DEGRADATION OF PERFLUOROALKYL ACIDS BY ENZYME CATALYZED OXIDATIVE HUMIFICATION REACTIONS

by

QI LUO

(Under the Direction of Qingguo Huang)

ABSTRACT

The historical mass production, long term utilization and direct disposal of perfluoroalkyl acids (PFAAs) resulted in the wide distribution of such chemicals in the environment. Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) are two predominant environmental PFAAs which could induce adverse effects in humans and the environment. However, this group of chemicals is extremely persistent and resistant to traditional degradation technologies. Thus developing a cost-effective and eco-friendly remediation technique is needed to resolve the concerns of PFAAs contamination. Our data showed that an enhanced natural process called enzyme catalyzed oxidative humification reactions (ECOHRs) is effective in transforming both PFOA and PFOS in the aqueous phase at environmentally relevant conditions. The decomposition of PFOA and PFOS with the addition of 1-hydroxybenzotriazole as the model mediator and laccase as the model enzyme both followed a pseudo-first order reaction.

Understanding the mechanisms of PFOA and PFOS transformation are critical in identifying optimal degradation conditions. High-resolution mass spectrometry results revealed that the primary degradation products were partially fluorinated shorter-chain alcohols and aldehydes. The proposed reaction mechanism involved the dissociation of COO^- and SO_3^- from

the PFCAs and PFASs respectively to form corresponding perfluoralkyl radicals, such radicals could further went through rearrangement or cross-coupling with the non-fluorinated compounds in the reaction solution.

Additionally, we investigated the PFOA degradation efficiency by ECOHRs in soil. In the system without addition of any mediator, the degradation of PFOA up to 24% over 12 weeks of reaction was observed indicating that soil organic matter could serve as mediator. Such process could further enhanced by supplying extra natural organic matters into the system to sustain the ECOHRs. We identified a natural organic matter, i.e. soybean meal as a great mediator which could convert 36% of PFOA after 20 weeks of incubation.

The data collected in our experiments suggested that both PFOA and PFOS could be effectively degraded into environmentally benign products during ECOHRs under mild reaction conditions. Therefore, ECOHRs has a promising future in the remediation of PFOA and PFOS.

INDEX WORDS: Enzyme catalyzed oxidative humificaiton reactions, perfluorooctanoic acid, perfluorooctanesulfonate, remediation, transformation mechanism

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DEDICATION

I dedicate this work to my lovely family – my mom, Shundi, dad, Zhiping, uncle, Xingping, and, in particular, my late aunt, Bonnie, who passed away in June 2010 after years of courageous fight with cancer. Without their support and encouragement, I cannot possibly make my way to UGA and complete this work.

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It is rather difficult to express in just few lines, my gratitude to all the people who helped me, in one way or another, to accomplish this work. I hope that those that I have mentioned realize that my appreciation extends far beyond the ensuing paragraphs.

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CHAPTER 1

INTRODUCTION

Perfluoroalkyl acids (PFAAs) are a group of compounds which have diverse applications and are widely distributed in the environment[1-7]. The extensive use of PFAAs is largely attributed to their extreme thermal and chemical stability. Such stability stems from the unique structural features of PFAAs in which the majority of the hydrogens in the carbon-carbon skeleton are replaced by fluorines. Among PFAAs, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are two typical and predominant PFAAs in the environment, and they are of the greatest concern due to their persistence, potential bioaccumulation, and toxicity to humans and animals[5]. PFAAs have been detected in nearly all environmental matrices, wildlife, and humans around the world[3, 5, 6, 8-11]. Environmental monitoring from the 1970s onward shows a strong upward trend of PFAAs in biota which is in broad agreement with the estimates of use and emission of PFAAs[12]. Studies have associated PFAAs with infertility, birth defects, higher cholesterol, thyroid diseases, increased cancer rates, and changes of lipid levels in the immune system and the liver[13-16].

The Science Advisory Board to the U.S. Environmental Protection Agency (EPA) has recommended that PFOA be classified as a likely human carcinogen [17]. PFOA and PFOS are included in the U.S. EPA Contaminant Candidate List 3[5]. The U.S. EPA recently implemented provisional health advisories for PFOA (0.4 μg/L) and PFOS (0.2 μg/L) in drinking water[18]. However, remediation of PFAAs contamination remains a significant challenge due to their persistence nature. My study focused on investigating the mechanisms of PFOA and PFOS

transformation during the enzyme-catalyzed oxidative humification reactions (ECOHRs), and examined the potential of using the ECOHRs as a remediation technology for PFAAs contamination in soil and water.

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CHAPTER 2

LITERATURE REVIEW

THE HISTORY OF PFAAS MANUFACTURING

Fluorine is the most electronegative element and can form strong bonds with almost all electropositive elements[1, 2]. The fluorinated hydrocarbon molecules rarely exist in natural condition; however, fluorinated organic molecules can be synthesized[3]. PFAAs have been manufactured for over a half century until the products had been phased out from the market in the United States since 2002[4]. Electrochemical fluorination (ECF) process is the predominant procedure used in PFAAs manufacturing[1, 5, 6]. This procedure involves mixing dispersed organic feedstock solution with anhydrous hydrogen fluoride, and then passing an electric current through the solution, causing the hydrogen atoms on the molecule to be replaced by fluorine[1]. For example, the 1-octanesulfonyl fluoride is a common organic feedstock to produce perfluorooctanesulfonyl fluoride (POSF)[1, 2, 7] which has been primarily used as precursor to make other fluorochemical products such as PFOS. The estimated production of the global POSF-derived products is approximately 43,500 tons from 1985 to 2002[2]. After 2002, the production of POSF sharply dropped due to the cessation of POSF production by Minnesota Mining and Manufacturing (3M). However, the dominant production was shifted to China by 2003 since there are no suitable replacements for POSF-derived products in certain industries such as semiconductors, medical devices, aviation, metal plating, pest control, and photographic processes[8]. In 2004, the production of POSF-based compounds in China was estimated to be < 50 tons[8]. In 2005, the global production of POSF-based compounds was between 73 and 162

tons[9], and Chinese production was over 200 tons by 2006[8]. The total historical global production was estimated at ~120,000 tons in 2009[2].

There are two major processes for manufacturing PFOA. One is through the ECF fluorination as described above[6, 10, 11]. The other one is through telomeriation process which includes two steps[10]. First, the organoiodine compounds react with unsaturated tetrafluoroethylene to produce the intermediate products. Then the intermediate products are oxidized by SO_3 to yield the PFOA as final products. Purified products could be obtained by further distillation[12].

PHYSICAL-CHEMICAL PROPERTIES OF PFAAS

The physical-chemical properties of PFAAs determine their fates and behaviors in the environment. PFAAs have unique chemical properties because the majority of hydrogens on the carbon-carbon skeleton are substituted by fluorines. It is worth noting that all POSF-derived substances would ultimately be degraded to PFOS[1] while the telomer-based polymers could be breakdown to PFOA[12]. Thus, studying the physical-chemical properties of PFOS and PFOA is particularly important in understanding the fate and transport of PFAAs in the environment. This section summarizes the physical-chemical properties of PFOA and PFOS and discusses their possible environmental behaviors.

The molecular structures of PFOA and PFOS are presented in Figure 2.1, and their major physic-chemical properties are summarized in Table 2.1. Fluorine is the most electronegative element having a reduction potential of 3.6 eV thus forming highly polarized fluorine-carbon bond by overlap the 2*s* and 2*p* orbitals of fluorine with the corresponding orbitals of carbon[13]. The carbon-fluorine bond is known as the strongest single covalent bond with a average bond energy of ~ 117 kcal/mole[14, 15]. Therefore, it is thermodynamically unfavorable to oxidatively

replace the fluorine atom with any other atom. In addition, the fluorine atoms, which are much larger than hydrogen atoms in size, form a dense hydrophobic layer surrounding carbon-carbon bonds which prevents them from being attacked by oxidative reagents. As a result, PFOA and PFOS are extremely resistant to degradation such as metabolism, hydrolysis, and photolysis in natural conditions.

The reported pKa value of PFOA is 0.6 which is lower than its corresponding hydrocarbon analogue[16]. However, no direct empirical pKa value is available for PFOS. The calculated pKa of PFOS are usually negative values. The low pKa values of PFOA and PFOS indicate that both chemicals are nearly completely dissociated in natural environment. It was reported that only 3-6 in 100, 000 molecules were PFOA molecules at pH 7 while about 6% was remained as PFOA molecules at pH 4[10].

The formation of micelle and liquid crystal phase significantly influenced the solubility of PFOA in water. The solubility of PFOA in water increased from 4.1 to 9.5 g/L when temperature raised from 22 ℃ to 25 ℃[17]. Such sharp change of solubility was due to the formation of micelles. The estimated solubility of PFOS was 570 mg/L in water[1]. The solubility of PFOS in water is also impacted by the ion strength. Only 12.4 mg of PFOS can be dissolved in one liter seawater when the temperature is around 22-23 ^{o}C[18].

Although the partition of PFOA and PFOS in octanol-water is extremely difficult to measure because of their surface active properties[19], information regarding the sorption of PFOA and PFOS to soil and organic carbon is available[20-23]. It was reported that the soil-water partition coefficient (K_D) for PFAAs correlated with the organic carbon content (OC) in soil[21]. A K_{oc} value ranging from 52 to 17 L/kg were reported for PFOA[22]. Another study investigated the sorption behavior of PFOA in four different soil types (0.8-5.8% OC) which confirmed the above

conclusions[24]. The K_D values of PFOA (9 to 230 L/kg) were linearly correlated with OC content in soil[25]. The sorption isotherms of PFOA were linear over a wide PFOA concentration range[26]. A strong inverse relationship between OC content and desorption was also found[25]. Besides the OC content, the K_D value also varies with carbon-chain length of PFAAs[21]. More information about the sorption of PFOA and PFOS was discussed in the "Fate and Transport" section.

The vapor pressures (VP) for PFAAs have been measured at wide temperature range (59.25 to 190.80 ℃)[27]. It was shown that VP decreased with the increasing of the chain length of PFAAs. PFOA and PFOS are usually considered non-volatile. The calculated VP for PFOA is 4.2 Pa at 25 ^{o}C. The volatilization of PFOA from water is pH dependent[28].

APPLICATION OF PFAAS

The PFAAs have many important manufacturing and industrial applications. For example, surface coating of fabrics and carpets, treating paper and packaging materials, formulating aqueous film forming foams (AFFFs)[2, 4, 10, 29].

Protective coating

PFAAs have been used on a wide range of fabrics and materials for surface protection due to their unique properties of repulsing both oil and water[18]. They are applied as a coating to the surface of fabric to create a protective barrier. Textile and apparel treatment accounts for nearly 50% of PFAAs consumption[30]. Carpet industry is the second largest consumer of PFAAs. PFAAs are applied during or post the carpet manufacturing process to incorporate the resistance to soil, water, and oil into the carpet. The city of Dalton in Georgia is the center of carpet manufacturing in North America which involves extensive use and discharge of PFAAs[31]. It was reported that the carpet and carpet treatment products usually contain PFAAs at levels ranging from 0.04-14100 ng/g[32].

Paper treatment

PFOS-related substances have been used to treat different types of paper[30]. The major type of perfluorinated substances used in paper treatment are phosphate derivatives of N-ethyl perfluorooctane sulfonamidoethanol (N-EtFOSE)[1]. It was reported that the food industry consumed 160 tons of N-EtFOSE in the Europe Union in 2000 alone[30]. The main purpose is to impart the property of resistance to grease, oil and water on paper and paper board substrates. Such products have been used in food-contact materials such as plates, food containers, bags and wraps, folding cartons, carbonless forms and masking papers [30]. They are mainly applied during the paper making process.

Fire fighting foams

The AFFFs contained 1-5% of fluorochemicals[33]. AFFFs are fire extinguishing agents that was developed by the Navy in 1960s. PFAAs are the key components to effectively combat fires occurring close to explosive materials. The primary advantage of using PFAAs in the AFFFs is that it could prevent the occurrence of reignition[33-36]. The municipalities (i.e., fire departments), the hydrocarbon-processing industry (i.e., oil refineries), and the military sectors are major consumers of AFFFs due to the presence of large quantities of flammable materials in these areas. Approximately 75% of AFFFs are utilized by the military, whereas the municipal entities and hydrocarbon-processing industry account for 13% and 5%, respectively[16, 37].

Other applications

PFAAs, especially PFOS-related products, are good acid mist suppressants because they are able to lower the surface tension. As such, PFAAs are used in chromium plating, anodizing, and acid pickling to prevent the formation of mists that may contain potentially harmful components. PFAAs also have multiple applications in the semi-conductor industry, usually

related to photolithography, a process which the circuits are produced on semi-conductor wafers. Such process needs the presence of photoresists, materials whose composition is altered on exposure to light, making them either easier or more difficult to remove and thus allowing structures to be built up in the wafer. The PFOS-related substances can form part of the photoresist itself, acting as a photoacid generator to chemically amplifying the effect of photo exposure.

OCCURRENCE OF PFAAS IN ENVIRONMENT

There are two primary routes for PFOA and PFOS entering to the environment. The direct sources are discharge from manufacture plants and release from commercial or industry application, distribution, and disposal processes[2, 31, 38]. The major indirect sources are the transformation of PFAAs precursors to PFOA and PFOS[39-45].

Discharge from manufacturing plant

Direct emission of PFAAs from manufacturing and chemical plants is one important pathway for PFAAs entering the environment[2, 10, 19, 46, 47]. The global historical industrial emissions of total perfluorinated carboxylic acids (PFCAs) from direct and indirect sources were estimated to be 3,200-7,300 tons[10]. The predominant (~ 80%) PFCAs in environment were from fluoropolymer manufacture and usage emission while the indirect source was only a minor contributor[10].

A study investigated the distribution of PFAAs in the environment near a fluorochemical industrial park in China[48]. At the industrial park, the highest concentrations of PFOA were 48 ng/g and 668 ng/L in the sediment and the river water, respectively; the highest PFOA concentration in the groundwater was 524 ng/L; and the PFOA levels in drinking water from the public water supply system ranged from 1.3 to 2.7 ng/L. The PFOA and PFOS levels in a river

receiving discharges from a fluorochemical facility in Decatur, Alabama were found up to 114 and 394 ng/L, respectively[49]. Another study provided a profile of PFAAs in the ambient environment of a PFAAs manufacturing facility[50]. Decreasing of PFAAs concentrations with increasing distance from the manufacturing plant was found in both soil and water, indicating that the production site was the primary source of PFAAs in this region.

Additionally, extensively utilization of PFAAs in various industries also contributes to the presence of these chemicals in environment. PFAAs are used as surface protection of carpets due to their abilities of repelling both oil and water. The city of Dalton in Georgia is the center of carpet manufacturing in North America[31]. Approximately 90% of the carpets worldwide were produced there. Due to the extensive use of PFAAs, the carpet industry in the northwestern Georgia was identified as a regional PFAAs contamination source[51]. The PFAAs concentrations were very high (PFOA, 253–1,150 ng/L; PFOS, 192–318 ng/L; other PFAAs, 15.8–369 ng/L) in the downstream receiving runoff from a wastewater land application system surrounding the carpet manufacturing plant[31].

Release from commercial and industry application

The AFFFs could be classified into different types based on their formulas. Flourine-free foams and telomers formulated foams are both used for Class B fire fighting in many countries. Flourine-free foams are formulated without fluoro-chemicals which do not contain fluoro-surfactants or fluoropolymers. Telomer-based foams do not use PFOA as a major ingredient either, but may still contain trace level of PFOA as an unintended consequence of the manufacturing process. The third type of AFFFs was formulated with PFOS. Although some effort has been made to replace the PFOS-based foams, most facilities continue to rely on their existing stockpiles, thus, large quantities of this type of foam are still in use. Repeated usage of

AFFFs in a variety of situations resulted in the release of PFAAs into the ambient environment. PFOA and PFOS concentrations up to 290 and 1,820 ng/g respectively was found near a fire fighting training field[52]. In 2003, more than 40,000 liters of AFFFs was used for combating a fire occurred at an oil storage facility in Japan[53]. The usage of such large quantity of AFFFs during this fire combat led to the release of PFAAs to the environment along with oil and oil-related chemicals. PFAAs including 4.15 ng/L of PFOA and 42.3 ng/L of PFOS were detected in samples collected one month after the fire in surface water, and 2.87 ng/g PFOA and 8.06 ng/g PFOS in soil[54]. Another study documented an accidental release of 22,000 liters of AFFFs at an airport which resulted in introducing of large amount of perfluorinated surfactants was into the nearby creeks[55]. The total perfluoroalkane sulfonate (PFSAs) concentrations in creek water ranged from < 0.017 to 2,260 μg/L while the PFCAs concentrations ranged from < 0.009 to 11.3 μg/L.

Groundwater at military bases was also influenced by fire-fighting-training activities[56, 57]. The concentration of PFCAs was up to 7,090 μg/L in the groundwater of a military base[57]. Another study reported the detection of PFAAs such as PFOA (up to 105 ng/L) and PFOS (4.0-110 ng/L) in the groundwater surrounding a fire-fighting pit[56]. Backe et al.[58] developed a large-volume direct injection high-performance liquid chromatography-mass spectrometry method to quantified eight typical PFAAs in the groundwater samples influenced by military training. The concentrations were reported ranging from 8.6 to 220,000 ng/L and 12 to150,000 ng/L for PFOA and PFOS, respectively[58].

Kärrman et al. [59] reported the concentrations of PFOA and PFOS in various environmental matrices including soil, water, and sediment near a fire fighting training area. Soil collected from this area contained 1.4 ng/g PFOA and 273 ng/g PFOS[59]. High concentrations of

PFOS (1,427-2,078 ng/L) were found in the seepage water at the training ground while the PFCAs levels were between 97 and 560 ng/L. PFOS ranging from 35 to 88 ng/g was detected in the sediment sampled from a lake receiving water from the fire training areas while PFOA was not detected[59].

Disposal

The majority wastes generated during the POSF production at the 3M Decatur, Alabama production plant were in the form of solid wastes. Approximately 90% of such wastes had been incinerated or disposed in landfills; 9% was discharged as wastewater, and 1% were emitted in the form of air [60]. Many studies measured the PFAAs concentrations in the environment which was affected by the disposal of wastewater treatment plant (WWTP) sludges and manufacture wastes[61-65]. PFOA concentrations was up to 16.8 ng/g in a former sludge disposal area[63]. PFOA ranging from 91.3 to 543 ng/g and PFOS ranging from 313 to 694 ng/g were found at a former manufacturing plant wastes disposal area in Cottage Grove, Minnesota. PFOA was detected in all soil samples collected from a former trench area at levels up to 26,100 ng/g[66]. Very high PFOS concentrations (2,800 to 19,300 ng/g) were found in 11 out of 12 samples collected from the backfill material in the former trenches. PFAAs concentrations up to 6,231 ng/L were also determined in the leachates[67].

FATE AND TRANSPORT OF PFAAS

The environmental fate and transport of PFAAs are very complicated due to their unique characteristics and the complex compositions of PFAAs sources. The interplay of various processes including physical transport and multimedia partitioning determines the fate and transport of PFAAs in the environment[4]. Volatile PFAAs can evaporate to the atmosphere, while

ionic PFAAs such as PFOA and PFOS can enter the aquatic environment directly from the product manufacturing processes, supply chains, product use, and disposal[4].

Aquatic phase

Distribution in the sediment-water system

The distribution of PFAAs between water and sediment is important in controlling the behavior of PFAAs in the aquatic environment. The occurrence of PFAAs in surface water has been widely reported[68-71]. The concentrations of PFOA and PFOS in sediment were usually at nanogram per gram level[23, 27, 72-76]. The mobility of PFAAs is related to the length of their fluorinated carbon chain. A study found that the sediment/water partition coefficient for PFAAs increased with the increase of carbon-chain length[23]. Therefore the shorter-carbon chain PFAAs are more mobile and more likely to cause water contamination than the longer-carbon chain PFAAs[23, 77].

A long term monitoring study at an area impacted by accidental release of AFFFs revealed that accumulation of PFOS in fish occurred even ten years after the accident[55]. The field-based K_D values were determined with log values being 1.00-1.85 for PFCAs and 0.54-1.65 for PFSAs[55]. Generally, higher concentration of PFOS was found in the sediment than in the water while PFOA level was higher in the aquatic phase than in the sediment, which reflected the difference in K_D between the two compounds[55, 78-80].

The adsorption of PFAAs to the sediment was also impacted by the pH of the system[23]. Organic carbon dominated the sorption process at high pH value. Martin et al.[81] hypothesized that the accumulation of PFOS in *Dipoeria*, a predominantly pelagic feeder, was due to the adsorption of PFOS to the organic matter in sediment. At neutral pH, the sorption was controlled by electrostatic interactions[23, 26, 82, 83]. Therefore, the effect of pH should be taken into account

when predicting the transport of PFAAs in aquifers, especially at sites impacted by landfill or WWTP sludge land application, as these processes could potentially change the pH of the environment[26].

As we known, the use of AFFFs for firefighting combats led to the release of significant amount of PFOS into the environment. Determining the extent to which co-existed organic pollutants would affect the fate and environmental transport of PFOS is very crucial for that the PFOS often appear along with fuel oil. A study showed that oil was a strong sorbent for PFOS, and such sorption is independent of solution parameters such as pH and ionic strength, suggesting that hydrophobic interactions between the hydrophobic moieties of PFOS and oil played a dominant role in the sorption interaction[84]. This finding could help us better understand the fate of PFAAs in the light non-aqueous phase liquids (LNAPL). On one hand, PFAAs are surfactants and can increase the transfer of hydrocarbons from LNAPL via emulisification. On the other hand, certain PFAAs may also be sorbed into LNAPL which would reduce the concentrations of PFAAs in water.

Bioaccumulation in aquatic animals

The concern over the presence of PFAAs in the natural environment is that they can bioaccumulate though food chains[79, 85-91]. A large number of biota samples contained quantifiable amount of PFAAs[22]. In combination with their high persistence and widespread in the environment, PFAAs have becoming a big environmental issue[22].

The bioconcentration factor (BCF) or bioaccumulation factor (BAF) values of PFAAs were estimated based on the data collected from empirical research on PFAAs in fish and aquatic invertebrates[22, 92]. A few conclusions could be drawn from these data. First, the BCF and BAF values for PFAAs increase with the increase of fluorinated carbon chain length. Compounds with

a fluorinated carbon chain length longer than eight are generally more bioaccumulative than those with carbon chain length shorter than seven[91]. Second, PFSAs are generally more bioaccumulative than PFCAs of the same fluorinated carbon chain length. For instance, in comparison to PFOS, PFOA tends to have less retention in many biological matrices since PFOA contains a carboxylate group while PFOS has a sulfonate group[91]. Third, PFCAs with seven fluorinated carbons or less are not considered bioaccumulative according to regulatory BAF/BCF "B" criteria of 1,000–5,000 L/kg[91, 93, 94].

Accumulation of PFAAs through food webs contributes significantly to the detection of PFAAs in human serum, blood, and other tissues. Human exposure to PFAAs through direct consumption of drinking water was another key component[95-97]. The average exposure to PFOS and PFOA in Taiwan via water consumption ranged from 0.16 to 220.15 and 0.43 to 12.50 ng/kg body weight/day for adults and 0.13 to 354.30 and 0.35 to 20.17 ng/kg body weight/day for children[98].

Soil

Distribution in groundwater-soil systems

Moreover, landfill and land application of WWTP sludge could introduce the PFAAs to soil[99]. PFAAs have been found presented in soil at different levels[74, 100, 101]. One concern over releasing PFAAs to soil is that they can leach to the ground water or enter the surface water with runoff due to their good mobility and water solubility[102-104]. Once the PFAAs are released to the groundwater, interaction with aquifer soil is likely the primary process that impacted the PFAAs transport[59, 105, 106]. The detection of PFAAs in the seepage water near a training facility using AFFFs suggested that leak of contaminants from the training ground has occurred[59]. The groundwater contained a relatively high level of PFOS at concentrations ranging from 1,427 to

2,078 ng/L[59]. The PFOS concentrations in the groundwater of an Air Force Base in the U.S. were between 4,000 and 110, 000 ng/L[56]. The concentrations of PFCAs with carbon-chain length of five to eight were ranging from 155 and 560 ng/L while the carbon-chain length of nine to eleven were detected at lower level (3.8-28 ng/L). High concentration of PFOS (up to 1,905 ng/g) was quantified in soil surrounding the training ground. The observed PFOS concentration decreased with increasing of distance from the source area. Such pattern indicated the movement of these chemicals was not only vertically but also horizontally[59].

Bioaccumulation of PFAAs in plants

The frequent detection of PFAAs in WWTP sludge amended soil raises the concern of transporting PFAAs from the contaminated soil to plants. A recent study reported the uptake of PFAAs and telomer alcohols from contaminated soil by different kinds of grasses[107]. Plenty of study results suggested that uptake PFAAs from soil to plant had occurred, particularly in the vegetative compartments of the crops occurred[108]. Stahl et al.[109] reported that the concentrations of PFAAs in rye grass, grain, and potatoes changed with the concentrations of PFAAs in the soil. Other studies about the distribution of PFOA and PFOS in different plant compartments confirmed that the uptake of PFAAs differed from plant species and genres of PFAAs[110]. The concentrations of PFOA and PFOS in different plant compartments, regardless of the plant species, were the highest in the vegetative compartments and sequentially decreased in the peelings and peeled edible parts[110]. Carryover of PFAAs through nutrient solution to lettuce was found mainly retaining at the non-edible compartment, i.e. the root[111].

A group of biological samples collected from the Mai Po Marshes Nature Reserve in Hong Kong were examined as well. The environmental distribution and biomagnification of PFAAs in the subtropical food web were evaluated[112]. Trophic magnification was observed for

PFOS in this food web. The risk assessment results suggested that the PFAAs concentration in water bird livers were unlikely to pose adverse biological effects to animals[112]. In another study, more than 200 food product samples (green beans, apples, pork, milk, chicken, eggs, bread, fish, and ground beef) were analyzed for the concentration of PFAAs[61]. PFOS was only detected in one ground beef and four milk samples with the maximum concentration of 0.852 ng/g.

The above section summarized the occurrence of PFAAs in the environment as a result of their manufacturing, applications, and disposals. Detection of PFAAs was frequently reported at concentrations of nanogram per liter to microgram per liter in water, and nanogram per gram to microgram per gram in soil and sediment.

TOXICITY OF PFAAS

The detection of PFAAs in the environment attracts tremendous attention from both public and scientific communities because they have potential adverse effects to the wildlife and human beings[18, 113, 114]. People are exposed to PFAAs through diet, water, or from use of commercial products. Monitoring of PFAAs in populations across the world demonstrated that PFAAs were ubiquitously present in human serum, blood, and tissue[17, 115]. In North American, the reported concentration in human serum ranged from 0.7 to 56.1 ng/mL and < 1.2 to 88 ng/mL for PFOA and PFOS, respectively[17]. It was found that workers exposed to the working environment with PFAAs generally had one order of magnitude higher PFOA and PFOS levels in their serum than the general population.

PFAAs have toxic effects on mitochondrial, microsomal, and cytosolic enzymes and protein, as well as lipid metabolisms[116, 117]. PFAAs can also cause peroxisome proliferation, and induce accumulation of triglycerids in liver[118], uncoupling of mitochondrial oxidative phosphorylation, and reduce thyroid hormone in circulation[58].

Toxicity of PFOA

Freshwater algae are very important components of aquatic food chains. Pollutants could be accumulated and/or adsorbed by them and transferred to other organisms, thus resulting in potential adverse effects to the aquatic ecosystem[119]. In a 24-h toxicity test of a green alage *Anabaena* CPB4337, the growth inhibition half maximal effective concentration (EC_{50}) of PFOA was reported at 19.81 mg/L[120]. According to a recent study, the acute 48-96 h EC_{50} of ammonium perfluorooctanoate (APFO) were higher than 400 mg/L for algae *Pseudokirchneriella subcapitata*[121], and the lowest chronic no observed effect concentration (NOEC) was 12.5 mg/L for inhibition of the growth and biomass[121].

[121121121121121121121121127127]The toxicity of PFOA on a marine microalgae *Isochrysis galbana* was assessed on early life stages with a reported acute EC_{50} value of 163.6 mg/L. Some other algae such as the green alga *Chlorella vulgaris*, the diatom *Skeletonema marinoi*, and the blue-green algae *Geitlerinema amphibum* were also tested for their response to PFOA, and the reported EC_{50} values ranged from 248.4 to 977.2 mg/L[122].

PFOA toxicity exhibits sex-related difference in certain animals. Repeat-dosage of PFOA to male rats induced adverse effects on the acyl-coA hydrolase, stearoyl-coA desaturase, 1-acylglycerophosphocholine acyltransferase and carnitine acyltransferase activities as well as elevated the peroxisomal-oxidation in the livers while none of these effects were observed in female rats[52]. The two-generation reproductive toxicity studies were also conducted in rats over a 70-day with PFAAs dosage of 1-30 mg/kg[123, 124]. The results showed that the body weight decreased while liver and kidney weight increased at all dosage levels in the parental male rats. However, female rats did not show similar changes and no reproductive endpoints were impacted by PFOA dosage. Despite the much longer biological half-life of PFOA in male versus female

rats, the 50% lethal dosage (LD_{50}) is approximately the same in both genders (430–680 mg/kg) which was on the same magnitude in mice[123, 124].

Among the toxic effects caused by PFOA, the hepatic toxicity is relatively well studied[114]. It is believed that the primary target organ of PFOA in the mice is liver. As shown in the previous study, exposure of mice to 10 mg/kg PFOA could lead to the increase of liver weight from 1.1 to 2.1 g while the decreased of the male rat body weight by 23.4% over 10-day dietary treatment was observed with PFOA dosage up to 5 mg/kg[114]. PFOA are expected to be a peroxisome proliferators which is known for inducing hepatocellular carcinomas in rats and mice[125]. Activation of the peroxisome proliferator-activated receptor-alpha (PPAR-α) was associated with the dosage of PFOA to the mice in the dietary[126]. In the meantime, these compounds can also act as tumor promoters by inhibiting gap-junctional intercellular communication. The positive association between the serum levels of PFOA and chronic kidney disease has been found in human being regardless age, sex, ethnicity, and body weight[127]. PFOA was also found to be an endocrine disruptor[4]. It was found that the levels of steroid hormones in rats were altered after exposed to PFOA[19, 61]. PFOA can also interfere the endocrine system indirectly via ovarian effects[18].

Data collected so far demonstrate that PFOA has potential carcinogenicity of PFOA to certain animals, but not sufficient enough to draw a conclusion on the carcinogenic potential of PFOA on humans. The considerations include: 1) the PPAR-α activation for liver tumors in rodents was considered not relevant to humans because of the decreased sensitivity to PPAR-α agonism in humans comparing to rodents; 2) hepatic cell proliferation was absent in a 6-month study of PFOA administration in cynomologous monkeys, the species considered the closest in physiology to humans; 3) a strong association between PFOA exposure and tumors in human

studies was absent in humans; 4) as well as differences in quantitative toxicodynamics between rats and humans; and 5) the view that mammary fibroadenomas reported in female rats were equivocal based on their comparable rates of occurrence relative to a historical control group.

Toxicity of PFOS

PFOS is able to induce moderate to acute toxicity to rats by oral exposure. The determined LD_{50} was 251 mg/kg[118]. Based on the oral toxicity studies, a NOEC and Low-Observed-Effect Level (LOEL) for second-generation offspring was 0.1 and 0.4 mg/kg bodyweight/day, respectively[118]. Studies focused on the reproductive effects of PFOS in rats estimated a NOEL of 47 μg/g serum or 72.5 μg/g liver which correspond to a dietary concentration of approximately 15 μg/g[128]. Exposure of rats and rabbits to PFOS concentration higher than 5 mg/kg/day resulted in reducing of body weight gain, feed consumption, litter size, and fetal weight[129]. According to the developmental toxicology study, the maternal NOEL of PFOS for rabbits was 0.1 mg/kg/day, and the developmental NOEL was 1 mg/kg/day[63]. Exposure to PFOS could decrease body weight, cholesterol level and increase liver weight in rodent. Repetitive dosage of PFOS in rodents and nonhuman primates have shown a dose-response curve for mortality[130]. A two-year bioassay revealed that PFOS could cause elevation of hepatocellular adenomas in Sprague-Dawley rats at 20 ppm in the diet[118, 131]. Similar pattern of effects has been described in repeat-dose studies of PFOA in rodents as well[28, 132]. A variety of assays suggested that neither of PFOA or PFOS is mutagenic[18, 113, 117]. A two-year carcinogenicity study observed significant increases in hepatocellular adenomas in both male and female rats at 1 mg/kg bw/day of PFOS dosage. Due to the lack of evident in the genotoxicity tests, a report concluded that the carcinogenic effect of PFOS was due to a threshold-mediated non-genotoxic mechanism[133].

The individual and combined toxicity of PFOA and PFOS to zebrafish embryos were also investigated[134]. PFOS was found to be more toxic than PFOA in the single toxicity testing. In the mixtures, PFOS and PFOA showed complex interactive effects that changed from additive to synergistic effect, and then to antagonistic effect, and at last turned over to synergic effect again, with increased molar ratios of PFOS[134]. Neither the concentration addition model nor the independent-action model could appropriately predict the combined effects when strong interactive effects existed. Although the interactive effects of PFOS and PFOA affected their combined toxicity, the trend of the mixture toxicity still showed an increase with increasing molar ratios of PFOS in the mixture[134].

