Hydrogen Peroxide and Antioxidant Enzymes Moderate Interaction of the Carbon and Oxygen Cycles at the Redoxcline

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HYDROGEN PEROXIDE AND ANTIOXIDANT ENZYMES MODERATE INTERACTION OF THE CARBON AND OXYGEN CYCLES AT THE REDOXCLINE

by

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DEDICATION

I dedicate my dissertation work to my family. I am extremely grateful to my loving parents, Xinhai Wang and Xiping Jin who instilled in me the love of learning from an early stage. My parents have been constant cheerleaders through academic and personal endeavor in my life. Thanks for mom and dad for always believing in me and offering their endless support, encouragement and sacrifices for educating and preparing me for my future. I am also thankful to my sister, Xiaoxiao Wang for her support and being a companion to my parents when I am far away from home.
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ABSTRACT

Hydrogen peroxide is a reduced form of dioxygen produced in natural waters from a manifold of abiotic and biological processes. Hydrogen peroxide is highly redox active and it often serves to initiate the formation of reactive oxygen species in the environment. It typically exists in natural waters at concentrations ranging from as low as ~ 1 nanomolar in “blue water” marine environments to as high as 10 micromolar near actively effluxing sediments. It has the potential to cause significant toxicity in aquatic organisms, which have evolved a collection of antioxidant enzymes including peroxidase, catalase, and superoxide dismutase. Work in this thesis is focused on the interaction of hydrogen peroxide and the peroxidase enzyme at redox interfaces, including the sediment/water column interface, saturated and unsaturated vadose zone, and pseudointerface in dense cyanobacterial communities. Initial field work shown documents the importance of oxic/anoxic interfaces at sediment surfaces as abiotic factors that force enhanced formation of peroxidases relative to total biomass in the system. This is shown in a detailed characterization of a field site disturbed by the storm surge of a passing hurricane; a “pump and probe” experiment that briefly transformed a salt marsh dominated by tidal water movement to a continuously submerged environment. The effect of such disruptions was further explored by examining the effect of naturally occurring levels of peroxide and peroxidase on anthropogenic contaminants in the presence of varying salinity and varying levels of biogenic dissolved organic carbon. At concentrations of peroxide and peroxidase representative of those in natural soils model contaminants were effectively condensed to
high molecular weight, insoluble polymers. Resulting immobilization was explored further at higher concentrations of the two species to explore their potential as remediation agents for the purposeful removal of organic contaminants in groundwater. Although increasing salinity, corresponding to the coastal flooding described in the earlier field work, had little effect on net removal the addition of biogenic natural carbon, it had statistically significant and positive effects on contaminant removal. Work in the thesis also examined the role of dense cyanobacterial mats, separate from sediments, in promoting the formation of peroxide and peroxidases. Field examination of the harmful algal bloom *Microseira wolleii* showed no peroxide but high levels of a novel peroxidase; sufficient levels to account for the difficulty in peroxide detection. The mats were characterized for peroxidase content and it was determined that sufficient peroxidase was present to afford the cyanobacteria a plausible defense mechanism for exogenous peroxycarbonate that is often applied as an algaecide for *Microseira* species and other harmful algal blooms. *Microseira wolleii* peroxidase was also evaluated for its ability to affect organic contaminants, including some algaecidal agents.
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CHAPTER 1
INTRODUCTION

Water is one of the most important natural resources on earth, but only less than 1% of that resource is available for human consumption.\textsuperscript{1} The precious resource suffers from water pollution coming from human activities all over the world. The continuous release of organic products, such as industrial wastes, herbicides, dyes and pharmaceuticals to natural waters causes a loss of water quality for consumption and activities related to human health.\textsuperscript{2} Some organics are unable to be sufficiently removed and become persistent organic pollutants and accumulate in lakes, rivers and aquifers, entering the drinking water, usually at level of µg L\textsuperscript{-1} to ng L\textsuperscript{-1}.\textsuperscript{3, 4} Most of these pollutant are toxic and can pose a hazardous risk for human health and other living beings, making developing effective and powerful remediation technologies an important subject to discuss.

In recent years, researchers have focused on remediation of organics in terrestrial environment, particularly attention given to bioremediation due to its environmental friendliness, mild condition requirements, high removal efficiency, and lack of toxic by-product formation.\textsuperscript{5-7} Such enzymatic treatment is achieved though reaction between peroxidase and hydrogen peroxide to form radical center which can promote the oxidation reaction.\textsuperscript{8, 9} The resulting formation of a radical during the oxidation step can produce dimers and eventually lead to the formation of polymer. Research has been underway for the last decade about this enzymatic oxidation process, however, the extrapolation of such
laboratory-derived results to environmental remediation and water treatment is imprecise. This is due to optimization, acclimation and high cell density cultures employed in most biochemical studies, which are rarely met in real world bioremediation scenarios. In this thesis, an attempt is made at understanding the nature of enzymatic treatment processes which may influence and, in some instances, remove the organic pollutant and control the harmful algal blooms (HABs) under environmental conditions.

1.1 Reactive Oxygen Species

The free radicals hydroxyl (•OH) and superoxide (•O₂⁻), the electronically excited singlet oxygen and the relatively stable hydrogen peroxide (H₂O₂), collectively known as Reactive Oxygen Species (ROS), have been playing important roles in the degradation of organics in the aquatic and sediment environment.¹⁰ ROS are generated as a by-product of other photochemical reactions and have all been measured in marine and some freshwater environment.¹¹-¹⁷ However, these transient species are highly reactive due to the presence of an unpaired or excited electron, and are short lived (diffusion controlled) as they quickly react with themselves or other component in the environment.¹⁸,¹⁹

1.1.1 Superoxide radical

Superoxide radical is the one-electron reduced form of triplet O₂ and is unique among the ROS, the potentials of their redox couples with H₂O₂ and O₂, respectively, theoretically permit not only oxidation of ferrous iron, but also reduction of many forms of ferric iron to the ferrous state at neutral pH. However, superoxide is inherently unstable due to the self-reaction. It undergoes protonation to form its conjugate acid, hydroperoxyl radical with pKa 4.8.²⁰

\[
• \text{O}_2^- + H^+ \rightleftharpoons HO • \text{O}^-
\]  

(1)
Disproportionation reactions occur slowly (on the order of seconds) between either two hydroperoxyl radicals or hydroperoxyl and superoxide various as a function of as follows:

\[ \text{HO} \cdot^2 + \text{HO} \cdot^2 \rightarrow \text{O}_2 + \text{HOOH} \quad (2) \]
\[ \text{HO} \cdot^2 + \cdot \text{O}_2^- \rightarrow \text{O}_2 + \text{HOO}^- \quad (3) \]

1.1.2 Hydroxyl radical

Hydroxyl radical is the most reactive radical in the environment. It plays an important role in atmospheric chemistry, but its role in aquatic environments is less clearly understood.\(^2^1\) From the past studies, photolysis of NO\(_3^-\) and NO\(_2^-\) are important •OH sources only in some upwelling areas, and in Fe-rich freshwaters, the Fenton reaction should be a significant •OH source.\(^2^2\), \(^2^3\) In seawater, the major source appears to be the dissolved organic matter (DOM) photochemical produced •OH in seawater.\(^2^4\) However, many sinks exist for •OH in seawater. For example, Br- can remove up to 93% of •OH production, and its products such as bromine-containing radicals can attack DOM.\(^2^5\) Other 7% of •OH produced reacts directly with other components including DOM.\(^2^5\) Thus, in seawater DOM plays a dual role as both source and sink for •OH.

\[ \text{NO}_3^- + \text{hv} \rightarrow \text{NO}_2^- + \frac{1}{2} \text{O}_2 \text{hvH}_2\text{O} \rightarrow \text{NO} + \text{HO}^- + \cdot \text{OH} \quad (4) \]
\[ \text{H}_2\text{O}^+ \rightarrow \text{H} \cdot + \cdot \text{OH} \quad (5) \]
\[ \text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{HO}^- + \cdot \text{OH} \quad (6) \]

These processes result in a steady state concentration of hydroxyl radical. In natural waters it contains between 10\(^{-15}\) and 10\(^{-18}\) M, and readily reacts with organics at an
essentially diffusion-controlled rate in a non-selective manner, making it an excellent oxidant for broad spectrum contaminant removal.\textsuperscript{25}

1.1.3 Hydrogen peroxide

Hydrogen peroxide is the most stable ROS and an important component of natural waters and act as a reactive transient to impact the redox chemistry in surface water.\textsuperscript{26} It is abundant in the atmosphere gas phase and is transported to soils and surface water by rain in a concentration range between $10^{-7}$ and $10^{-11}$ M.\textsuperscript{27-29} H$_2$O$_2$ is a long lived ROS as it has half-lived on the order of hours, except in the presence of transition metals where H$_2$O$_2$ quickly decomposes through the mechanism seen in eq. 2.\textsuperscript{30} In aqueous environment, photochemical reaction can produce free electrons or excited state that can react with organic matter (OM) to reduce dissolved oxygen to superoxide.\textsuperscript{31}

$$0M + O_2 + \text{hv} \rightarrow 0M^{+} + \cdot O_2^{-}$$

Some reduced metals can react with dioxygen to form superoxide, but this reaction is often inhibited under acidic conditions and in the presence of chloride.\textsuperscript{26} Generated superoxide radicals can undergo disproportionation and yield H$_2$O$_2$, or also react with Fe(II) to further reduced to H$_2$O$_2$ in acidic conditions, depending on the relative concentration of superoxide to Fe(II) at the time of its generation.

$$\text{Fe(II)} + O_2 \rightleftharpoons \text{Fe(III)} + \cdot O_2^{-} \quad K_1 = 13 \text{ M}^{-1}\text{s}^{-1}$$

$$K_{-1} = 1.5 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \quad (8)$$

$$2 \cdot O_2^{-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (9)$$

$$\text{Fe(II)} + \cdot O_2^{-} + 2H^+ \rightarrow H_2O_2 + \text{Fe(III)} \quad K = 1.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$$

(10)
1.2 Peroxidases

Peroxidases are a functionally defined class of enzymes designed to provide catalytic antioxidant protection that are essentially ubiquitous in the oxic portion of the biosphere. The require co-factors, or sacrificial reductants, to perform their function with the highest efficiency. In order to carry out this function under the widest range of conditions peroxidases are not particularly selective with respect to cofactors and are important in the environmental processing of natural and anthropogenic chemicals. Peroxidases are widely distributed in nature, such as being produced by animals, plants, and microbes (bacteria, fungi and yeast). Many plant sources for peroxidases production have been reported such as horseradish roots, papaya (Carica papaya), banana (Musa paradisiaca), bare (Acorus calamus) and so forth. Because of their unselective use of cofactors peroxidases have been applied as tools to enhance the reactivity of several chemicals with hydrogen peroxide for a large number of applications such as decomposition of pollutants, decolorization of synthetic dyes, paper-pulp industry for lignin degradation, sewage treatment and also as biosensors. They are also involved in the scavenging of ROS that exist in microbial surrounding area or plant, which are capable of causing oxidative damage to the cell.

