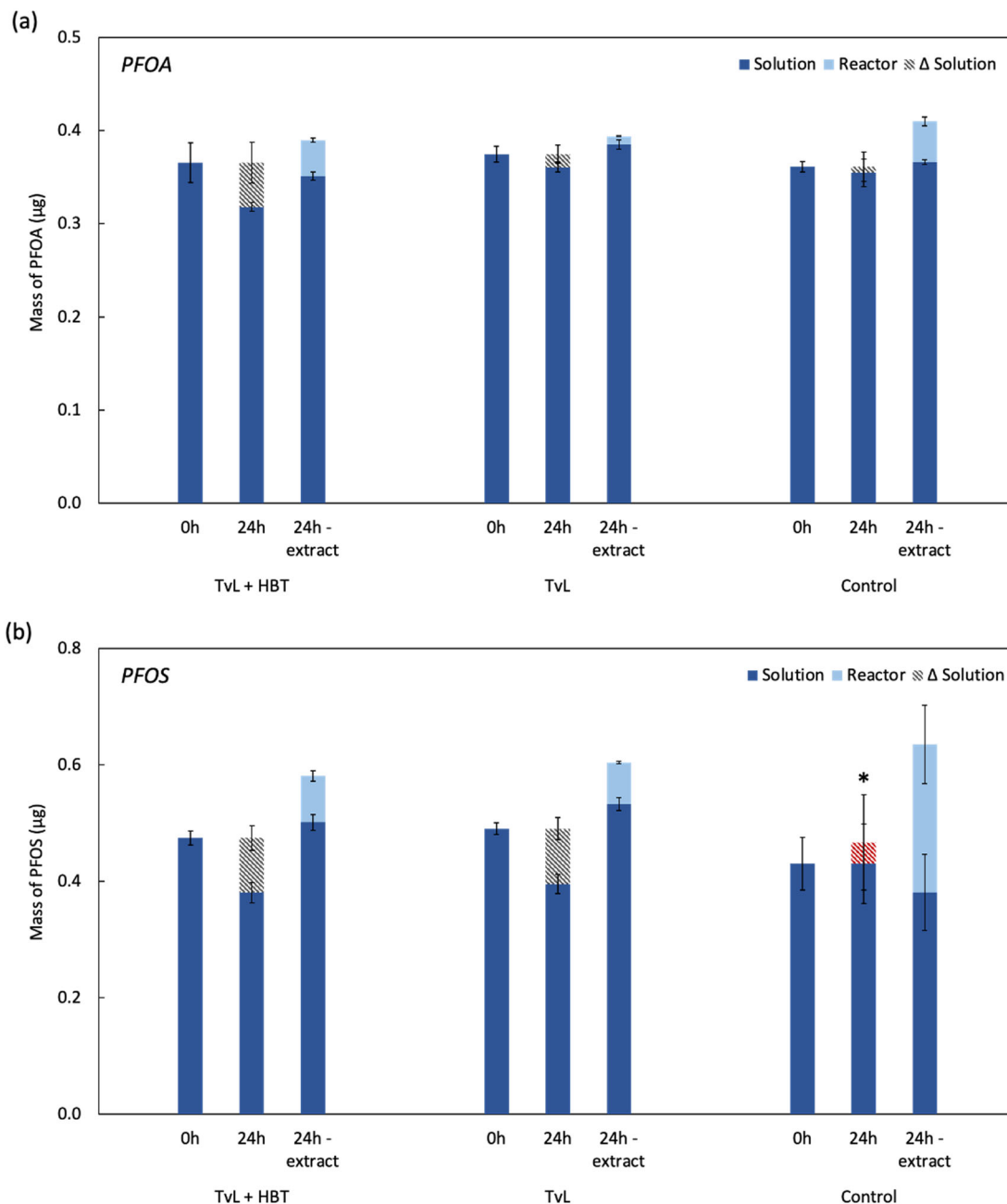
Figure 24. Reactors were amended with TvL and HBT twice daily. a) Concentration of PFOA and b) Concentration of PFOS monitored via removal of an aqueous aliquot from the reactors; c) Mass of PFOA and d) Mass of PFOS in reactors calculated from detected concentration and total solution volume at the beginning of the experiment, at the 96 hour time point, and in the extracted solution volume at the 96 hour time point. Error bars are standard deviations of triplicate reactors.