The U.S. EPA has included PFOA and PFOS in its Contaminant Candidate List (CCL 3) and Unregulated Contaminant Monitoring Rule 3 (UCMR 3) List[94]. Monitoring of these chemicals in the public water systems was conducted since 2013. The U.S. EPA Office of Water has also established Provisional Health Advisories for PFOS (0.2 μg/L) and PFOA (0.4 μg/L) based on a risk assessment performed by the European Food Safety Authority[17]. The U.S. EPA Region 4 also established Residential Soil Screening Levels of 16 mg/kg for PFOA and 6 mg/kg for PFOS[135].

Researchers have made great progress during the past few years in understanding the occurrence of PFAAs in the environment and toxicology of PFAAs to wildlife and humans. Many previous studies have proved that the occurrence of PFAAs is worldwide. However, more efforts need to be put into other remaining questions. For example, more research is needed to understand the biological basis for the species differences in elimination of PFAAs. It is also important to determine whether any of these effects is species-specific, thereby providing a sound basis for health risk assessment of these chemicals as a class.

CURRENT DEGRADATION TECHNOLOGIES

PFAAs are hard to degrade in the natural environment due to their extreme stability. A limited number of studies have been done to study the degradation of PFAAs. The advanced oxidation processes (AOPs) such as alkaline ozonation, peroxone, or Fenton's reagent have little success in degrading PFAAs[33]. These methods mainly rely on the oxidation power of hydroxyl radicals which limited their applications in the degradation of PFAAs. The hydroxyl radicals normally react with saturated organics through a hydrogen-atom abstraction, or react with unsaturated organics via an oxidative addition reaction. However, PFAAs have no hydrogen available for abstraction, or a double bond for addition, leaving direct electron transfer the only possible pathway which is unfortunately less potent of hydroxyl radical.

The photolytic oxidation methods were found effective in PFAAs degradation. Persulfate photolysis is viable in decomposing PFAAs of various chain lengths[136, 137]. The sulfate radical generated during the photolysis has higher reduction potential (2.3 V) than hydroxyl radical (1.9 V), thus making it a stronger electron transfer oxidant. PFOA degradation by sulfate radical has half-life time on the order of hours with 15% of total fluorine was converted to fluoride. The degradation was initiated by extracting one electron from the carboxylate terminal group to the sulfate radical and followed by Kolbe decarboxylation[137]. However, persulfate photolysis is subjected to the interference of other dissolved organic species which could quench the sulfate radicals. Concentration of PFOA that exceeds the total organic matter is required to observe significant PFOA degradation. As a result, the persulfate photolysis is not suitable for virtual environmental applications[138]. Direct persulfate oxidation of PFOA was also investigated. Researchers observed 93.5% degradation of PFOA in aqueous phase after 30 hours of reaction with heating the system to 85 ^{o}C[139]. The sequential losing of CF_2 units from PFOA was

identified as the degradation mechanism since shorter-carbon chain PFCAs were detected during the degradation.

Phosphotungstic acid could serve as a photocatalyst to degrade PFOA as well[81]. The $PW_{12}O_{40}^{3-}$ could adsorb light and be converted to photo-excited state ($PW_{12}O_{40}^{3-*}$). Similar to sulfate radical, an electron can be transferred directly from PFOA to $PW_{12}O_{40}^{3-}$, and then PFOA could decarboxylate to form perfluoroheptyl radical. Oxygen then accepts electron from phosphotungstic acid ($PW_{12}O_{40}^{4-}$) to return it to its photoactive state[81].

Another photocatalyst – TiO_2, was found to be effective in degrading PFOA but did not work for PFOS[140]. The TiO_2 could adsorb PFOA and direct oxidize PFOA by TiO_2 valence band hole. A photo-Kolbe decarboxylation mechanism similar to phosphotungstic acid was proposed[21, 140]. The reaction rate was pH dependent. This reaction required 60 h to achieve 50% PFOA removal at extremely low pH value[140].

Direct photolysis of PFOA and PFOS occurs only under very rough reaction conditions[81, 141]. Under the vacuum UV (VUV, < 200 nm) radiation, the photolysis of PFOA achieved a half-life of 90 min with 12% of defluorination ratio[81]. Another photolysis study reported that the degradation rate for PFOS was lower than PFOA under VUV with a half-life of 5.3 day[141].

Sonochemistry was another approach which has been studied for PFOA and PFOS as well. Effectively degradation of PFOA and PFOS to fluoride and sulfate as the primary ionic products was observed[44, 142, 143]. The decomposition rate was not limited by other organic species that co-existed in the reaction system[142, 143].

In addition, electrochemical decomposition of PFOA has been reported in many studies[144, 145]. Under the optimal reaction condition, over 99% of PFOA was decomposed with a first-order rate constant of 1.93/h[146]. However, the matrix effect is a big concern for the

electrochemical decomposition. Both inorganic and organic compounds may impact the oxidation efficiency. Niu et al. [145] conducted an electrochemical mineralization experiment of PFCAs with Ce-doped porous nanocrystalline PbO_2 film electrode. The degradation followed the pseudo-first-order kinetics. Up to 96.7% removal of PFCAs after 90 min was observed. The possible degradation pathways were proposed as well. The reaction was initiated by directly transferring one electron from PFCA to the anode leading to the decarboxylate of PFCAs. Then PFCAs radicals further degraded through two different possible reaction pathways[145, 146]. It can either combine with the hydroxyl radical generated from the water electrolysis or interact with oxygen produced from water electrolysis[146].

ENZYME-CATALYZED OXIDATIVE HUMIFICATION REACTIONS

As discussed above, all current degradation techniques either involve high energy inputs (light source, high-power ultrasound, high temperature and high pressure, etc.) or special devices. Earlier studies reported that the enzyme-catalyzed oxidative humification reactions (ECOHRs) can effectively degrade various persistent organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and PFOA, in solution at environmental relevant conditions[147-149]. This finding introduces a novel pathway to transform PFAAs. ECOHRs refers to a class of oxidative reactions that are crucial in natural organic matter humification processes[150]. This process is based on the fact that certain chemicals can serves as natural humic acid precursors and be incorporated into humus. These reactions are ubiquitous in soil systems, and usually catalyzed by extracellular enzymes produced by certain white/brown rot fungi[150, 151].

One natural function of white/brown rot fungi is to degrade lignin. Lignin is a three-dimensional complex heteropolymer of aromatic alcohols with its accurate structure has not been

fully determined[152]. It is an integral part of the secondary cell walls of plants and quite abundant on the planet[153]. Lignin is the most persistent natural organic matter[154]. White/brown rot fungi is reported the most efficient lignin degrader in nature, no other microorganism have been described to mineralize lignin as efficient as white/brown rot fungi. Lignin degradation by white-rot fungi has been intensively studied during the last thirty years in relation to biotechnical applications such as biopulping, biobleaching, treating of pulp mill effluents, and soil bioremediation[150].

Such special capability of white/brown rot fungi is mainly attributed to certain extracellular enzymes such as lignin peroxidase, manganese peroxidase, and laccases, which are produced by white-rot fungi. These enzymes can effectively convert the natural or anthropogenic chemicals containing phenolic or aromatic anime moieties into active intermediates such as radicals or quinones. These intermediates are unstable and usually short-lived. They may undergo further oxidation or reduction. Possible pathways for these radicals to interact is to couple with each other or bound covalently into natural organic matters[141, 147, 148, 155-157]. In the meantime, active intermediates may also attack other inert chemicals, such as lignin, and thus cause their degradation and consequently being incorporated into the natural humification process.

Laccase are a group of typical phenol-oxidase. They use molecular oxygen as an oxidant and have very broad substrates specificity[158]. They initiate the reaction by extracting electrons from phenolic substrates, and then transport these electrons to oxygen and reduce the oxygen to water[158]. The products of oxidation can undergo non-enzymatic reactions such as cross linking of monomers, degradation of polymers and ring cleavage of other chemicals. Laccase are found in a variety of fungi including *Trametes versicololr, Pleutrotus ostreatus, Rhizoctonia praticola, etc.*

The function of different fungal laccase is basically the same, i.e. all laccases are able to transforming phenolic substrates. However, there are differences in the quantitative oxidation efficiencies among fungal laccase. These variations may be due to differences in optimal pH, temperature, and other characteristics of the enzyme[150].

Previous studies showed that ECOHRs process could degrade PAHs[148] and PCBs[149]. It was found that nearly fourteen PAHs could be oxidized by laccase from *Trametes versicolor* in vitro[159]. Some research reported that the oxidation of certain PAHs such as pyrene and benzo[α]anthracene increased significantly with the presence of certain small molecules[160]. The addition of such small molecules could facilitate the degradation of certain persistent chemicals which are not direct substrates of laccase. Such systems were usually called laccase-mediator system. During this process, the laccase converts those mediators into radicals as described above, and then these active radicals could further attack other persistent organic pollutants and lead to their degradation and detoxification[161].

A recent study reported that PFOA could be degraded by ECOHRs mediated with horseradish peroxidase in the presence of 4-methoxyphenol under room temperature at pH 7. Such reactions did not occur in the absence of the 4-methoxyphenol, suggesting that phenoxyl radical that generated during the ECOHRs led to the decomposition of PFOA. They proposed that the transformation of PFOA in this reaction system was likely initiated by abstracting an electron from the head C-C bond on PFOA, leading to its breakdown through Kolbe decarboxylation. It is not fully clear yet why phenoxyl radicals appear to be more effective in transforming PFOA than hydroxyl radicals generated in AOPs, but it may be related to the longer life time of phenoxyl radicals and its relative hydrophobicity.

Based on a thorough review of previous researches, we designed a serial of experiments to systematically investigate the degradation of PFOA and PFOS during ECOHRs in both the aqueous phase and the soil to identify factors controlling PFOA and PFOS degradation during ECOHRs and find optimum conditions. We also explored the possible reaction pathways and mechanisms that led to PFOA and PFOS transformation during ECOHRs.

Our study confirmed that ECOHRs could effectively transform the PFOA and PFOS to more environmental benign products. The degradation mechanisms were also proposed by identifying critical reaction products. The data collected from our experiment provides guideline for the future application of ECOHRs in the remediation of PFAAs.

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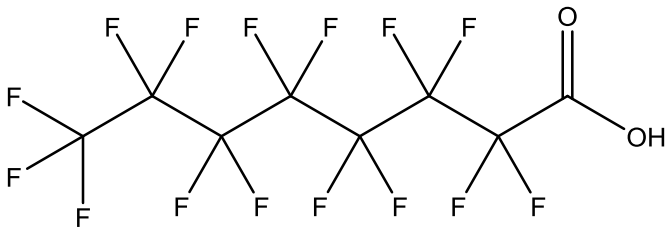
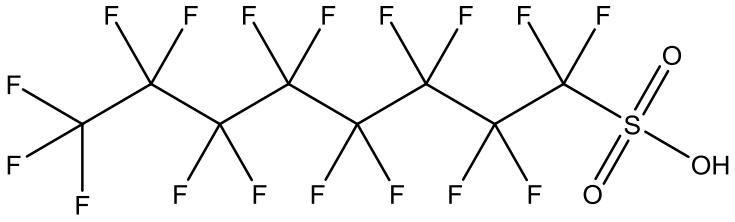
PFOA



Perfluorooctanoic acid

PFOS



Perfluorooctanesulfonic acid

Figure 2.1 Molecular structures of perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS)

Table 2.1 Physical-chemical Properties of PFOA and PFOS[1].

Chemical	PFOA	PFOS
CAS	335-67-1	2795-39-3
Molecular weight	414	500
Melting point	45-50	> 400
Boiling point	189-192	Not measurable
Vapor pressure (mm Hg @ 20 ^{O}C)	0.017	2.48×10^{-6}
Water solubility (g/L)	3.4-9.6	0.570
pKa	2.6	< 0
Half-Life	> 92 years	> 41 years

CHAPTER 3

LACCASE CATALYZED DEGRADATION OF PERFLUOROOCTANOIC ACID [1]

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ABSTRACT

This study examined the decomposition of perfluorooctanoic acid (PFOA) in enzyme catalyzed oxidative humification reactions (ECOHRs). ECOHRs are a class of reactions that are ubiquitous in the environment. Approximately 50% of PFOA in a mineral buffer solution was decomposed with the addition of laccase and 1-hydroxybenzotriazole after 157 days with a pseudo-first order rate constant of 0.0044/day (r^2=0.89). No shorter-carbon chain perfluorocarobxylic acids were detected as degradation products during the experiment. However, partially fluorinated shorter-chain alcohols and aldehydes were identified by high-resolution mass spectrometry. These partially fluorinated compounds were likely products resulting from PFOA degradation via a combination of free radical decarboxylation, rearrangement and coupling processes. Fluoride was detected in the reaction solution, and the concentration indicated a 28.2% defluorination ratio during the treatment. This finding suggests that PFOA may be transformed during humification, and ECOHRs can potentially be used for the remediation of PFOA.

INTRODUCTION

Perfluorocarboxylic acids (PFCAs) are a group of compounds that have extreme thermal and chemical stability[2] due to the strong fluorine-carbon bond (~ 110 kcal/mol)[3, 4]. Owing to such unusual characteristics, PFCAs have been used in nearly every aspect of daily lives[5, 6]. Although the excellent chemical inertness is a huge advantage in the application of PFCAs, it also causes considerable environmental concerns because of their ubiquitous presence in the environment and toxicity to animals[7-9].

High concentrations of PFCAs were frequently detected at sites impacted by aqueous film-forming foam in firefighting practices, with a median concentration of 26 μg/L in

groundwater and 21 μg/g in soil samples[9]. The National Health and Nutrition Examination Survey (NHANES) conducted by Centers for Disease Control and Prevention (CDC) from 1999 revealed that PFCAs such as perfluorooctanoic acid (PFOA) were present in all the human serum samples in the U.S[10]. PFCAs are associated with carcinogenicity, infertility, birth defects, and reduced immune function at pars-per-million levels[1, 11-13]. PFOA has been included in the EPA Contaminant Candidate List 3[14] and is a potential candidate of Substance of Very High Concern under the European chemical regulation[15]. Several studies reported using electrochemical, photolytic or sonochemical oxidation, and catalyzed hydrogen peroxide propagation to breakdown PFCAs[16-20]. However, these approaches require high energy inputs and/or special devices, thus limiting their applications.

For potential remediation applications, it is desirable to identify an approach that can decompose PFCAs under naturally relevant conditions. Enzyme-catalyzed oxidative humification reactions (ECOHRs) could serve such a role but have not been well examined. ECOHRs refer to an important class of reactions that are facilitated by extracellular enzymes such as peroxidases and phenoloxidases to mediate the polymerization of small molecule humic precursors into humic substances in the environment[21]. These enzymes oxidize phenolic or anilinic substrates into radical and quinone intermediates that are further covalently bond with each other via coupling[22]. The active intermediates formed during ECOHRs can also attack other inert compounds such as polychlorinated biphenyls (PCBs)[23] and polycyclic aromatic hydrocarbons (PAHs)[24], thus incorporating them into humification and leading to their decomposition and detoxification. In an earlier study, PFOA was effectively transformed in ECOHRs mediated by horseradish peroxidase (HRP)[25]. Laccase is another enzyme that mediates ECOHRs[26], and in comparison to HRP, laccase is more suitable for possible in-situ remediation

applications because it can maintain its activity over a long period of time[27] and uses oxygen instead of hydrogen peroxide as an electron acceptor.

The objective of this study was to investigate the possibility and mechanisms of PFCA degradation by ECOHRs. To this end, we have conducted a series of experiments and characterizations using laccase as a model humification enzyme, 1-hydroxybenzotriazole (HBT) as a model mediator, and PFOA as the target chemical. HBT can be oxidized via laccase catalysis to form radicals[28], and has been used as a laccase mediator to degrade PAHs[24].

MATERIAL AND METHODS

Standards and reagents

Laccase from *Pleurotus Ostreatus* (EC 420-150-4), 1-hydroxybenzotriazole (HBT), and 2,6-dimethoxyphenol (DMP) were purchased from Sigma Aldrich (St. Louis, MO). The 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO) was purchased from Enzo Life Sciences (Farmingdale, NY). The PFCAs with the total carbon number from C4 to C11 and the surrogate standard perfluoro-n-[13C8]-octanoic acid (M8PFOA) were obtained from Wellington Laboratories (Ontario, Canada). The buffer salts, including cupric/magnesium/manganese sulfates (see Appendix A, Buffer Solution for a full list), were ACS grade and from Fisher Scientific (Pittsburgh, PA). HPLC grade acetonitrile, methanol, and dichloromethane were also from Fisher Scientific.

Experimental setup

The reactions were conducted in 100 mL of a mineral buffer (Appendix A, Material and Methods) containing 1.0 μM PFOA (414 μg/L). The initial enzyme activity was 1 U/mL and the initial HBT concentration was 0, 2, or 20 μM in different treatment. Laccase solution was freshly prepared and assayed. One unit of laccase activity is defined as the amount of enzyme that

causes one unit change in absorbance at 468 nm per minute of a DMP solution at pH 3.8 in a 1-cm light path cuvette[29] (See Appendix A, Materials and Methods and Figure S3.2). It is noted that the enzyme activity level used in this study (1 U/mL) fell in a common range (e.g., 0.265 - 15.9 U/mL) employed in previous laccase bioremediation studies[24, 30, 31] (see Appendix A, Laccase Activity Assay for detail).

All reactors were incubated in a shaker at 22 ^{o}C, and laccase and HBT were supplemented every six days (Appendix A, Experimental Setup). Three 0.5 mL aliquots of solution were taken from each reactor for quantification of C4-C11 PFCAs and HBT simultaneously at pre-selected time intervals. Correction of concentrations was performed to compensate the variation of solution volume caused by evaporation and supplementation. For product identification purposes, a positive control that contained only PFOA without enzyme or HBT and a negative control that did not contain PFOA but with repeated enzyme and HBT additions were also incubated and processed along with the treatment reactors (Appendix A, Experimental Setup). An additional experiment was conducted to compare PFOA degradation in ECOHRs in the absence and presence of DIPPMPO, a spin trap that can effectively scavenge HBT free radicals, and the detail is also provided in the Supporting Information (Appendix A, Experimental Setup).

Chemical analysis

The samples were spiked with M8PFOA as a surrogate standard before being subjected to solid phase extraction with HLB cartridge following a procedure reported in earlier studies[32, 33] with minor modification. PFCAs and HBT (Table S3.1) were quantified by Waters 2690 HPLC coupled with a Micromass Quattra tandem mass spectrometer (HPLC-MS/MS) (Waters,

Milford, MA). Details can be found in the Supporting Information (Appendix A, Material and Methods).

Identification of reaction products

Samples (5 mL) were taken after 157 days from selective treatments, and negative and positive controls. Each sample was extracted with 1 mL dichloromethane with vigorously shaking for 15 min. The extractants were reconstituted in methanol and analyzed by an Orbitrap Elite high resolution mass spectrometer (HRMS) (Thermo Scientific, San Jose, U.S.). The identification of the reaction products was based on element compositions and product ion spectra (MS/MS) (Appendix A, Material and Methods). A Dionex ICS-1000 ion chromatograph was used to quantify fluoride (Appendix A, Material and Methods).

RESULTS AND DISCUSSION

Removal of PFOA in aqueous solution

As shown in Figure 3.1A, no appreciable degradation of PFOA was found in the solution with laccase only. However, significant reduction of PFOA concentration was achieved with periodical addition of HBT (see Figure S3.1A for statistical analysis result), indicating the key role of HBT in this process. It is known that laccase converts HBT to its free radicals that can either be quenched via self-coupling or oxidation to benzotriazole[34] or attack other non-substrate chemicals,[35, 36] such as PFOA in this case. Continuous consumption of HBT was observed in the treatment systems (Figure S3.3). With periodic additions of 2 μM or 20 μM HBT (named as 1-2 and 1-20 treatments in Figure 3.1, respectively), the total quantities of PFOA removed in these two systems were not significantly different (0.0495 and 0.0505 μmole, respectively), even though the total HBT consumption was significantly different between the systems (2.28 μmole and 37.0 μmole, respectively). This is not surprising because it is known that the increase of

mediator concentration in an ECOHRs system can lead to increased radical quenching that outcompetes the degradation of the target inert chemical[23]. Thus, increasing HBT concentration does not necessarily enhance PFOA degradation proportionally. The ratio between the removed PFOA quantity and the total HBT consumption was 2.17% and 0.14% in the 1-2 and 1-20 treatment, respectively.

The PFOA degradation time-course data in Figure 3.1A was fitted to the pseudo-first-order rate model (Appendix A, Kinetic Analysis) and the results are displayed in Figure S3.3. The pseudo-first-order rate constant (k) was 0.0042/day ($r^2 = 0.84$) and 0.0044/day ($r^2 = 0.89$) for 1-2 and 1-20 treatment, respectively, corresponding to the half-lives of 165 and 157 days.

Reactions in the presence of DIPPMPO

An additional experiment was conducted to compare PFOA degradation in ECOHRs in the absence and presence of DIPPMPO, a spin trap that can effectively scavenge HBT free radicals[37] (Appendix A, Experimental Setup), and the results are presented in Figure 3.1B. In this additional experiment run, the 1-0 and 1-20 treatments without DIPPMPO addition essentially repeated the trends obtained in the earlier experiment shown in Figure 4.1A. The remaining PFOA was 93 ± 4.5% and 66 ± 7.6% for 1-0 and 1-20 treatment, respectively after 108 days (Figure 3.1B), while those were 95 ± 4.6% and 56 ± 4.0% in the earlier run at 105 days (Figure 3.1A). PFOA degradation was however significantly suppressed in the 1-20 treatment with DIPPMPO addition (see Figure S3.1B for statistical analysis result). This is strong evidence that laccase-produced HBT radicals played a key role in degrading PFOA.

Identification of degradation products

The degradation products of PFOA in ECOHRs systems were analyzed with HRMS (mass accuracy < 5 ppm) which allowed accurate determination of element compositions.

Possible PFOA degradation products (Table 3.1) were first identified by comparing the mass spectrum of the 1-20 treatment sample after 157 d of incubation with those of corresponding positive (PFOA only) and negative (no PFOA but with laccase and HBT) controls. No products were confirmed with authentic standards because no authentic standards were available. These products identified in the study are the ones having relatively high MS responses (Table 3.1). Products 3 (212%) and 4 (277%) have very high relative intensities comparing to PFOA (100%), followed by Product 6 (87.5%). It should be noted however that the peak intensities of different chemicals are strongly dependent on their molecular structures, and do not quantitatively reflect the concentrations of the chemicals in the absence of standards. Structures of selected products were further deduced according to their product ion spectra without standards (MS/MS) (Figure 3.2). For those compounds (product 5, 7, 9 and 10) not showing distinct product ions, speculated structures were given based on possible reaction pathways and element compositions. As shown in Table 3.1, most of the identified products were partially fluorinated alcohols/aldehydes and fluoroalkyl substituted aromatic compounds. The aromatic ring in product 9 and 10 was attributed to the reactions with an amino acid which entered the reaction system as an impurity in the laccase solution. This amino acid was determined as phenylalanine (Figure S3.5). Similarly, the amine in product 1 was probably from the amino acid residues in the enzyme solution. The double bond in product 2 and 4 may be formed from free radical rearrangement as reported in earlier study[25]. Release of fluoride was considered as an important indicator of PFOA degradation. Fluoride concentration in the sample after 157 days of incubation was 35.5 ± 2.18 μg/L while its levels were below the detection limit (1.5 μg/L) in both controls. The fluoride concentration would be 125.7 μg/L if complete mineralization of PFOA occurred. Therefore,

28.2% of PFOA defluorination was achieved in this study by a calculation method reported earlier[16].

Mechanisms of PFOA degradation

Based on the results discussed above, the mechanism of PFOA degradation in ECOHRs can be proposed (Figure 3.3). The reaction was initiated by transferring an electron from the carboxyl head group of PFOA to an HBT radical followed by Kolbe decarboxylation to form a perfluoroheptyl (C_7F_{15}) radical. The C_7F_{15} radical can go through hydrolysis with concurrent elimination of an HF molecule and a fluoride to form $C_6F_{13}COOH$. This Kolbe decarboxylation cycle was well recognized as the mechanism of eliminating CF_2 unit from PFCAs in electrochemical and persulfate oxidation processes[16, 38], leading to the formation of a perfluoroalkyl acid with one CF_2 unit less. Each process involves the generation a corresponding perfluoroalkyl radical. Alternatively, shorter-chain perfluoroalkyl radical can also be formed directly from longer-chain radical via free radical rearrangement[25]. The HBT radicals not only initiated PFOA decarboxylation but also converted other non-fluorinated organic chemicals in the solution to free radicals which cross-coupled with the perfluoroalkyl radicals to form the products (Table 3.1) that are either partially fluorinated or contain a perfluroalkyl moiety. Such free radical coupling is common to laccase-mediated reactions[39, 40]. These non-fluorinated chemicals were most likely the impurities such as amino acids or fermentation by-product alcohols (e.g., 1-propanol, 1-hexanol, 1,5-pentanediol) in laccase solution which was confirmed by HRMS analysis (Figure S3.5 and S3.6).

To summarize, this study indicates that ECOHRs can effectively transform PFOA to shorter-chain, partially fluorinated products in the presence of HBT. Since these products are seemingly analogues of PFCA precursors, it is possible that some of them may be further

transformed to their corresponding shorter-chain PFCAs under extreme oxidative conditions, but these partially fluorinated products, as well as shorter-chain PFCAs, are believed to be more environmentally benign[41]. It should be noted that laccase substrates with phenolic functional groups are abundant in natural organic matter, which may serve as mediators for ECOHRs[42]. Such natural processes may only effect very slow PFOA degradation given the low PFOA concentrations and variable humification enzyme activities in the natural environment, but it is possible to enhance the humification reactions through engineering approach for remediation purposes.

SUPPORTING INFORMATION AVAILABLE

Additional experimental details, figures, and table are available in Appendix A.

ACKNOWLEDGEMENTS

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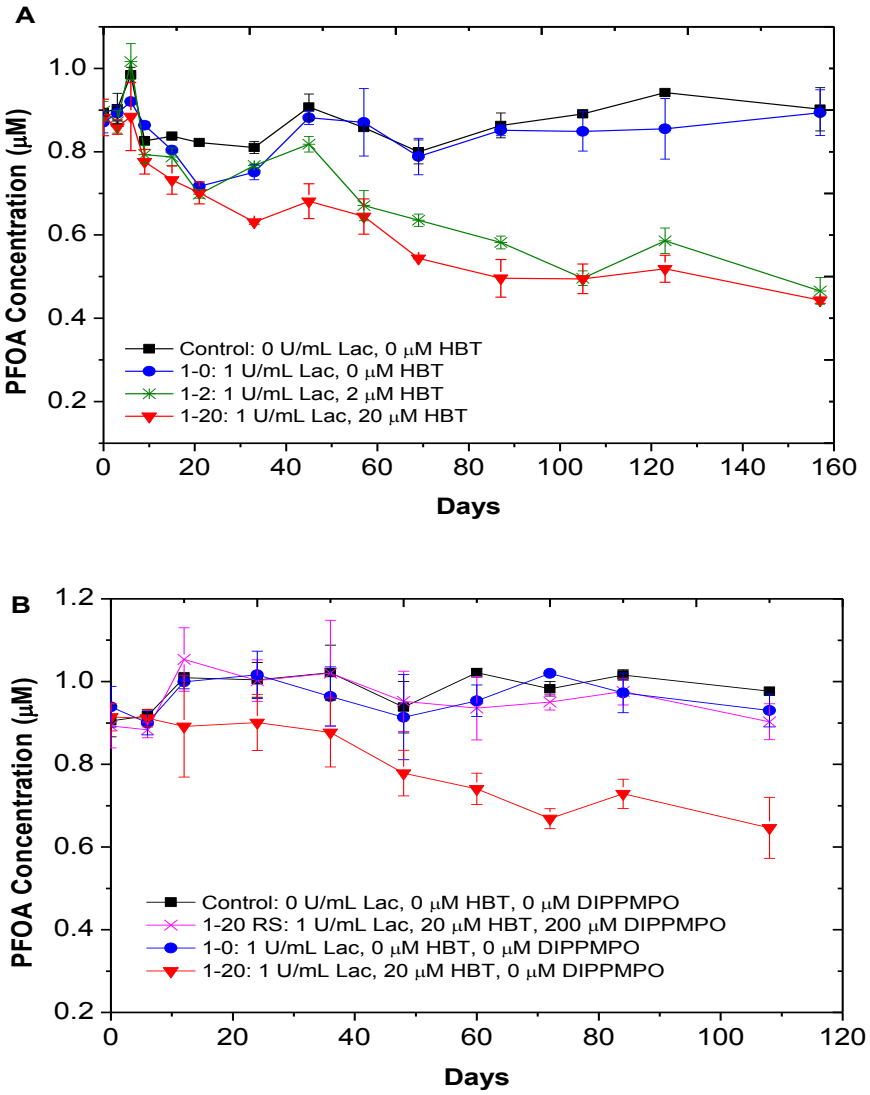


Figure 3.1. (A) Change of PFOA concentration in ECOHRs over time. Control: the positive control sample to which no laccase or HBT was added; 1-0: 1 U/mL laccase added every 6 d but no HBT; 1-2: 1 U/mL laccase and 2 μM HBT added every 6 d; 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d. (B) Change of PFOA concentration in ECOHRs with or without the addition of DIPPMPO as a HBT radical scavenger. 1-20 RS: 1 U/mL laccase, 20 μM HBT, and 200 μM DIPPMPO added every 6 d.

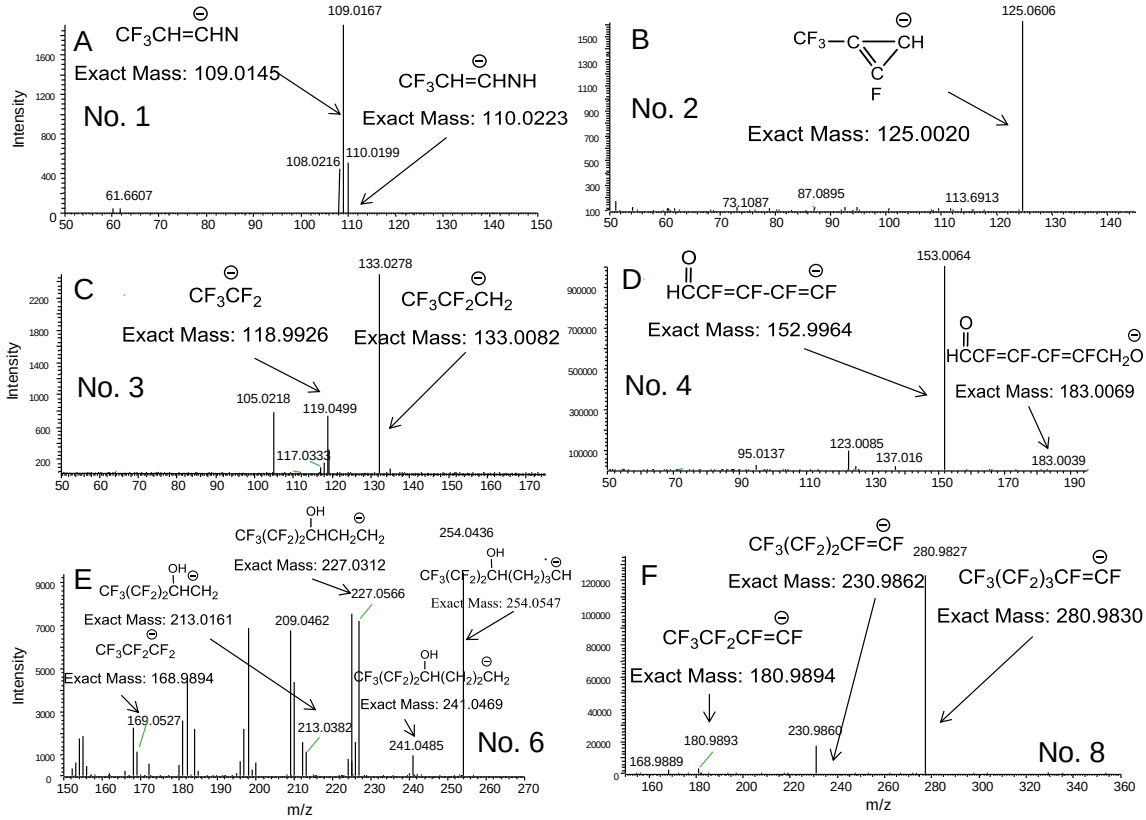


Figure 3.2. MS/MS spectra of PFOA degradation products given by high resolution mass spectrometry and their possible transition ions. (A) Product No. 1: $C_4H_4F_3ON$, measured m/z= 138.0164; (B) Product No. 2: $C_4H_4F_4O$, measured m/z= 143.0128; (C) Product No. 3: $C_4H_5F_5O$, measured m/z= 163.0186; (D) Product No. 4: $C_6H_4F_4O_2$, measured m/z= 183.0062; (E) Product No. 6: $C_8H_{14}F_4O_2$, measured m/z= 271.0558; (F) Product No. 8: $C_7HF_{13}O$, measured m/z= 346.9742.