One of the most industrial produced and commonly used is horseradish peroxidase (HRP), a heme-containing enzyme with a molar mass of approximately 44 KDa. It can catalyze a variety of reactions such as the oxidation of a variety of organic chemicals by hydrogen peroxide. Horseradish peroxidase reactions can be simply summarized in figure 1.1.
Figure 1.1 The catalytic cycle of Horseradish Peroxidase. RH represents a substrate susceptible to peroxidase actions and R• represents the free radical formed. Later the formation of radical species (R•) may result in the formation of oligomeric or polymeric structure that can act as substrates in subsequent steps.

Despite being seen as unstable and delicate structures, enzymes can be resistant as any inorganic catalyst if used in an appropriate way and can achieve product mixtures unachievable in abiotic processes. The reaction conditions in the lab should avoid high temperatures, extreme pH values, high substrate and product, oxidant and organic solvent concentrations. Temperature is important operation factor in enzymatic reactions since it affects the structural integrity of the enzyme. In a study concerning HRP thermal stability, reported HRP activity was maintained high over the range from 30 to 45°C, while at 60°C the free enzyme lost about 75% of its activity in 5 mins. The stability of enzymes also depend on the pH which is usually maintained in a reaction with the use of buffer solutions. HRP has an isoelectric point at 9, and its activity peaks at pH 6-8, near the physiological pH. Inactivation of peroxidases by oxidant H₂O₂ is also concerning as a consequence of its inappropriate use. Studies have found the exposure of HRP to a high concentration of H₂O₂ may lead to an irreversible “suicide inactivation” with the formation of a highly reactive radical that may trigger the destruction of the prosthetic group.
Thus, to ensure the fully utilized enzyme functionality, H₂O₂ can be added continuously to the reaction to keep low its concentration. Under the optimum condition, including the physiological conditions, peroxidases are able to perform the normal functionality and used as a biological catalyst to replace the chemical catalyst for eco-friendly purpose.

1.3 Thesis focus

As the biosphere has progressed from the pre-oxic condition through the Great Oxidations it has developed standing reservoirs of a kinetically stable but thermodynamically unstable mix of dioxygen and reduced carbon. This thesis is focused on studying the role of peroxidases as environmental catalysts that allow those reservoirs to interact in a kinetically facile manner in natural environments. An essential component of this process, hydrogen peroxide, is typically found at relatively high (micromolar) concentrations at the edge of the oxic/anoxic interfaces those regions were of particular interest in these studies. Work progressed at a series of field sites and also in laboratory models of field sites that could be transient or difficult to access directly, such as the edge of the photic zone in a HAB or deep underground at the oxic/anoxic interface in groundwater. Overall, understanding the interaction of enzyme and ROS in different circumstances (oxic/anox interface in costal sediment, groundwater and photic zone in a HAB) requires knowledge about the process of modelling them in the laboratory. This thesis thus focuses on evaluating the environmental importance of the peroxidase and ROS interaction in a series of laboratory tests. The main aims of the study are to investigate: 1) The impact of high elevated peroxide concentration induced by hurricane on the peroxidase activity in costal soil matrix. 2) The interaction of peroxide formed naturally at the interface along with corresponding induced enzyme formation can have significant consequences to
dissolved organic contaminants. 3) Dense mats of photosynthetic algae promote the bacterially driven development of sharp redox gradients at the edge of the photic zone, which can mimic the conditions that lead to ROS formation at the sediment water interface.

To fulfil these aims, the thesis is divided into four chapters (including this introduction chapter) with content of each chapter summarized below.

Chapter 2. Hurricane-Driven Increase in Antioxidant Enzyme Expression by a Sediment Biofilm Community

In this chapter, we explored situations where forced mechanical mixing at the oxic/anoxic interface promotes elevated peroxide levels that lead to enhanced expression of antioxidant enzymes, particularly peroxidase. The extreme weather event (Hurricane Irma, shown in Fig. 1.2) caused flooding to have resulted in significant accumulation of peroxide-forming reductive equivalents in sediments isolated from the atmosphere. Here we show the storm-driven oxygen restriction can result in sustained production of reactive oxygen species when flooded soils are re-exposed to oxygen as storm surges pass, inducing oxidant stress in sedimentary biofilm communities relative to before the hurricane. Transects of a Sporobolus alterniflorus (formerly known as Spartina alterniflora)-dominated salt marsh tidal creek in South Carolina (off Sol Legare Island) were monitored for peroxide formation potential, total protein and peroxidase enzymes before and after the landfall of Hurricane Irma. Post-hurricane peroxide formation potential in sediments increased a factor of 1-4 relative to pre-hurricane levels with the largest increases in the deepest waters. Total sedimentary organic carbon was constant before and after landfall. The ratio of peroxidases to total protein increased significantly in the areas of the transect routinely exposed to changing redox conditions.
Figure 1.2 Satellite image of Hurricane Irma approaching the study site and followed the coastline of the Southeastern US (track of the followed the purple line, approximately, toward the Charleston SC metropolitan area). The study site (yellow pin) was on Sol Legare Island, approximately two kilometers inland from Folly Island, SC. Hurricane Jose, which is visible in the lower right, did not affect the study site.
Chapter 3. Saltwater intrusion improves remediation efficiency for coastal soils by peroxidase-mediated polymerization

In this chapter, we explored how in carbon rich environments the interaction of carbon, peroxidase, and background levels of hydrogen peroxide can result in assembly of complex organic structures, too large be taken up into the organic phase. We examined this assembly process as a possible in-situ polymerization strategy to manage pollutant risk by converting mobile contaminants to immobile oligomers with low solubility. This process, analogous to humification, was achieved through a peroxidase-mediated oxidation process performed under hydrogen peroxide and peroxidase conditions similar to those found in coastal soils. Fluoroanilines and halophenols, alone and in mixtures, were used as model contaminants. Their transformation by oxidation to high molecular weight, insoluble oligomers was monitored for several hours. These products are capable of sequential oxidation to molecular weights approaching 1000 Da, detected by mass spectrometry. The maximum solubility of these products was significantly reduced relative to the starting materials, resulting in their direct immobilization. Resulting polymers have been characterized with respect to their affinity for still-dissolved residual parent contaminants, as well as their inability to release the contaminant back to the aqueous phase. Their function as adsorbents has been quantified relative to an equal mass of powdered activated carbon and their adsorption constant (K) has been determined to be approximately $6.9 \times 10^{-3}$, less than that of activated carbon but still quite capable of adsorptive removal of dissolved organic contaminants. The process has also been characterized under the saltwater intrusion environment and showed effective treatment with the presence of seawater.
Chapter 4. *Microseira wollei* peroxidase: An anti-algicidal enzyme produced by a harmful algal bloom

In this chapter, we capitalized on a field study of *Microseira wollei* (*M. wollei*) to infer the presence of a previously unknown peroxidase. In our field work we observed that photosynthetic *Microseira* fiber mats experienced dissolved oxygen variation from highs of 16 mg/L (where O$_2$ was actively bubbling from lake water) to undetectable over distance of less than 5 cm. Given that every other redoxcline we studied showed marked hydrogen peroxide evolution at such conditions we examined three different geographically distinct *Microseira* mat communities (separated by at least 3 km, shown in Fig. 1.3) for hydrogen peroxide, but none was detected (est. detection limit 100 nM). Therefore, it was hypothesized that *Microseira* was expressing a peroxidase enzyme, the characterization of which was the focus of this chapter. This is the first report of a peroxidase enzyme derived from a HAB species and the first report of a peroxidase in *M. wollei* from Lake Wateree. Extraction and partial purification of peroxidases were performed and resulted peroxidases were studied in terms of pH optima, substrate specificity and the activity over the range of hydrogen peroxide. Most importantly, data from the experiment in this thesis increase our fundamental understanding of peroxide-based algaecide and algal antioxidant enzyme relationship, and provide more accurate predication to support the effective and ecologically safe use of peroxide-based algaecide in drinking water resource management.
1.4 Overview of experimental approach

This thesis focuses primarily on the interaction between enzyme and peroxide occurring in coastal soil oxic/anoxic interface, saturated and unsaturated vadose zone and photic zone in a HAB. These processes have been investigated in laboratory experiments under well-controlled conditions close to the conditions existing in natural environment. In all cases, the results obtained from the laboratory studies have been used to model the conditions under different environments.

The detailed description of the preparation of reagents and the experimental methods employed is provided in the relevant chapters. However, an overview of three
major experimental techniques used in this work, UV and visible spectroscopy, Fluorescence spectroscopy and liquid chromatography with tandem mass spectrometry (LC-MS/MS) are described briefly here.

The UV and visible spectroscopy were used for standardization of horseradish peroxidase by pyrogallol assay, using the extinction coefficient of 12.0 mM⁻¹ cm⁻¹ at 420 nm. Hydrogen peroxide stock solutions (30% v/v) was diluted (1000-fold) freshly and standardized by UV-Vis absorption, using its extinction coefficient of 18.6 M⁻¹ cm⁻¹ at 254 nm. Protein content was determined with Bradford assay, measured by UV-Vis absorption at 595 nm and then compared with a standard curve (Fig. 1.4 A) generated using bovine serum albumin.

Fluorescence spectroscopy was used for measurement of hydrogen peroxide concentration and peroxidase activity using Amplex Red method. The fluorescence emission intensity of the product resorufin, produced by oxidation of Amplex Red, was measured on a Spectra-Max M5 microplate reader at an excitation wavelength of 530 nm and emission wavelength of 585 nm. The value was then compared with a standard curve generated using corresponding reagent (horseradish peroxidase shown in Fig. 1.4 B, hydrogen peroxide shown in Fig. 1.4 C).

LC-MS/MS (Waters Acquity UPLC® coupled with a Xevo triple quadrupole mass spectrometer with an electrospray ionization sources) was used for measurement of all organic contaminants removal rate. Contaminant quantify method was based on multiple reaction monitoring mode by monitoring the mass transition. Resulted target analyte peak area was compared with the standard calibration curve, for example, 4-chlorophenol calibration curve are shown in Fig. 1.4 D. The mass spectra of polymer products were
obtained by collecting precipitated particles, dissolution in excess acetonitrile and injection into the LC-MS/MS with mass scan mode.