The enzyme-only conditions do not appear to promote significant sorption of PFOA compared to PFOS, while the enzyme-mediator conditions appear to promote sorption of both

substrates. Molecular docking studies have shown that changes in the enzyme oxidation state significantly change certain structural characteristics of the protein.⁵⁴ It is possible that the binding mechanism of PFOS is relatively unaltered regardless of the enzyme oxidation state (i.e., non-specific hydrophobic driving forces) while the binding affinity of PFOA is affected by the enzyme oxidation state (i.e., specific interaction with a protein side chain), hence indicating sorptive behavior only in the presence of the enzyme and mediator. The apparent increase in PFOA and PFOS, particularly in the enzyme-free treatment conditions, may be due to sorption to the reactors which was not accounted for in the solution aliquot taken at the initial timepoint.

To further evaluate sorption to the reactor container and the enzyme, and differentiate these two sorption processes, we conducted an experiment in which enzyme-containing solution and reactors were extracted separately with basic methanol. The results of this experiment are summarized in Figure 25.

Figure 25. Reactors were treated with a single dose of 2 U/mL (60 mg) TvL and 2 mM HBT. Detected mass of a) PFOA and b) PFOS was measured from a solution aliquot immediately after reactor preparation (0h) and after 24 hours (24h) of incubation; Δ Solution is calculated from the mass detected in the aliquot between 0-24 hours (i.e., the mass attributed to protein sorption). At 24 hours, the protein-buffer solution and the reactor were extracted separately with basic MeOH. The mass of PFAAs from the extracted solutions was measured for comparison. Error bars are



the standard deviation of triplicate reactors. *Note: Δ Solution for the PFOS control is negative and is plotted overlaying the 24h value.

We observed an apparent PFOA loss in the TvL/HBT treated reactors of $18 \pm 2\%$ compared to the total PFOA mass extracted (Figure 25a, Table 2); for PFOS, we observed an apparent loss of $34 \pm 4\%$ compared to the total PFOS mass extracted (Figure 25b, Table 2). In the reactors containing only the TvL enzyme, we observed similar losses for PFOS of $35 \pm 3\%$ compared to the total PFOS mass extracted; for PFOA, however, substantial mass loss was not observed. Upon extracting PFOS from the reactors, we observed that PFOS sorption to the

reactor was greater in the enzyme-free control ($40 \pm 12\%$ of total mass) relative to the TvL/HBT ($14 \pm 2\%$ of total mass) and TvL ($12 \pm 1\%$ of total mass) treatments (Table 2), suggesting that sorption to the protein was more favorable. Sorption of PFOA to the reactor was more similar among the treatments and control (Table 2).

The difference in mass detected in the 0h and 24h subsample (Δ Solution) can be reasonably attributed to enzyme sorption. For PFOA, a $13 \pm 6\%$ mass loss was observed in solution treated with TvL/HBT, while substantial mass loss was not observed in the TvL treated or control reactors. For PFOS, a $20 \pm 5\%$ mass loss was observed in solution treated with TvL/HBT, and a $19 \pm 4\%$ mass loss was observed in solution treated with TvL only. As shown in Figure 25, the Δ Solution mass (hashed bar) indicates a complete mass balance, within error, compared to the total mass extracted from the protein-buffer solution.

Table 2. PFOA and PFOS mass recovery (mass detected in Subsample, Solution extract, Reactor extract, and Δ Solution, calculated as the difference in mass detected in the subsample at 0h and 24h) compared to total extracted mass.

PFOA		Mass Recovered	Error
TvL + HBT			
	<i>Subsample (24 hours)</i>	82%	1.7%
	<i>Solution extract</i>	90%	1.2%
	<i>Reactor extract</i>	10%	0.6%
	Δ <i>Solution</i>	13%	6.1%
TvL			
	<i>Subsample (24 hours)</i>	92%	1.8%
	<i>Solution extract</i>	98%	1.3%
	<i>Reactor extract</i>	2%	0.1%
	Δ <i>Solution</i>	4%	2.6%
Control			
	<i>Subsample (24 hours)</i>	87%	3.6%
	<i>Solution extract</i>	89%	0.6%
	<i>Reactor extract</i>	11%	1.2%
	Δ <i>Solution</i>	2%	4.4%