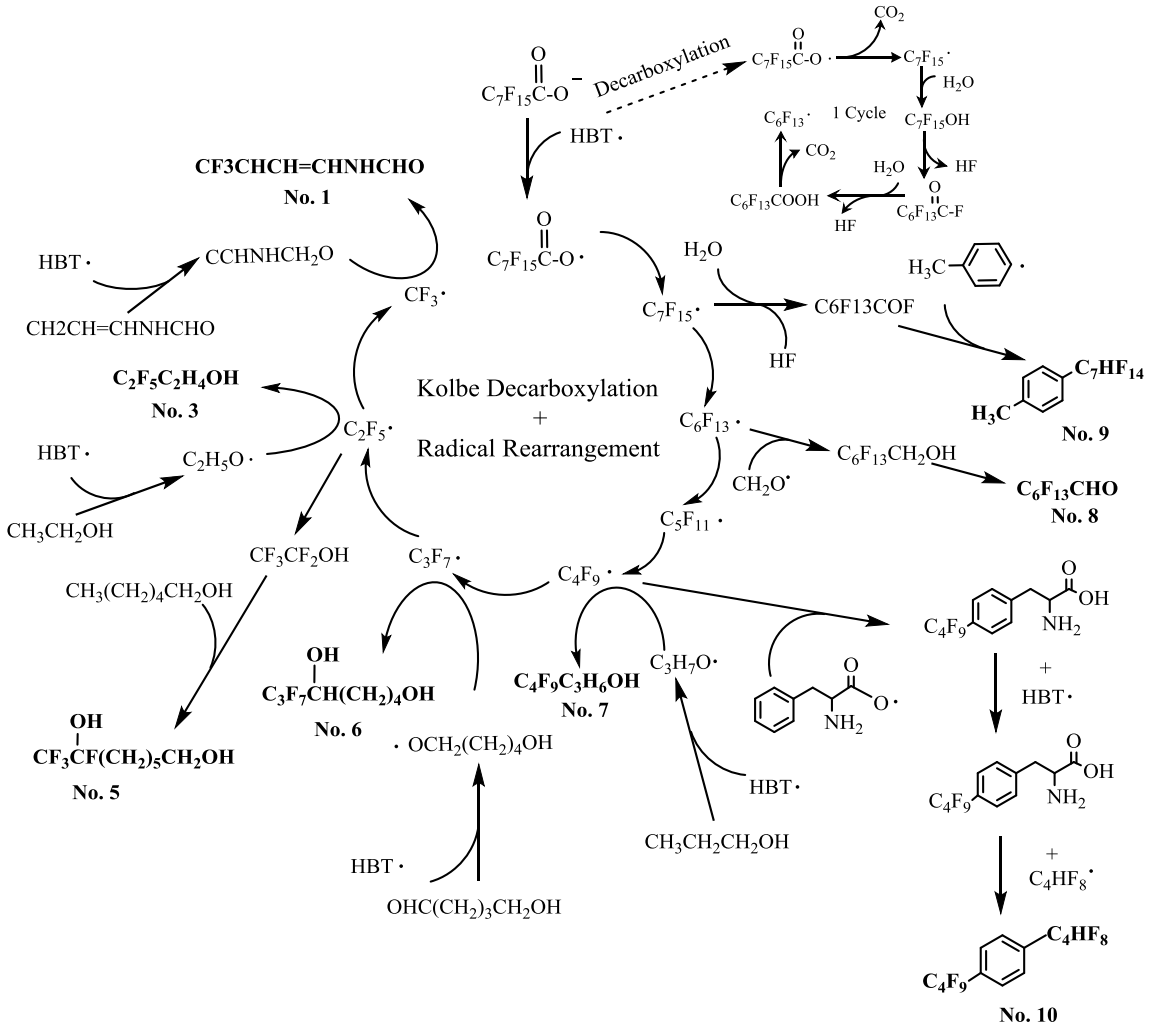
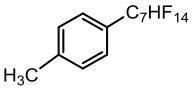
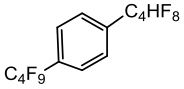
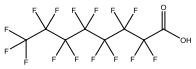


Figure 3.3. Proposed PFOA degradation pathways during ECOHRs treatment with laccase and 1-hydroxybenzotriazole.

Table 3.1. Molecular formulas, theoretical and measured deprotonated molecule weight $[M-H]^-$, mass accuracy (ppm), possible structures, and HRMS responses of PFOA degradation products from ECOHRs.

No	Formula	$[M-H]^-$		Mass accuracy (ppm)	Possible structure	Relative intensity[1]
		Theoretical	Experimental			
1	$C_4H_4F_3ON$	138.0167	138.0164	2.0	$CF_3CH=CHNHCHO$	0.029%
2	$C_4H_4F_4O$	143.0120	143.0128	0.0	CF_3-CH=CF-CH_2OH	15.4%
3	$C_4H_5F_5O$	163.0182	163.0186	-2.3	$CF_3CF_2CH_2CH_2OH$	212%
4	$C_6H_4F_4O_2$	183.0069	183.0062	3.9	$HCCF=CF-CF=CFCH_2OH$ (O)	277%
5	$C_8H_{14}F_4O_2$	217.0852	217.0856	-2.0	$CF_3CF(CH_2)_5CH_2OH$ (OH)	38.8%
6	$C_8H_{11}F_7O_2$	271.0569	271.0558	4.1	$CF_3(CF_2)_2CH(CH_2)_3CH_2OH$ (OH)	87.5%
7	$C_7H_7F_9O$	277.0275	277.0270	1.8	$C_4F_9C_3H_6OH$	14.0%
8	$C_7HF_{13}O$	346.9742	346.9742	-0.1	$CF_3(CF_2)_5CHO$	1.99%
9	$C_{14}H_8F_{14}$	441.0324	441.0326	-0.4		14.3%
10	$C_{14}H_5F_{17}$	495.0041	495.0040	0.3		14.0%
PFOA	$C_8F_{15}O_2H$	412.9659	412.9648	-2.6		100%

1: Relative intensity equals to the absolute intensity of product divided by the absolute intensity of PFOA measured by HRMS.

CHAPTER 4

FACTORS CONTROLLING THE RATE OF LACCASE-MEDIATED PERFLUOROOCTANOIC ACID DEGRADATION: THE ROLE OF METAL IONS [2]

[2] Luo, Q.; Wang, Z.; Feng, M.; Lu, J.; Chiang, S.-Y. D.; Liang, S.; Lu, J.H.; Woodward, D.; Huang, Q. To be submitted to *Environmental Science & Technology*.

ABSTRACT

The study investigated degradation of perfluorooctanoic acid (PFOA) in enzyme-catalyzed oxidative humification reactions (ECOHRs) mediated by laccase with 1-hydroxybenzotriazole (HBT) as mediator. The reaction rates were examined under various conditions with different initial PFOA concentrations, laccase and HBT dosages in different buffer solutions. PFOA degradation followed pseudo-first order kinetics, and the rate constants (k) were similar for the systems with high (100 μM) and low (1 μM) initial PFOA concentration under an optimum treatment condition, at 0.0040 day^{-1} (r^2 = 0.98) and 0.0042 day^{-1} (r^2= 0.86), respectively. The metal ions contained in the reaction solution appeared to have a strong impact on PFOA degradation. Differential UV-vis spectrometry revealed that the metal ions that can complex with PFOA, e.g., Cu^{2+} and Fe^{3+}, facilitated PFOA degradation. These metal ions can probably bridge negatively charged PFOA and laccase, so that the HBT radicals (BTNO) released from laccase surface can reach and react with PFOA. Fluoride and partially fluorinated compounds were detected as PFOA degradation products using ion chromatography and high resolution mass spectrometry. The structures of the products suggest the reaction pathways involving BTNO-initiated radical decarboxylation, rearrangement and cross-coupling.

INTRODUCTION

A large quantity of perfluoroalkyl acids (PFAAs) had been produced to meet the massive demand in various important industrial applications such as surfactant, fire retardant, and surface treatment[1, 2]. The wide applications of PFAAs are attributed to their extreme thermal and chemical stability as well as their hydrophobic and lipophobic nature[3]. These physical-chemical properties are derived from the unique structural features of PFAAs in which all hydrogens in the carbon-carbon skeleton are replaced by fluorines[4]. PFAAs enter the environment primarily

through emission from manufacturing facilities[5], utilization of PFAA-based products[6], and transformation from polyfluoroalkyl precursors[7]. Relatively high concentrations of PFAAs have been frequently detected near the manufacturing plants and disposal sites. For example, the wastewater collected from the firefighting training areas where the aqueous film-forming foams (AFFFs) were applied contained up to 3.35 mmole L^{-1} of PFAAs[8]. The total concentration of PFAAs was up to 0.063 mmole kg^{-1} in the soil sampled from a former industrial waste disposal site[9].

Perfluorooctanoic acid (PFOA) is a PFAA that has drawn tremendous attention from both the public and the scientific community due to its potential adverse effects to human health and the environment[10]. The potential health risks from exposure to PFOA include hepatic, reproductive, developmental, immunological, and endocrine system toxicity[11]. In 2008, the European Food and Safety Authority has established a tolerable daily intake value for PFOA of 1.50 μg kg^{-1} body weight[12]. The U.S. Environmental Protection Agency (EPA) plans to eliminate PFOA emission from the U.S. by 2015[13]. Numerous studies have been devoted to develop treatment and remediation techniques for PFOA contamination. The conventional advanced oxidation processes (AOPs) have been proven ineffective in PFOA degradation[14]. Other techniques such as electrochemical, photolytic, and sonochemical oxidation either involve high energy inputs or require special devices to achieve effective PFOA degradation[15]. It is thus of an urgent need to develop a feasible treatment and remediation strategy to address PFOA contamination.

Earlier studies indicated that enzyme catalyzed oxidative humification reactions (ECOHRs) were effective in degrading PFOA under environmentally relevant conditions, and thus potentially feasible for remediation applications[16, 17]. ECOHRs are indeed involved in

natural humification processes mediating degradation of lignocellulosic materials as well as polymerization of small humic precursor molecules into humic substances. A number of extracellular enzymes such as peroxidases and phenol oxidases can mediate ECOHRs in soil[18].

Laccases are a group of phenol oxidases that mediate ECOHRs, and well studied for their roles in the natural wood delignification process[19]. Laccases can directly react with substrates containing phenolic or anilinic functional groups to convert them into active intermediates such as radicals and quinones[20, 21]. These active intermediates can further react with recalcitrant organic matters such as lignin, the most persistent natural organic material in the environment, to cause their degradation. Such laccase-mediator systems have been shown capable of degrading persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs)[22] and polycyclic aromatic hydrocarbons (PAHs)[23]. The mediators such as 1-hydorxybenzotriazole (HBT), vanillin, ferulic acid, and syringaldehyde, etc. have been used in these studies because of their high efficiency and low environmental impact[24]. These model mediators also contain functionalities that are commonly present in natural organic matter.

Our recent study revealed that 1 μM PFOA in a mineral buffer was transformed about 50% to partially fluorinated compounds via ECOHRs in a laccase-HBT system after 157 days of incubation[16]. It is proposed that the benzotriazole nitroxyl radical (BTNO) generated from HBT during ECOHRs could induce the decarboxylation of perfluorocarboxylic acids (PFCAs) to form perfluoroalkyl radicals. The perfluoroalkyl radicals then further reacted with other non-fluorinated free radicals that were also generated by ECOHRs to form partially fluorinated cross-coupling compounds.

In this study, we systematically evaluated the kinetics of PFOA degradation in laccase-HBT systems with key conditions varied, including PFOA and HBT concentrations and ionic

compositions in the solution, in an attempt to identify factors controlling the reaction rate. It was found in particular that the multivalent ions presented in the solution played a significant role, and the mechanism was elucidated. The results provide a basis to assess the feasibility of ECOHRs in PFOA remediation and means to optimize the process for potential application.

EXPERIMENTAL SECTION

Chemicals and reagents

Perfluorooctanoic acid (PFOA), laccase from *Pleurotus Ostreatus* (EC 420-150-4), 1-hydroxybenzotriazole (HBT), and 2,6-dimethoxyphenol (DMP) were purchased from Sigma Aldrich (St. Louis, MO). Perfluorocarboxylic acids (PFCAs) with total carbon-chain length from C4-C11 and a surrogate standard perfluoro-n-[^{13}C8]-octanoic acid (M8PFOA) were obtained from Wellington Laboratories (Ontario, Canada) (Appendix A, Table S3.2). Cupric/magnesium/manganese sulfates, citric acid and sodium citrate were from Fisher Scientific (Pittsburgh, PA). All organic solvents were HPLC-grade and also from Fisher Scientific, including acetonitrile, methanol, and dichloromethane.

Experimental setup

The PFOA degradation experiments were carried out in polypropylene bottles at 22 ^{o}C with continuous shake at 120 rpm in an incubator (Innova 42, New Brunswick Scientific). Each reactor contained 100 mL solution with PFOA initial concentration at 1 or 100 μM. The solution was prepared in a mineral buffer, a citric buffer, or a solution containing different metal ions (Cu^{2+}, Mg^{2+}, and Mn^{2+} at 0.1, 1.0, or 10 mM, respectively, or Fe^{3+} at 200 μM) for comparison. The major components in the mineral buffer included $CuSO_4$, $MgSO_4$, and $MnSO_4$. The pH for the mineral buffer and the citric buffer were both 4.5. The metal ion solution was prepared without additional pH buffering, and the determined pH values for each metal ion solution were

listed in Table S3.1. The details for preparation of each solution were provided in Appendix A. Each reactor also contained 0, 20 or 100 μM HBT and was dosed with 1 U mL^{-1} laccase to initiate ECOHRs treatment. Every six days during the treatment, the reactor was repeatedly dosed with freshly prepared enzyme stock solution to replenish the laccase activity at 1 U mL^{-1}, while at the same time 0, 20 or 100 μM of HBT was also added to the corresponding treatment. Blank control samples without laccase or HBT addition were also prepared and processed at the same time with the same volume of HPLC water and acetonitrile supplemented instead of enzyme and HBT stock solution.

Samples were withdrawn from each reactor at preselected time intervals. For the experiment with initial PFOA concentration at 1 μM, triplicates of 0.5-mL solution were sampled from each reactor and mixed with 0.5 mL of 0.5 μM M8PFOA as a surrogate standard. For the experiment with initial PFOA concentration at 100 μM, triplicate 0.1-mL samples were taken, and each was diluted with 0.9 mL HPLC water, from which a 0.1-mL subsample was taken and mixed with 0.5 mL of 0.5 μM M8PFOA. All mixtures were subjected to solid phase extraction (SPE) cleanup as reported in our previous study[16] (a brief description is provided in Appendix B) and then analyzed for PFOA and HBT concentrations described below. The variation of concentrations caused by solution evaporation and supplement was adjusted by weighting the reaction solution before and after each supplement event. A 20-μL solution from each reactor was taken every six days for laccase activity assessment using a method reported by Park et al[25]. One unit of laccase activity is defined as the amount of enzyme that causes one unit change in absorbance at 468 nm per minute of a DMP solution at pH 3.8 in a 1 cm light path cuvette[25] (details is included in Appendix B).

Chemical analysis

At selective time, 2-mL solution was taken from the reactor for fluoride analysis. The concentrations of fluoride ion were measured using an ion chromatography method which was reported in our previous study[16] (a description provided in Appendix B). Quantitative analysis of PFOA and HBT was performed with a Waters AQCUITY I-class UPLC system coupled with a XEVO TQD mass spectrometer (Waters, Milford, MA). The separation was carried out on a Waters UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters, Milford, MA) at 40 ℃ using a gradient composition of solvent A (5 mmol L^{-1} ammonium acetate in HPLC water) and solvent B (5 mmol L^{-1} ammonium acetate in methanol). The flow rate was 0.3 mL min^{-1} with a gradient program lasting for 10 min: 0−0.5 min, hold at 10% B; 0.5−8 min, linearly increased B from 10% to 95%; 8−8.1 min, a linear decrease from 95% to 100%; 8.1−9 min, linearly reduced B to 10%, and then equilibrium at 10% B for 1 min. Electrospray ionization was operated in a negative mode with the parameters set as capillary voltage at -1.0 kV, desolvation temperature at 400 ^{o}C, source block temperature at 150 ^{o}C. Nitrogen (> 99.999% purity, Airgas) was used as desolvation gas with a flow rate of 550 L $hour^{-1}$. PFOA, M8PFOA, HBT, and seven additional PFCAs (C4-C11) were monitored simultaneously using multiple reaction monitoring. The precursor and transition *m/z* values of all the monitored PFCAs and HBT as well as their detection limits are listed in Table S3.2.

Identification of reaction products

High-resolution mass spectrometry (HRMS) was used to identify reaction products in selected treatment reactors. To this end, a positive control reactor that contained only PFOA without enzyme or HBT addition and a negative control reactor that did not contain PFOA but with repeated enzyme and HBT additions using the same regime as for the treatment reactor

were also prepared, processed, and analyzed along with the treatment reactors. At the end of incubation, 1 mL solution was taken from each reactor and extracted with 1 mL dichloromethane. The extractant was then reconstituted in 20-μL methanol and analyzed using an Orbitrap Elite HRMS with full scan (resolution R= 250,000 at *m/z* 400, for *m/z* = 10 to 1000) at ESI negative mode (Thermo Scientific, San Jose, U.S.)

By comparing the mass spectra, the *m/z* peaks that were detected in the reaction samples but not in the negative or positive controls were identified as possible products. The element composition of a possible product was assigned by its accurate *m/z* value using a formula generator program (Molecular Weight Calculator by Matthew Monroe) with the mass error range set at 5 ppm that matches the range of the HRMS used in this study, and the conventional molecular mass rules, including the carbon-hydrogen ratio and nitrogen rule, were employed to exclude unreasonable element compositions. The structure of a possible product was further deduced from its corresponding fragment ion spectra obtained from tandem mass spectrometry on the Orbitrap Elite HRMS with tandem mass fractionation (resolution R = 60 000 at *m/z* 400, for *m/z* = 100 to 1000) (Thermo Scientific, San Jose, U.S.).

Differential UV-vis Spectrometry

A differential UV-vis spectrometry method[26] was used to probe possible interactions between metal ions and PFOA. To this end, a 2-mM metal (Cu^{2+}, Mg^{2+} or Mn^{2+}) sulfate solution was mixed with a 2-mM PFOA solution by different ratios to prepare a series of 25-mL mixtures in citric buffer solution with the pH controlled at the values equal to those of unbuffered solutions containing the corresponding metal ions at 10 mM, i.e. pH 4.9 for Cu^{2+}, pH 7.0 for Mg^{2+}, and pH 6.5 for Mn^{2+}. The combinations of the metal ion and the PFOA molar concentrations were maintained at 0.8 mM for all mixtures. Additional reference solutions

without metal ion or PFOA were also prepared in the same manner. All mixtures and reference solutions were allowed to equilibrate for 24 hours prior to being scanned on a Beckman DU800 spectrophotometer for absorbance spectra at wavelengths from 200 to 800 nm. A differential absorbance spectrum (DAS) was constructed by the following equation:

$$\Delta A_{\lambda,\ DAS} = A_{\lambda,\ mixture} - A_{\lambda,\ PFOA} - A_{\lambda,\ cation}$$

where $A_{\lambda,\ mixture}$, $A_{\lambda,\ PFOA}$ and $A_{\lambda,\ cation}$ are the absorbance at λ wavelength of the mixture solution, the corresponding reference PFOA and cation solution, respectively.

RESULT AND DISCUSSION

Rate of PFOA removal during ECOHRs

Figure 4.1 depicts the change of PFOA concentration over time in a laccase-HBT reaction system in the mineral buffer, with PFOA initial concentrations at 1 or 100 μM, respectively. For both initial concentrations, PFOA concentrations remained stable in the systems with addition of only 1 U mL^{-1} of laccase but no mediator, which were essentially no different from the control systems to which no enzyme or HBT were added. PFOA degradation was evident when both laccase and HBT were added repetitively. For the system with PFOA initial concentration at 1 μM, the removal of PFOA was approximately 43.5% with the addition of 1 U mL^{-1} laccase and 20 μM HBT (named as 1-20 in Figure 4.1) after 156 days of incubation; while that for the system with PFOA initial concentration at 100 μM was 46.8%. The PFOA degradation data in Figure 4.1 were fitted well to the pseudo-first-order rate model. The reaction rate constants (k) for 1 μM PFOA (0.0042 day^{-1}, $r^2 = 0.86$) and 100 μM (0.0040 day^{-1}, $r^2 = 0.98$) systems in the 1-20 ECOHR treatment were similar to each other. This result suggests that the reaction rate is not limited by the increase of PFOA initial concentration, which has ramification in potential application of ECOHRs for PFOA remediation. It is notable that the 1-20 treatment

for the system with PFOA initial concentration at 1 μM replicates the condition that we have tested in our previous study[16], in which PFOA removal was 50% after 157 days of treatment with a pseudo-first-order rate constant of 0.0044 day^{-1} (r^2 = 0.89) that is very close to this study (0.0042 day^{-1}), indicating reasonable reproducibility of the experiment.

Effect of mediator concentration

The removal of PFOA (initial concentration = 100 μM) during ECOHRs in the mineral buffer was evaluated with HBT addition at two different dosages, 20 μM or 100 μM, as depicted in Figure 4.1B, specified as 1-20 or 1-100 treatment, respectively. PFOA removal was 46.8% in the 1-20 treatment while that for the 1-100 treatment was 38% at day 156. The pseudo-first order reaction rate constant (k) was 0.0040 day^{-1} (r^2 = 0.98) and 0.0030 day^{-1} (r^2 = 0.93) for 1-20 and 1-100 treatment, respectively, corresponding to the half-lives of 173 and 231 days. The consumption of HBT over the reaction period was also quantified (Figure S1A, Appendix B). These data indicated that, although about four times of HBT was converted by laccase in the 1-100 treatment (201.2 μmole) than in the 1-20 treatment (51.7 μmole), the increase of HBT dosage did not necessarily enhance the chance of BTNO to attack and react with PFOA. Instead, the production of excessive BTNO instantaneously may stimulate radical quenching pathways and thus mitigate PFOA degradation.

The laccase activities in the reaction solutions were also monitored during the incubation period, and are shown in Figure S1B (Appendix B). It is evident that laccase deactivated more rapidly in the systems with higher HBT concentration, in the order 1-100 > 1-20 > 1-0 treatment. This is reasonable because the radicals generated from the active substrates of an ECOHR enzyme can deactivate the enzyme, known as a suicide mechanism[27]. Therefore, the balance between laccase stability and the productivity of BTNO governs the ECOHR efficiency in these

reaction systems. Based on the data, the 1 U mL^{-1} laccase and 20 μM HBT combination (1-20 treatment) offered the most PFOA removal.

The impact of solution composition

We have made an important observation that the PFOA removal occurring in the mineral buffer (pH 4.5) as shown in Figure 4.1A was not evident in a citric buffer solution (pH 4.5) as seen in Figure 4.2. Apparently, the compositions of the buffer solutions had significant influence in the reaction rates. Likely, the metal ions included in the mineral buffer solution, 1.60 mM of Cu^{2+}, 0.203 mM of Mg^{2+}, and 0.002 mM of Mn^{2+}, may play an important role in facilitating PFOA degradation in ECOHRs. Several earlier studies have indicated that the presence of metal ions such as Fe^{3+} enhanced the photolytic decomposition of PFCAs[28-30].

We then evaluated PFOA degradation in a series of solutions that each contained an individual metal ion, Cu^{2+}, Mg^{2+} or Mn^{2+}, at different concentrations under the same laccase and HBT regime as the 1-20 treatment in the mineral buffer solution. As shown in Figure 4.3A, PFOA degradation was evident in the presence of Cu^{2+}, and it increased with the increase of Cu^{2+} concentration. Little, if any, PFOA degradation was observed in the Mg^{2+} or Mn^{2+} solutions regardless of their concentrations (Figure 4.3B and 4.3C). An earlier photochemical degradation study also showed that Cu^{2+} enhanced PFOA degradation more than Mg^{2+} and Mn^{2+} ions[30].

The role of metal ions

We hypothesized that the enhancement effect of divalent metal ions was related to their charge interactions with the enzyme and PFOA in the ECOHRs systems. Both PFOA (pKa 0.6)[31] and laccase (pI 4.0)[32] were negatively charged in all examined conditions. They thus repulsed each other due to the electrostatic force. Meanwhile, the short lifespan of BTNO[33, 34] limited its chance of reaching PFOA after being released from the enzyme. It is possible that certain

divalent metal ions, such as Cu^{2+}, could bridge negatively charged PFOA and laccase by forming complexes which enabled the BTNO radicals released from laccase to attack PFOA.

We further assessed the interactions between PFOA and metal ions using the differential absorption spectra[26, 35] as described in the experimental section. A differential absorbance spectrum was obtained by subtracting the absorbance of the ion solution and the PFOA solution from that of their mixture solution, and a series of such differential spectra were acquired for mixture solutions with PFOA and the metal ion at different ratios.

The UV-vis spectra of PFOA at different concentrations are presented in Figure S4.2 (Appendix B) exhibiting a signature absorbance at 212 nm. The differential absorbance spectra (DAS) of Mg^{2+}-PFOA and Mn^{2+}-PFOA (Figure 4.4B and Figure 4.4C) both have a distinct band with peak wavelength at 212 nm, which corresponds with signature absorbance of PFOA. For the Cu^{2+}-PFOA DAS, a similar trend at the wavelength 212 nm was also observed. However, unlike Mg^{2+}-PFOA and Mn^{2+}-PFOA, the Cu^{2+}-PFOA DAS had an intensive negative peak at 265 nm which varied as a function of the component ratio. The negative peak with such variation suggested the change of electronic density in the molecules caused by the formation of a complex between Cu^{2+} and PFOA[26, 36]. The result supports the notion that a divalent ion (e.g. Cu^{2+}) that complexes with PFOA may facilitate PFOA degradation; while that does not complex with PFOA (e.g. Mg^{2+} and Mn^{2+}) at the examined conditions has no impact on PFOA degradation.

An earlier study indicated the formation of a complex between Fe^{3+} and PFOA[30]. We therefore further tested PFOA degradation in ECOHR systems in the presence of ferric ion, and the results are shown in Figure 4.5. Continuous degradation of PFOA was observed, and it followed the pseudo-first order kinetics ($k = 0.0032$, $r^2 = 0.82$) in the 1-20 treatment system in a

solution containing 200 μM Fe^{3+}. PFOA removal reached 35% after 108 days of incubation which is similar to that in the mineral buffer (38% after 114 days).

PFOA degradation products

The concentration of fluoride was quantified using ion chromatography in the ECOHR samples with PFOA initial concentration at 100 μM in the mineral buffer, and the results are displayed Figure 6. It shows that the fluoride concentration increased along with PFOA degradation (shown in Figure 4.1B) in both 1-20 and 1-100 treatment samples. At day 42, the fluoride concentration in 1-100 treatment (3.79 mg L^{-1}) was nearly twice of that in 1-20 treatment (1.99 mg L^{-1}) which corresponds to the PFOA removal of 20.3% and 12.4% respectively. When PFOA removal in 1-20 treatment (30.5%) caught up with 1-100 treatment (34.1%) after 96 days of incubation, the fluoride concentrations in these two samples also reached a similar level (3.90 mg L^{-1} and 4.75 mg L^{-1} respectively). Therefore, the corresponding defluorination ratios at day 96 were 44.8% and 48.9% for 1-20 and 1-100 treatment respectively based on the equation given in the previous research[37].

Possible degradation products were also identified using HRMS in the 1-20 treatment sample with 100 μM initial PFOA concentration after 156 days of incubation, as described in experimental section. A total of 12 products were identified (Table 4.1), and the molecular structures were deduced from elemental composition determined by the accurate mass of the molecular ion (< 5 ppm) and the fragment ion spectra from tandem mass spectrometry. All fragment ion spectra of degradation products were summarized in supporting information Figures S4.3 and S4.4. The same HRMS technique has been used in our earlier study to identify reaction products in a 1-20 treatment system with 1 μM initial PFOA concentration in the mineral buffer[16].

All products identified in this study (Table 4.1) are partially fluorinated or contain perfluroalkyl moieties, which are similar to those identified in the earlier study[16], although are not of exactly the same structures. Such products were likely formed from a combination of free radical decarboxylation, rearrangement and cross-coupling processes as proposed in the earlier study[16]. Namely, the reaction is initiated by transferring an electron from PFOA to BTNO radical followed by Kolbe decarboxylation cycles and/or radical rearrangements to form shorter-chain fluoroalkyl radicals, including ones with double bonds. The BTNO radicals do not only initiate PFOA decarboxylation but also convert other non-fluorinated organic chemicals in the solution to free radicals, which then cross-couple with the fluoroalkyl radicals to form the products (Table 4.1) that are either partially fluorinated or contain a perfluroalkyl moiety. It is worth noting that the molecular structures of products No. 6 and 12 contained HBT moieties (Table 4.1; Appendix B, Figures S4.3 and S4.4). This is direct evidence of cross-coupling between BTNO and PFOA derived fluoroalkyl radicals. This kind of structures was not found in the earlier study, probably due to the much lower PFOA concentration.

The detection of fluoride and partially fluorinated compounds in the product solution confirmed PFOA degradation during ECOHRs, and verified the pathways involving BTNO-initiated radical decarboxylation, rearrangement and cross-coupling, although the products detectable may vary depending on reaction conditions, such as PFOA initial concentration and incubation time. PFOA degradation during ECOHRs followed the pseudo-first-order kinetics, and the rate is strongly impacted by the metal ions present in the solution. Differential absorbance spectra revealed that the metal ions that can form a complex with PFOA at the examined conditions, e.g. Cu^{2+} and Fe^{3+}, can bridge between negatively charged PFOA and laccase thus facilitating PFOA degradation.

SUPPORTING INFORMATION AVAILABLE

Additional experimental details, figures, and table are available at Appendix B.

ACKNOWLEDGEMENTS

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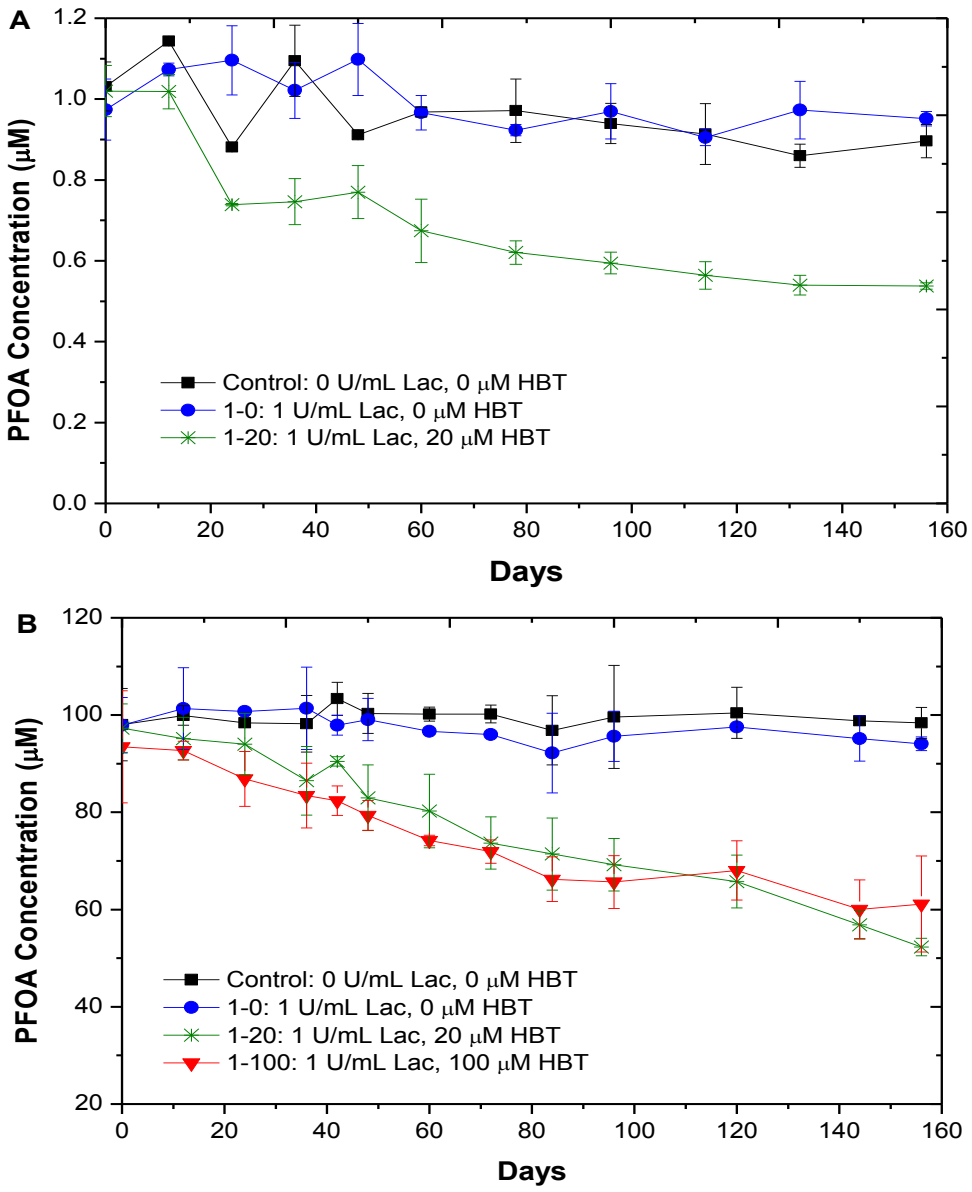


Figure 4.1. Change of PFOA concentration in laccase-mediated ECOHRs over time. (A) PFOA initial concentration of 1 μM; (B) PFOA initial concentration of 100 μM. Control: the positive control to which no laccase or HBT was added; 1-0: 1 U/mL laccase added every 6 d but no HBT; 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d; 1-100: 1 U/mL laccase and 100 μM HBT added every 6 d.