Figure 1.4 Illustration of major calibration curves used in this work. Calibration curves were freshly prepared prior to the experiment with sample size n = 3. (A) A set of standardized Bovine serum albumin were measured by Bradford assay. Amplex Red calibration curve for (B) hydrogen peroxide and (C) horseradish peroxidase. (D) 4-Chlorophenol calibration curve from LC-MS/MS with monitoring the mass transition from 127.0987 Da to 90.9576 Da due to loss of one chlorine atom (instrument condition: BEH C18 column, 12 V collision energy, ES- mode).
CHAPTER 2

HURRICANE-DRIVEN INCREASE IN ANTIOXIDANT ENZYME EXPRESSION BY A SEDIMENT BIOFILM COMMUNITY\(^1\)

Abstract

The oxidation of Fe(II) by atmospheric oxygen leads to the formation of reactive oxygen species, including superoxide, hydrogen peroxide, and hydroxyl radicals. Extreme weather events such as hurricanes can cause flooding, resulting in significant accumulation of peroxide-forming reductive equivalents in sediments isolated from the atmosphere. Here we show the storm-driven oxygen restriction can result in sustained production of reactive oxygen species when flooded soils are re-exposed to oxygen as storm surges pass, inducing oxidant stress in sedimentary biofilm communities relative to before the hurricane. Transects of a *Sporobolus alterniflorus*-dominated salt marsh tidal creek in South Carolina (off Sol Legare Island) were monitored for peroxide formation potential, total protein and

\(^1\)F. J. Wang, T. M. Makris and J. L. Ferry, Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208. To be submitted to *Estuarine, Coastal and Shelf Science*
peroxidase enzymes before and after the landfall of Hurricane Irma. Sedimentary biofilm communities in the top centimeter of sediment were sampled at a series of locations indexed against their position relative to the deepest point of a tidal creek and at a minimum horizontal distance of 1 m. Sites were varied based on their typical oxygen exposure, from an initial site that was continuously submerged to a site on the landward margin of the salt marsh that was typically submerged less than four hours per day. Site characterization included sedimentary total organic carbon, hydrogen peroxide formation potential sedimentary protein content, and total peroxidase enzyme quantification. The physical impacts of Irma’s landfall included destruction of local built environments and movement of *Sporobolus* detritus but no physical modification of the underlying tidal creek bank system. Post-hurricane peroxide formation potential in sediments increased a factor of 1-4 relative to pre-hurricane levels with the largest increases in the deepest waters. Total sedimentary organic carbon was constant before and after landfall. The ratio of peroxidases to total protein increased significantly in the areas of the transect routinely exposed to changing redox conditions.

2.1 Introduction

The formation of reactive oxygen species (ROS) is initiated in sedimentary biofilms chiefly by surficial photochemical processes or the oxidation of biogeochemically generated Fe(II) by atmospheric oxygen (Fig 2.1).\(^{52, 53}\) The ROS include singlet oxygen (\(^{1}\text{O}_2\)), superoxide (\(\text{O}_2^-\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), and hydroxyl radical (\(\text{HO}^-\))\(^{19, 54}\). Photoexcited natural organic matter (NOM) is capable of generating the four listed ROS with the concomitant formation of organic radicals (R\(\cdot\))\(^{31, 55}\). Photoexcited nitrate can decompose to yield the oxide radical anion, which would rapidly protonate at pHs below
11 to yield hydroxyl radical. The photoreduction of iron and the biogeochemical reduction of iron both lead to the formation of ferrous complexes that can be oxidized by atmospheric oxygen to yield superoxide, with subsequent oxidation leading to the formation of hydrogen peroxide. The lifetimes of singlet oxygen and hydroxyl radical in seawater are on the order of nanoseconds to microseconds, but the lifetime of superoxide (at pH 8.2) and H$_2$O$_2$ can be considerably longer (minutes to days). The environmental persistence of the latter ROS is sufficient to induce affected microbial populations to express antioxidant enzymes, including superoxide dismutase, catalase, and peroxidase.

![Figure 2.1 Simplified schematic depiction of the most important ROS-related process in the aqueous environment.](image)

Sediment-associated antioxidant enzymes are an important part of the estuarine microbiome and can exist as intracellular or extracellular substances across the oxic/anoxic interface; including interfaces found in the rhizosphere of aquatic plants. Peroxidases
are particularly important in this role since they function not only as antioxidants, but also as biosynthetic tools.\textsuperscript{65} For example, the haloperoxidases deactivate hydrogen peroxide by forming oxo-Fe complexes with hypervalent iron centers. In marine systems the resulting oxo-Fe complexes react with ambient halides to produce hypohalites (e.g. the production of HOCl from hydrogen peroxide and chloride in seawater).\textsuperscript{65} The conversion of ambient peroxide to hypohalites allows the organism to effectively engage in allelopathic interactions with other microbes or can also enable the generation of halogenated secondary metabolites.\textsuperscript{66} In either case the peroxide is removed from the local environment of the cell.

Biofilm communities are particularly dependent on antioxidant enzymes as their high metabolic consumption of oxygen can lead to sharp redox gradients in a small volume and their frequent presence in high light intensity environments can also promote the photoformation of ROS.\textsuperscript{67} The production of antioxidant enzymes is known to occur in single species biofilms and more complex multispecies communities.\textsuperscript{68} \textit{Pseudomonas aeruginosa} biofilms generate extracellular catalase as a response to UVA exposure, for example.\textsuperscript{69} Applied peroxides, as would be encountered during disinfection or in proximity to intense natural sources like Fe(II)-containing groundwater seeps, also provoke the production of catalase in biofilms.\textsuperscript{70} Interestingly, ROS are often used in multispecies biofilms as an antimicrobial strategy. Indeed, multispecies microbial communities often express antioxidant enzymes as part of a more complex strategy of ROS derived allelopathy.\textsuperscript{71-74}

Flooding due to extreme weather events can inundate coastal soil, resulting in temporary anoxia and accumulation of ROS precursors such as Fe(II) or hydrogen sulfide
in porewaters.\textsuperscript{75} The reintroduction of oxygen as flood waters recede can then inject a burst of ROS into the system, production analogous to that seen during oxidative reperfusion in tissues with corresponding oxidative stress.\textsuperscript{76, 77} This type of flood-induced, temporary redoxcline is short-lived but can cause significantly enhanced peroxidase expression even in vascular plants.\textsuperscript{77}

The current work focused on determining the role of extreme weather events as a source of oxidative stress in sedimentary biofilms, as indexed by the ratio of antioxidant enzymes to total protein. Landfalling hurricanes are typically associated with storm surges that cap marsh sediments with seawater for periods of time significantly longer than a typical tide cycle.\textsuperscript{78-80} These floodwaters are typically particulate laden with low optical pathlengths. Coupled with the relatively low surface insolation during hurricanes, these conditions suggest photochemical processes will not be a significant a source of ROS compared to sedimentary processes.\textsuperscript{52, 81-83} Here we report the interaction of a sediment surface biofilm transect with a sudden change in total oxygen driven by passage of the storm surge brought by Hurricane Irma. The effects of the storm surge on the potential for producing peroxide from sedimentary biofilms and the relationship between the relative production of peroxidase and protein content are presented.

2.2 Experimental Methods

2.2.1 Materials

Horseradish peroxidase (Type II, salt-free, lyophilized powder, 150 U/mg), bovine serum albumin (lyophilized powder, \textgeq 96\%), sucrose (99\%), dithiothreitol (DTT, 99\%) and ethylenediaminetetraacetic acid (EDTA, 99.5\%) were acquired from Sigma-Aldrich. 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red, AR) was purchased from American
Advanced Scientific Inc and stored at -4°C until use. Bugbuster® HT Protein Extraction Reagent was acquired from EMD Millipore Corp. Bradford Dye Protein Reagent was purchased from BIO RAD. Hydrogen peroxide was obtained as a 30% solution from BDH and was standardized by UV-Vis absorption, using its extinction coefficient of 18.6 M⁻¹ cm⁻¹ at 254 nm.²⁶ Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, 99%) was from Molecular Sigma Biology. Diethylenetriamine pentaacetate (DTPA, 99%) was from Acros Organics. All other salts (99.5%) were obtained from Fisher Scientific. All chemicals were used as received. Solutions were made in Barnstead E-pure (18 MΩ cm⁻¹) water.

2.2.2 Site description and sampling

Samples were collected from five sites in a (Sites A, B, C, D, and E; stretching from the center of a tidal creek to the landward edge of the marsh) mature Sporobolus alterniflorus-dominated marsh and saline tidal creek on the eastern shore of Sol Legare Island, South Carolina (32°41'25.74"N, 79°57'10.62"W, Fig. 2.2). This area, which typically experiences two tidal cycles/day with samples sites C, D, and E exposed to atmosphere at low tide, was inundated Sept 11-12, 2017, by (at maximum) approximately 2 meters of storm surge from the passage of Hurricane Irma. Pre-storm “baseline” samples were collected on Sept 7th and post-storm on Sept 14th at the earliest date civilian access was restored to Sol Legare. All the collections were made during low tide. Five sediment sites were sampled at each time with a 1 cm surface scrape. The sites corresponded to a transect between the minimum and maximum O₂ exposure conditions expected in the marsh system, including a permanently inundated sediment from the bottom of the tidal creek (A), a site exposed to air and water at the edge of the creek (B), the creekward edge...
of the *Sporobolus* (C), in the middle of *Sporobolus* growth (D) and at the *Sporobolus*-dry land margin (E). The sites were located along the marsh with a minimum distance of 1m between each site. Samples were immediately frozen and transported on dry ice to the laboratory, where the sample were refrigerated at -80°C until enzymatic analyses were carried out.

![Diagram of sediment sampling sites](image)

**Figure 2.2 Depiction of sediment sampling sites.** Sediment scrapes (1 cm depth max) were collected at sites representing different redox regimes (sample sites indicated at A-E). All samples were frozen at -80°C and stored at that temperature until processed in the laboratory.

### 2.2.3 Analytical Methods

#### 2.2.3.1 Sediment characterization

The water content of the sediment was determined by comparing the mass of the wet sediment subsample with the mass of sample dried to a constant mass in a ventilated oven at 60°C. The total organic carbon content of the sediment sample was determined by the loss of mass on ignition (LOI) method with burning 2 g of dry subsample at 470°C for 6 h, with additional detail in Fig. 2.3.

#### 2.2.3.2 Protein analysis

The sediment protein extraction method was adapted from Singleton and co-workers. Frozen sediment samples, 1.00 g (approximate, each sample determined...
individually) were weighed into microcentrifuge tubes (1.5 mL capacity) in which 1 ml of extraction buffer had been added. The buffer solution contained 50 mM Tris-HCl, 10% sucrose, 2 mM DTT, 4 mM EDTA, 10% Bugbuster® and the pH of final suspension was adjusted to 7.6 with ammonium hydroxide. Prior to extracting the protein from the soil sample, the centrifuge tubes were closed and mixed for 30 s on a vortex mixer. Efficient protein recovery was obtained based on published protocols for sediment protein extraction.85 Following extraction, total protein was determined with the Bradford assay, in which the transmissivity of a mixture of protein co-extracted with the Bradford Dye Protein Reagent was measured with a UV-Vis spectrometer at 595 nm.48 These values were then compared with a standard curve generated using bovine serum albumin.