PFOS		Mass Recovered	Error
TvL + HBT			
	<i>Subsample (24 hours)</i>	66%	3.5%
	<i>Solution extract</i>	86%	3.3%
	<i>Reactor extract</i>	14%	1.6%
	Δ <i>Solution</i>	20%	4.5%
TvL			
	<i>Subsample (24 hours)</i>	65%	3.1%
	<i>Solution extract</i>	88%	2.5%
	<i>Reactor extract</i>	12%	0.4%
	Δ <i>Solution</i>	19%	4%
Control			
	<i>Subsample (24 hours)</i>	73%	16.5%
	<i>Solution extract</i>	60%	13.5%
	<i>Reactor extract</i>	40%	12.1%
	Δ <i>Solution</i>	– 8%	19.0%

Conclusions & Implications

While attractive as a bio-based treatment method for PFAS remediation, laccase-mediator systems appear to be unsuccessful at reliably degrading the perfluorinated compounds PFOA and PFOS. Previous studies had shown promising transformation results of PFOA and PFOS in a treatment system with the high redox laccase species, *Pleurotus ostreatus* and the nitroxyl mediator HBT.^{5,13} We were unable to reproduce transformation with multiple other enzymes and mediators tested, namely, *Trametes versicolor* and HBT, despite the multiple literature reports of this system for contaminant degradation and our own success at replicating the *Trametes versicolor* and HBT system for treatment of another prevalent contaminant, carbamazepine.

A thorough investigation of the proposed, multi-step oxidation mechanism, including confirming the enzyme activity and activity retention, the enzyme-mediator reaction (i.e., radical generation), and the reactivity of the radical mediator towards a proxy substrate, carbamazepine, led us to conclude that the oxidative mechanism and reaction conditions were effective. However, lack of substantial removal of the target perfluorinated substrates (PFOA, PFOS), indicated that the system was not effective at oxidizing these species. This was not altogether surprising, given the recalcitrant nature of the compounds, and the extremely stable C–F bonds required to break for transformation. Additionally, laccase enzymes on their own typically react directly with substrates containing phenol or aniline functional groups (i.e., native to lignin), while nitroxyl-type mediators typically react directly to oxidize alcohols,²⁹ both of which have substantially lower bond strengths and redox potentials than the perfluorinated compounds.

While we did not observe transformation of PFOA and PFOS, close examination of the treatment reactors did reveal a removal artifact that we attributed to protein sorption. Analysis of the substrate mass contained in the protein-buffer solutions, and residual substrate mass on the reactor container, revealed that sorption to both enzyme and the reactor container contributed to apparent concentration decreases in the reaction system. These concentration decreases could appear as a removal artifact of 20-30%; however, extraction of the reactors resulted in full recovery of the substrates at the end of treatment times. Sorptive removal of PFAAs is a previously reported phenomenon, and can be attributed to the hydrophobicity of the PFAAs as well as specific interactions between PFAA functional groups (i.e., carboxylate) and protein side chains.

This project set out to learn if laccase-mediator systems could be applied as a robust, bio-based treatment strategy for PFAA transformation. In particular, we sought to combine enzyme based treatment with well studied chemical oxidation mechanisms (e.g., HAPPO), to achieve complete mineralization of PFAAs in situ. We considered that the multiple components of the laccase-mediator system (enzyme, mediator, buffer, metals) lend themselves to a highly tunable system, which we thought could be optimized for kinetics and treatment efficiency. Ultimately, we found that laccase-mediator systems could not reliably transform PFAAs, but rather, that laccase enzymes remove PFAAs via adsorption. Enzyme adsorption technologies may not be feasible on a scale necessary to treat PFAAs, however, further investigation of the enzyme-PFAA binding modes and interactions may provide inspiration to novel sorptive materials capable of selective PFAA removal from complex contaminant matrices.

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Appendices

A. List of Publications

Steffens, S. D.; Antell, E. H.; Cook, E. K.; Rao, G.; Britt, R. D.; Sedlak, D. L.; Alvarez-Cohen, L. An Artifact of Perfluoroalkyl Acid (PFAA) Removal Attributed to Sorption Processes in a Laccase Mediator System. *Environ. Sci. Technol. Lett.* **2023**, *10*(4), 337-342.