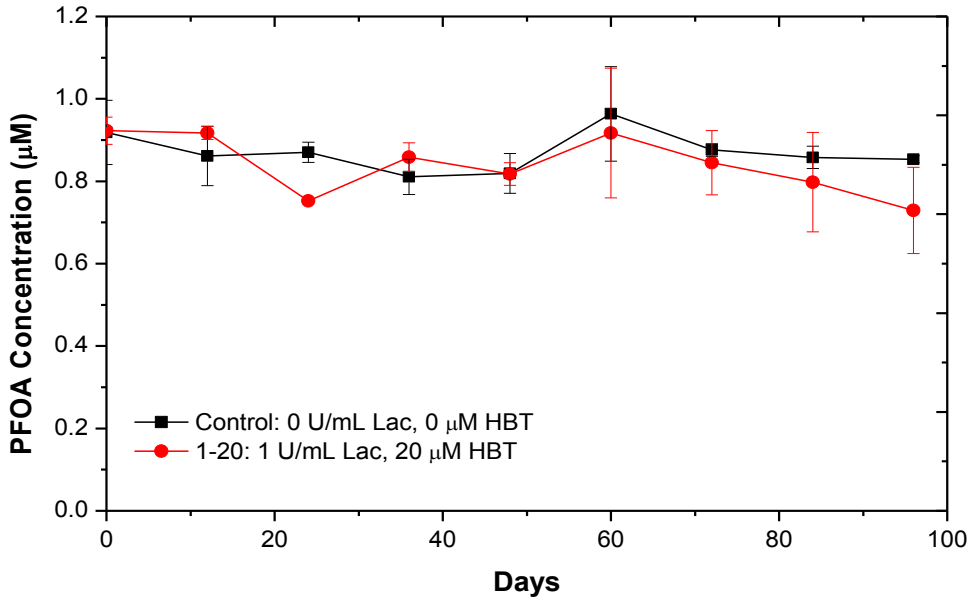


Figure 4.2. Change of PFOA concentration during ECOHRs treatment in pH 4.5 citric buffer with PFOA initial concentration of 1 μM. Control: the positive control sample to which no laccase or HBT was added; 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d.

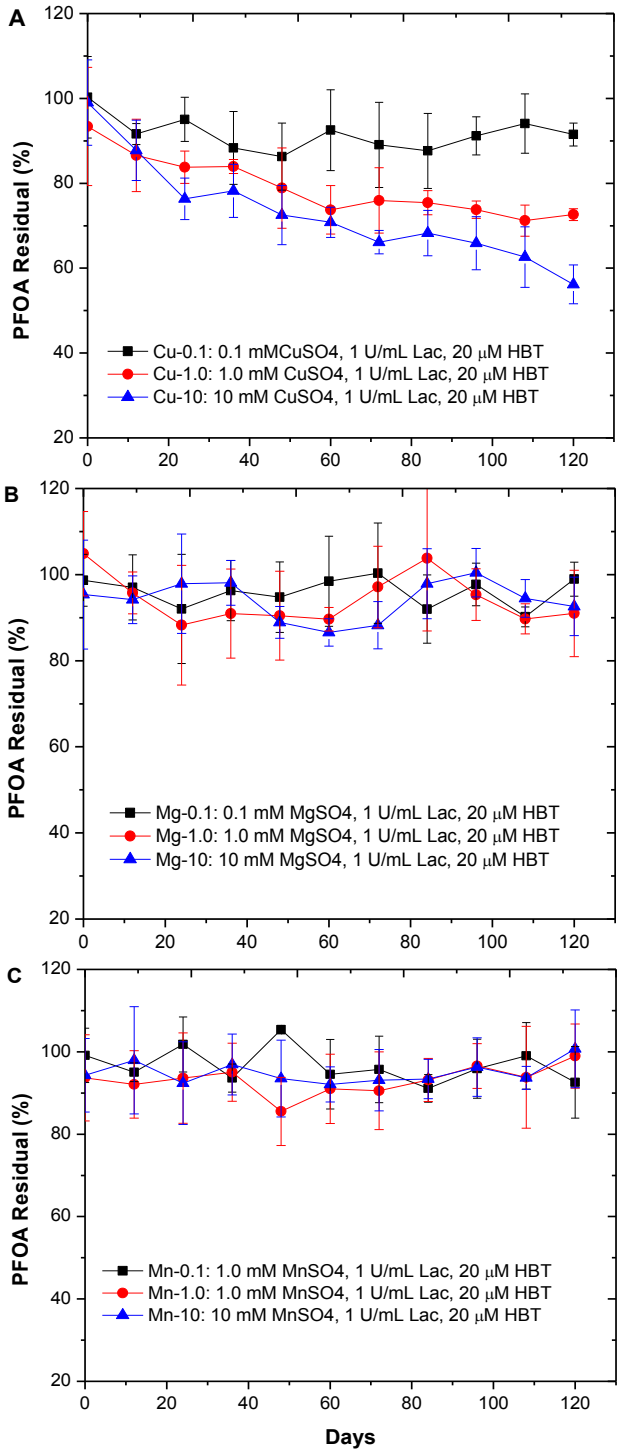


Figure 4.3. Change of PFOA residual in 1 U/mL laccase and 20 μM HBT induced ECOHRs over time with the presence of 0.1/1.0/10 mM cations: (A) Cu^{2+}; (B) Mg^{2+}; and (C) Mn^{2+}. The PFOA residual is calculated by dividing the PFOA concentration in the reaction sample by that in the control (without laccase or HBT) sampled at the same time.

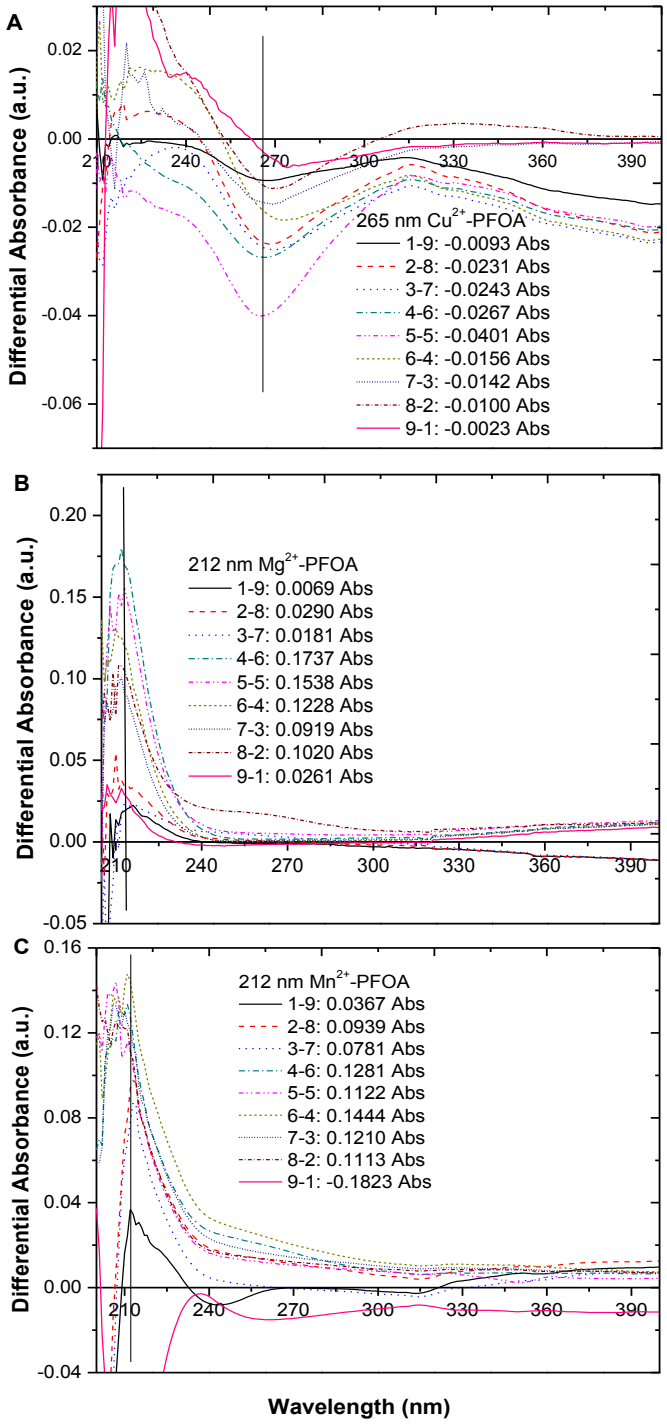


Figure 4.4. UV-vis differential absorbance spectra (DAS) calculated according to the data recorded at varying metal ion to PFOA ratios while maintaining their combined concentrations at 0.8 mM at all time. (A) Cu^{2+}-PFOA DAS determined at pH 4.9; (B) Mg^{2+}-PFOA DAS determined at pH 6.2; (C) Mn^{2+}-PFOA DAS determined at pH 5.7.

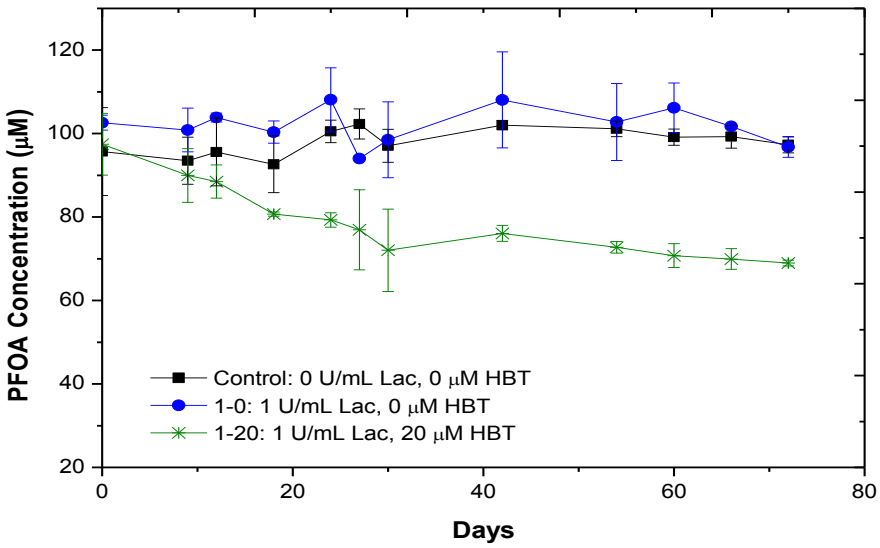


Figure 4.5. Change of PFOA concentration over time in laccase-mediated ECOHRs with addition of 200 μM Fe^{3+}. The initial PFOA concentration is 100 μM. Control: the positive control to which no laccase or HBT was added; 1-0: 1 U mL^{-1} laccase added every 6 d but no HBT; 1-20: 1 U mL^{-1} laccase and 20 μM HBT added every 6 d.

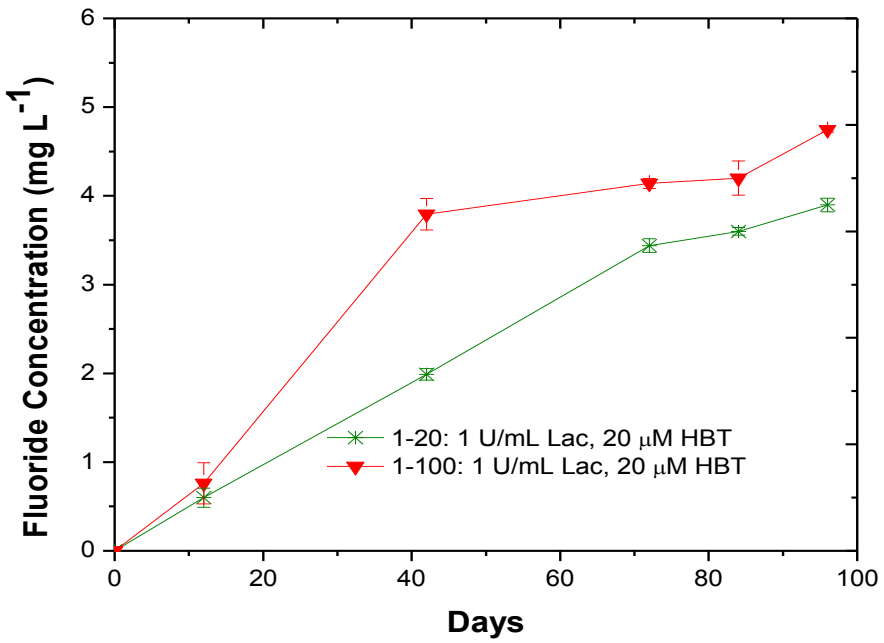
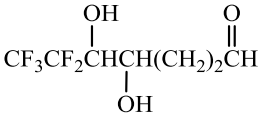
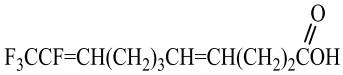
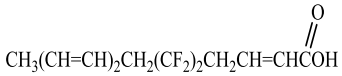
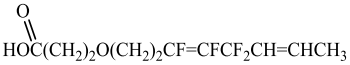
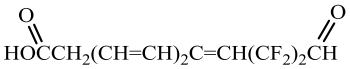
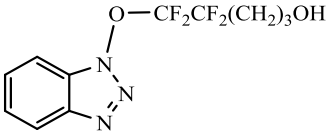
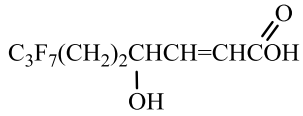
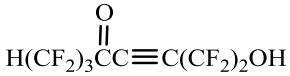
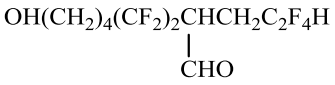
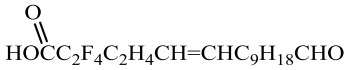
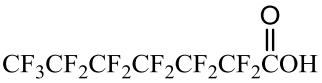
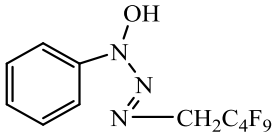


Figure 4.6. Change of fluoride concentration over time during the degradation of PFOA (initial concentration = 100 μM) as mediated by laccase in the presence of HBT. 1-20: 1 U mL^{-1} laccase and 20 μM HBT added every 6 d; 1-100: 1 U mL^{-1} laccase and 100 μM HBT added every 6 d.

Table 4.1. Molecular formulas, theoretical and measured deprotonated molecule weight $[M-H]^-$, mass accuracy (ppm) and possible structures of PFOA degradation products from ECOHRs.

No	Formula	$[M-H]^-$		Mass accuracy (ppm)	Possible structure
		Theoretical	Experimental		
1	$C_7H_8F_5O_3$	235.0404	235.0394	-0.2	
2	$C_{11}H_{13}F_4O_2$	253.0852	253.0848	1.4	
3	$C_{11}H_9F_4O_3$	265.0488	265.0477	4.1	
4	$C_{11}H_{13}F_4O_3$	269.0800	269.0793	2.9	
5	$C_{11}H_9F_4O_4$	281.0437	281.0432	1.8	
6	$C_{11}H_{10}F_4N_3O_2$	292.0709	292.0705	1.4	
7	$C_9H_8F_7O_3$	297.0362	297.0374	-4.2	
8	$C_8HF_{10}O_2$	318.9816	318.9801	5.0	
9	$C_{11}H_{13}F_8O_2$	329.0788	329.0795	-2.2	
10	$C_{17}H_{25}F_4O_3$	353.1739	353.1722	5.0	
11	$C_7F_{13}O_2$	362.9691	362.9698	-2.0	
12	$C_{11}H_7F_9N_3O$	368.0445	368.0457	-3.2	

CHAPTER 5

LACCASE INDUCED DEGRADATION OF PERFLUOROOCTANOIC ACID IN SOIL[3]

[3] Luo, Q.; Lu, J.; Zhang, H.; Wang, Z.; Feng, M.; Chiang, S.-Y. D.; Woodward, D.; Huang, Q. To be submitted to *Environmental Science & Technology*.

ABSTRACT

The universal distribution of perfluoroctanoic acid (PFOA), which can induce undesirable effects to humans and animals, attracts much attention from both the public and the scientific communities. Degradation of PFOA in aqueous phase has been studied extensively while limited information for PFOA decomposition in soil is available. In this research, we have demonstrated that PFOA could be effectively transformed by enzyme catalyzed oxidative humification reactions (ECOHRs) in soil at environmental relevant conditions. During 12-weeks of incubation, we found that the reductions of PFOA up to 24.6% were detected without adding 1-hydroxybenzotriazole as mediator indicating soil organic matter itself could serve as a natural mediator. To accelerate the PFOA degradation in soil, we evaluated the PFOA degradation efficiencies using soybean meal as a natural mediator. Approximately 36% of PFOA was transformed after twenty-weeks of incubation in such soil slurry-soybean meal system. This is a very important finding since the application of other techniques in PFOA soil remediation was limited by the requirement of high energy input and complicated soil characters. Degradation of PFOA by ECOHRs, on the other hand, is an enhanced natural process which has great potential for future soil PFOA remediation.

INTRODUCTION

Soil is an important reservoir for persistent organic pollutants (POPs)[1]. The interactions between soil, water and atmosphere are ways of redistributing these contaminants in different environmental matrices[2], especially for the perfluorocarboxylic acids (PFCAs) which are comparatively more mobile and persistent than their corresponding hydrocarbon analogues[3]. PFCAs contamination in soil draws much attention during the past decades. The major concerns over soil PFCAs contamination are leaching to groundwater[4], transportation to surface water

with runoff[5], bioaccumulation over food chains[6], as well as direct or indirect exposure to wild animals[7]. The soil PFCAs contamination is typically due to the aqueous film-forming foams (AFFFs) application[8], PFCAs-based product wastes landfill[9, 10], and atmospheric wet deposition[11]. PFCAs have been detected in most soil environment at nanogram per gram dryweight soil[12], particularly in the areas where AFFFs have been routinely used[13]. It was found that the historical fire-fighting training fields were found polluted by high concentrations of PFCAs[14]. At the former Minnesota Mining and Manufacturing Company (3M) production process wastes disposal area in Cottage Grove, Minnesota, PFOA concentration ranged from 91.3 to 543 ng/g[10].

Although various techniques have been developed targeting at PFOA degradation in aqueous phase, most of them are not appropriate for soil remediation. For example, the electrochemical oxidation is limited by the complicated electrodes field installation process. The direct photolytic degradation is less effect in the soil than in the water due to the lack of light penetration in soil. The sonochemical degradation involves intensive energy input and special equipment. The persulfate radicals are found effective in PFOA degradation; however they are nonselective radicals which only would work in the situation that PFOA concentration is much higher than other co-contaminants.

Enzymes, on the other hand, are catalysts bearing excellent properties such as high activity, selectivity and specificity that enable to catalyze complex chemical processes under environmental friendly conditions[15]. Enzyme catalyzed oxidative humification reactions (ECOHRs) have been proven to be effective in the degradation of PFOA[16] with the presence of laccase enzyme and 1-hydroxybenzotriazole (HBT). The primary issues regarding the utilization of enzyme-mediator system in remediation are the instability of free enzyme, the high cost of

purified enzyme, and the need of extra additives in the system. Adsorption of enzyme to the soil is an enzyme immobilization process which could improve enzyme stability and make them become more resilient to the fluctuation of environment conditions than its free form[17]. Therefore, we expected that the soil remediation of PFOA by ECOHRs could benefit from such enzyme stabilization process. Using crude enzymes which are commercially available at acceptable price for remediation instead of purified ones could reduce the cost as well. It is known that ECOHRs is a serial of enhanced natural process which is ubiquitously present in the environment. Some natural organic materials containing phenolic or anilnic functional groups could serve as mediators as well. Therefore, we could utilize cheap and widely available natural organic materials such as soybean meal to replace the synthetic mediator such as HBT to further reduce the remediation cost.

In current study, a serial of experiments have been conducted to examine the PFOA degradation by ECOHRs in soil slurries. Three laccase species with different impurities were tested at various dosages. The primary objectives were to verify the efficiency of ECOHRs in PFOA degradation in soil with the presence of different mediators. We also conducted additional experiments to monitor the change of laccase activity in soil over months to determine whether the laccase stability could be improved after been introduced to the soil.

MATERIALS AND METHODS

Soil preparation

The soil samples used for the soil slurry experiments were sterilized by autoclaving three times, 60 minutes each time, at 121^{o}C within a three-day period. The soil samples were incubated at room temperature for 24 hours after each autoclaving events. To prepare PFOA loaded soil samples, 100 g soil was spiked with PFOA stock solution in methanol, which was

then left uncovered under a fume hood with vigorous mixing to evaporate the solvent and yield a soil sample containing 0.5 μg /g PFOA. The properties of the soil used in this study are presented in Table 5.1.

Enzyme

Three laccase species were tested in these experiments. Two purified laccase species from *pleurotus ostreatus* (PO), *trametes versicolor* (TV) were purchased from Sigma Aldrich (St. Louis, MO), and one crude laccase solution concentrated from the fermentation broth of the fungus *Pycnoporus sp*. SYBC-L3 (PS) (activity of ~ 873 U/mL) was provided by a research collaborator (Jiangsu, China). We have previously conducted studies to characterize the crude enzyme and study its uses in decontamination and biofuel production[18, 19]. The enzyme exhibited great stability even in the crude liquid concentrated form, retaining near 90% activity after one year of shelf storage at room temperature[20].

Natural organic mediator

Soybean meal was purchased from Eureka Springs Organics and used as model natural organic mediator in the current study. Soybean meal is an important soybean oil extraction byproduct which is available worldwide at low price. It has already been used in soil amendment. Our analysis showed that soybean meal contained different metal ions and high concentration of organic materials (Table 5.1). We chose the soybean meal as a model natural organic mediator because degradation of PFOA was observed in the solution prepared with soybean meal extraction.

Incubation experiment

The incubation was conducted in 20-mL polypropylene tubes. Each tube contained 1 g of soil (preloaded with 0.5 μg PFOA) and 1.5 mL of HPLC water. Different enzyme addition

strategies were evaluated: one-time addition of high dosage of laccase and multi-addition of low dosage of laccase to the system. Control experiments were also prepared without laccase addition. All tubes were incubated at room temperature throughout the experiment, with openings covered with paraffin film to allow exchange of oxygen while restricting water evaporation. Each tube was thoroughly mixed using a mechanical shaker at the beginning of the incubation and then mixed manually twice a day during the entire incubation period. At selected time intervals, triplicate tubes were sacrificed for PFOA analysis. To this end, the content in each tube was first spiked with 0.125 μg of M8PFOA as internal standard, frozen at -18 ^{o}C, and then freeze dried (Labconco freeze drier) for further extraction of PFOA from soil as described below.

Extraction of PFOA from soil

A 0.5 g portion of the freeze dried sample was mixed with a 5 mL mixture of dichloromethane and methanol (2:1, v:v), ultrasonicated for 30 minutes (Cole Parmer, Ultrasonic Processor), and then centrifuged for 10 minutes at 200 g to collect the supernatant. The same extraction procedure was repeated three times, with all supernatants combined and blown to dry under a gentle nitrogen flow and reconstituted in 2 mL methanol. The solution was then loaded onto a 0.45 μm cellulose acetate membrane (VWR International, Radnor, PA) that was preconditioned with 2 mL of methanol. The collection tube was rinsed twice with 1 mL methanol each time and the rinsants were then passed through the membrane as well. All the filtrates were collected and blown with nitrogen gas to 1 mL for HPLC-MS/MS quantification as described previously. The recovery of PFOA for this method was determined to be 79 ±2.4%.

Chemical analysis

PFOA quantitative analysis was done with a Waters AQCUITY I class UPLC system coupled with a XEVO TQD mass spectrometer (Waters, Milford, MA). The separation was

carried out by a Waters UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters, Milford, MA) at 40 ℃ using a gradient composition of solvent A (water of 5 mmol L^{-1} ammonium acetate) and solvent B (methanol of 5 mmol/L ammonium acetate). The flow rate was 0.3 mL/min and the gradient program lasted for 10 min: 0−0.5 min, hold at 10% B; 0.5−8 min, linearly increase B from 10% to 95%; 8−8.1 min, a linear decrease from 95% to 100%; 8.1−9 min, linearly reduced B to 10%, and then equilibrium at 10% B for 1 min. Electrospray ionization was operated in a negative mode with the parameters set as capillary voltage at -1.0 kV, desolvation temperature at 400 ^{o}C, source block temperature at 150 ^{o}C. Nitrogen (> 99.999% purity, Airgas) was used as desolvation gas with the flow rate of 550 L/hour. In addition to PFOA, M8PFOA, HBT, and seven additional PFCAs (C4-C11) were monitored simultaneously using multiple reaction monitoring. The precursors and transitions *m/z* values of all the monitored PFCAs and HBT as well as their detection limits were listed in Table 5.2.

Assessment of enzyme activity in soil slurry

Additional experiments were carried out to monitor the change of laccase activity over time in soil slurry. Each reactor contained 1.0 g autoclaved soil, 1.5 mL HPLC water, and 2 units of laccase. The enzyme was added to soil first in 0.2 mL water and then 1.3 mL water was added to bring the total volume of water to 1.5 mL. All three enzymes including PO, PS, and TV, were tested separately. Samples were collected and measured for enzyme activities at predetermined time intervals by sacrificing the reactors. During each sampling, liquid and solid phases were separated by centrifuging them at 8000 rpm for 5 minutes. The supernatant was then removed with pipette to determine the laccase activity in the liquid phase as described previously. Then the solid phase was rinsed three times with HPLC water. The enzyme activity was measured each time till there is no activity was detected in the rinse water. The combined activity in the

supernatant and rinse water were equal to the enzyme activity remain in the liquid phase. Laccase activity in the liquid phase was measured using the same method reported by Park et al. via oxidation of 1 mM 2, 6-dimethoxyphenol (DMP) in a citrate phosphate buffer (pH 3.8), with the absorbance change was measured at 468 nm[21]. One unit is defined as the amount of enzyme that causes a unit absorbance change per minute in 3.4 ml of this solution in cuvette with 1 cm light path.

In order to measure laccase activity in the solid phase, 3 ml of 100 mM 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in phosphate buffer (pH 6.0) was added directly to the solid phase sample in each tube, which was then vortexed and centrifuged to allow for 10 minutes of color development. Laccase activity was then quantified by the rate of absorbance change at 420 nm[22]. The laccase activity thus measured in the ABTS unit was then converted to the DMP unit by a calibration curve prepared by measuring known amounts of laccase using both DMP and ABTS methods. ABTS, instead of DMP, was used as a substrate for laccase activity measurement in the solid phase because ABTS did not adsorb on soil but DMP did. The equation below showed the relationship between the laccase activities measured by these two methods:

$$Activity\ (measured\ by\ DMP) = 2.65 \times Activity\ (measured\ by\ ABTS)$$

Statistical analysis

Analysis of Variance (ANOVA) has been performed to compare the PFOA concentrations of the same treatment over different sampling times as well as the different treatments at the same sampling time. ANOVA was conducted on SAS using a linear model with the significant difference $\alpha = 0.05$ followed by a least significant difference test.

RESULTS AND DISCUSSION

PFOA degradation in soil slurry

The initial PFOA concentration was 0.5 μg/g soil, pH was unadjusted (pH 5.1), and no chemical mediator was added. Figure 5.1 showed the change of PFOA concentration over time in the soil slurry system. Three enzymes: PO, TV, and PS, have been tested separately, each using three different addition modes specified below, along with controls to which enzyme was not added.

(1) one single addition of 2 U/g soil at the beginning of incubation

(2) one single addition of 20 U/g soil at the beginning of incubation

(3) repeated additions of 2 U/g soil every 2 days during incubation.

PFOA residual (%) was calculated by dividing PFOA concentration in reaction samples (with enzyme addition) by that in control samples (without enzyme addition) measured at the same time. A decrease trend of PFOA concentration was obvious in systems with PO by all three enzyme addition modes. The PFOA removal could also be seen in systems with single addition of PS or TV enzyme at 20 units. However, PFOA reduction under all soil slurry conditions does not appear to be pronounced. The PFOA residual was 81.2% (18.8% reduction), 82.0% (18% reduction), and 75.4% (24.6% reduction) after 12 weeks of incubation in systems with 20 units of PO, PS or TV enzyme respectively. The poor degradation efficiencies might due to the depletion of soil organic matters [23] over long incubation time in the soil [23].

In order to facilitate the degradation of PFOA, another batch of experiment was tested to determine the efficiency of ECOHRs by using HBT as synthetic mediator. PFOA concentration was gradually decreased over 12 weeks in the presence of different types of lacasse and various concentrations of HBT (Figure 5.2). All PO-treated samples demonstrated the degradation of

PFOA with 82.9% residual (17.1% reduction) for PO 20/4 and 83.0% (17% reduction) for PO 20/4-0.4/4 HBT after 12 weeks of incubation. Degradation efficiencies (8% for PS 20/4 and 7% for PS 20/4-0.4/4 HBT) were found much lower in PS-treated samples than in PO-treated samples with or without the addition of HBT. These data indicated that addition of HBT at 0.4 μmole level did not significantly facilitate the degradation of PFOA in soil.

As shown in Figure 5.3, significant reductions of PFOA were found in all samples with the addition of laccase and soybean meal. At the first four weeks, nearly 27% of PFOA were removed from the PO 60 (60 U PO/g soil) system while 18%, 17%, 14%, and 10% for samples treated with PO 20/4 (20 U PO/g soil per four week), PS 20/4 (20 U PS/g soil per four week), TV 20/4 (20 U TV/g soil per four week), and PS 60 (60 U PS/g soil) respectively. After 20 weeks of reaction, further degradation of PFOA was observed in all treatments. Approximately 36% of PFOA was removed from the PS 60 (60 U PS/g soil) system, while the removal efficiency slows down for the PO 60 sample. This was probably due to the fact that PS was more stable than PO in the slurry system. The stability of different enzymes was discussed in the following section. For other treatments, addition of 20 unit of enzyme every four weeks clearly facilitates the degradation of PFOA. The removal increased to 29%, 31%, 26% and 34% for PO 20/4, PS 20/4, TV 20/4, and PO 60, respectively while PFOA concentration in the control samples without laccase remained at 96% during the whole reaction period.

Enzyme activity in soil slurry

The change of laccase activity in soil slurry system was monitored in separate experiment and the results are shown in Figure 5.4. One observation is that the total apparent enzyme activity was immediately reduced after being introduced to the soil slurry system. For example, a total of 1.71 units of PO enzyme in solution were added to the soil slurry, the enzyme activity

determined right after the addition was 0.23 and 0.25 units in the liquid and solid phase respectively, which equalled to a total of 0.48 units of apparent enzyme activity. This is probably because the large portion of PO enzyme was adsorbed to soil and became immobilized, and it is known that the apparent activity of enzyme usually reduced after immobilization[17].

Another observation is that the enzyme in solid phase is more stable than in liquid phase (Figure 5.4). For example, the PO enzyme activity was 0.23 units at time zero in liquid phase and reduced to 0.07 units at day 28; while the PO enzyme activity in solid phase at time zero and day 28 was 0.25 and 0.18 units respectively. We also recorded the change of laccase activity in the system with addition of HBT. As shown in Figure 5.4, having HBT in the system did not significantly impact the laccase stability in the soil slurry system.

The 36% reduction of PFOA in soil slurry after 20 weeks of incubation with 20 units laccase and 50 mg soybean meal addition suggested that ECOHRs is effective in the degradation of PFOA in soil. It is noted that even without the addition of mediator such as HBT and soybean meal, the degradation of PFOA was still observed which indicated that SOM could serve as mediator. However, the active sites in SOM may become exhausted over time during ECOHRs, thus limiting the PFOA degradation. To overcome such limitation, we proposed to use the soybean as natural organic mediator due to its suitable composition of high organic matter and various metal ions. The results showed that laccase-soybean meal system was able to effectively transform the PFOA in soil which may have a promising future in soil remediation of PFCAs.