2.2.3.3 Peroxidase assay

Samples were analyzed for peroxidase enzyme activity using a modified version of the Amplex Red method.49, 50 Frozen (-80°C) samples were allowed to warm to standard temperature and homogenized. Approximately 2.00 grams of the homogenate (the mass of each sample was determined individually) were transferred into 20 mL borosilicate EPA vials and suspended in 10.0 mL phosphate buffer (50 mM, pH 7.5; pH adjusted by dropwise addition HCl; exact sample mass recorded for calculation purposes). All measurements were performed in a darkened laboratory (red light) to avoid photoactivation of transition metals in the sediment. Amplex Red (2.00 mM) and H2O2 (3.00 mM) in phosphate buffer (50 mM, pH 7.5) were used to quantify peroxidase activity. This was determined by monitoring the kinetics of resorufin evolution in a stirred sediment suspension, followed fluorometrically in aliquots drawn from the reactor every five minutes over a thirty-minute development process.86, 87 The intensity of fluorescence emission from centrifuged aliquots
(Dade Immufuge, 3225 rpm, 2 mins) was determined at an excitation wavelength of 530 nm and emission wavelength of 585 nm on a Spectra-Max M5 microplate reader.

Peroxidase activity in the unknowns was quantified against standardized solutions of horseradish peroxidase. Peroxidase standards were calibrated using the pyrogallol method (which was not itself directly suitable for sediment enzyme determinations because of the known reaction between pyrogallol and some sediment components such as Fe(III)). Peroxidase activity in the sample was then determined by the slope of resorufin evolution and expressed as $\times 10^{-8}$ g HRP standard g dry wt$^{-1}$. Peroxidase recovery standard assays were also performed on thermally inactivated sediments to determine the efficiency of peroxidase extraction by this technique. Pyrogallol-calibrated peroxidase standards were added to the thermally inactivated sediments and analyzed by Amplex Red technique (vide supra) to determine the recovery efficiency of the technique (minimum of three replicates/condition). The extraction procedure recovered approximately 35% of exogenous horseradish peroxidase spiked into heat-sterilized sediments if extracted within 60 min of addition. Extraction efficiency decreased significantly with longer sample hold times (Fig. 2.5) so samples were extracted immediately upon thawing. The total peroxidase in the sample was then determined by multiplying the recovered peroxidase by the inverse of the average recovery to account for incomplete recovery.

**2.2.3.4 Hydrogen peroxide formation potential**

Previous work has shown that hydrogen peroxide itself has a very brief lifetime in redox-active sediments, therefore it is unrealistic to expect even frozen samples to contain repeatably assayable peroxide. However the reduced sulfur and iron species that drive the formation of peroxide in soils are stable at reduced temperatures, so it was reasonable to
thaw frozen sediments in aqueous solution to re-initiate peroxide formation in the laboratory and gain insight into the potential of a given sediment to generate this reactive oxygen species. Freshly thawed sediments (2.00 +/- 0.10 grams) were suspended in 10 mL phosphate buffer (50 mM, pH 7.5), and aerated for approximately 30 min. At discrete time intervals overlaying solution was withdrawn and centrifuged (Dade Immufuge, 3225 rpm, 2 mins) immediately. Peroxide formation was measured in the resulting mixture using the Amplex Red technique (which differed from the Amplex Red technique for peroxidase through the application of solutions with known peroxidase activity but no exogenous peroxide). The supernatant samples were tested with Amplex Red by adding equal volume ratio of supernatant, 0.01 M DTPA in phosphate buffer (50 mM, pH 7.5), and mixture of 100 µM Amplex Reagent and 2 U/mL HRP standard. The fluorescence emission intensity of the resorufin produced by this process was measured on a Spectra-Max M5 microplate reader at an excitation wavelength of 530 nm and emission wavelength of 585 nm. Each measurement was accompanied by a control with same initial inorganic condition but no sediment.

2.3 Results and Discussion

2.3.1 Hurricane Irma

Hurricane Irma made landfall near Folly beach in Charleston SC on Sep 11th, 2017. As the first category five hurricane in the 2017 Atlantic hurricane season, Irma caused extensive and catastrophic damage to the coastal environment. During the landfall, the storm surge at Sol Legare Island was approximately 2.2 m above the typical low tide minimum. The sampled transect was inundated by seawater for approximately 48 hrs.
Samples were taken within 48 hrs before landfall and as soon as possible after the storm surge retreated and civilian access was restored to the area.

2.3.2 Variations in soil TOC

Although Irma’s landfall caused significant changes in the built environment on Sol Legare it caused relatively minor changes to the transect area at the geostructural level (i.e. the positions of Sporobolus beds and tidal creeks as shown in figure 2.3 were essentially constant in a before and after landfall comparison). Sites A, B, and C, corresponding to the middle of the creek channel, the creek edge at low tide, and the seaward edge of the Sporobolus dominated area respectively had the highest organic content at approximately 6-10% organic C in the top cm scrape. Scrapes from inside the Sporobolus growth (Sites D and E) had relatively little surficial organic C (1-2%), presumably indicating the role of light availability in supporting photosynthetic biofilms at the other locations. Regardless of the organic carbon measured at any location, the pre- and post Irma landfall measurements were within approximately <10% variation (Fig. 2.4). This change indicated that essentially no “fresh” sediments had been exposed by passage of the storm.
Figure 2.3 The sampling environment changed after the hurricane. The storm surge associated with Hurricane Irma affected the built environment around Sol Legare Island but had little to impact on the physical structure of the surrounding marsh. (A) before landfall; (B) after landfall.

Figure 2.4 Total organic carbon content of sediment surface scrapes. Organic carbon for the five sites was determined by mass loss from dry samples on ignition, n=3 per sample shown. Comparison shows no statistically significant changes in organic carbon pre and post Irma. (mean ± SD)
2.3.3 Sediment Protein Content

The sedimentary protein content along the transect before and after the hurricane was determined. Similar to total organic carbon, there was very little net change in protein content before and after hurricane landfall (Fig. 2.5). However, the protein profile across the transect varied, with an apparent decrease in the protein content of samples D and E relative to the observed mid-channel. The change was small relative to the error intrinsic to the method, but speculatively the creek channel could be more dominated by biofilm derived, polysaccharide rich detritus than the samples from inside the Sporobolus growth.97

![Graph 2.5] (Sediment protein measurements. Sediment protein content was determined across the transect before and after landfall (n = 3). The pre and post hurricane protein content variation was not significant at the 95% level of confidence.)

2.3.4 Soil Peroxidase across the transect

Peroxidase activity (normalized to horseradish peroxidase) was determined in heat treated, sterilized soils that had been pre-spiked with horseradish peroxidase. Spiked soils served as recovery standard assays for peroxidase in soils in this study. Recovery was not
quantitative and peaked at 35.0 ±4.8% if recovery was performed in the first thirty minutes after spiking into the sample (Fig. 2.6)

**Figure 2.6 Recovery standard analyses.** Recovery standards for horseradish peroxidase from solution and sediment suspensions were conducted at multiple time points to map the stability of possible enzymes during the extraction process. Maximum recovery efficiency was obtained from freshly prepared samples, and all subsequent natural samples were evaluated corresponding to the most efficient recovery (35.0 ±4.8) %. Enzyme activity decline rapidly in the soil sample relative to the blank (mean ± 95% CI) and was essentially undetectable after an hour.

### 2.3.5 Determining sedimentary H$_2$O$_2$ formation potential

Anoxic salt marsh sediments are well known to accumulate reduced transition metals and sulfides, which on exposure to air can reduce ambient $\text{O}_2$ to ROS. For examples, previous field studies at the same site demonstrated the formation of ROS in *Sporobolus* rhizospheres, which are well known to conduct $\text{O}_2$ deep into the anoxic portions of the sediment column (20+ cm below the sediment surface in some cases). The current work explores how storm-surge driven inundation further isolates surface biofilm communities from oxygen, allowing facultative anaerobes at the sediment/water interface to accumulate significant stores of reductive equivalents. Site A in this study is continuously inundated
and too deep underwater to sustain vascular aerial plants but experienced significant water flows during the tidal exchange so was unable to accumulate significant peroxide precursors before storm. However during the storm surge it was protected under the deepest water and accumulated significant precursors, as indicated by a three-fold increase in peroxide formation potential after the storm had passed (Fig. 2.7). Sites B through D also experienced increases in peroxide formation potential but not to such an extent, presumably because the abundance of crab burrows at site B and Sporobolus at C and D. Site E is anomalously high according to this model but is also frequently buried in a mulch of dead *Sporobolus* washed up at the marsh edge. It could be that it was more isolated from the atmosphere under this mulch layer than anticipated.

![Figure 2.7](image)

**Figure 2.7 Hydrogen peroxide formation potential measurements. Comparison of the H$_2$O$_2$ (µM) generated from suspension and aeration of sediments before and after landfall of Hurricane Irma at the five sediment in a Sporobolus-dominated salt marsh on Sol Legare Island near Folly Beach, SC (mean ± 95% CI).**

### 2.3.6 Soil enzyme expression before and after hurricane

Total peroxidase activity was normalized against soil protein to investigate the oxidative stress response of the community (Fig. 2.8 and insert). Interestingly, the sites
normally exposed to the greatest daily changes in oxygen concentration showed the
greatest ability to adapt to the high peroxide formation potential imposed by the storm
surge, showing an peroxidase activity to total protein ratio that varied from 2.6 (±1.4) times
the original sediment to 0.6 (±0.4) times the original sediment pre and post hurricane.
Overall with the grassy edge facing the tidal creek (site C), was the most responsive to
oxidative stress, and the bottom of creek (site A), which was consistently suboxic or anoxic,
was the least responsive, presumably due to its stable position on the redox cline. As the
site E experienced the least impact proven by hydrogen peroxide formation potential, the
enzyme expression was not changed after the landfall.