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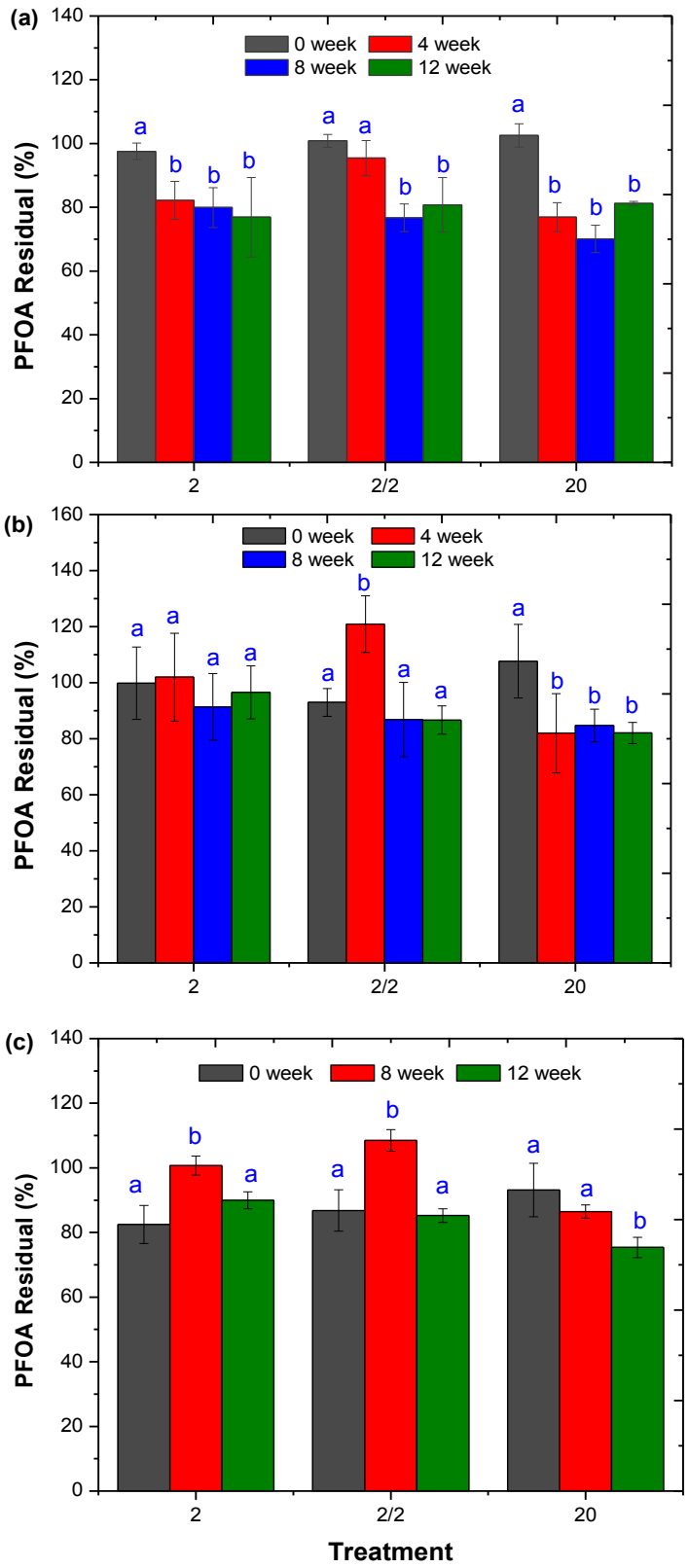

(a)
0 week
4 week
8 week
12 week
PFOA Residual (%)
140
120
100
80
60
40
20
0
a
b
b
b
a
a
b
b
a
b
b
b
2
2/2
20
(b)
0 week
4 week
8 week
12 week
PFOA Residual (%)
160
140
120
100
80
60
40
20
0
a
a
a
a
a
b
a
a
a
b
b
b
2
2/2
20
(c)
0 week
8 week
12 week
PFOA Residual (%)
140
120
100
80
60
40
20
0
a
b
a
a
b
a
a
a
b
2
2/2
20
Treatment

Figure 5.1. PFOA residual (%) in soil slurry with different laccase(a) PO, (b) PS, (c) TV additions: 2 or 20 units only at the beginning of incubation or 2 units every 2 days (2/2). PFOA residual (%) was calculated by dividing PFOA concentration in reaction samples by that in control samples (without enzyme addition) measured at the same time. Values are the means of six replicates, and error bars represent standard deviations. Analysis of variance (ANOVA) was performed to evaluate different treatments at each sampling time, and bars with the same letters are not considered to be statistically different according at $\alpha = 0.05$.

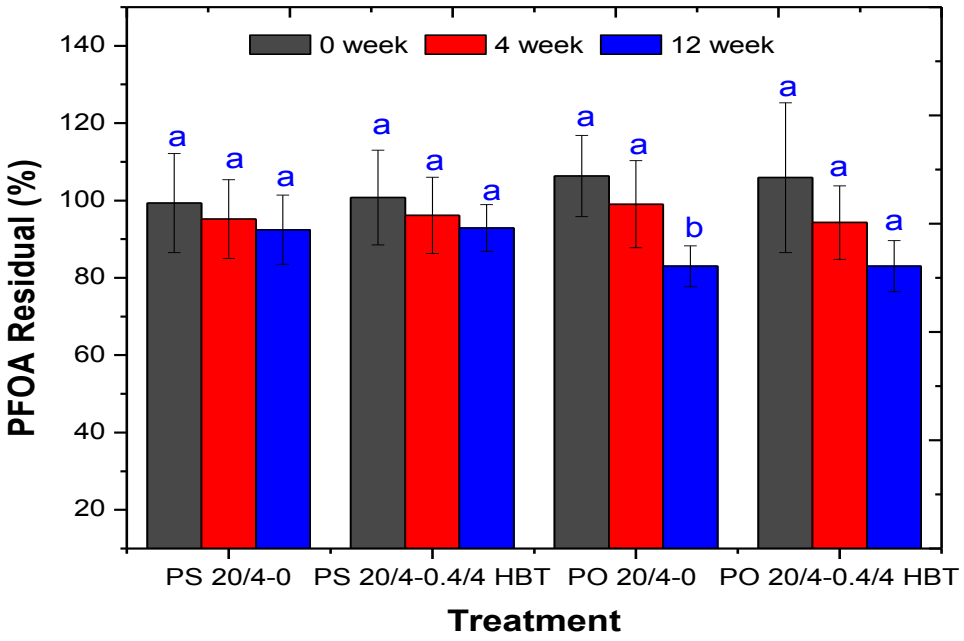


Figure 5.2. PFOA residual (%) in soil slurry containing 1 g soil and 1.5 mL HPLC water. PS 20/4-0: 20 U of PS was added every four weeks. PS 20/4-0.4/4: 20 U of PS and 0.4 μmole HBT were added to the reactor every four weeks. PO 20/4-0: 20 U of PO was added every four weeks. PO 20/4-0.4/4: 20 U of PO and 0.4 μmol HBT were added to the reactor every four weeks. PFOA residual (%) was calculated by dividing PFOA concentration in reaction samples by that in control samples (without enzyme addition) measured at the same time. Values are the means of six replicates, and error bars represent standard deviations. Analysis of variance (ANOVA) was performed to evaluate different treatments at each sampling time, and bars with the same letters are not considered to be statistically different according at $\alpha = 0.05$.

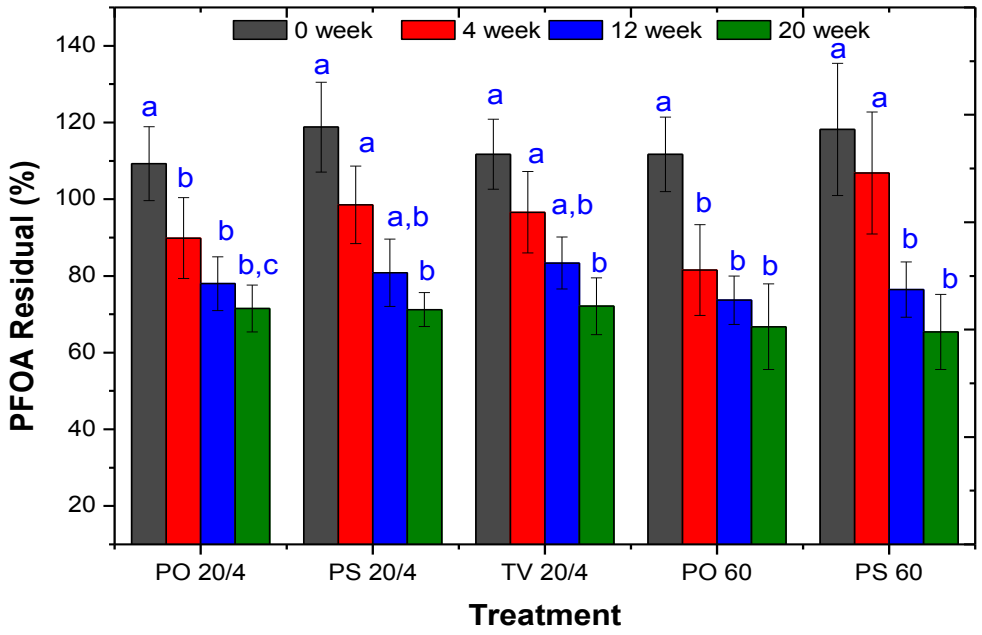


Figure 5.3. PFOA residual in percentage in soil slurry containing 1 g soil, 50 mg soybean meal, and 1.5 mL HPLC water. PO 20/4: 20 U of PO was added every four weeks. PS 20/4: 20 U of PS was added every four weeks. TV 20/4: 20 U of TV was added every four weeks. PO 60: 60 U of PO was added to the reactor at the beginning of the experiment only. PS 60: 60 U of PS was added to the reactor at the beginning of the experiment only. PFOA residual (%) was calculated by dividing PFOA concentration in reaction samples by that in control samples (without enzyme addition) measured at the same time. Values are the means of six replicates, and error bars represent standard deviations. Analysis of variance (ANOVA) was performed to evaluate different treatments at each sampling time, and bars with the same letters are not considered to be statistically different according at $\alpha = 0.05$.

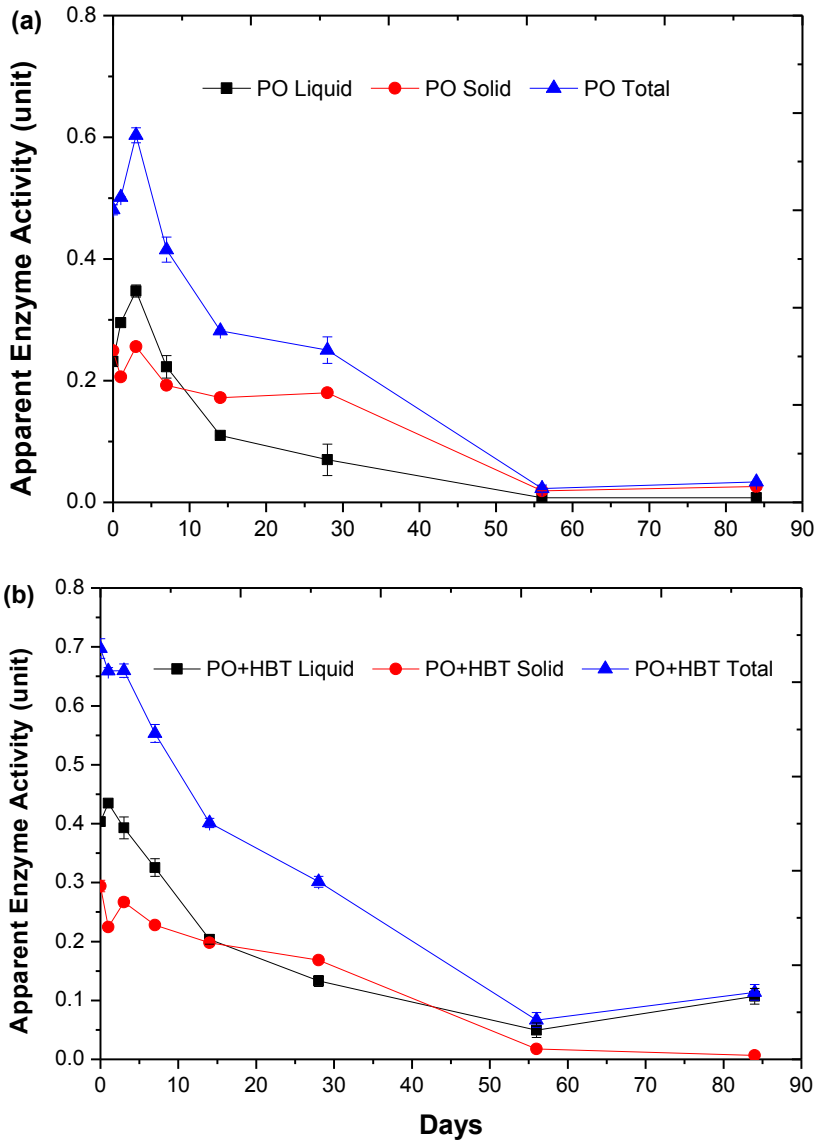
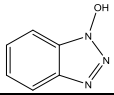


Figure 5.4. Change of laccase activity in soil slurry over time. (A) Addition of 1.71 units PO laccase only at time zero (PO). (B) One system included 0.04 µmol HBT with 1.71 units PO laccase added initially (PO+HBT). The enzyme activity was measured separately in liquid and solid phases and both were expressed in DMP units. The total enzyme activity is calculated by addition of the enzyme activities in liquid and solid phases.

Table 5.1. Total organic carbon and different metal concentrations in extract solutions.

Extract Solution	Total organic carbon (ppm)	Na (ppm)	Mg (ppm)	Al (ppm)	Ca (ppm)	Fe (ppm)	Cu (ppm)
Soil	172.9	4.05	3.74	1.89	9.47	3.93	< 0.008
Soybean meal	1865.0	< 1.00	78.33	< 0.080	78.67	< 0.6	0.0411

Table 5.2. The molecular formula, retention time, precursor ion, transition ion, and detection limit of the PFCAs and HBT monitored in UPLC-MS/MS analysis.

Chemicals	Molecular Formula	RT (min)	Precursor ion m/z	Transition ion m/z	Detection limit (μg/L)
PFBA (C4)	F-$(CF_2)_3$COOH	2.6	213	169	0.018
PFPeA (C5)	F-$(CF_2)_4$COOH	4.5	263	219	0.027
PFHxA (C6)	F-$(CF_2)_5$COOH	5.5	313	269	0.022
PFHpA (C7)	F-$(CF_2)_6$COOH	6.1	363	319	0.008
PFOA (C8)	F-$(CF_2)_7$COOH	6.6	413	369	0.011
PFNA (C9)	F-$(CF_2)_8$COOH	7.02	463	419	0.15
PFDA (C10)	F-$(CF_2)_9$COOH	7.32	513	469	0.10
PFUA (C11)	F-$(CF_2)_{10}$COOH	7.63	563	519	0.012
HBT		0.44	134	105	0.019

PFBA: perfluoro-n-butanoic acid; PFPeA: perfluoro-n-pentanoic acid; PFHxA: perfluoro-n-hexanoic acid; PFHpA: perfluoro-n-heptanoic acid; PFNA: perfluoro-n-nonanoic acid; PFDA: perfluoro-n-decanoic acid; PFUA: perfluoro-n-undecanoic acid; HBT: 1-hydroxybenzotriazole.

CHAPTER 6

PERFLUOROOCTANOIC ACID ECOTOXICOLOGICAL EFFECTS OF ON FRESHWATER MICROALGAE *CHLAMYDOMONAS REINHARDTII* AND *SCENEDESMUS OBLIQUUS*

INTRODUCTION

The presence of perfluorinated alkyl acids (PFAAs) in the environment is a growing concern because PFAAs such as perfluorooctanoic acid (PFOA) are globally distributed, extremely persistent and toxic to animals and environment[1-4]. PFAAs have been detected in various environmental matrices, with the concentrations in natural water from nondetectable to μg/L level[5-7]. The detection of PFOA was associated with the degree of industrialization and population density. For example, PFOA was quite prevalent in the samples collected from the Baltic Sea, the Mediterranean, the Great Lakes and Asian coasts areas[8-11]. Although PFOA is found in environment at low levels, continuous exposure to such relatively low concentrations may increase the risk of health effects due to its bioaccumulative property[11].

Toxicological studies on PFOA are primarily focused on rodents and mammals due to the concern about PFOA accumulation in higher organisms and their associated potential toxicity. Some reports regarding the toxicity of PFOAs on aquatic species such as *Daphnia magna*[12], minnow (*Gobiocypris rarus*)[13], tilapia (*Oreochromis niloticus*)[14], salmon (*Salmo salar*)[15], *Paracentrotus lividus* and *Psetta maxima*[16] have been published in recent years. However, the ecotoxicity of these chemicals in the aquatic environment are not fully understood.

Microalgae are primary producers that have been widely used as ecological indicators in polluted aquatic environments[17]. They have relative short generation times but quite sensitivity to the impact of pollutants[18]. According to a recent study, the 24-h EC_{50} of PFOA was 19.81 mg/L for the *Anabaena* CPB4337[19]. The acute freshwater aquatic toxicity studies on ammonium perfluorooctanoate (APFO) revealed that the 48 to 96 h LC/EC_{50} values were greater than 400 mg/L while no observed effect concentration (NOEC) was 12.5 mg/L for inhibition of the growth and biomass to algae *Pseudokirchneriella subcapitata*. The effects of PFOA on early life

stages of a marine microalga *Isochrysis galbana* was also assessed with the acute EC_{50} value of 163.6 mg/L[16].

Although collecting information regarding the LC/EC_{50} values are important for toxicity assessment, other data of growth and physiological responses are needed to better understand the environmental risk of these chemicals[20]. Moreover, PFOA is bioaccumulative. There will be great potential for PFOA to transfer from microalgae to other aquatic organics in the higher level of food chain if PFOA could absorb or uptake by microalgae. Therefore, information regarding the uptake and sorption of PFOA on algae is very import in assessing the ecological risk of PFOA in aquatic system.

In present study, we reported a systematic acute toxicity study of PFOA on two freshwater green algae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*. The *C. reihardtii* is ubiquitously present in aquatic system and frequently used as a eukaryotic model organism to study responses to different environmental factors[21, 22]. *S. obliquus* is a green algae has already been used to evaluated the toxicity of chemicals via growth inhibition tests[23]. The main purpose of this study was to study the effect of PFOA on the growth and physiological process of the microalgae as well as determine the uptake and sorption of PFOA by the selected microalgae after 8-d exposure.

MATERIALS AND METHODS

Chemicals

PFOA (> 98% purity) of analytical grade was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals used in this study, including trichloroacetic acid, thiobarbituric acid, sulphosalicylic acid, ninhydrin were all of analytical grade and obtained from Fisher Scientific (Pittsburgh, PA).

Microalgae culture

The freshwater microalgae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* was provide by UTEX Culture Collection of Algae, the University of Texas at Austin. The *C. reinhardtii* was cultured in modified High Salt Medium (HSM)[24] and *S. obliquus* was incubated in HB-4 medium[25] (Appendix C) under standard lighting and temperature with the initial pH of 6.8. The cultures conditions were as follow: 25 ± 1 ℃, 16-h light/8-h dark photoperiod with illumination provided by cool white fluorescence lights at 85 - 90 μmol photo/m^2 s irradiance. The cells were subculturing every five days to maintain in exponential growth. The cell number linearly related with chlorophyll *a* (chl *a*), thus the algal biomass could be calculated indirectly using spectrophotometric monitoring of the algal growth at wavelength 680 nm[26].

Growth inhibition test

Appropriate amount of PFOA was added into 100 mL culture solution with initial cell densities of 1×10^4 cells/mL for *C. reinhardtii* and 5×10^3 cells/mL for *S. obliquus.* The test initial PFOA concentrations were 0, 1, 3.16, 10, 31.6, 100, 316, and 1000 mg/L for different treatments. All control and exposure groups were operated in five replicates. The whole test lasted for 96 hours with sampling every 24 hours. At each sampling point, spectrophotometer (DU 640B Spectrophotometer, Beckman, USA) examination was used to monitor the microalgae cells growth at 680 nm. The 96-h EC_{50} and EC_{10} values were estimated by nonlinear regression fit using Software GraphPad Prism 5 (GraphPad Software, San Diego California USA).

Acute toxicity test

The 100-mL culture solutions with cellular concentration of 5×10^6 cells/mL were mixed with certain amount of PFOA to reach final nominal concentrations of 0, 5, 10, 20, and 40 mg/L, respectively. These groups were tested in triplicates under the same incubation conditions

described above. The algal cells density i.e. OD_{680} value was determined following the OECD guideline 201[27] using the Beckman DU 640B Spectrophotometer (Beckman, USA).

Lipid peroxidation

Thirty mL culture was taken and centrifuged at 7232 ×g for 15 min from the flasks at the end of 8-day exposure experiment. Each algal sample frozen in liquid nitrogen was ground and dissolved in 3 mL of 0.1% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 12857 ×g for 30 min and 2 mL of the supernatant was mixed with 2 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was then heated at 95 ℃ for 30 min, chilled on ice, and centrifuged at 10000 ×g for 5 min. The absorbance of the supernatant was measured at 532 nm. The value for nonspecific absorbance at 600 nm was subtracted[28]. The malonaldehyde (MDA) content was presented as nmol/10^6 cells.

Proline assay

The proline content was extracted and quantified using the method as described by Bates et al.[29]. Briefly, 30-mL culture was collected by centrifuge at 7232 ×g for 15 min. Each algal sample was mixed with 3% (w/v) aqueous sulphosalicylic acid, and then measured by ninhydrin reagent. Absorbance of the fraction with toluene aspired from the liquid phase was read at 520 nm against a blank of reagents (Appendix C).

PFOA phase distribution analysis

A HPLC-MS/MS was used to determine the concentrations of PFOA distributed in cultural media, surface of microalgae and microalgae. After 8-d exposure, three mL of the culture was collected and centrifuged at 7232 ×g for 15 min. The supernatant was collected for the determination of residual PFOA concentration in the culture medium. The precipitate was then repeatedly washed three times with 5 mL distill water each time. The PFOA in the washing

solutions in combined with the residual PFOA concentration in cultural medium was considered as the adsorbed PFOA to the algae. The uptake amount of PFOA by algae was calculated according to equation (1). Control groups which were prepared at the same initial PFOA concentrations in culture media without microalgae cells were also measured at the same time to determine the possible removal of PFOA from adsorption to glass containers.

Amount of uptake, uptake percentage, and sorption percentage was calculated by the follow equations, respectively:

$$U_A = \frac{(M_N - M_8 - M_{AC} - M_{AM})}{N} \quad (1)$$

$$U_P(\%) = \frac{(M_N - M_8 - M_{AC} - M_{AM})}{M_N} \times 100 \quad (2)$$

$$A(\%) = \frac{M_{AM}}{M_N} \times 100\% \quad (3)$$

where U_A is the uptake amount of PFOA by the microalgae (μg/10^6 cells), U_P is the uptake percentage (%) by the microalgae, A is the sorption percentage onto the microalgae (%), M_N is the amount of PFOA that was initially added to each flask (mg), M_8 is PFOA left in the medium after 8-d incubation (mg), M_{AC} is PFOA adsorbed by the container (mg), M_{AM} is PFOA in the washing solutions and accounted as PFOA adsorbed by the microalgae (mg), and N is the amount of the microalgae at day eight (10^6 cells).

The HPLC-MS/MS measurement was performed using Waters Alliance 2690 HPLC with an Ascentic C_{18} reversed phase column (250 × 4 mm, 5 μm; Supelco, St. Louis, MO). The HPLC mobile phase consisted of acetonitrile (A) and 2 mM of ammonium acetate in HPLC water (B). The flow rate was 0.3 mL/min with mobile-phase gradient as follows: 0 to 5 min, changing A from 40% to 80%, and then increasing A to 90% from 5 to 10 min and holding at this gradient

for 15 min, returning to 40% of acetonitrile in 1 min and equilibrating for 4 min after each run. A mass spectrometer (Waters Micromass QuattroMass) with an electrospray source operated in the negative ion mode was used to detect PFOA. The multiple reaction monitoring (MRM) mode was used to identify and quantify PFOA. The capillary voltage was -3.01 kV, cone voltage - 20 kV, source temperature 100 ℃, and desolvation temperature 300 ℃. The nebulizer was set at 25 L/h and desolvation gas at 235 L/h. the collision energy was 11 eV.

PFOA in samples was identified by comparing the retention time with the PFOA standards and by confirmation ion in the MRM: *parent ion=413, daughter ion = 369*. The retention time for PFOA is 10.95 min. External five point calibration curves for this method were generated using standard PFOA samples ($r^2 \geq 0.997$) (Appendix C). For high level PFOA including 10, 20, and 40 mg/L groups, the samples were firstly diluted with distilled water to lower the PFOA concentration to approximately 4 mg/L before the HPLC-MS/MS measurement.

Statistical analysis

The data shown are the arithmetic means ± corresponding standard deviations. The EC_{50} and EC_{10} were estimated by simulating the corresponding inhibition data with Logistic dose-response model. Any reported significant difference in the study was based on the results of one-way analysis of variance (ANOVA) with the software Origin 7.0, taking $P < 0.05$ as significant and $P < 0.01$ as highly significant.

RESULTS AND DISCUSSION

Microalgae growth inhibition and EC_{50} /EC_{10} values

OECD guidelines recommend using freshwater alga growth inhibition tests for hazardous assessment and classification of chemicals. The test endpoint is inhibition of growth (the change of cell density) during the exposure period. The advantage of such endpoint is that it is an

integral parameter showing inhibitory effect of chemicals on all cellular metabolism[30, 31]. Previous studies reported various EC_{50} values of PFOA to different microalgae. For example, the EC_{50} values for *Anabaena* CPB4337[19] and *Pseudokirchneriella subcapitata* were 19.81 mg/L and > 100 mg/L, respectively[32]. Another study reported the EC_{50} values of approximately 200 mg/L for green algae *Chlorella pyrenoidosa* and *Selenastrum capricornutum*[20]. As shown in Figure 6.1, growth inhibition effect on the two microalgae: *C. reinhardtii* and *S. obliquus* increased with the incensement of PFOA concentration within the tested PFOA concentration range. We observed the death of both microalgae exposed to 1000 mg/L PFOA within the first 24 h of the test. Different from the previous results, the 96-h EC_{50} for *C. reinhardtii* and *S. obliquus* were 51.9 ±1.0 and 44.0 ± 1.5 mg/L PFOA in our study. The 96-h EC_{10} was 5.77 ± 0.22 mg/L for *C. reinhardtii* and 4.89 ± 0.31 mg/L for *S. obliquus*. The difference in morphology, cytology, physiology and genetics among different algal species may cause varied sensitivity to the same chemical substance[33, 34].

Although the EC_{50} and EC_{10} values for both microalgae were similar, the growth behaviors were different for them (Figure 6.2). Compared with the control, *C. reinhardtii* in all treated groups decreased after 2nd day, especially in the group treated with 40 mg/L PFOA (Figure 6.2A). On day 8, the cell number in the group 40 mg/L was approximately 1.78×10^6 cells/mL, which was significantly lower ($P < 0.01$) than the control (3.21×10^6 cells/mL). However, the growth of *S. obliquus* was not inhibited by PFOA as much as *C. reinhardtii* (Figure 6.2B). In addition, the growth of *S. obliquus* had a longer lag phase than *C. reinhardti,* indicating *C. reinhardtii* was more sensitive to PFOA than *S. obliquus*. After 8-d exposure, PFOA can inhibit the growth of *C. reinhardtii*, especially at relatively high concentrations.

However, the data from the growth showed that *S. obliquus* was not significantly affected (Figure 6.2).

The contents of MDA and proline

Beside the growth inhibition test, we also performed the assays to determine the biochemical response of the algae which could help us improve our understanding the mechanism of the adverse effects of PFOA to microalgae. Both MAD and proline content were used as indicators of PFOA-induced stresses in microalgae. In biological and chemical systems, the occurrence of peroxidation is usually quantitatively correlated with malonaldehyde (MDA) production, thus MDA has been widely used to determine the degree of oxidative stress[35, 36]. The reactive oxygen species (ROS) in the organisms can cause membrane lipid peroxidation which results in the formation of various breakdown products and the MDA is one of them[37].

Proline is a low-molecular weight compound concentrated on the algal cell membrane[38]. It tends to accumulate when the algae cells experience oxidative stress like increasing of ROS, disruption of membrane integrity and enzyme stability[39, 40]. Proline has been used to evaluate the oxidative stresses in microalgae[41]. Accumulation of proline in green microalga *Chlorella vulgaris* following copper exposure was also reported. Xu et al. [20] suggested the PFOA concentrations at 30 mg/L and 60 mg/L were able to elevate the activities of superoxide dismutase and catalase in *Chlorella pyrenoidosa* and *Selenastrum capricornutum* after 8-d exposure. The two antioxidant enzymes induced by PFOA could prevent ROS from damaging cells.

As shown in Figure 6.3, the amount of MDA in cells of the two microalgae after 8-d exposure. For both algae, PFOA had no significant ($P > 0.05$) effects on MDA content when the exposure concentration was lower than 10 mg/L. However, significant accumulation of MDA

content in the group 20 and 40 mg/L for both algae were observed. The proline contents are shown in Figure 6.4. For *C. reinhardtii*, the contents of proline in all exposure groups, except the group 5 mg/L, were significantly ($P < 0.01$) higher than the control (Figure 6.4A). However, the proline in the *S. obliquus* was only significantly ($P < 0.01$) impacted by the PFOA of 40 mg/L (Figure 6.4B). The data showed that the defense responses were induced in these two algae at PFOA concentration around 20 mg/L, which was less than half of EC_{50} values.

PFOA absorption and uptake by microalgae

The PFOA ratio of sorption and uptake by the algae may also influence the toxicity and its ecological risk in the aquatic food chain. Therefore, these two aspects were also investigated. Freshwater algae are very important components of aquatic food chains. Pollutants could be accumulated and/or adsorbed by them and transferred to higher tropic level organisms, leading to potential adverse effects to the aquatic ecosystem. It is known that PFOA can be readily absorbed via ingestion but poorly eliminated and resistant to biodegradation[42 43]. For PFOA in the algae culture, its fate could be four aspects: sorbed by the container, sorbed by the algae, uptake by the algae, and remained in the culture medium.

In order to rule out the influence from the absorption of PFOA by the glassware, we prepared a serial of media controls which contain the same concentrations of PFOA in media but without the presence of microalgae. The results showed that 34, 34, 30, and 32% of PFOA was sorbed by the containers for the group 5, 10, 20, and 40 mg/L PFOA, respectively. The concentrations of PFOA left in the culture medium are shown in Table 4.1. The data suggested that PFOA could be sufficiently removed from the microalga surface by triple rinsing with distilled water. It was found that PFOA concentration in the rinsate of the 3rd time rinsing was less than 97% of that in the 1st rinsate. For instance, the four portions of PFOA in the group 5

mg/L of this study were 34%, 7.3%, 8.9%, and 49.8%, respectively. The portion sorbed by the microalgae was ranged 5.5-7.5%. The uptake percentage exceeded 10% when the exposure dosage was between 5-20 mg/L, suggesting a significant threat to the aquatic ecosystem. The highest uptake percentage was found in the group 10 mg/L (approximately 18%), and the lowest was in the group 40 mg/L (about 4%). The amount of uptake (U_A) was 1.26 μg/10^6 cells for *C. reinhardtii* and 0.70 μg/10^6 cells for *S. obliquus* in the group 20 mg/L. When exposure dosage reached 40 mg/L, uptake percentage decreased, while the amount of accumulated PFOA had no significant changes. The reasons for this might be that the algae have an inherent capability for PFOA uptake which could be inhibited when PFOA reached certain level. Meanwhile, the algae growth was also depressed at 40 mg/L which could influence the uptake of PFOA.

Microalgae has potential for removal of nitrogen, phosphorus, metal and organic pollutants[44-46]. In term of interaction between microalgae and PFOA, the processes might be involved the initial sorption of PFOA onto the cell wall followed by uptake by the algae. Since it is possible for PFOA to be bioaccumulated through aquatic food chain once it was absorbed and uptake by microalgae, the exposure to low levels of PFOA should not be overlooked.

CONCLUSION

We have investigated two primary freshwater microalgae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* in response to PFOA toxicity. The EC_{50} and EC_{10} values and the oxidative stress response of the algae to the PFOA were reported in this study. The EC_{50} values for *C. reinhardtii* and *S. obliquus* were 51.9 ± 1.0 and 44.0 ± 1.5 mg/L PFOA, respectively. The EC_{10} values were 5.77 ± 0.22 mg/L for *C. reinhardtii* and 4.89 ± 0.31 mg/L for *S. obliquus*. We observed significant growth inhibition when *C. reinhardtii* was incubated with PFOA concentration ranging from 5 - 40 mg/L, while *S. obliquus* was not impacted by PFOA

concentration up to 40 mg/L. We also found that PFOA was able to induce the increasing the malonaldehyde and proline levels in both microalgae at the concentration of approximately 20 mg/L. The absorption and uptake of PFOA by the microalgae was also studied. The adsorption of PFOA by microalgae ranged from 5.5 to 7.5% after 8-d exposure to PFOA concentration between 5 - 20 mg/L. The uptake of PFOA by microalgae indicated potential bioaccumulation of PFOA in the aquatic ecosystem via the food chain.

SUPPORTING INFORMATION AVAILABLE

Additional experimental details, figures, and table are available in Appendix C.

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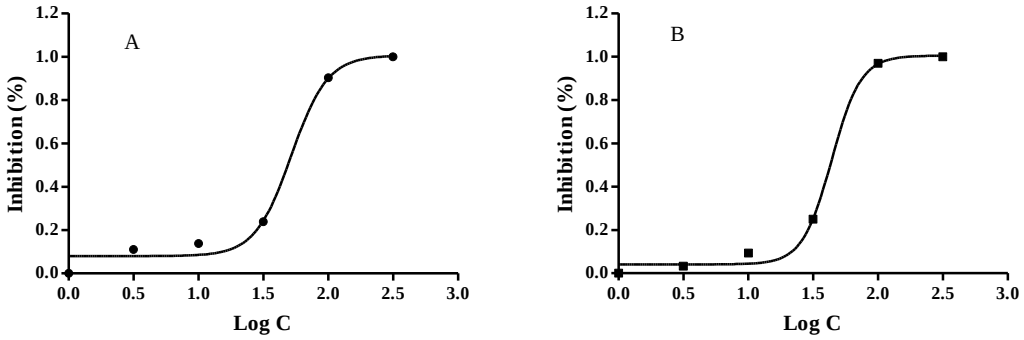

Figure 6.1. Nonlinear regression fits of inhibition and Log C for A: *Chlamydomonas reinhardtii* and B: *Scenedesmus obliquus*.

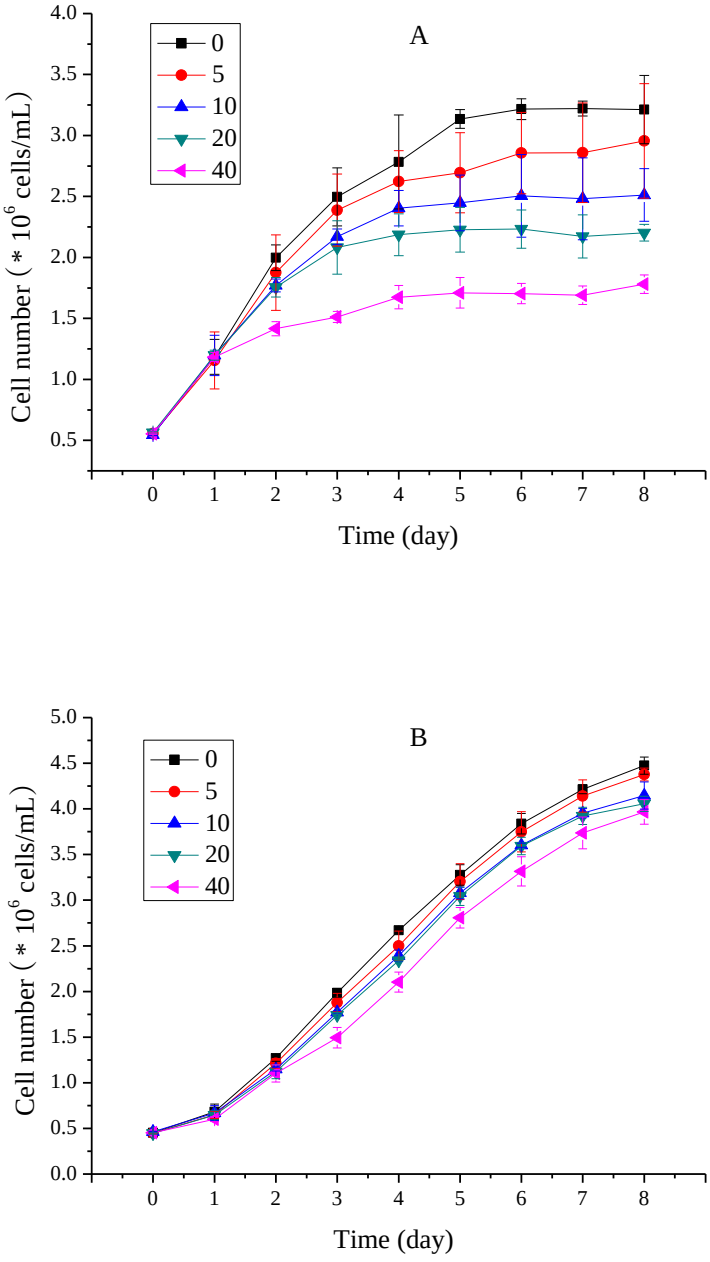

Figure 6.2. Growth kinetics of *Chlamydomonas reinhardtii* (A) and *Scenedesmus obliquus* (B).

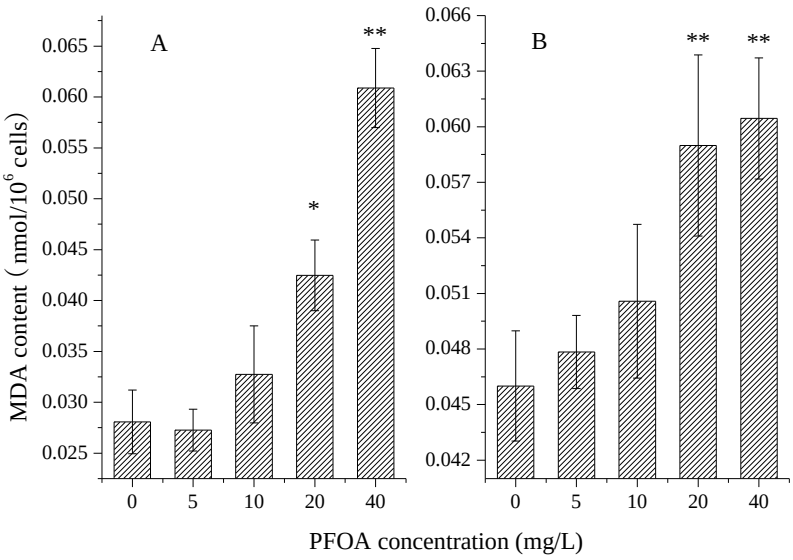

Figure 6.3. Level of lipid peroxidation products (MDA) measured as thiobarbituric acid reactive substances in *Chlamydomonas reinhardtii* (A) and *Scenedesmus obliquus* (B) exposed to different concentrations of PFOA.

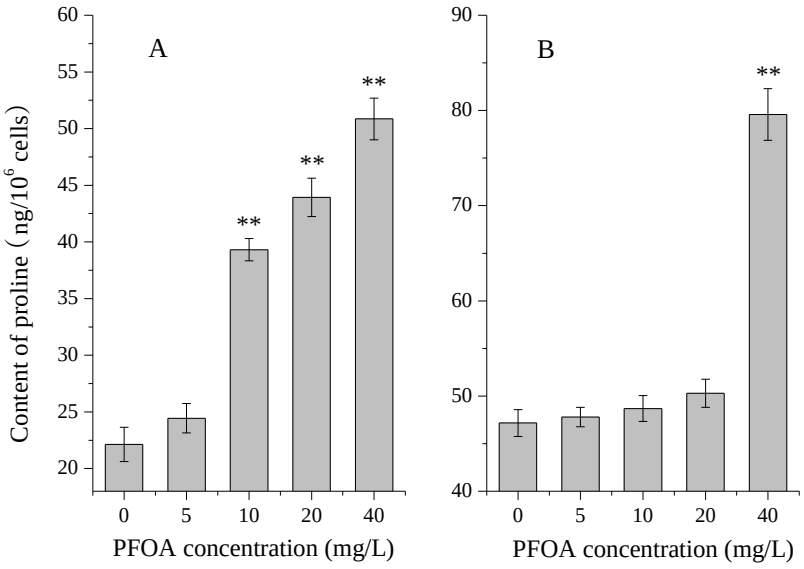

Figure 6.4. The contents of proline in *Chlamydomonas reinhardtii* (A) and *Scenedesmus obliquus* (B) after 8-d exposure to PFOA with different concentrations.

CHAPTER 7

DEGRADATION OF PERFLUOROOCTANESULFONATE CATALYZED BY LACCASE [4]

[4] Luo, Q. and Huang, Q. To be submitted to *Environmental Science & Technology*.

ABSTRACT

This research investigated the degradation of perfluorooctanesulfonate (PFOS) by laccase-induced enzyme catalyzed oxidative humification reactions (ECOHRs) using 1-hydroxybenzotriazole (HBT) as mediator. Approximately 59% of PFOS was transformed over 162-day incubation with the initial PFOS concentration of 1.0 μM. The degradation followed the pseudo-first order with reaction rate constant of 0.0043/d (r^2 = 0.86) which was on the same magnitude as the one reported for perfluorooctanoic acid (PFOA) degradation (0.0044/day, r^2 =0.89). It was also found that the presence of certain metal ions like Cu^{2+} than Mg^{2+} could facilitate the degradation of PFOS by ECOHRs. High resolution mass spectrometry results showed that ECOHRs transformed the majority of PFOS to partially fluorinated compounds. These products could be generated through the dissociation of SO_3^- from PFOS following with radical rearrangement and cross-coupling between the perfluoroalkyl radicals and other co-existed non-fluorinated compound radicals. This finding suggested that ECOHRs may have a great potential for removing PFOS from contaminated environmental waterbody.

INTRODUCTION

Perfluoroalkyl acids (PFAAs) represent a group of persistent man-made organic chemicals with fully fluorinated carbon backbone attached with sulfonic, carboxylic, or phosphonic acid functional group[1]. They attract much attention because of their ubiquitous presence in the environment, resistance to the degradation and toxicity to animals[2, 3]. PFAAs act quite differently from their corresponding hydrocarbon analogues due to the high energy of carbon-fluorine bond and nucleophilic property of fluorine[4]. PFAAs have extremely high thermal and chemical stabilities. The dual hydrophobic and oleophobic character of PFAAs make them appropriate for water and oil repellency applications[5]. The extensive usage of these chemicals in

industrial and daily consumer products, such as fluoropolymer manufacturing, firefighting foam forming, and semiconductor productions[6], leads to their frequent detections in various environmental matrices[7]. Exposure to PFAAs such as perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) is universal. For example, PFOS concentrations up to 2730 ng/g wet weight were found in the polar bear in the North American and European Arctic[8, 9]. The global distribution of PFAAs in the ocean at nanogram per liter level was also reported in a survey conducted in 2014[10]. Authorities like U.S. Environmental Protection Agency (EPA) have classified two PFAAs i.e. PFOA and PFOS as emerging contaminants which have potential threats to the human health and the environment[11]. PFOS, in particular, is able to induce a variety of undesirable effects in animals including carcinogenesis, infertility, endocrine disruption, etc.[12] The toxicity, mobility and bioaccumulation potential of PFOS drive the need to develop effective treatment methods for PFOS remediation[13].

Adsorption via granular activated carbon is currently the most widely applied techniques to physically remove the PFOS from the field[14]. However, this technique shows little success in the field demonstration and still requires other recycle or degradation approaches to achieve complete detoxification of PFOS[15]. Several oxidation methods have been examined for the degradation of PFOS. For instance, high-power sonochemical was able to effective transform PFOS into PFOA and other short-carbon perfluorocarboxylic acids (PFCAs)[16]. Photolysis was also effective in decomposing PFOS with reaction rate constant of 0.13/d[17]. Decomposition of PFOA by reduction was reported as well. Reductants such as vitamin B12 and zero-valent iron have been used to reduce PFOS. However, zero-valent iron induced PFOS degradation needs to be conducted at very harsh reaction conditions[18] while vitamin B12 performed poorly in

breaking down the linear PFOS[19]. Therefore, applications of these techniques described above are either restricted by the complicate operation procedures, high energy cost, or low efficiency.

A novel scheme involves a natural occurring oxidative humification process shed a light on the remediation of PFOS. The reaction of oxidative humification has significant environmental implication because it can lead to the detoxification of xenobiotics[20]. As reported, many pollutants like chlorinated phenols and polycyclic aromatic phenols can form irreversible binding with natural organic matter (NOM) through enzyme catalyzed oxidative humification reactions (ECOHRs)[21, 22]. This process is catalyzed by widely distributed extracellular phenoloxidases and peroxidases in the environment[23, 24] and could be enhanced by addition of such extracellular enzymes and organic compounds in to the contaminated sites[25, 26]. The ECOHRs is a free-radical process involving loss of an electron from the phenolic or anilinic substances which results in the formation of active intermediates such as free radicals and quinones. These radicals could undergo self-polymerization, or oxidize a variety of other persistent organic matters which could not be directly activated or oxidized by the enzyme[24].

Laccase may be the most applicable oxidases because they are able to produce reactive radicals, maintain their activities for a long period of time, and only require oxygen as electron acceptor[27]. The small molecules containing phenolic or anilinic functional groups which can be directly converted by laccase into radicals are called mediators[28, 29]. Laccase-mediator systems are found effective in the dechlorination of chlorinated phenols, cleavage of aromatic ring, mineralization of polycyclic aromatic hydrocarbons (PAHs), decolorization of pulp mill effluent, and detoxification of textile dyes[30].

We have demonstrated that laccase induced ECOHRs could effectively transform the PFOA, a predominant PFAA in environment, using the 1-hydroxybenzotriazole (HBT) as

mediator[31]. We also proposed that the reaction pathway of PFOA degradation by ECOHRs as following: first, the HBT radical (BTNO) converted the PFOA anion to PFOA radical, and then the PFOA radical went through Kolbe decarboxylation, free radical rearrangement and coupling processes to form the final degradation products which were mainly partially fluorinated compounds. These degradation products were identified as less environmental toxic than PFOA. Based on the previous findings, we expected that ECOHRs could be one effective in breaking down the PFOS under naturally relevant conditions as well.

Herein, we reported the degradation of PFOS by ECOHRs in the aqueous phase. The primary objectives of this research were to study the efficiency of ECOHRs degradation of PFOS with the presence of HBT and investigate the degradation mechanisms. We evaluated the PFOS degradation kinetics by monitoring the PFOS concentration change over time under various reaction conditions. The degradation mechanisms were also proposed by identifying critical degradation products. Since the co-contamination of PFOA and PFOS are frequently occurred, ECOHRs provide a promising remediation technique which is able to simultaneously breakdown the PFOA and PFOS under environment relevant conditions.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals used in the experiments were reagent grade or higher and used as received. Laccase from *Pleurotus Ostreatus* (EC 420-150-4), 1-hydroxybenzotriazole (HBT), and 2,6-dimethoxyphenol (DMP) were from Sigma Aldrich (St. Louis, MO). The 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO) was purchased from Enzo Life Sciences (Farmingdale, NY). Perfluoroctanesulfonate (PFOS) was purchased from Inpofine Chemical Company (Hillsborough, NJ), perfluoroalkane sulfonates (PFSAs) and PFCAs and surrogate

standard sodium perfluoro-1-[$^{13}C_8$]-octanesulfonate (M8PFOS) were obtained from Wellington Laboratories (Ontario, Canada) (see Table S7.1 for a full list). The cupric/magnesium sulfates were obtained from the Fisher Scientific (Pittsburgh, PA). All the HPLC-grade organic solvent including acetonitrile, methanol, and dichloromethane were also purchased from Fisher Scientific (Pittsburgh, PA). Milli-Q water (18.2 MΩ/cm resistivity) was prepared using the Nanopure Stand purification system (Thermo Scientific, San Jose, U.S.)

Experimental setup

The working solution was prepared by dissolving PFOS in solutions containing 10 mM metal cation i.e. Cu^{2+} or Mg^{2+} to obtain the PFOS initial nominal concentration of 1.0 μM. The original pH values of the working solution were 4.9 and 6.2 for Cu^{2+} and Mg^{2+} solution, respectively. Seventy-two reactors were prepared for each cation group. Each reactor included 10 mL working solution, 60 μL of 167 U/mL laccase stock solution (1 U/mL), and 20 μL of 10 mM HBT dissolved in acetonitrile (20 μM HBT) or 20 μL acetonitrile (0 μM HBT). The reactor with addition of 60 μL of Mill-Q water and 20 μL of acetonitriles instead of laccase and HBT were designated as positive controls. Degradation experiments were conducted using time-sequenced, multiple-addition scheme at 22 ^{o}C with continuously shaking at 120 rpm in an incubator (Innova 42, New Brunswick Scientific). The whole experiment lasted for 162 days. Every six days, the reactors were dosed with the same amount of freshly prepared laccase stock solution (167 U/mL) and HBT in acetonitrile (10 mM) or acetonitrile as the starting point. At each sampling event (every 12 days), sets of nine reactors in which triplicates from each treatment and positive control were sacrificed and diluted with appropriate quantities of Mill-Q water to unify the final volume to 12.16 mL for all reactors. Accurate 0.5-mL solution was withdrawn from each reactor and alkalified with 60 μL of 200 μM NaOH and then spiked with 0.5 mL of 0.5 μM M8PFOS

before subjected to solid phase extraction (SPE) cleanup as reported in previous study[31]. The process was briefly described in the Appendix B. Laccase activity assay was reported by Park et al.[32] One unit of laccase activity is defined as the amount of enzyme that causes one unit change in absorbance at 468 nm per minute of a DMP solution at pH 3.8 in a 1 cm light path cuvette[32] (a description provided in Appendix D).

An additional experiment was conducted to compare PFOA degradation in ECOHRs in the presence of DIPPMPO, a spin trap that can effectively scavenge HBT free radicals[33]. To this end, one group of reactors was set up and processed using the same procedure as described above with 20 μM HBT added every six days, except that 200 μM DIPPMPO was added to one of the two reactors each time along with the HBT. Addition of DIPPMPO was made in 40 μL of a 500 mM stock solution in Mili-Q water, and the same amount of water was added to the other reaction without DIPPMPO addition.

Chemical analysis

The PFOS quantitative analysis was done with a Waters AQCUITY I class UPLC system coupled with a XEVO TQD mass spectrometer (Waters, Milford, MA). The separation was carried out by a Waters UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm, Waters, Milford, MA) at 40 ℃ using a gradient composition of solvent A (water of 5 mmol/L ammonium acetate) and solvent B (methanol of 5 mmol/L ammonium acetate). The flow rate was 0.3 mL/min and the gradient program lasted for 10 min: 0−0.5 min, hold at 10% B; 0.5−8 min, linearly increase B from 10% to 95%; 8−8.1 min, a linear decrease from 95% to 100%; 8.1−9 min, linearly reduced B to 10%, and then equilibrium at 10% B for 1 min. Electrospray ionization was operated in a negative mode with the parameters set as capillary voltage at -1.0 kV, desolvation temperature at 400 ^{o}C, source block temperature at 150 ^{o}C. Nitrogen (> 99.999% purity, Airgas) was used as

desolvation gas with the flow rate of 550 L/hour. In addition to PFOS, M8PFOS and HBT, additional PFSAs and PFCAs with total carbon-chain length ranging from C4 to C11 were monitored simultaneously using multiple reaction monitoring. The precursors and transitions *m/z* values of all the monitored PFSAs, PFCAs and HBT as well as their detection limits were listed in Table S7.1.

Identification of reaction products

The process using high resolution mass spectrometer (HRMS) to identify reaction products was described in our previous research[31]. Briefly, a positive control that contained only PFOS without enzyme or HBT and a negative control that did not contain PFOS but with repeated enzyme and HBT additions were also incubated and processed along with the treatment reactors. At the end of incubation, 10 mL solution was taken from each reactor and repetitively extracted with 1 mL dichloromethane for four times. The extractants were then combined and reconstituted in 40 μL methanol and analyzed by an Orbitrap Elite HRMS (Thermo Scientific, San Jose, U.S.). Details are provided in the Appendix B.

By comparing the spectra of the reaction and the control samples, we were able to filter out those accurate *m/z* values that were only presented in the reaction samples but could not be detected in neither negative nor positive controls. The element composition for each potential product *m/z* values were proposed by formula generator program given error window of 5 ppm (Molecular Weight Calculator by Matthew Monroe). Some other conservative rules such as carbon-hydrogen ratio and nitrogen rule were employed to exclude the unreasonable element compositions. The structure of each potential reaction product was further deduced from its corresponding fragment ions spectra obtained from the Orbitrap Elite high resolution tandem mass spectrometer.

RESULTS AND DISCUSSION

ECOHRs degradation of PFOS

The efficiency of PFOS degradation by ECOHRs was evaluated with initial PFOS concentration of 1.0 μM at natural relevant conditions. We chose to add cations such as Cu^{2+} and Mg^{2+} to the reaction systems because we have found that, in the previous case, metal ions played an important role in the degradation of PFOA by ECOHRs[31]. Figure 7.1 shows the change of PFOS concentration over time in the presence of laccase and HBT. The PFOS decomposition in the Cu^{2+} solution was faster than in the Mg^{2+} solution. The degradation percentages were 59.0 ± 7.42 and 34.5 ± 7.27 for Cu^{2+} and Mg^{2+} solution respectively, after 162 days of incubation while no degradation was observed in the systems with addition of only 1 U/mL of laccase but no HBT regardless the cation category. The decomposition of PFOS in both treatments follows the pseudo-first-order kinetic. The reaction rate constants (k) were 0.0043/d (r^2 = 0.863) and 0.0027/d (r^2 = 0.925) for Cu^{2+} and Mg^{2+} solution respectively, corresponding to the half-lives of 161 and 257 days.

We expect that BTNO was able to abstract electron from the head C-C bond of PFOS in the similar way of degrading PFOA. In order to evaluate the role of BTNO, the ECOHRs degradation of PFOS in the presence of DIPPMPO, which is known as a radical scavenger for BTNO, was carried out in the Cu^{2+} solution. The results were summarized in Figure 7.2. PFOA degradation was significantly suppressed in the 1-20 treatment with DIPPMPO addition while approximately 59% reduction was found in the 1-20 treatment without DIPPMPO over 162 days of incubation. This phenomenon indirectly confirmed our hypothesis that BTNO could attack PFOS which further led to its degradation.

In addition, we documented the consumption of HBT in the ECOHRs-treated groups (Figure 7.3a) with periodic additions of laccase and 20 μM HBT (named as 1-20 in Figure 7.1). Continuous consumption of HBT was found in both ECOHRs-treated groups. We found that the total quantities of HBT converted in Mg^{2+} and Cu^{2+} groups were not significantly different (5.79 $\pm$0.90 and 6.38 $\pm$0.72 μmole for Mg^{2+} and Cu^{2+} system, respectively), however the PFOS transformation efficiency was significantly higher in the Cu^{2+} system (k = 0.0043/d) than in the Mg^{2+} system (k = 0.0027/d). This finding suggested that the degradation efficiency of PFOS by ECOHRs, somehow, was impacted by the types of cations in the system which needed further investigation.

Identification of degradation products

By comparing the high resolution mass spectrum of the 1-20 treatment sample after 162 d of incubation with those of corresponding positive (PFOS only) and negative (no PFOS but with laccase and HBT) controls, the accurate *m/z* values which only presented in the 1-20 treatment samples could be determined. The element compositions of the PFOS degradation products were determined according to the accurate molecular weights given by HRMS (mass accuracy < 5 ppm). Possible PFOS degradation products structures were then deduced from their fragment ion patterns (Table 7.1). Selected MS/MS spectra are shown in Figure S7.1 (Appendix D). For those products containing the ^{32}S element such as product No. 1, 2 and 15, their corresponding ^{34}S isotope intensities were also used to further verify the product structures. However, most of the products were not confirmed with authentic standards because no authentic standards were available.

The formation of short-carbon chain PFCAs and PFASs as PFOS degradation products was reported in other studies[34]. Although none of short-carbon chain PFCAs was identified as

final reaction product in our research, the accumulation of one short-carbon chain PFASs – perfluorohexanesulfonate (PHxS) was observed in the ECOHRs-treated reaction solution. As shown in Table 7.1, most of the identified products were partially fluorinated alcohols/aldehydes and fluoroalkyl substituted aromatic compounds. The CF=CF double bond in products No. 1, 8 and 12 could be formed from free radical rearrangement as reported in earlier study[35]. Another observation is that the molecular structures of products No. 10 and 15 contained HBT moieties which directly confirmed the interaction between the BTNO and short-carbon chain fluoroalkyl radicals. For example, the product No. 10 could form via cross-coupling between BTNO and $CF_2CF_2NHC_3H_7$ radical while the product No. 15 could generated from the interaction between the BTNO moiety and the $C_2H_4NHC_2F_4(CH=CH)_2COOH$ radical.

Mechanisms of the ECOHRs degradation of PFOS

On the basis of the obtained facts, we proposed a possible reaction pathway of PFOS degradation by ECOHRs (Figure 7.4). The proposed reaction pathway involved transferring an electron from the sulfonic head group of PFOS to a HBT radical to generate a C_8F_{17} radical. Such radical could further go through hydrolysis with concurrent elimination of an HF molecule and a fluoride to form the PFCAs or PFASs with one unit CF_2 less (Figure 7.4). This cycle was found in the PFOA degradation by electrochemical and persulfate oxidation as well[17, 36]. Then the repetition of dissociation of the COO^- and SO_3^- from the short-carbon chain PFCAs and PFASs could lead to the formation of corresponding short-carbon chain perfluoroalkyl radicals. Alternatively, these shorter-chain perfluoroalkyl radicals can also be formed directly from longer-chain radical via free radical rearrangement[35]. In the meantime, the BTNO could transform the coexistent non-fluorinated compounds into radicals. These two groups of radicals

could further couple with each other which is a common process during the laccase-mediated reactions[37, 38].

This research showed the effective degradation of PFOS by ECOHRs in the presence of HBT under natural environment condition. The transformation products are mainly the short-carbon chain, partially fluorinated products. Since most of the products are seemingly analogues of PFSA precursors, it is possible that some of them may be further oxidized to shorter-chain PFSAs under certain conditions, but these partially fluorinated products, as well as shorter-chain PFSAs, are believed to be more environmentally benign. The natural organic materials in the environment contained abundant phenolic functional groups which could serve as a good mediator[26]. Therefore, the remediation of PFOS could be facilitated through addition of natural organic materials for remediation purposes.

SUPPORTING INFORMATION AVAILABLE

Additional experimental details, figures, and table are available at Appendix D.

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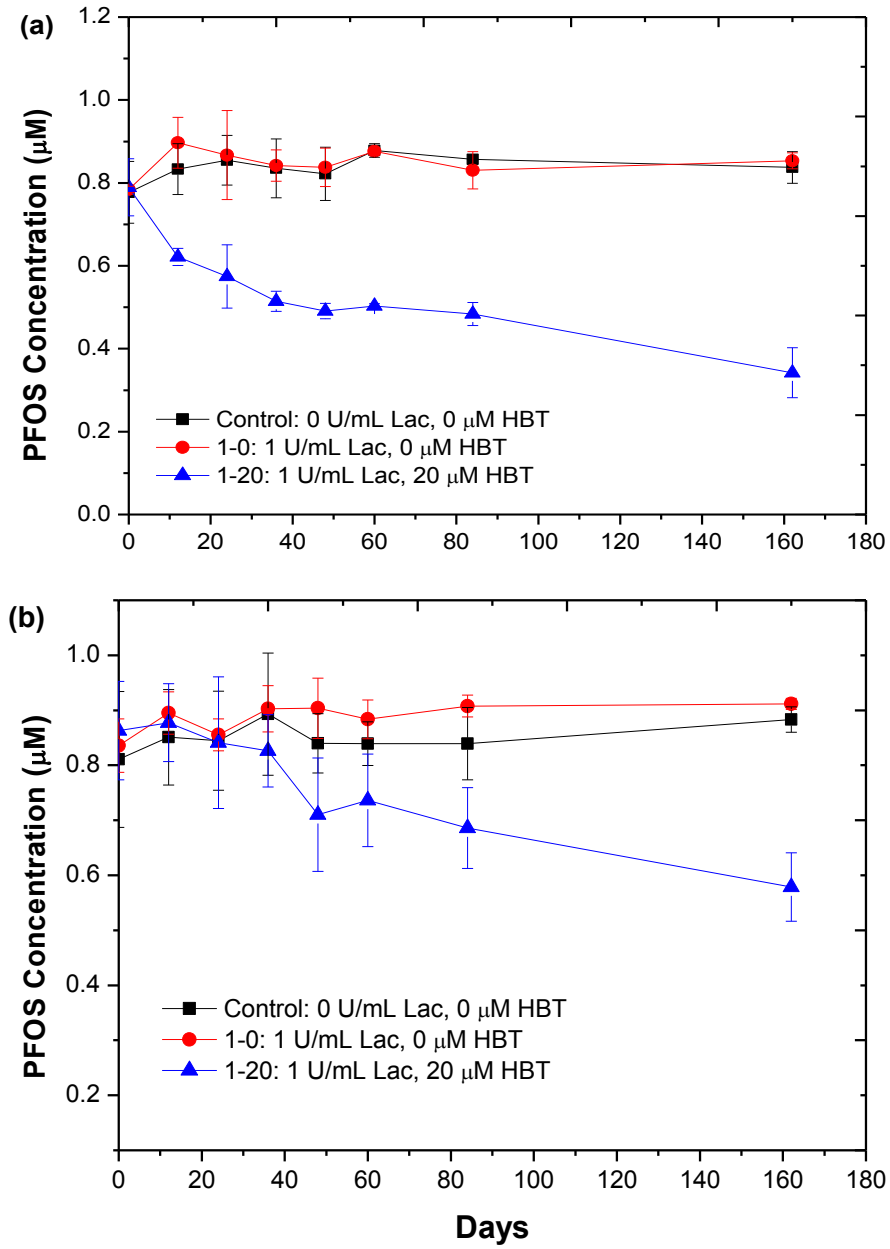


Figure 7.1. Change of PFOS concentration in ECOHRs over time with the presence (A) Cu^{2+} in the solution; (B) Mg^{2+} in the solution. Control: the positive control sample to which no laccase or HBT was added; 1-0: 1 U/mL laccase added every 6 d but no HBT; 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d.

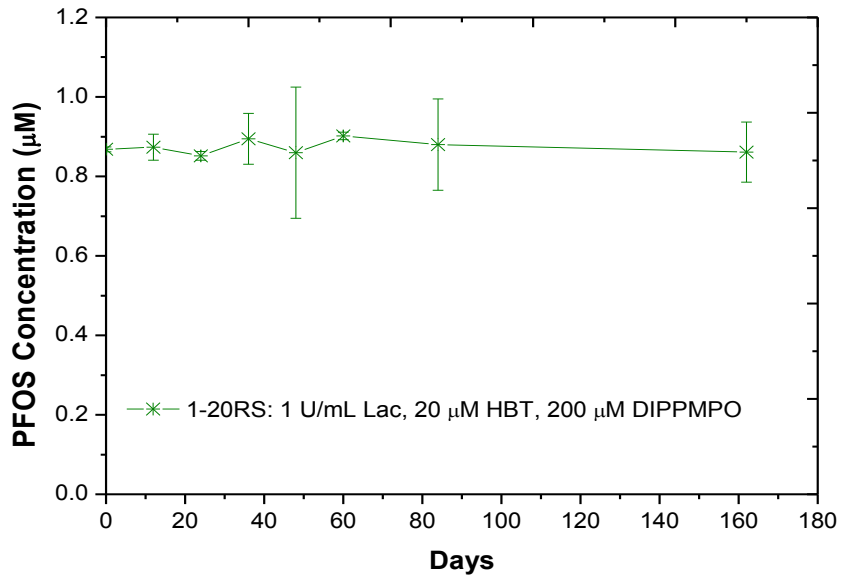


Figure 7.2. Change of PFOA concentration in ECOHRs over time with the addition of DIPPMPO as a HBT radical scavenger. 1-20 RS: 1 U/mL laccase, 20 μM HBT, and 200 μM DIPPMPO added every 6 d.

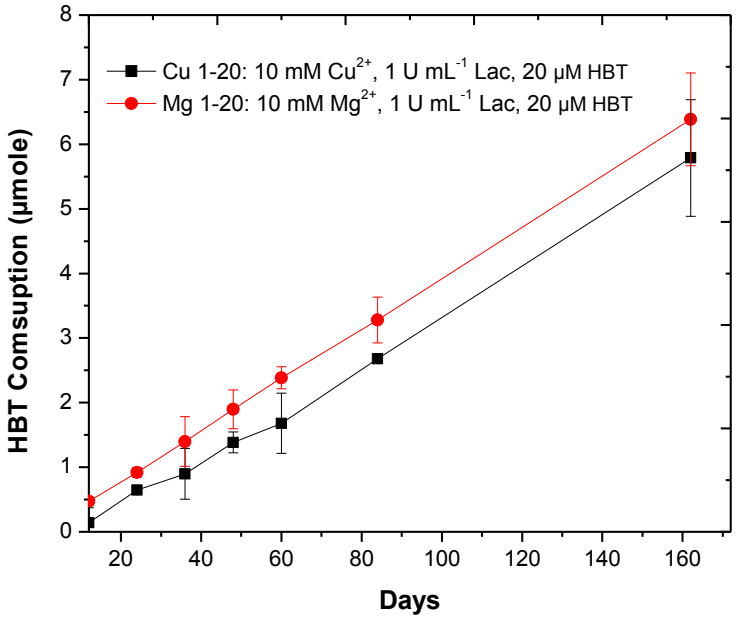


Figure 7.3. The cumulative amount of HBT consumed during ECOHRs. Cu 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d with the presence of 10 mM Cu^{2+}; Mg 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d with the presence of 10 mM Mg^{2+}.