Figure 2.8 The fraction of total protein expressed as enzyme. The fraction of total
protein expressed as peroxidase increased significantly after the storm surge
retreated, indicating that the microbial community could respond quickly to the
increased peroxide formation potential in surface sediments. A site-normalized before
and after comparison (insert) shows that the microbial communities most frequently
challenged by oxidative stress responded more fully. (mean ± SD)
2.4 Conclusion

The release of anoxic sedimentary pore waters through movement of the tidal prism or groundwater efflux is known to result in the generation of locally high levels of reactive oxygen species.\textsuperscript{79} This has been shown to be a result of the air oxidation of Fe(II) species and the redox cycling of Fe(II)/(III) species supported by co-occurring, kinetically facile reductants like sulfide or dissolved organic matter.\textsuperscript{52, 83, 98} Although it is known that antioxidant enzymes are a necessity for life at redox interfaces it was not known how responsive biofilm communities could be to rapid changes in the local redoxcline. The passing of Hurricane Irma provided an opportunity for a rare “pump and probe” experiment in an environmental system to observe the timescale over which established communities could respond to substantial changes like those observed in this study. The current work shows that significant change in the rate of antioxidant enzyme expression were observable over a timespan of just four days. Novel QAQC work reported in this paper, examining recovery standards in sedimentary biofilm analyses, suggested that changes in enzyme expression as a result of redox stress may be significantly higher than previously thought based on analyses that did not contain the appropriate recovery based correction factor.
CHAPTER 3

IN-SITU POLYMERIZATION OF GROUNDWATER CONTAMINANTS LEADS TO THEIR IMMOBILIZATION AS INSOLUBLE BY-PRODUCTS

Abstract

Point sources of organic contaminants in groundwater and soils are sometimes present at sufficiently high concentrations that cross-linking or polymerization of source material may be a more economical remediation alternative than exhaustive oxidation. Here we show that peroxidase enzymes and hydrogen peroxide at naturally occurring and elevated levels relative to those that naturally occur in soils are sufficient to induce the polymerization of several halogenated aromatic chemicals. High molecular weight products precipitated from solution resulting in net removal of contaminants concurrent with the production of high-molecular, non-polar phases capable of adsorbing unreacted starting material. Product mixtures were shown to consist of $n$-mers up to $n = 10$ by qualitative mass spectrometry. Organic precipitates were characterized by dynamic light scattering. Individual particles were typically 3-10 microns in diameter, containing the

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equivalent of approximately $10^9$ molecules starting material per particle, and under appropriate mixing conditions could continue to aggregate loosely to diameters of approximately millimeter scale. Newly formed product phases were tested against adsorbents used in water remediation (e.g. powdered activated carbon, zeolites, etc) for scaling and observed to be 10-15% as effective at sequestering model contaminants as designed materials. The precipitation rate was sensitive to the initial concentration of added peroxidase and hydrogen peroxide and varied positively with their increase. The effects of added dissolved organic matter and ionic strength on contaminant removal were also determined to explore the effects of saline, surface water intrusion as observed in many coastal groundwaters. Although both processes contributed positively to contaminant removal the effect of added organic matter was significantly higher than the effect of increasing ionic strength. These outcomes were repeatable across several types of peroxidases, including those isolated from vascular plants (horseradish peroxidase), marine algae (*Lynbyga majascula*) and freshwater cyanobacteria (*Microseira wollei*).

3.1 Introduction

The United States’ need for clean water resources (all sectors) is expected to grow by 15%-25% above the current use level by the year 2050.\textsuperscript{99} Surface water resources alone are not sufficient to meet this need, but unfortunately many groundwater sources are contaminated with organic chemicals so their utility is limited. For context, the National Research Council’s recent report *Alternatives for Managing the Nation’s Complex Contaminated Groundwater Sites* documents over 120,000 sites in the United States with soil/water contamination at levels too high for safe use of the associated groundwater and therefore requiring some form of remediation.\textsuperscript{100} Groundwater remediation strategies are
successful when their application results in reduction of dissolved contaminants to or below the maximum level mandated by regulation. This functional definition of remediation success has encouraged development of multiple strategies for answering different needs, defined by the soil matrix, flow conditions, exposure risk etc.\textsuperscript{100, 101}

Remediation approaches for organic contaminants tend to emphasize physical removal followed by disposal, or degradation through metabolic processes or chemical modification of contaminants. Phase transfer/sequestration involves phase transfer from the aqueous phase to the gas phase or an adsorbent such as activated carbon. This may remediate the groundwater but still leaves the original mass of toxic material intact (now associated with a new phase) and require additional treatment, such as the remediation of spent activated carbon.\textsuperscript{102, 103} Chemical remediation strategies, either \textit{in-situ} via injected oxidants or reactive barriers or \textit{ex-situ} in the case of pump-and-treat strategies are designed to rapidly transform contaminants and ideally mineralize them (i.e. drive the process until the contaminants have been rendered to carbon dioxide, water and mineral acids).\textsuperscript{104-109} \textit{In-situ} injection strategies like \textit{in-situ} chemical oxidation (ISCO) are particularly interesting methods because they minimize physical disturbance of the contaminated site, can be applied at depth through wells or push-points and may achieve the highest rates of contaminant removal per volume of treated soil.\textsuperscript{100} Drawbacks are that chemical remediation, and in particular reactions with strong oxidants, are often unselective, require large stoichiometric excesses of reactant, and are prone to the production of water-soluble and potentially toxic by-products that themselves require further application of oxidant.\textsuperscript{110-113}
A possible alternative to exhaustive oxidation of organic contaminants is selective, partial oxidation under conditions that promote oxidative condensation, ultimately leading to the conversion of contaminants to their corresponding n-mers with the ultimate goal of forming stable, immobilized solid-phase polymers with low toxicity. Vinyl chloride is an illustrative example; the starting material is a volatile contaminant commonly associated with the microbial degradation of haloethylenes that is extremely toxic to humans, but the condensed product polyvinyl chloride is so insoluble and non-toxic that it is frequently used in water distribution systems as a plumbing material. The potential of in-situ polymerization to immobilize semi-volatile, unsaturated organics can be readily visualized by modeling the aqueous solubility of the resulting n-mers using a structural subunit estimation method (Fig. 3.1). The maximum solubility of a series of haloethylenes and halophenols was modeled across a molecular weight range from a monomer to heptamer for each of the model contaminants. If a nominal maximum solubility limit of five micromolar is applied as the goal, it becomes apparent that this can be achieved with as few as six condensations for most contaminants.
Figure 3.1 Estimated solubility maxima (mg/L) for (A) polymerized chloroethylenes and (B) polymerized halophenols. Assuming the polymer was terminated with H abstracted from dissolved organic matter native to groundwater. The solubilities of the proposed products were calculated based on the structural-subunit estimation method (EPI Suite™).

The current work tests the hypothesis that oxidative condensation of organic contaminants under environmentally relevant conditions can lead to the formation of stable, non-polar solid phases. Chlorophenols were chosen as model substrates because the solubility of their monomers is among the highest of the semi volatile contaminants, making them among the most challenging substrates to remove from water through polymerization. The peroxidase-catalyzed one electron oxidation of chlorophenols by hydrogen peroxide was selected as a selective polymerization tool because it was among the less expensive one electron oxidant systems that are applicable to soils. The role of added halides and natural organic matter is also explored to account for remediation in coastal environments that may experience saline intrusion.
3.2 Methods

3.2.1 Materials

All reagents were used as received. 4-Chlorophenol (4CP, 99%), 2,4-dichlorophenol (24DCP, 99%), 4-fluoroaniline (4FA, 99%), 2,4-difluoroaniline (24DFA, 99%), horseradish peroxidase (lyophilized powder, 150 units/mg), bovine liver catalase (lyophilized powder, 2000-5000 units/mg protein) were acquired from Sigma-Aldrich. 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex red) was purchased from American Advanced Scientific Inc, and the latter three reagents were stored in a desiccator at -5°C. Hydrogen Peroxide (30%, w/w) were obtained from BDH. 1,2-Dichlorobenzene (12DCB) and 1,4-dibromobenzene (14DBB) were acquired from Aldrich Chem Co. Methyl tert-butyl ether (MTBE, 99.9%), diethylenetriamine pentaacetate (DTPA, 99%) and zeolite (without alumina acid sites, ratio SiO$_2$/Al$_2$O$_3$=1000) were purchased from ACROS Organics. Activated carbon (black powder, surface area 1300-1400 m$^2$/g) were purchased from Strem Chemicals. Dimethyl sulfoxide (DMSO, 99.9%) and all other salts (99.5%) were obtained from Fisher Scientific. All chemicals were used as received. The standard humic acid (HA, Cat. No. 1R101N), also known as natural organic matter (NOM), was acquired from International Humic Substances Society (IHSS) which were extracted from Suwannee river, the elemental composition was as follows: C 52.47%, H 4.19%, O 42.69%, N 1.10%, S 0.65%, P 0.02%.

All aqueous solutions were prepared with Barnstead E-pure (18 MΩ cm$^{-1}$) deionized (DI) water, and all the pH values of buffer solutions were adjusted by using sodium hydroxide (0.1 M) and hydrochloric acid (0.1-3 M). Phosphate buffer solution (PBS) was prepared with 0.05 M dibasic sodium phosphate heptahydrate and pH adjusted
to 7.50 ± 0.05 and store at room temperature. DTPA solution (0.01 M) was prepared in 0.05 M sodium phosphate buffer (pH 7.50 ± 0.05) and stored at room temperature.

3.2.2 Model contaminants removal studies

Four model contaminants, 4-chlorophenol (100 mM), 2,4-dichlorophenol (20 mM), 4-fluoroaniline (100 mM) and 2,4-difluoroaniline (100 mM), were all prepared in DI water. Reaction mixtures contained, in a final volume of 8 mL, 2 mM of model contaminants, and 1 U/mL of HRP enzyme (standardized by pyrogallol assay) in PBS. The reaction was initiated by adding 2 mL H$_2$O$_2$ solution (10 mM, standardized by UV-Vis absorption, using its extinction coefficient of 18.6 M$^{-1}$ cm$^{-1}$ at 254 nm). Aliquots were withdrawn and transferred to vials with buffered solution containing catalase (10 units/mL) to decompose the H$_2$O$_2$ and quench the reaction. The removal studies were performed in the absence and presence of 30 mg/L humic acid (HA). All the reactions in this paper were carried out with replicate reactors, n = 3 per experiment.

3.2.3 Characterization and measurements

3.2.3.1 Mass spectrometry

The concentration of XPs was monitored using a Waters (Milford, MA, USA) Acquity UPLC® coupled with a Xevo triple quadrupole mass spectrometer (MS/MS) equipped with an electrospray ionization sources (ESI). The column was a Waters Acquity UPLC® BEH C18 (50 mm × 2.1 mm id, 1.7 µm) column. The mobile phase consisted of 0.1% formic acid in water (aqueous A) and acetonitrile (organic B), with the following gradient program; for 4CP and 24DCP, 60% A hold for 0.1 min, decrease to 10% at 1.8 min and hold for 0.1 min, then back to 60% A at 2.00 min; for 4-fluoroaniline and 2,4-difluoroaniline, 90% A hold for 0.1 min, decrease to 40% at 1.5 min and hold for 0.3 min,
then back to 90% A at 2.00 min. Multiple reaction monitoring mode was used to quantify the XPs by monitoring the mass transition. For example, 4CP showed a mass transition from parent 127.0987 Da to daughter 90.9576 Da with loss of a chlorine atom with 12 V collision energy at ES- mode. 24DCP showed a mass transition from 160.8682 to 88.9553 Da with loss of two chlorines at 18 V collision energy, and a transition from 160.8682 to 124.8787 with one chlorine fragmented at 16 V collision energy at ES- mode. 4FA fragmented with loss of one fluorine atom and NH$_2$ group with a transition from 111.7611 to 74.9020 at 28 V collision energy at ES+ mode, and 24DFA fragmented through loss of two fluorine with transition from 129.7517 to 89.9222 at 12 V collision energy at ES+ mode. The mass spectra of condensation products were obtained by collecting precipitated materials, dissolution in excess acetonitrile and injection into the LC-MS/MS using the same mobile phase mixture, but with a different gradient program, starting with 50% A hold for 0.1 min, decreasing to 20% A at 2 min and hold for 1 min, then back to 50% A at time 5 min.