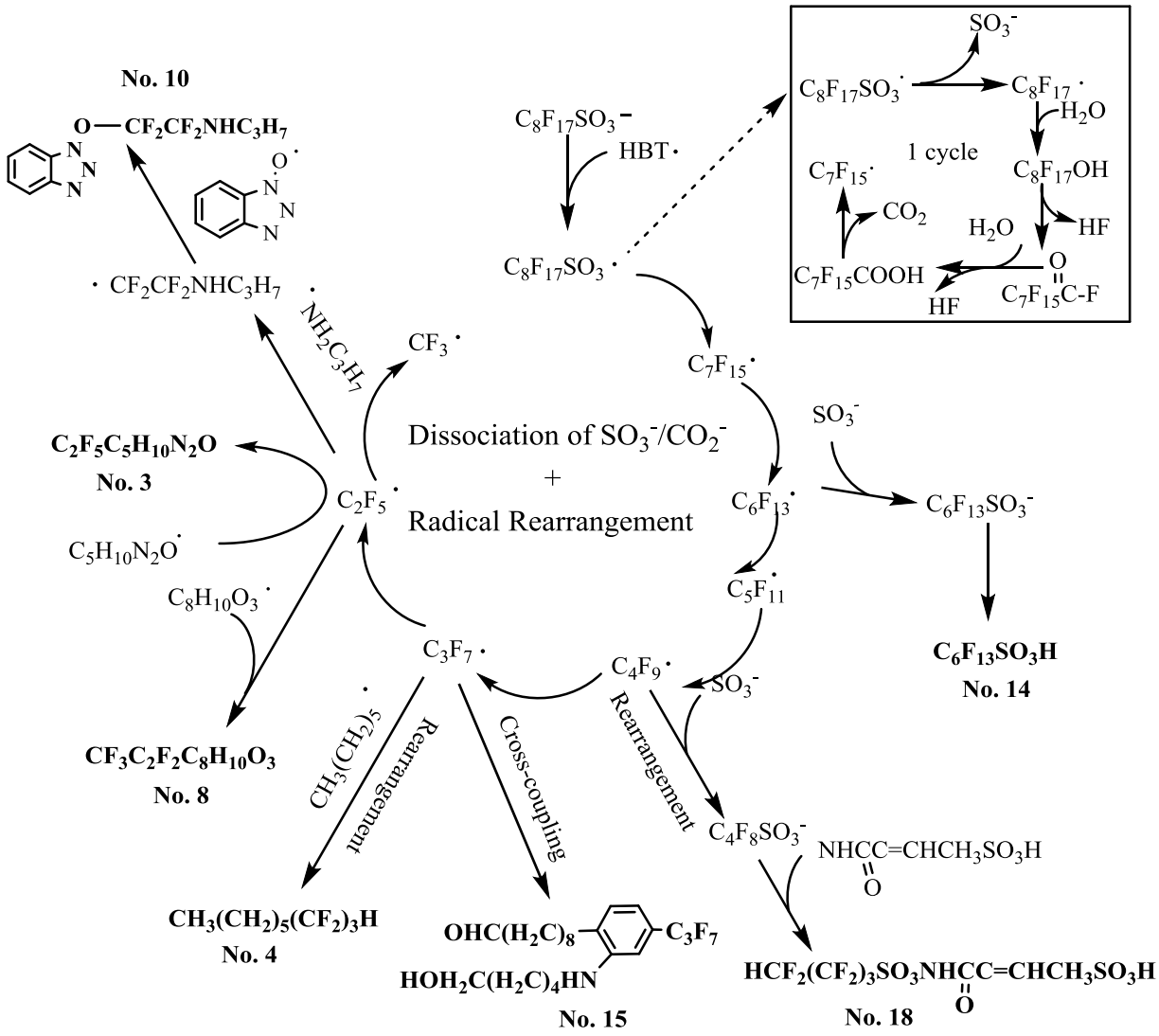
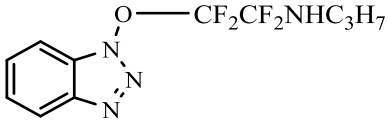
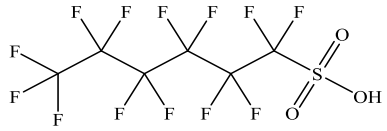


Figure 7.4. Proposed PFOS degradation pathways during ECOHRs treatment with laccase and 1-hydroxybenzotriazole

Table 7.1. Molecular formulas, theoretical and measured deprotonated molecule weight $[M-H]^-$, mass accuracy (ppm) and possible structures of PFOS degradation products from ECOHRs.

No	Formula	$[M-H]^-$		Mass accuracy (ppm)	Possible structure
		Theoretical	Experimental		
1	$C_4H_7F_2N_3OS$	182.0200	182.0207	-4.0	$HS\text{-}CF{=}CF(CH_2)_2NHN{=}NOH$
2	$C_6H_8F_2N_2O_2S$	209.0196	209.0204	-3.7	$HSCF_2NHCH{=}CHCH_2N{=}CHCO_2H$
3	$C_7H_{11}F_5N_2O$	233.0713	233.0721	-3.3	$CF_3CF_2NH(CH_2)_3NHCH_2CH{=}O$
4	$C_9H_{14}F_6$	235.0921	235.0917	1.9	$CH_3(CH_2)_5(CF_2)_3H$
5	$C_{10}H_{15}F_4NO$	240.1012	240.1022	-4.4	$C_3H_7(CF_2)_2CH(CH_3)CH_2CH_2O$
6	$C_7H_{11}F_5N_4O$	261.0775	261.0782	-2.5	$CF_3CF_2N{=}NO(CH_2)_5N{=}NH$
7	$C_9H_{10}F_4N_2O_3$	269.0549	269.0557	-2.9	$HOCCHCH(CH_2)_3N{=}N(CF_2)_2CO_2H$
8	$C_{10}H_{11}F_5O_3$	273.0550	273.0541	3.3	$CF_3CF{=}CFCH{=}CHCH_2CH_2CHCH_2COH$ (OH, O)
9	$C_8H_{12}F_4N_2O_4$	275.0655	275.0668	-4.8	$HOC(CH_2)_3NHNHCHC_2F_4COH$ (OH, O)
10	$C_{11}H_{12}F_4N_4O$	291.0869	291.0883	-4.8	
11	$C_{11}H_{10}F_4N_2O_3$	293.0549	293.0567	-5.0	$HOOCF_2CF_2C$–C$_6$H$_4$–$NHNHCH_2COH$
12	$C_{14}H_{24}F_4O_2$	299.1634	299.1620	4.7	$CH_2OH(CH_2)_8CH(CHF)CF{=}CFH$ (OH)
13	$C_{20}H_{24}F_4O_3$	387.1583	387.1572	2.9	HOC–C$_6$H$_4$–$C_2H_2CF_2(CH_2)_4CHOCF_2C_3H_7$ (OH)
14	$C_6HF_{13}SO_3$	398.9361	398.9361	0.0	
15	$C_{16}H_{16}F_4N_4O_4$	403.1029	403.1045	-3.9	$HOOC$–C$_6$H$_4$–$N{=}NNHC_2H_4NHC_2F_4(CHCH)_2COH$ (O)
18	$C_8H_7F_8NO_7S_2$	443.9458	443.9461	-0.7	$HCF_2(CF_2)_3SO_3NHCC{=}CHCH_3SO_3H$ (O)
19	$C_{22}H_{32}F_7NO_2$	474.2243	474.2251	-1.7	$OHC(H_2C)_8$–C$_6$H$_4$–$(CF_2)_3CHFNH(CH_2)_4CH_2OH$

CHAPTER 8

CONCLUSIONS

This study investigated the degradation of two predominant perfluoroalkyl acids (PFAAs) in the environment – perfluoroocatnoic acid (PFOA) and perfluoroctanesulfonate (PFOS) with the enzyme catalyzed oxidative humification reactions (ECOHRs). The degradation mechanisms of PFOA and PFOS by ECOHRs were also explored using the high resolution mass spectrometer (HRMS).

PFOA and PFOS are two emerging contaminants which have adverse effects to animals and could biocumulate through the food chain. The toxicity of PFOA to two typical green microalgae has been examined. The 96-h EC_{50} values were determined to be 51.9 $\pm$1.0 and 44.0 $\pm$1.5 mg/L for *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*, respectively. PFOA was able to induce oxidative stress in the microalgae. Increase of malonaldehyde and proline level was observed with the present of 20 mg/L PFOA after 8-day incubation. As primary producer, microalgae can be consumed by other aquatic organisms. Therefore, the sorption and uptake of PFOA by microalgae may influence the toxicity and its ecological risk in the aquatic ecosystem. Although low levels of PFOA (less than 27% in this study) was sorbed or uptake (approximately 10%) by these algae, the possibility of bioaccumulation of this chemical along the aquatic food chain should not be overlooked.

The extreme stability of fluorine-carbon bond in the PFOA and PFOS makes them resistant to traditional remediation techniques. Current developed methods for PFOA and PFOS degradation either involve high energy input or special devices. ECOHRs, as a natural process,

may have a great potential in degrading the PFOA and PFOS since effective transformation of variety of persistent organic pollutants (POPs) such as polychlorinated biphenyl (PCBs) and polycyclic aromatic hydrocarbons (PAHs) by ECOHRs has been found. In our study, ECOHRs was proven to be effective in the decomposition of PFOA in both aqueous and soil phase. Transformation of approximately 49% of PFOA was found in the aqueous phase with periodic addition of laccase and 1-hydroxybenzotriazole (HBT) as mediator after 157 days. The pathway of ECOHRs transforming PFOA was also investigated. Transferring one electron from the carboxylic head of PFOA to the HBT radical was the first step of converting the PFOA anion to the perfluoroalkyl radicals. These radicals could future go through Kolbe decarboxylation and hydrolysis to form shorter-carbon chain perfluorocarboxylic acids (PFCAs) while releasing fluoride. However, our results indicated that shorter-carbon chain PFCAs were not the major degradation products because these shorter-carbon chain PFCAs could further activated by HBT radicals and then cross couple with other co-existed non-fluorinated compound radicals leading to the formation of partially fluorinated compounds as major products. We also found that the metal ions such as Cu^{2+} and Fe^{3+} in the reaction system played an important role in the PFOA degradation. They facilitated the degradation of PFOA by serving as a bridge between the PFOA and HBT radicals and bring them to proximity. We further identified a natural organic matter, i.e. soybean meal as mediator that could be applied in the soil PFOA remediation. The degradation of PFOA up to 36% was found in the soil with 20 U/g laccase and 50 mg/g soybean meal.

PFOS is another widespread PFAA in the environment which attracts tremendous attention due to its toxic effects to animals and humans. Our results suggested that ECOHRs could successfully transformed 59% of PFOS during 162 days of incubation. The reaction

mechanism was considered similar to the one proposed in the PFOA degradation since partially fluorinated alcohols/aldehydes/acids were still the dominant degradation products. Co-contamination of PFOA and PFOS is often occurred, developing a method that could simultaneously degrade PFOA and PFOS under natural conditions is critical in the remediation of PFAAs.

This study provides important information in potential application of ECOHRs in the PFOA and PFOS remediation in water and soil. The results helped researchers understand a possible fate and transport route of the PFOA and PFOS in the natural environment. The reaction kinetic data validated the effectiveness of ECOHRs in PFOA and PFOS degradation and provide valuable guide in incorporating such process in future remediation. Furthermore, the adjustment of various reaction factors such as type of laccase enzyme, mediator concentration, addition of metal ion, during the study facilitated the optimization of the systems which could enhance the effectiveness of ECOHRs process in the future field application. Knowledge of ECOHRs degradation pathways could assist in predicting potential byproducts of the degradation reaction and in potentially identifying dead-end products that could also provide evidence of ECOHRs success.

APPENDIX A

SUPPORTING INFORMATION:

LACCASE CATALYZED DEGRADATION OF PERFLUOROOCTANOIC ACID

MATERIAL AND METHODS

Buffer solution

A mineral buffer solution was prepared by dissolving 0.6 g $NaNO_3$, 0.4 g $CuSO_4 \cdot 5H_2O$, 0.101 g NaH_2PO_4, 0.02 g Na_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, and 0.0034 g $MnSO_4$ in 1 L Milli-Q water. The pH of this mineral buffer is 4.5. This buffer recipe was derived from a formula that has been used for fungal cultivation[1] with the concentration of each component reduced 10 times. This buffer solution was used in all experiments to examine PFOA degradation in ECOHRs.

Experimental setup

The reactions were conducted in 100 mL of the mineral buffer containing 1.0 μM PFOA. The initial laccase enzyme activity was 1 U/mL and HBT concentration was 0, 2, or 20 μM in the different treatments. The reactors were incubated at 22^{o}C and shaken at 120 rpm in a shaker incubator (Innova 42, New Brunswick Scientific). Every six days during the incubation, the laccase activity was monitored, and an appropriate amount of enzyme solution (~200 U/mL) in Milli-Q water was added to the reactor to bring the enzyme activity back to its original level (Figure S4.2). A 40 μL stock solution of 0, 5 or 50 mM HBT in acetonitrile was also added to the 0, 2, or 20 μM HBT treatment reactor, respectively, to supplement the mediator. Parafilm was used to cover the flasks to limit water evaporation and removed for 30 minutes every six days to allow for air exchange. At select times, three 0.5 mL aliquots of solution were taken from each reactor for PFOA quantification. All reactors were weighed before and after each sampling and solution supplement to account for the change of solution mass that was factored into PFOA concentration calculation to adjust for the dilution or concentration effect due to solution supplement or evaporation.

For product identification purposes, a positive control that initially contained only 1.0 μM PFOA and a negative control that initially contained 1 U/mL laccase and 20 μM HBT but without PFOA were also prepared and incubated along with the treatment samples. Every six days when the enzyme and HBT supplements were made to the treatment samples, the same amounts of enzyme and HBT were also added to the negative control, while the same volumes of Milli-Q water and acetonitrile were added to the positive control.

An additional experiment was conducted to compare PFOA degradation in ECOHRs in the absence and presence of DIPPMPO, a spin trap that can effectively scavenge HBT free radicals[2]. To this end, two reactors were set up and processed using the same procedure as described above with 20 μM HBT added every six days, except that 200 μM DIPPMPO was added to one of the two reactors each time when HBT addition was made. Addition of DIPPMPO was made in 40 μL of a 500 mM stock solution in acetonitrile, and the same amount of acetonitrile was added to the other reaction without DIPPMPO addition. A positive control and a treatment with 1 U/mL laccase and 0 μM HBT addition every six days as described above were also repeated in this experiment run.

Solid phase extraction

Each 0.5-mL PFOA sample was first spiked with 0.5 mL of 0.5 μM M8PFOA in Milli-Q water as the surrogate standard. The samples were then subjected to solid phase extraction (SPE) (Oasis HLB SPE cartridges, 3 cc, 60 mg, Waters, Milford, MA) as described in earlier studies[3, 4] with minor changes. The SPE cartridge was conditioned with 3 mL methanol, two 3 mL aliquots of Milli-Q water sequentially, followed by loading the sample, then rinsed with 3 mL MILLI-Q water and blown to dry under vacuum. The cartridge was then eluted with 1 mL methanol for

three times and then 1 mL acetonitrile for two times. All eluents were combined, and the mixture was blown to 1 mL with nitrogen gas for PFOA and HBT quantification.

Recovery of the method has been evaluated using a standard addition method by adding 1 μM PFOA to selected sample aliquots before being processed as described above. The standard addition recovery was 98.76 ± 10.3%. In addition, the recovery of the surrogate standard M8PFOA was 96.7 ± 6.14% with great consistency during the entire experiment period.

PFCAs and HBT quantification

PFOA quantitative analysis was done with a Waters 2690 HPLC system coupled with a Micromass Quattro tandem mass spectrometer (Waters, Milford, MA). An Ascentic C18 column (25 × 4 mm, 5 μm, Supelco, St. Louis, MO) was used for HPLC separation with a mobile phase consisting of a water solution containing 2 mM ammonium acetate (A) and methanol (B) at a flow rate of 0.4 mL/min by a gradient program lasting 17 min: 50% B at time 0, linearly increasing to 90% B at 3 min, held 90% B until 14 min, reduced to 50% B at 14.1 min, and then held at 50% B for 3 min. Electrospray ionization was operated in a negative mode for PFOA detection with the parameters set as capillary voltage at 11 V, cone voltage 20 V, desolvation temperature at 300 ^{o}C, source block temperature at 100 ^{o}C. Nitrogen (> 99.999% purity, Airgas) was used as the nebulizer and drying gas with the flow rates at 34 and 198 L/hour, respectively. Multiple reaction monitoring (MRM) was used at the transition *m/z* = 413 > 369 for PFOA, *m/z* = 421 > 376 for M8PFOA.

Besides PFOA, eight additional PFCAs (C4-C11) and HBT were also monitored simultaneously using multiple reaction monitoring at the transitions *m/z* = 213 > 169 (perfluoro-n-butanoic acid, PFBA), *m/z* = 263 > 219 (perfluoro-n-pentanoic acid, PFPeA), *m/z* = 313 > 269 (perfluoro-n-hexanoic acid, PFHxA), *m/z* = 363 > 319 (perfluoro-n-heptanoic acid, PFHpA), *m/z*

= 463 > 419 (perfluoro-n-nonanoic acid, PFNA), *m/z* = 513 > 469 (perfluoro-n-decanoic acid, PFDA), *m/z* = 563 > 519 (perfluoro-n-undecanoic acid, PFUA), *m/z* = 134 > 105 (HBT). The precursors and transitions m/z values of all the PFCAs and HBT monitored and their detection limits were listed in Table S3.1.

Reaction product identification

The high resolution mass spectrometer with full scan and tandem mass fractionation was performed using an Orbitrap Elite (resolution R = 60 000 at *m/z* 400, for *m/z* = 100 to 1000) from Thermo Scientific (San Jose, U.S.) with ESI negative mode.

The fluoride concentration was quantified using an ion-chromatograph (ICS-1000, Dionex, USA). The system was equipped with an autosampler (sample injection volume: 10 μL), a pump, a degasser, a guard column, and a separation column (Dionex IonPac AS 12A, 4 mm i.d × 200 mm, USA) operating at 30 ^{o}C. The mobile phase was a solution containing 15 mM KOH at a flow rate of 1 mL/min. The retention time for fluoride is 3.1 min. An external six-point calibration curve was generated by measuring standard fluoride samples with concentration ranged from 5 to 500 μg/L.

Laccase activity assay

Laccase activity was assayed using 2,6-dimethoxyphenol (DMP) as substrate. One unit of activity of laccase equals the amount of enzyme that causes an absorbance change in 468 nm at a rate of 1.0 unit/min in 3.4 mL of 1 mM 2,6-dimethoxyphenol in citrate-phosphate buffer (pH 3.8) in a 1-cm light path cuvette[5]. All activities were reported as measured by this method in this study.

We have also assayed laccase activity using a method with 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate to enable comparison with some earlier studies. Laccase activity was measured by monitoring the oxidation of 3 mL 100 mM ABTS substrate solution at 420 nm in 0.1 M Phosphate buffer (pH 6). One unit of laccase activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol ABTS per min[6]. By our measurements, the laccase activity measured by the two methods can be converted using the following equation:

Activity (measured by DMP) = 2.65 × Activity (measured by ABTS)

The laccase activity used in this study (1 U/mL by DMP method) is equivalent to 0.377 U/mL (by ABTS method).

Statistical analysis

Analysis of Variance (ANOVA) has been performed to compare the PFOA concentrations of the same treatment over different sampling times as well as the different treatments at the same sampling time. The results are shown in Figure S3.1. ANOVA was conducted on SAS using a linear model with the significant difference $\alpha = 0.05$ followed by a least significant difference test.

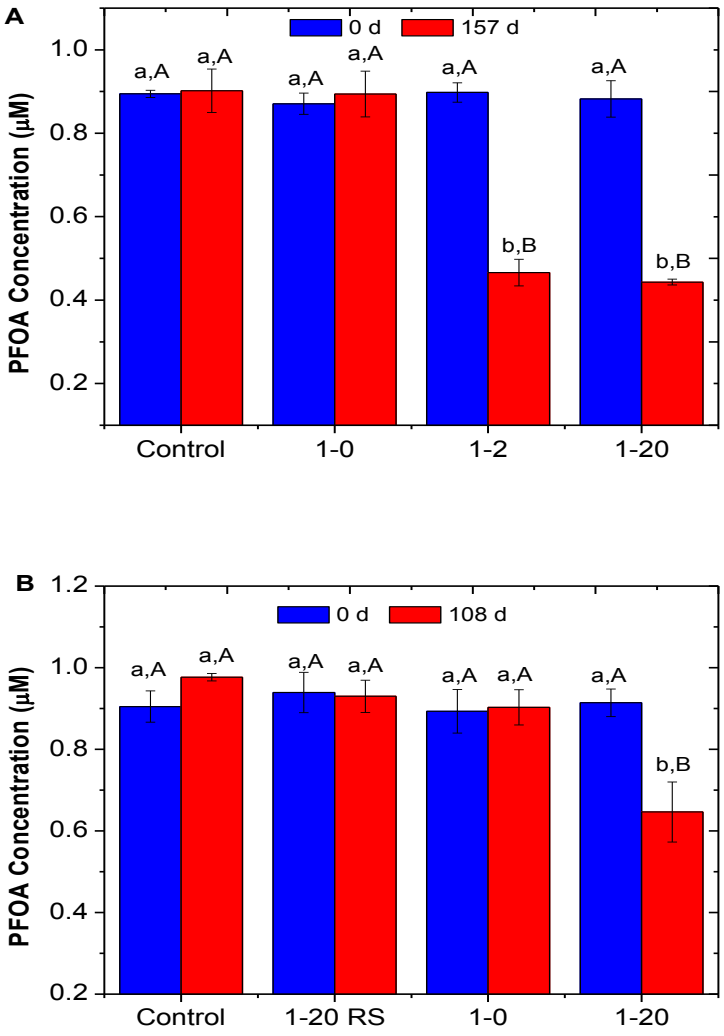


Figure S3.1. (A) PFOA concentrations at 0 and 157 d in the ECOHRs experiment presented in Figure 4.1A in the paper; (B) PFOA concentrations at 0 and 108 d in the ECOHRs experiment with and without the addition of DIPPMPO from Figure 4.1B in the paper. The same letter indicates no statistical difference at α=0.05, with the lower case for the comparison of the same treatment over different sampling times, and upper case for the different treatments at the same sampling time. Control: the positive control sample to which no laccase or HBT was added; 1-0: 1 U/mL laccase added every 6 d but no HBT; 1-2: 1 U/mL laccase and 2 μM HBT added every 6

d; 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d; 1-20 RS: 1 U/mL laccase, 20 μM HBT, and 200 μM DIPPMPO added every 6 d.

LACCASE ACTIVITY AND HBT CONSUMPTION DURING ECOHRS TREATMENTS

Laccase activity and HBT concentration were monitored during the ECOHRs for which the PFOA degradation is shown in Figure 4.1A in the paper. Figure S3.2 displays the residual laccase activity that was measured at each sampling time. Figure S3.3 presents the cumulative HBT consumption during the reaction. HBT consumption was calculated by subtracting the residual HBT quantity in 1-2 or 1-20 treatment from the total quantity of HBT that had been added to the corresponding treatment at the sampling time.

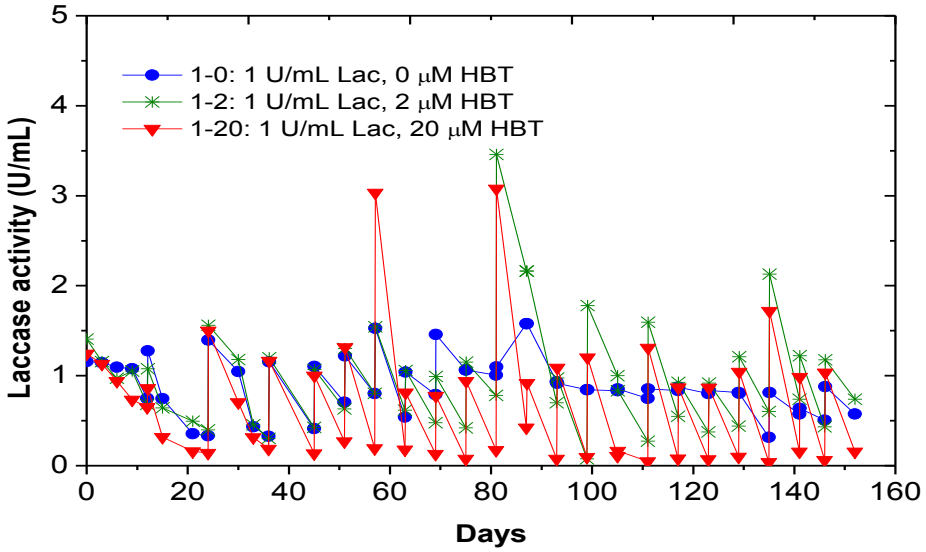


Figure S3.2. Change of laccase activity during ECOHRs treatment (PFOA degradation data presented in Figure 4.1A in the paper). 1-0: 1 U/mL laccase added every 6 d but no HBT; 1-2: 1 U/mL laccase and 2 μM HBT added every 6 d; 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d.

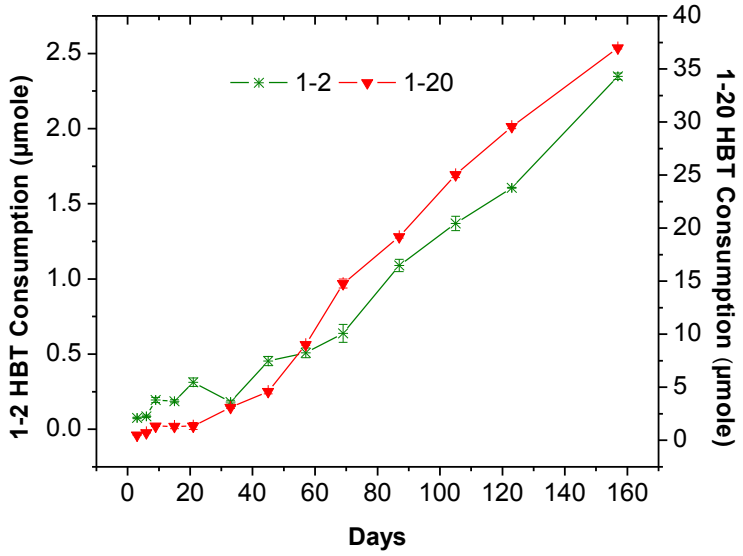


Figure S3.3 The cumulative amount of HBT consumed during ECOHRs (PFOA degradation data presented in Figure 4.1A in the paper). 1-2: 1 U/mL laccase and 2 µM HBT added every 6 d, 1-20: 1 U/mL laccase and 20 µM HBT added every 6 d.

KINETIC ANALYSIS

A kinetic analysis was conducted on PFOA degradation (the time-course data in Figure 4.1A in the paper) by data fitting to the pseudo-first-order rate equation in the form ln (C_t/C_0) = kt, where C_0 and C_t are PFOA concentrations at time 0 and t, respectively, and k is the pseudo-first-order rate constant, and the results are shown in Figure S3.3.

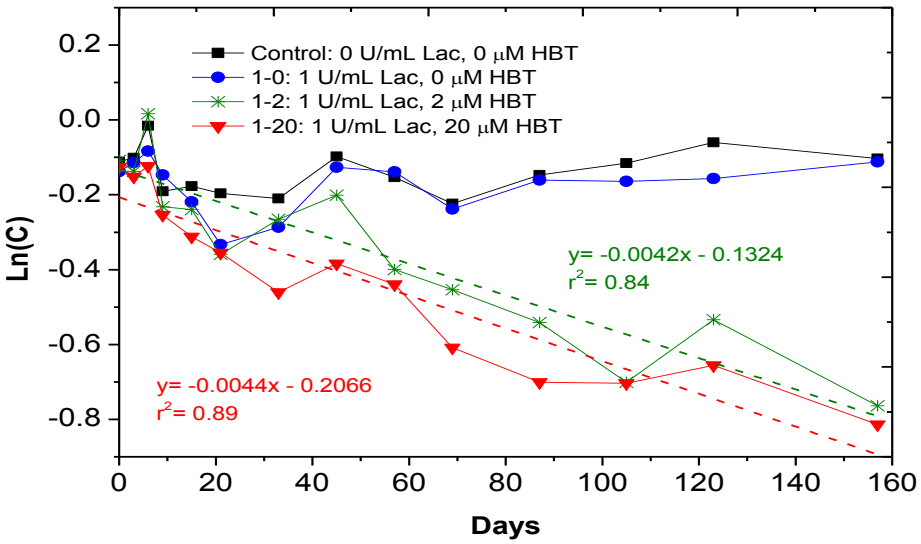


Figure S3.4. Pseudo-first-order rate model fit for the PFOA degradation data (Figure 4.1A in the paper), with the rate constant (k) of 0.0042/day (r^2 = 0.84) and 0.0044/day (r^2 = 0.89 for the 1-2 and 1-20 treatment, respectively. Control: the positive control sample to which no laccase or HBT was added, 1-0: 1 U/mL laccase added every 6 d but no HBT; 1-2: 1 U/mL laccase and 2 μM HBT added every 6 d; 1-20: 1 U/mL laccase and 20 μM HBT added every 6 d.

Selected chemicals identified by HRMS

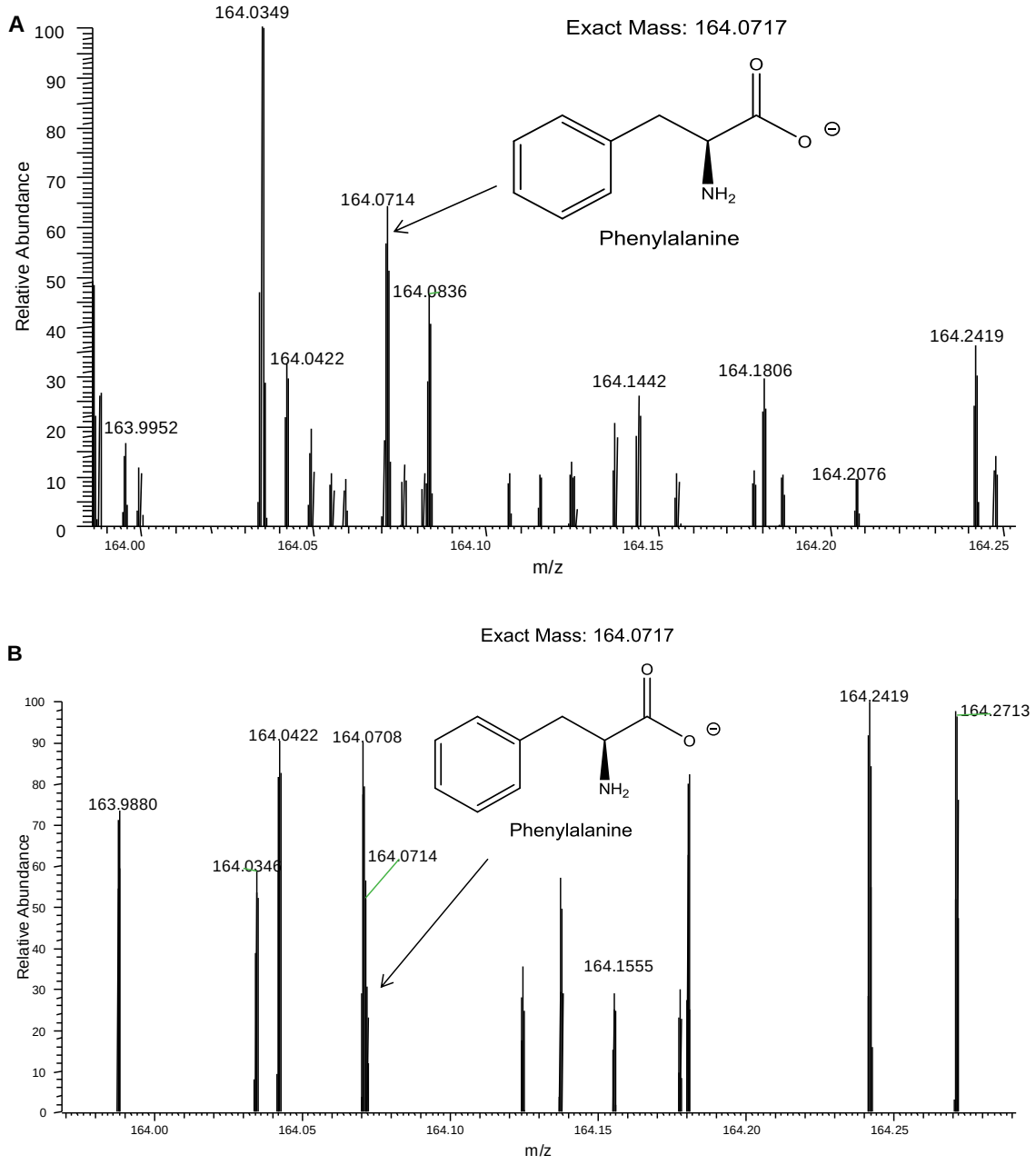


Figure S3.5. The accurate m/z of phenylalanine measured in the treatment sample (A) and negative control (B) with laccase and HBT.