3.2.3.2 Hydrogen peroxide and peroxidase measurement

Hydrogen peroxide measurements were performed by using the Amplex Red method as previously reported. In brief, an indicator solution was made by mixing 100 µL of 10 mM Amplex Red/DMSO solution, 200 µL of 10 units/mL horseradish peroxidase and 9.7 mL of PBS. 100 µL of sample and 100 µL of DTPA solution was transferred into a microplate followed by the addition of 100 µL of the indicator solution to each sample. After 30 minutes of incubation in the dark, samples were excited at 530 nm, and the emissions at 585 nm were measured. Peroxidase measurements were also performed by using amplex red method, except changing the composition of indicator solution. The
indicator solution to test peroxidase activity was made by mixing 100 µL of 10 mM Amplex red/DMSO solution, 200 µL of 15 mM hydrogen peroxide and 9.7 mL of PBS. Spectroscopic measurements for all observations were performed using a 96-well glass plate on a Molecular Devices™ SpectraMax M5 Multi-mode Microplate Reader.

### 3.2.3.3 Dynamic light scattering

Dynamic light scattering studies of the resulting polymers at laboratory condition in aqueous solution were conducted using Malven Instruments Zetasizer Nano ZS instrument equipped with a 4 mW He-Ne laser operating at λ = 632.8 nm. The reaction conditions are prepared with 2 mM of substrate mixed mixing with 1 units/mL horseradish peroxidase in PBS and initiated by adding 2 mM H₂O₂ solution. Through agitation by converting the cuvette, samples are continuously measured over the time course of one hour.

### 3.2.4 Experimental conditions

Enzymatic induced oxidation polymerization was conducted under environmental conditions to test the contaminants removal ability. The desired steady state concentration of hydrogen peroxide and peroxidase were controlled by constantly injecting two steady streams of stock solution of hydrogen peroxide and peroxidase controlled by a peristaltic pump. Concentration of hydrogen peroxide and peroxidase were also measured by Amplex red method every 30 mins to maintain their balance at environmental level (HRP at ~10 mU/mL, H₂O₂ at around micromolar range). Physical evaporation of the phenol in time-course experiments was calculated by comparing the initial and final concentrations of the
controls. The results of 4CP removal rate is calibrated against the evaporation rate and divided by initial concentration to get $A/A_0$ across time range of 4 hours.

### 3.2.5 Product absorptivity

Condensation products generated new stationary phases in the studied systems, and their potential to serve as adsorbents was measured and indexed against that of powdered activated carbon and a model zeolite adsorbent. 12DCB was used as the indicating probe for these assays. Freundlich adsorption isotherms were obtained for 12DCB in carbone-standardized suspensions of synthesized particles (determined by total organic carbon analysis) and reference suspensions of powdered activated carbon or zeolite. 12DCB loss was monitored post-centrifugation in supernatant, using liquid-liquid extraction to remove 12DCB from the supernatant and GC with electron capture detection to determine 12DCB in the extraction solvent. Extracting solvent was MTBE. GC (Agilent, MA, USA, carrier gas: helium) conditions were: 60°C hold for 1 min, then 300°C at 15°C/min, hold 1.5 mins.

### 3.2.6 Effects of modeled seawater intrusion

Seawater was obtained from Charleston Harbor (Charleston, SC) and stored in a darkened laboratory for usage within three months. Salinity of sampled seawater was measured at 33 parts per thousand. Condensation reactions were performed at varying total salinity levels obtained from seawater dilution. Given the complexity of seawater a more straightforward ionic strength adjuster made of potassium nitrate was also used in a parallel series of experiments and the outcomes compared for emergent properties of seawater on the system. Potassium nitrate (2 M), was prepared in PBS with ionic strength calculated including buffer as 2.133 and titrated to a set of ionic strength with buffer solution to mimic
the saltwater intrusion ionic strength. Four conditions were prepared as buffer adjusted with ionic adjuster, seawater titration, NOM added into the buffer with ionic strength adjusted and NOM added into the titration of seawater. The final volume of the reactors was 5 mL solution held in 8mL EPA vials with stir bars. Initial conditions included 1 mM of 4CP, 0.57 units/mL HRP and 30 mg/L NOM if applicable in an ionic strength pre-diluted conditions, reactors were initiated by adding H₂O₂ (at final concentration of 1 mM). Blank reactors were prepared with the same condition except adding DI water rather than H₂O₂. After one hour of stirring, solutions were withdrawn from the reactor and quenched by adding catalase (10 units/mL) before injecting them on to the LC-MS/MS.

3.2.7 Association of 4CP to particulate material

The enzymatically condensed phase was analyzed for its capacity to retain 4CP. Bulk 4CP particles were synthesized by adding 4.3 mM of 4CP, 7.3 units/mL HRP and 5 mM H₂O₂ into a 200 mL reactor containing 10 mL seawater, or 30 mg/L HA. The condensation proceeded for one hour then centrifuged to recover particles (Fisher AccuSpin™ 1/1R centrifuge, 4,000 rpm, 25 min, 20°C). The 4CP mass balance was determined in solution and used to calculate the mass of 4CP converted to particulate material. Collected particles were washed with two 10 mL aliquots of seawater twice and recovered via additional centrifugation, with the final suspension made up to a volume of 3000 microliters. 100 microliters of this suspension were added to solutions and the final volume brought up to 5.00 mL with concurrent adjustments to pH, ionic strength, etc. Suspensions were stirred for three hours and the solution phase concentration of released, unreacted 4CP determined.
3.2.8 Testing different enzyme including novel *Microseira* Peroxidase

Several different peroxidase enzymes were tested for their ability to condense model contaminants, including those obtained from horseradish, *Lyngbya majascula* (a marine algae) and *Microseira wolleii* (freshwater cyanobacteria). Enzymes were prepared freshly except for that the *Microseira wolleii* enzyme, which was pre-extracted and stored in the -80°C freezer and thawed to room temperature before the experiment. All enzymes were quantified by ABTS assay.\(^{116}\) 4CP was added to all the reactors at the same concentration at 1 mM. Hydrogen peroxide was the same for HRP and marine-derived *Lyngbya spp* enzyme at 1 mM. However, to overcome the low activity/mass in *Microseira wolleii* enzyme 5 mM H\(_2\)O\(_2\) were added. Aliquots were withdrawn and quenched by 10 units/mL catalase solution at time 1 hour after constant mixing and analyzed immediately.

3.3 Results and Discussion

3.3.1 Process characterization

The model contaminant 4CP was stable in solutions of all the tested peroxidase types prior to the addition of hydrogen peroxide. After peroxide addition the formation of particulates was apparent and rapid under every condition tested (Fig. 3.2) with concurrent loss of 4CP. The concentration of 4CP in the solution phase stabilized after approximately five minutes and correlated negatively (\(r^2 = 0.984\)) with increasing peroxidase activity, indicating that peroxidase was probably experiencing suicide inactivation at the high concentrations of peroxide in these proof-of-concept experiments.\(^{45, 46, 117, 118}\)
Figure 3.2 Comparison of 4CP removal efficiency under different loadings of HRP. 4CP was rapidly but not exhaustively removed under the experimental conditions shown (Initial reactor conditions are 2mM 4-chlorophenol and various activities of HRP in pH 7.5 phosphate buffer, reaction initiated by adding 2 mM H$_2$O$_2$.)

The role of suicide inactivation was examined in a series of experiments monitoring the percent removal of 4CP with time. After the initial removal phase (as shown in Fig. 3.3) either additional hydrogen peroxide or 4CP were added to the system. The addition of a second bolus of hydrogen peroxide had no effect on 4CP removal at later times, same as the addition of 4CP and hydrogen peroxide resulted in no additional 4CP removal. This indicated the HRP enzyme has been deactivated in first 5 mins reaction time by hydrogen peroxide.
Figure 3.3  HRP was an effective catalyst for 4CP oxidation until suicide inactivation. Initial reactor conditions include 2mM 4-chlorophenol and 1 Units/mL HRP, reaction initiated by adding 2 mM H$_2$O$_2$. After inactivation new additions of 4CP and peroxide resulted in no reaction.

3.3.2 The effect of Suwannee River Humic Acid on the stability of hydrogen peroxide and peroxidase

The natural environment contains more variables that need to be taken into consideration and due to their reactivity, they might change the polymerization mechanisms. Aquatic HA standard acquired from IHSS were included in this study due to its highly chemically reactive yet recalcitrant with respect to biodegradation property in the nature. To confirm the involvement of HA may not interfere with the individual reactant, control experiments were performed regarding to the stability of HRP and H$_2$O$_2$ under the presence of HA. The enzyme maintained essentially 100% activity in HA solution over the course of five hours (Fig. 3.4 A), indicating that although HA may participate in the experiments in other ways it does not participate by directly consuming enzyme. With the
presence of HA, hydrogen peroxide concentration maintained largely unaffected, with less than 20% degradation over 5 hours stirring (Fig. 3.4 B). Thus, the HA has no inhibition effect on enzyme activity, and small negative impact on H$_2$O$_2$ concentration which is not significant on the timescale of our experiments.

Figure 3.4 Stability test for enzyme and H$_2$O$_2$ in the presence of NOM. (A) The HRP activity remains stable over 5 hours. ([HRP]$_0$ = 10 mU/mL, [HA] = 30 mg/L, pH 7.5) (B) H$_2$O$_2$ concentration dropped less than 20% over 5 hours. ([H$_2$O$_2$] = 7 µM, [HA] = 30 mg/L, pH 7.5)
3.3.3 Model contaminant removal study

The broader utility of enzymatic immobilization to remove contaminant was tested by examining the reactivity of three additional halophenols in this system, including two fluorinated molecules (4CP, 24DCP, 4FA, 24DFA). Control experiments of all four contaminants without initiation step were performed to calculate the percentage removal rate for enzymatic oxidation process. 4CP has the highest removal percentage, 90% have been removed within 15 mins reaction time (Fig. 3.5 A). Followed by 24DCP, 4FA and 24DFA with removal percentage varying from 30-70%. In general, the final removal percentage trend shows that fluoroanilines had the lower removal efficiency compare to chlorophenols. Moreover, the less the number of halogen atoms attached to benzene ring showed the better efficiency regarding to the removal process. Adding humic acid to the same condition experiment results in no changes on the 4CP removal efficiency (Fig. 3.5 B). However, 24DCP removal degree was improved significantly, which indicates the NOM has the positive effect on removing chlorophenols. Adding NOM into fluoroanilines reactor showed the negative effect on removing rate, which indicate the presence of NOM is not promoting the fluoroanilines removal efficiency. These graphs of contaminants removal kinetics were all reached equilibrium after 30 mins from initiation step, which proved the inactivation time for enzymes in these substrates were the same.
Figure 3.5 Contaminants removal rate comparison. (A) Reactor initial condition: 2 mM of substrates, 1 U/mL HRP, 50 mM phosphate buffer pH 7.5. Initiated reactor by adding 2 mM H$_2$O$_2$ at T=0.01m. (B) Same condition except with 30 mg/L HA added.
3.3.4 Particle size kinetic measurement

To further investigate the physical properties of those formed precipitates, particle sizes have been kinetically measured under both conditions, without and with NOM. The size of 4CP derived polymeric aggregates growing rapidly and reached the detection limit (6000 nm) within 15 mins under no presence of NOM (Fig. 3.6 A). Meanwhile, 24DCP achieved slightly less but still large particle size within one hour of reaction time. However, the fluoroanilines derived polymeric aggregates grown into smaller sizes that were just above the detection limit (10 mm).

With the presence of NOM, the size of resulting polymer aggregates was significantly smaller (Fig. 3.6 B). Therefore, NOM has a significant effect on the overall process; interacting with anilines to inhibit overall condensation and inhibiting aggregation for phenols and amines. This indicates that addition of NOM was unable to help with the size growing, including for the fluoroaniline particles.
Figure 3.6 Particle size kinetic measurements for the polymerized products. Instrument detection limit represented by horizontal line. (A) Reactor initial condition: 2 mM of substrates, 1 U/mL HRP, 50 mM phosphate buffer pH 7.5. Initiated reactor by adding 2 mM H₂O₂ at T=0.01m. (B) Same condition except with 30 mg/L HA added.
3.3.5 Mass spectrometry to determine the products molecular distribution

To illustrate the generated particle products and proposed mechanism of structure, the resulted particles were identified by LC-MS/MS. Proposed reaction mechanism shows that phenolate radicals are formed by removal of an electron and a hydrogen ion from the hydroxyl group (Fig. 3.7). The resulting phenolate radicals then couple to yield stable dimerized and polymerized product. The occurrence of such oxidative coupling reactions mediated by enzymes in soil can be altered from addition of humic substance that constitute a complex mixture of polymerized molecules. However, the chemical identification of these high-molecular-weight molecules in the natural environmental or mixing with humic acid is very complicated and sometimes impossible, and therefore it appears necessary to study the basic reactions with single reactants using enzyme system. Thus, we prepared the particles without the presence of NOM, and resulted oligomers were detected with molecular weights of up to 1200, corresponding to decamer (Fig. 3.8). This particle molecular weight determination is greatly limited by the instrument detection limit for current available mass spectrometric technique. Additionally, due to the chloric isotopic fractionation for the multiple number of chlorines attached in the higher molecular weight particles, the signal to noise ratio was too low to detect. However, it is clear that higher oligomers and polymers were formed. According to the product solubility estimation (Fig. 3.1 B), when n=5 which structurally corresponding to the pentamer, the solubility of product has been dropped under the drinking water limit (5μg/L) for halogenated compound. Thus, the mobile contaminants have been removed from aqueous phase and posed no risk to the safety of human drinking water.
Figure 3.7 Proposed mechanism and structure for possible polymerization. Circled structure represents a general structure for polymerized products.
Figure 3.8 4-Chlorophenol formed oligomers analyzed by mass spectrometry. Products were found shown in spectrum (A) ranging from monomer to trimer, and even up to hexamer shown in mass spectrum (B). (C) shows the molecular weight of products determined up to n=12, with possibility of characterization of higher molecular weight of polymers is unable to detect with the instrument.
3.3.6 Replication environmental conditions for the remediation

Microbes that are existing in the subsurface soil and vadose zone are known to produce extracellular enzyme such as peroxidase to battle the oxidative stress, caused by various reasons such as virus, pathogens, xenobiotics etc. Peroxidase that are native to the soil can react with an oxidant H$_2$O$_2$, which is also constantly produced in the environment due to the oxidation of Fe(II) by molecular oxygen. Those two things are generating in the constant rate while being consumed by various sinks. Thus, the concentration of natural enzyme and hydrogen peroxide were mostly maintained at steady state, typically at concentration level of mU/mL range for HRP and µM range for H$_2$O$_2$. Under the replication environmental conditions, the 4CP removals were conducted with steady state concentration maintained for both reactants. Results show that the 25% 4CP were removed after four hours of continuously adding HRP and H$_2$O$_2$ and mixing (Fig. 3.9 A). The removal process followed the pseudo first order decay which indicated the minimal HRP inactivation existed in the system. Results also show that comparatively lower amount of 4PC were removed with the presence of HA, being 15% after four hours reaction time (Fig. 3.9 B). This indicates that with the presence of NOM in nature the removal efficiency is relatively lower. However, comparing to the time span for natural attenuation of 4CP, typically around months, efficiency can be greatly increased by utilizing the peroxidase and peroxide reaction. Also, the resulted particles will stick to clay, silt and other soil components, which will then become part of the soil matrix.
Figure 3.9 Replicate the environmental condition for testing the potential for the removal rate of contaminants. 2 mM of 4CP were added initially as a model for contaminants with 30 mU/mL HRP. Reactor buffered at pH 7.5 with 50 mM phosphate buffer. Steady state of HRP and H$_2$O$_2$ added into the reactor along the reaction. (HRP: 50 mU/mL per hour, H$_2$O$_2$: 1 uM/min). 4CP concentrations have been normalized against the dilution factor and volatilization rate from a parallel control reactor. (A) Pseudo first order decay of 4CP. (B) With the presence of 30 mg/L NOM.
3.3.7 Adsorption of co-existence contaminant

Resulted particles have been tested in terms of its absorptivity to other co-contaminants. The adsorption data can be fitted into the Freundlich isotherm, eq. 1:

$$q = Kc^{1/n}$$

(1)

K and n are constants for a given adsorbate and adsorbent at a particular temperature. Higher the maximum capacity of an adsorbate results in higher the K value. q represents the amount of solute adsorbed per unit weight of solid at equilibrium (g/g). Equilibrium concentration of solute remaining in solution represented in the eq. 1 as c. Adsorbent (1,2-dichlorobenzene, 12DCB) was used in this study as a co-contaminant. Two commonly used adsorbate, activated carbon and zeolite, were added to serve as an absorptivity index against 4CP particles. The percentage of 12DCB adsorbed with varying the concentration of adsorbate after reaching equilibrium at time 4 hours were presented (Fig. 3.10). Fitting the adsorption isotherm to the Freundlich equation can generate K and n value for specific adsorbate. Results show through comparing the experiment generated K value (shown in Table 3.1), 4CP derived polymeric aggregates (4CP particles) are at around 15% removal capacity compared to the activated carbon, which is a promising adsorbate that is commonly used in remediation projects. This suggest the resulted particles have affinity towards other compounds and achieve a degree of co-remediation.
Figure 3.10 Affinity for still-dissolved residual contaminants test. The aliquot of sample was taken at reaction time 4 hours (reaction equilibrium reached), against different loadings of adsorbate.

Table 3.1 Freundlich Isotherm generated absorption constant regards to each adsorbate

<table>
<thead>
<tr>
<th>Adsorbate</th>
<th>n</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>4CP Particles</td>
<td>0.875</td>
<td>0.00692</td>
</tr>
<tr>
<td>Activated Carbon</td>
<td>0.600</td>
<td>0.0478</td>
</tr>
<tr>
<td>Zeolite</td>
<td>0.394</td>
<td>0.000395</td>
</tr>
</tbody>
</table>

Note: Freundlich adsorption isotherm suggests the constants for the given adsorbate and adsorbent (1,2-dichlorobenzene) at a temperature, where n is smaller, and K is larger, the better the adsorption capability.

3.3.8 Remediation under seawater condition

The impact caused by saltwater intrusion on pollutant enzymatic removal process are much less investigated in the literature. This study indicated that both ionic strength (altered by saltwater concentration) and NOM can affect the removal efficiency of organic pollutants. Increasing the ionic strength in seawater condition can improve the 4CP removal percentage up to ~12%, comparing to the control experiment which used buffer solution and its ionic strength was adjusted by potassium nitrate (Fig. 3.11). Moreover, addition of NOM to the buffer condition resulted in a greater removal percentage, 20% more of 4CP were removed comparing to the buffer control. The addition of seawater under
the presence of NOM showed the highest removal efficiency, 55% of 4CP were removed comparing to 50% with NOM in buffer condition. Results show changing ionic strength with the presence of NOM were not affecting the process, although reaching overall greater removal efficiency. These suggest that in areas with low presence of NOM, increasing ionic strength (more seawater mixed in the area) would result in a proportionally increase on removal efficiency. In areas with presence of high amount of NOM, the removal efficiency can be improved by mixing with seawater.

Figure 3.11 Testing the impact on the removal efficiency with varying ionic strength and NOM, in buffer and seawater condition.

3.3.9 Release of residual 4CP from generated particles

Enzymatic oxidation resulted particles in seawater condition, with and without NOM, have been tested regarding to their ability to release the 4CP back to the conditions with varying pH and ionic strength. A correlation between the amount of 4CP releases back to the environment and the conditions it applied to were observed (Fig. 3.12). However,
the overall number of released 4CP are much smaller comparing to the number of mobile contaminants that were synthesized and combined into particles, less than 1%. This indicates that the binding between the mobile contaminants and immobile polymers are strong and no reverse reaction happened after releasing 4CP particles back to conditions ranging from freshwater to seawater. Thus, preventing the polymeric aggregated from releasing mobile contaminants back to the environment to cause secondary contamination after saltwater intrusion faded.

Figure 3.12 Release of residual 4CP from generated particles. Collected particles were transferred back to initial pH and ionic strength conditions and release of residual 4CP monitored; <1% desorbed.