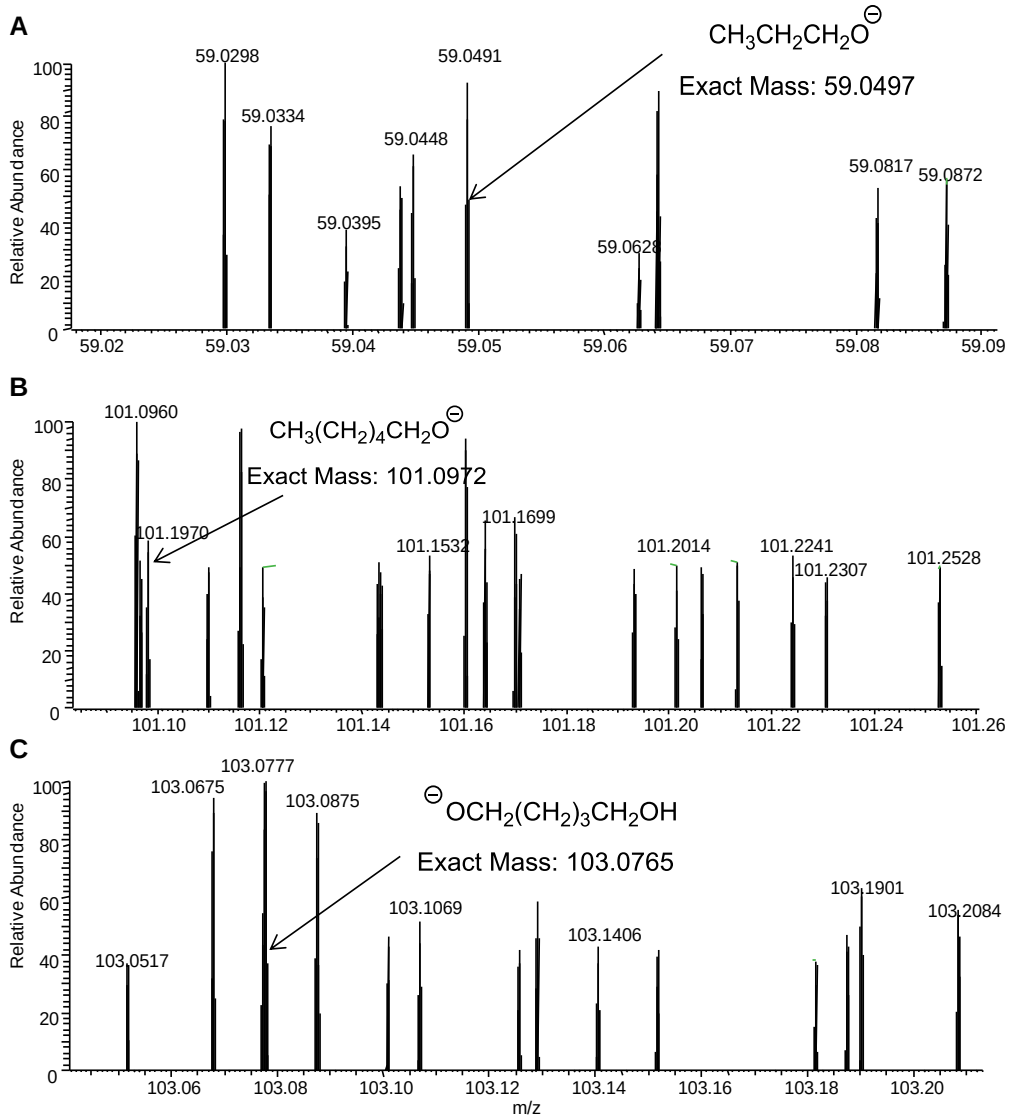
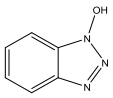


Figure S3.6. The measured m/z values of different alcohols identified in the negative control sample with laccase and HBT but no PFOA. A: 1-Propanol; B: 1-Hexanol; C: 1,5-Pentanediol

Table S3.1. The molecular formula, retention time, precursor ion, transition ion, and detection limit of the PFCAs and HBT monitored in HPLC-MS/MS analysis

Chemicals	Molecular Formula	RT (min)	Precursor ion m/z	Transition ion m/z	Detection limit (μg/L)
PFBA (C4)	$F-(CF_2)_3COOH$	9.23	213	169	1.01
PFPeA (C5)	$F-(CF_2)_4COOH$	10.12	263	219	0.13
PFHxA (C6)	$F-(CF_2)_5COOH$	10.53	313	269	0.07
PFHpA (C7)	$F-(CF_2)_6COOH$	10.93	363	319	0.06
PFOA (C8)	$F-(CF_2)_7COOH$	11.26	413	369	0.05
PFNA (C9)	$F-(CF_2)_8COOH$	11.58	463	419	0.09
PFDA (C10)	$F-(CF_2)_9COOH$	11.99	513	469	0.20
PFUA (C11)	$F-(CF_2)_{10}COOH$	12.24	563	519	0.27
HBT		4.51	134	105	4.05

PFBA: perfluoro-n-butanoic acid; PFPeA: perfluoro-n-pentanoic acid; PFHxA: perfluoro-n-hexanoic acid; PFHpA: perfluoro-n-heptanoic acid; PFNA: perfluoro-n-nonanoic acid; PFDA: perfluoro-n-decanoic acid; PFUA: perfluoro-n-undecanoic acid; HBT: 1-hydroxybenzotriazole.

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APPENDIX B

SUPPORTING INFORMATION:

FACTORS CONTROLLING THE RATE OF LACCASE-MEDIATED PERFLUOROOCTANOIC ACID DEGRADATION: THE ROLE OF METAL IONS

MATERIALS AND METHODS

Buffer solutions

A mineral buffer solution was prepared by dissolving 0.6 g $NaNO_3$, 0.4 g $CuSO_4 \cdot 5H_2O$, 0.101 g NaH_2PO_4, 0.02 g Na_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, and 0.0034 g $MnSO_4$ in 1 L HPLC water. The pH of this mineral buffer is 4.5. This buffer recipe was derived from a formula that has been used for fungal cultivation[1] with the concentration of each component reduced 10 times. A citric buffer of pH 4.5 was prepared with addition of 0.191 g anhydrous citric acid and 0.296 g dehydrate sodium citrate to 1 L HPLC water. The metal ion solutions were prepared by adding 2.50, 2.46, or 1.69 g of $CuSO_4$, $MgSO_4$, or $MnSO_4$ into 1 L HPLC water respectively.

Solid Phase Extraction

The samples were then subjected to solid phase extraction (SPE) (Oasis HLB SPE cartridges, 3 cc, 60 mg, Waters, Milford, MA) as described in earlier studies[3, 4] with minor changes. The SPE cartridge was conditioned with 3 mL methanol, two 3 mL aliquots of HPLC water sequentially, followed by loading the sample, then rinsed with 3 mL HPLC water and blown to dry under vacuum. The cartridge was then eluted with 1 mL methanol for three times and then 1 mL acetonitrile for two times. All eluents were combined, and the mixture was blown to 1 mL with nitrogen gas for PFOA and HBT quantification.

Recovery of the method has been evaluated using a standard addition method by adding 1 μM PFOA to selected sample aliquots before being processed as described above. The standard addition recovery was 98.76 ± 10.3%. In addition, the recovery of the surrogate standard M8PFOA was 96.7 ± 6.14% with great consistency during the entire experiment period.

Laccase Activity Assay

Laccase activity was determined using 2,6-dimethoxyphenol (DMP) as substrate. One unit of activity of laccase equals the amount of enzyme that causes an absorbance change in 468 nm at a rate of 1.0 unit/min in 3.4 mL of 1 mM 2,6-dimethoxyphenol in citrate-phosphate buffer (pH 3.8) in a 1-cm light path cuvette[5]. All activities were reported as measured by this method in this study.

We have also assayed laccase activity using a method with 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate to enable comparison with some earlier studies. Laccase activity was measured by monitoring the oxidation of 3 mL 100 mM ABTS substrate solution at 420 nm in 0.1 M Phosphate buffer (pH 6). One unit of laccase activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol ABTS per min[6].

By our measurements, the laccase activity measured by the two methods can be converted using the following equation:

Activity (measured by DMP) = 2.65 × Activity (measured by ABTS)

The laccase activity used in this study (1 U/mL by DMP method) is equivalent to 0.377 U/mL (by ABTS method).

Ion chromatograph for fluoride analysis

The fluoride concentration was quantified using an ion-chromatograph (ICS-1000, Dionex, USA). The system was equipped with an autosampler (sample injection volume: 10 μL), a pump, a degasser, a guard column, and a separation column (Dionex IonPac AS 12A, 4 mm i.d × 200 mm, USA) operating at 30 ^{o}C. The mobile phase was a solution containing 15 mM KOH at a flow rate of 1 mL/min. The retention time for fluoride is 3.1 min. An external six-point

calibration curve was generated by measuring standard fluoride samples with concentration ranged from 0.01 to 5 mg/L.

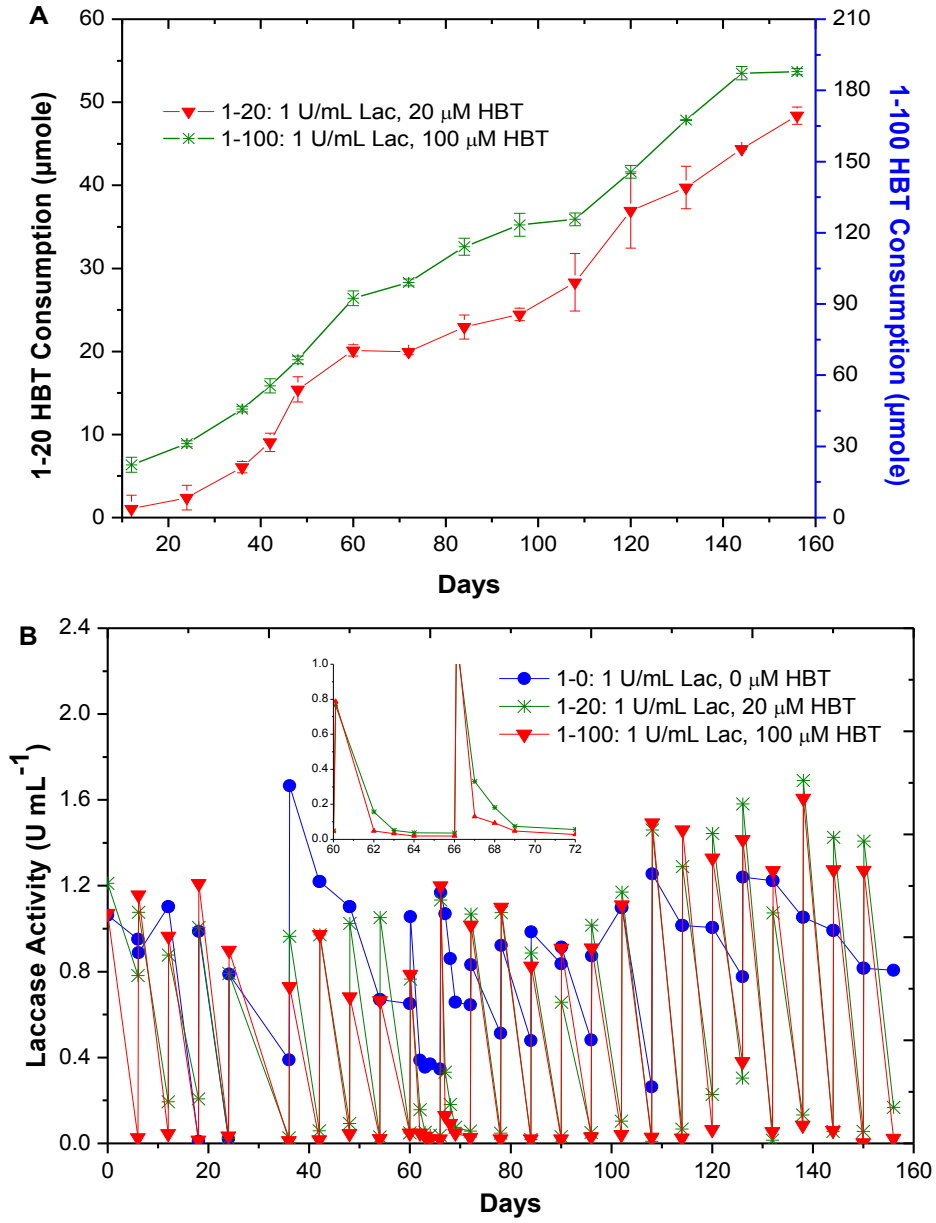


Figure S4.1. HBT consumption and laccase activity change in the ECOHR systems with PFOA initial concentration at 100 µM (PFOA concentration change shown in Figure 4.1B): (A) The cumulative amount of HBT consumed during ECOHRs; (B) Change of laccase activity during ECOHRs (Insert shows the daily change of laccase activity from day 60 to 72). 1-0: 1 U mL^{-1} laccase added every 6 d but no HBT; 1-20: 1 U mL^{-1} laccase and 20 µM HBT added every 6 d; 1-100: 1 U mL^{-1} laccase and 100 µM HBT added every 6 d.

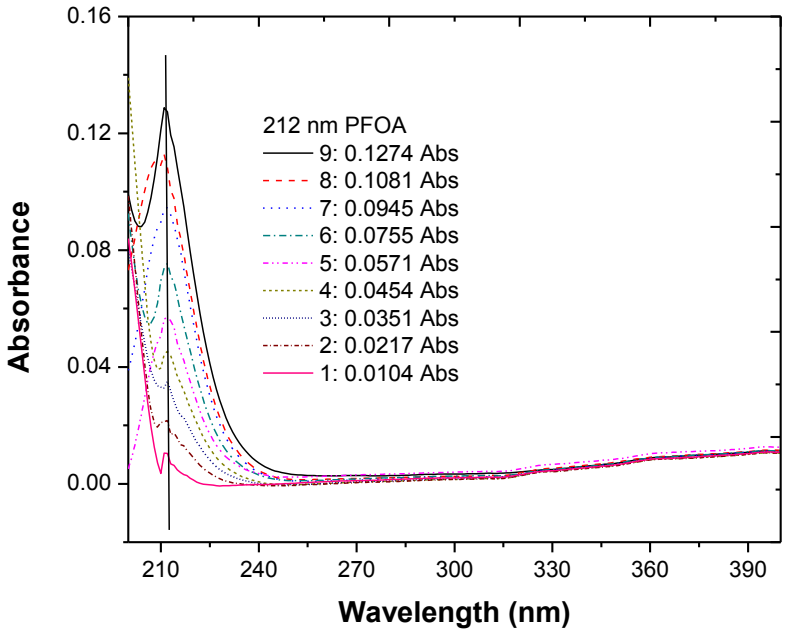


Figure S4.2. The scanned UV-vis absorbance spectra (190 to 400 nm) obtained at varying PFOA concentrations.

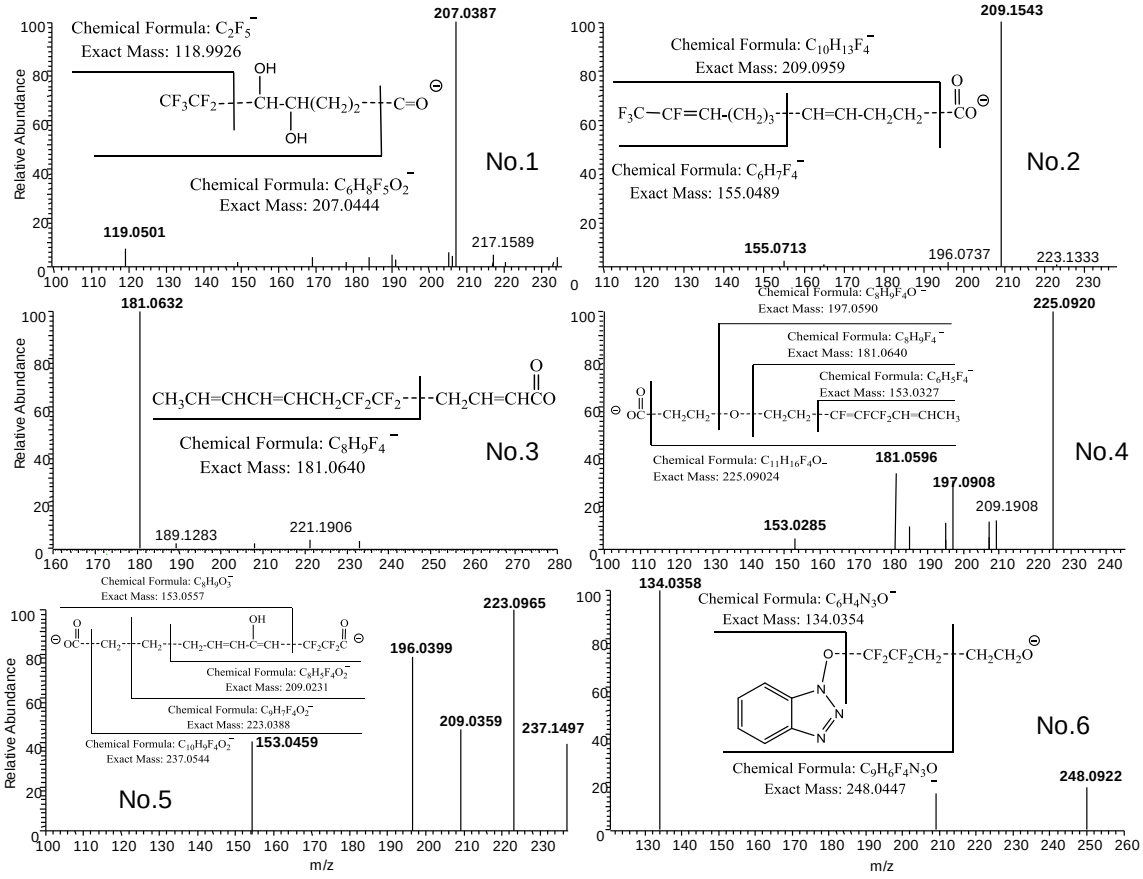


Figure S4.3. The MS/MS spectra of PFOA degradation products No. 1 to No. 6 given by high resolution mass spectrometry and their possible transition ions. Product No. 1: $C_7H_8F_5O_3$, measured m/z = 235.0394; Product No. 2: $C_{11}H_{13}F_4O_2$, measured m/z = 253.0848; Product No. 3: $C_{11}H_9F_4O_3$, measured m/z = 265.0477; Product No. 4: $C_{11}H_{13}F_4O_3$, measured m/z = 269.0793; Product No. 5: $C_{11}H_9F_4O_4$, measured m/z = 281.0432; Product No. 6: $C_{11}H_{10}F_4N_3O_2$, measured m/z = 292.0705.

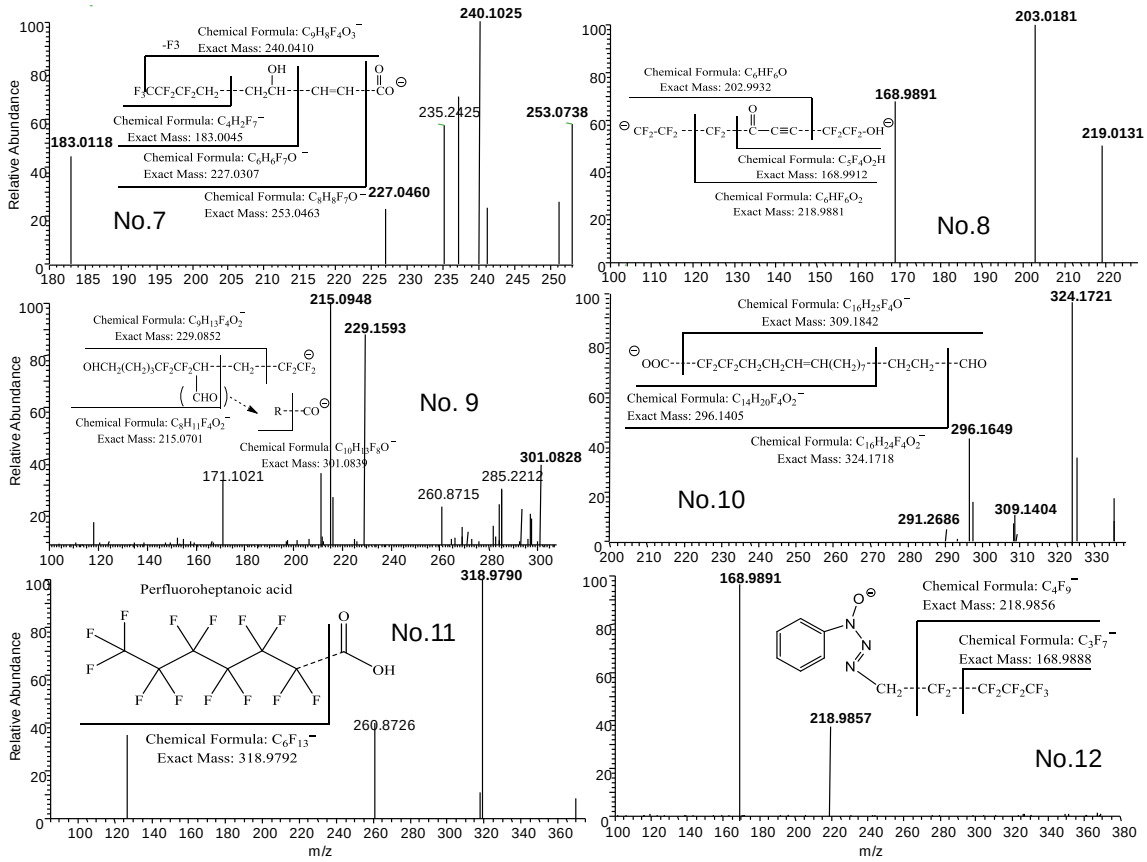
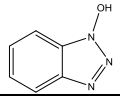


Figure S4.4. The MS/MS spectra of PFOA degradation products No. 7 to No. 12 given by high resolution mass spectrometry and their possible transition ions. Product No. 7: $C_9H_8F_7O_3$, measured m/z = 297.0374; Product No. 8: $C_8HF_{10}O_2$, measured m/z = 318.9801; Product No. 9: $C_{11}H_{13}F_8O_2$, measured m/z = 329.0795; Product No. 10: $C_{17}H_{25}F_4O_3$, measured m/z= 353.1722; Product No. 11: $C_7F_{13}O_2$, measured m/z = 362.9698; Product No. 12: $C_{11}H_7F_9N_3O$, measured m/z = 368.0457.

Table S4.1. The determined pH values for the individual metal ion solution at different concentrations.

Metal ion conc. (mM)	pH value			
	Cu^{2+}	Mg^{2+}	Mn^{2+}	Fe^{3+} (200 μM)
0.1	5.4	7.5	7.3	
1.0	5.2	7.3	7.0	2.9
10	4.9	7.0	6.5	

Table S4.2. The molecular formula, retention time, precursor ion, transition ion, and detection limit of the PFCAs and HBT monitored in UPLC-MS/MS analysis

Chemicals	Molecular Formula	RT (min)	Precursor ion m/z	Transition ion m/z	Detection limit (μg/L)
PFBA (C4)	F-$(CF_2)_3$COOH	2.6	213	169	0.018
PFPeA (C5)	F-$(CF_2)_4$COOH	4.5	263	219	0.027
PFHxA (C6)	F-$(CF_2)_5$COOH	5.5	313	269	0.022
PFHpA (C7)	F-$(CF_2)_6$COOH	6.1	363	319	0.008
PFOA (C8)	F-$(CF_2)_7$COOH	6.6	413	369	0.011
PFNA (C9)	F-$(CF_2)_8$COOH	7.02	463	419	0.15
PFDA (C10)	F-$(CF_2)_9$COOH	7.32	513	469	0.10
PFUA (C11)	F-$(CF_2)_{10}$COOH	7.63	563	519	0.012
HBT		0.44	134	105	0.019

PFBA: perfluoro-n-butanoic acid; PFPeA: perfluoro-n-pentanoic acid; PFHxA: perfluoro-n-hexanoic acid; PFHpA: perfluoro-n-heptanoic acid; PFNA: perfluoro-n-nonanoic acid; PFDA: perfluoro-n-decanoic acid; PFUA: perfluoro-n-undecanoic acid; HBT: 1-hydroxybenzotriazole.

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APPENDIX C

SUPPORTING INFORMATION:

ECOTOXICOLOGICAL EFFECTS OF PERFLUOROOCTANOIC ACID ON FRESHWATER MICROALGAE *CHLAMYDOMONAS REINHARDTII* AND *SCENEDESMUS OBLIQUUS*

MATERIAL AND METHODS

Culture media

The HB-4 medium was prepared by dissolving 0.200 g $(NH4)_2SO_4$, 0.030 g superphosphate, 0.080 g $MgSO_4$, 0.100 g $NaHCO_3$, 0.025 g KCl, 0.15 mL of 1% (w/v) $FeCl_3$ $6H_2O$ solution in 1000 mL distilled water.

High Salt Medium (HSM)

The HSM was prepared by mixing the following stock solutions:

1) Beijerinck's #2: 50 mL

NH_4Cl 5 g/500 mL

$MgSO_4 \cdot 7H_2O$ 0.2 g/500 mL

$CaCl_2$ 0.1 g/500 mL

2) Phosphate #2: 25 mL

KH_2PO_4 14.8 g/500 mL

K_2HPO_4 28.8 g/500 mL

3) AAP Trace Metal Solution 1 mL

Trace Metal Solution:

H_3BO_3 92.76 mg/500 mL

$MnCl_2 \cdot 4H_2O$ 207.69 mg/500 mL

$ZnCl_2$ 1.64 mg/500 mL

$FeCl_3$ $6H_2O$ 79.89 mg/500 mL

$Na_2EDTA \cdot 2H_2O$ 150.00 mg/500 mL

$CoCl_2 \cdot 6H_2O$ 1.30 mg/500 mL

$Na_2MoO_4 \cdot 2H_2O$ 3.63 mg/500 mL

$CuCl_2$ $2H_2O$ 0.006 mg/500 mL

APPENDIX D

DEGRADATION OF PERFLUOROOCTANESULFONATE CATALYZED BY LACCASE

Solid Phase Extraction

The samples were then subjected to solid phase extraction (SPE) (Oasis HLB SPE cartridges, 3 cc, 60 mg, Waters, Milford, MA) as described in earlier studies[1, 2] with minor changes. The SPE cartridge was conditioned with 3 mL methanol, two 3 mL aliquots of Milli-Q water sequentially, followed by loading the sample, then rinsed with 3 mL Milli-Q water and blown to dry under vacuum. The cartridge was then eluted with 1 mL methanol for three times and then 1 mL acetonitrile for two times. All eluents were combined, and the mixture was blown to 1 mL with nitrogen gas for PFOS and HBT quantification.

Recovery of the method has been evaluated using a standard addition method by adding 1 μM PFOA to selected sample aliquots before being processed as described above. The standard addition recovery was 83.69 $\pm$2.98% and 85.01 $\pm$2.63% for Cu^{2+} and Mg^{2+}solution respectively.

Laccase Activity Assay

Laccase activity was assayed using 2,6-dimethoxyphenol (DMP) as substrate. One unit of activity of laccase equals the amount of enzyme that causes an absorbance change in 468 nm at a rate of 1.0 unit/min in 3.4 mL of 1 mM 2,6-dimethoxyphenol in citrate-phosphate buffer (pH 3.8) in a 1-cm light path cuvette[3]. All activities were reported as measured by this method in this study.

We have also assayed laccase activity using a method with 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate to enable comparison with some earlier studies. Laccase activity was measured by monitoring the oxidation of 3 mL 100 mM ABTS substrate solution at 420 nm in 0.1 M Phosphate buffer (pH 6). One unit of laccase activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol ABTS per min[4].

By our measurements, the laccase activity measured by the two methods can be converted using the following equation:

Activity (measured by DMP) = 2.65 × Activity (measured by ABTS)

The laccase activity used in this study (1 U/mL by DMP method) is equivalent to 0.377 U/mL (by ABTS method).

Reaction Product Identification

The high resolution mass spectrometer with full scan and tandem mass fractionation was performed using an Orbitrap Elite (resolution R = 60 000 at *m/z* 400, for *m/z* = 100 to 1000) from Thermo Scientific (San Jose, U.S.) with ESI negative mode.

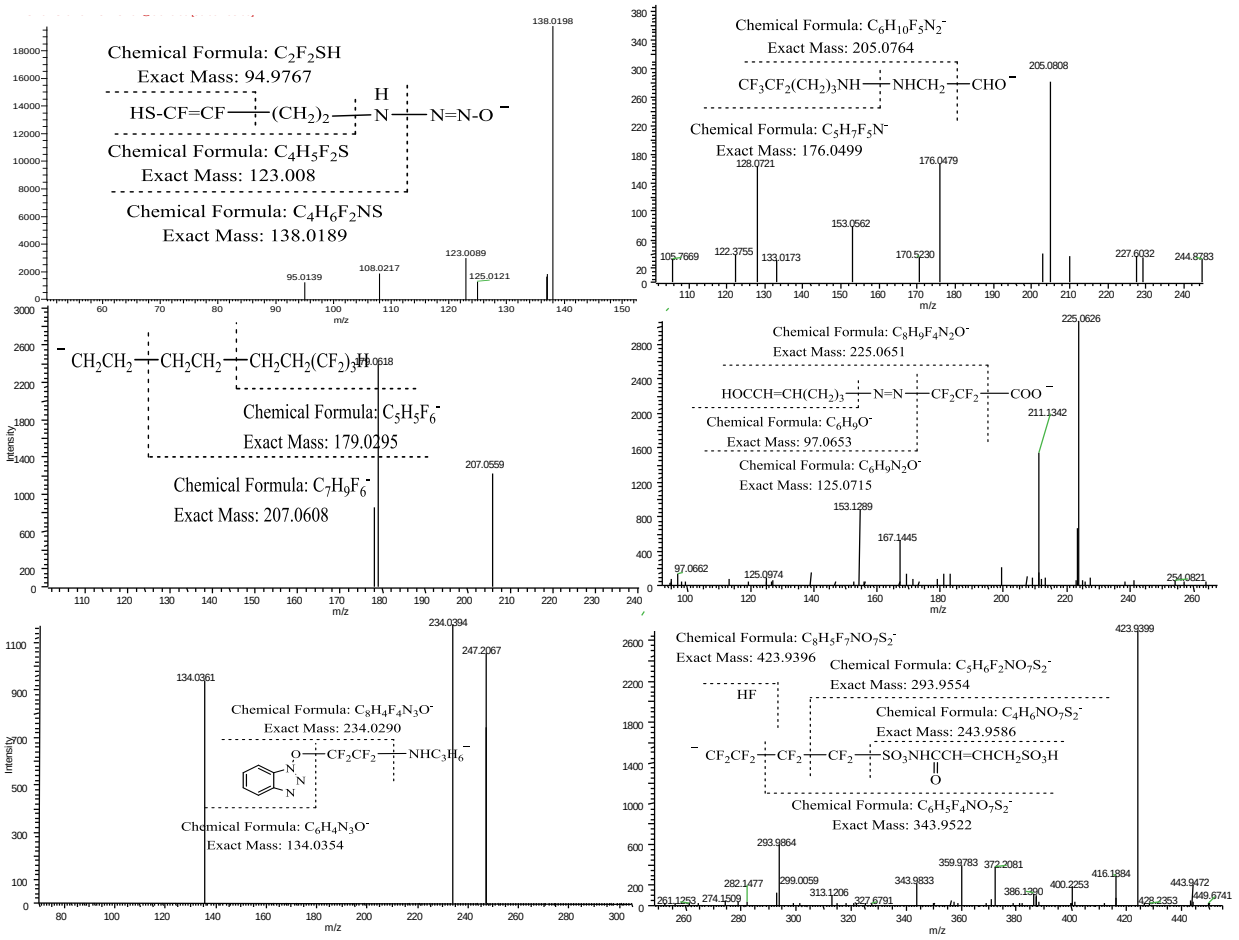
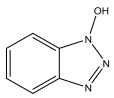


Figure S7.1. Selected MS/MS spectra of PFOS degradation products given by high resolution mass spectrometry and their possible transition ions. Product No. 1: $C_4H_7F_2N_3OS$, measured m/z = 182.0200; Product No. 3: $C_7H_{11}F_5N_2O$, measured m/z = 233.0713; Product No. 4: $C_9H_{14}F_6$, measured m/z = 235.0917; Product No. 7: $C_9H_{10}F_4N_2O_3$, measured m/z= 269.0549; Product No. 10: $C_{11}H_{12}F_4N_4O_2$, measured m/z = 291.0869; Product No. 18: $C_8H_7F_8N_3O_7S_2$, measured m/z = 443.9458.

Table S7.1. The molecular formula, retention time, precursor ion, transition ion, and detection limit of the PFCAs, PFASs and HBT monitored in UPLC-MS/MS analysis

Chemicals	Molecular Formula	RT (min)	Precursor ion m/z	Transition ion m/z	Detection limit (μg/L)
PFBA (C4)	$F\text{-}(CF_2)_3COOH$	2.6	213	169	0.018
PFPeA (C5)	$F\text{-}(CF_2)_4COOH$	4.5	263	219	0.027
PFHxA (C6)	$F\text{-}(CF_2)_5COOH$	5.5	313	269	0.022
PFHpA (C7)	$F\text{-}(CF_2)_6COOH$	6.1	363	319	0.008
PFOA (C8)	$F\text{-}(CF_2)_7COOH$	6.6	413	369	0.011
PFNA (C9)	$F\text{-}(CF_2)_8COOH$	7.0	463	419	0.15
PFDA (C10)	$F\text{-}(CF_2)_9COOH$	7.3	513	469	0.10
PFUA (C11)	$F\text{-}(CF_2)_{10}COOH$	7.6	563	519	0.012
PFBS (C4)	$F\text{-}(CF_2)_4SO_3$	4.8	299	99	0.021
PFHxS (C6)	$F\text{-}(CF_2)_6SO_3$	6.2	399	99	0.064
PFOS (C8)	$F\text{-}(CF_2)_8SO_3$	7.0	499	99	0.082
HBT		0.44	134	105	0.019

PFBA: perfluoro-n-butanoic acid; PFPeA: perfluoro-n-pentanoic acid; PFHxA: perfluoro-n-hexanoic acid; PFHpA: perfluoro-n-heptanoic acid; PFNA: perfluoro-n-nonanoic acid; PFDA: perfluoro-n-decanoic acid; PFUA: perfluoro-n-undecanoic acid; PFBS: perfluorobutaesulfonate; PFHxS: perfluorohexanesulfonate; PFOS: perfluorooctanesulfonate; HBT: 1-hydroxybenzotriazole.

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