3.3.10 Testing with novel algal enzyme

Novel enzymes derived from harvested *M. wolleii*, a harmful algal bloom persistently occurring in lake Wateree, were applied to the 4CP removal process with hydrogen peroxide. The commercially available marine-derived *Lyngbya spp* enzyme was
chosen to compare the removal efficiency with \textit{M. wollel} enzyme. Horseradish peroxidase was applied as a control study to compare and generate a removal efficiency index against other enzymes. Results show that the marine-derived \textit{Lyngbya spp} enzyme has the highest removal efficiency comparing to the horseradish peroxidase and the novel \textit{M. wollel} enzyme (Fig. 3.13). The latter two have the same removal efficiency of 4CP, which suggests that the \textit{M. wollel} enzyme derived from an environmental toxic HAB species can be applied for enzymatic oxidation removal of organic contaminants, and achieving comparable removal efficiency to HRP.

\begin{center}
\includegraphics[width=\textwidth]{figure3.13.png}
\end{center}

\textbf{Figure 3.13} \textit{M. wollel} enzyme collected from a harmful algal bloom was tested regarding to its effectiveness at 4CP removal. Others including marine-derived \textit{Lyngbya spp}, enzyme and horseradish peroxidase were also tested under the same condition. All three enzyme activities were measured using ABTS assay under the same condition.
3.4 Conclusion

Organic contaminants in groundwater pose risks to human health because of their mobility and toxicity. Here we apply in-situ polymerization strategies to manage risk by converting mobile contaminants to immobile oligomers with low solubility. This process has achieved significant removal rate under the steady state of hydrogen peroxide and peroxidase with environmental level. Detected products had masses ranging from dimers to larger than decamers, with the maximum solubility of these products significantly reduced relative to the starting materials with visible polymers forming in solution in minutes. Resulted polymers also show affinity towards the parent organic contaminant, less but comparable to activated carbon. Saltwater intrusion will enhance the remediation efficiency and the resulted particles binding to the contaminant are irreversible.
CHAPTER 4

MICROSEIRA WOLLEI PEROXIDASE: AN ANTI-ALGICIDAL ENZYME PRODUCED BY A HARMFUL ALGAL BLOOM

Abstract

In lakes and reservoirs, harmful algal blooms became an important stressor to the local species, especially in the case of CO₂ depletion caused by dense bloom. Large-scale mat-forming cyanobacterium, Microseira wollei (M. wollei), was present in the lake Wateree, SC where the water quality is a concern to lake users. This research represents the analysis of the first antioxidant enzyme derived from M. wollei, in comparison with horseradish peroxidase and marine-derived Lyngbya spp. peroxidase. The derived algal enzyme has ability to diminish the efficacy of peroxide-based algaecide in the remediation process. Thus, the study of algal enzyme response to the peroxide-based algaecide exposures are needed, to accurately predict the effective amount of applied algaecide and

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3F. J. Wang, S. P. Putnam, O. M. Manley, T. M. Makris, G. I. Scott and J. L. Ferry, Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208. To be submitted to Environmental Science & Technology
guide informed decisions regarding to the algal management. The derived *M. wolleii* peroxidase’s molecular weight was evaluated by SDS-PAGE assay and activity was measured as 1.40 units per gram of fresh dry algae against ABTS assay. Substrates such as guaiacol, pyrogallol, 4-aminopyrine/phenol, and pyrocatechol/aniline were also tested, and sensitivity results reported regarding the *M. wolleii* peroxidase were lower than ABTS. Since lake pH level varies across the day, the pH optima of this algal enzyme were also studied. Finally, the enzyme’s antioxidant ability was tested against hydrogen peroxide and peroxide-based algaeicide. This study suggests that the treatment of algae can be more effective when considering the present of antioxidant enzyme in algae. The provided information regarding to the algal enzyme based on preliminary results can allow water resource managers to select the most efficient algaeicide amount for desired algal control. Also, this study leads to the need to complement more definitively detect and characterize HAB species antioxidant enzyme.

### 4.1 Introduction

Recent years, harmful algal blooms (HABs) have expanded in scale worldwide as the climate change accelerates and eutrophication occurs due to increased nutrient availability.\textsuperscript{119-121} Among the HABs species, cyanobacteria often receive the most attention in freshwaters because of human health concerns associated with production of harmful toxins (e.g., hepatotoxin, dermatoxin, and neurotoxin).\textsuperscript{122-124} The mat-forming cyanobacterium, *M. wolleii*, is of specific concern in the United States because of its widespread distribution and invasive proliferation.\textsuperscript{125-127} Increased frequency of *M. wolleii* growths have been found across the US, including multiple reservoirs in the Southeast region.\textsuperscript{128-130} Dense odorous mats of *M. wolleii* can have an economic impact on the
recreational water quality, wildlife habitat, irrigation and property values. Beyond the direct economic consequences, *M. wolleii* also poses potential health risks for humans, domestic pets, livestock, and wildlife associated with freshwater resources by producing numerous toxins. Toxins produced by *M. wolleii* include neurotoxins (BMAA, anatoxin-a, *M. wolleii* toxins 1-6), hepatotoxins (cylindrospermopsins) and dermatotoxins (aplysia-toxin, lyngbyatoxin).\(^{125,127,135}\)

Lake Wateree, a hydroelectric reservoir in South Carolina, suffers from increasing infestations of *M. wolleii* and provide an opportunity to sampling the algae and evaluate the effectiveness of algaecide in the laboratory. Lake Wateree also serves as a drinking water source for several counties around the lake. Human exposed to such infested lake in many ways, for example, through contacting with the recreational water (e.g., swimming and fishing on foot), drinking contaminated water, or consuming food (e.g., fish and contaminated seafood).\(^{136}\) Thus, the dense *M. wolleii* growths have created concerns from local communities and water resource management over the health and economic perspective. Currently, nutrient reduction is arguably the best strategy to prevent the incidence of HABs, but almost impossible to achieve. Mechanical harvesting is an unlikely option to control HABs due to the irregular depth and shape of coves around the lake, as well as the requirement for large investment on staff and equipment. Additionally, mechanical harvesting would be needed multiple times a year and every year to effectively treat the infestation.\(^{137}\) Chemical algaecides are often used by water resource managers as an effective mitigation of problems in a short time frame.\(^{138}\) However, the selection of certain algaecides to deal with HABs problems must take into consideration their impact on the sustainability of the water system.
Copper-based algaecide formulations are commonly used for control of cyanobacteria but is toxic to a wide variety of plants and animals, and its residue can be built up in sediments and led to long term toxic effect to fish and other non-targeted organisms inhabiting in the lake.\textsuperscript{128, 139} Moreover, repeated applying copper-based algaecide can lead to resistance of phytoplankton communities.\textsuperscript{140, 141} Sodium percarbonate (Na$_2$CO$_3$ $\cdot$ H$_2$O$_2$), a granular powder that containing hydrogen peroxide (H$_2$O$_2$), is selective for cyanobacteria (vs. eukaryotic algae and higher plants) and poses no serious long-term effects on the system.\textsuperscript{142, 143} Sodium percarbonate acts quickly to dissociate into sodium carbonate and hydrogen peroxide, the exposure of the latter causes intercellular and extracellular damage that can adversely affect algal cells. Shortly after the application, typically within a few days, hydrogen peroxide is degraded to oxygen and water, and thus leaves no long-term chemical traces in the environment.

Several laboratory studies have found the antioxidant enzyme in algae can specifically decomposes H$_2$O$_2$.\textsuperscript{144-146} H$_2$O$_2$ occurs naturally in small concentrations in all surface waters and many organisms produce H$_2$O$_2$.\textsuperscript{29, 147, 148} As part of the protein in algae expresses antioxidant enzyme function due to its benefit to protect the algae from oxidative damage.\textsuperscript{149} Those oxidative damage are generally induced by light, native steady-state hydrogen peroxide, and microbial activity produced ROS and can significantly alter the life span of algae.\textsuperscript{39, 150, 151} Antioxidant enzymes in algae are essential proteins that maintain cell proliferation potential by protecting against oxidative stress by detoxifying ROS.\textsuperscript{152} For example, enzyme 2-Cys peroxiredoxin (PRX) was found in the raphidophyte \textit{Chattonella marina} as an antioxidant enzyme that specifically decomposes H$_2$O$_2$. The
light-induced oxidative stress would increase the generation of PRX as a more advanced system to protect *Chattonella marina* from ROS.\textsuperscript{153}

In this study, an attempt is made to understand the antioxidant enzyme which influence and protect the *M. wollei* algae from peroxide and provide laboratory results to the in-situ treatment through applying an effective concentration of algaecide. The enzyme derivatization work was done, and have been characterized in terms of substrate specificity, pH and hydrogen peroxide activity. One of the substrates most commonly used to assess peroxidase in biological samples is 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), which peroxidase can oxidize ABTS to ABTS\(^{++}\) colored blue-green, was used in this study as an index method for quantify between three enzymes. The aim of this study was to isolate *M. wollei* antioxidant enzyme from field samples followed by studies on the substrate’s specificity, influence of pH, and demonstrate the laboratory predictions for in-situ treatment using sodium percarbonate concentration at maximum efficacy for target *M. wollei* while minimizing risk for non-targeted species.

### 4.2 Materials and Methods

#### 4.2.1 Materials

Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, 99%) was from Molecular Sigma Biology. Horseradish peroxidase (Type II, salt-free, lyophilized powder, 150 U/mg), *Lyngbya* sp., recombinant peroxidase (3.4 units/mg), bovine serum albumin (lyophilized powder, ≥96%), sucrose (99%), dithiothreitol (DTT, 99%), aniline (99.0%) and ethylenediaminetetraacetic acid (EDTA, 99.5%) were acquired from Sigma-Aldrich. Bugbuster\textsuperscript{®} HT protein extraction reagent was acquired from EMD Millipore Corp. 
Bradford Dye Protein Reagent was purchased from BIO RAD. Hydrogen peroxide was obtained as a 30% solution from BDH and was standardized by UV-Vis absorption, using its extinction coefficient of 18.6 M\(^{-1}\) cm\(^{-1}\) at 254 nm.\(^{26}\) 1,2,3-Trihydroxybenzene (pyrogallol, 99%) was purchased from Matheson Coleman & Bell. Phenol (99%) and sodium percarbonate (avail. H\(_2\)O\(_2\) 20-30 %) were purchased from Aldrich Chemical Company, Inc. 10-Acetyl-3,7-dihydroxyphenoxazine (amplex red, AR) was purchased from American Advanced Scientific Inc. Tris(hydroxymethyl)aminomethane (Tris, 99%) and 2-Aminoethanesulfonic (taurine, 98%) were purchased from TCI Chemicals. Potassium bromide (99%) was acquired from Mallinckrodt. Sodium hypochlorite (12.5% w/w) was purchased from VWR. Diethylenetriamine pentaacetate (DTPA, 99%), guaiacol (99%), catechol (99%), and 4-Aminoantipyrine (98%) were purchased from ACROS Organics. DTPA solution (0.01 M) was prepared in 0.05 M sodium phosphate buffer (pH 7.50 ± 0.05) and stored at room temperature. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 98%), ammonium hydroxide (A.C.S. certified) and all other salts (99.5%) were obtained from Fisher Scientific. All chemicals were used as received. All aqueous solutions were prepared with Barnstead E-pure (18 MΩ cm\(^{-1}\)) deionized (DI) water.

4.2.2 Source of *M. wollei* sample collection.

Lake Wateree was created in 1920 for the operation of Wateree Hydroelectric Station. The man-made lake has approximately 13,864 surface acres and 181 miles of shoreline and is the second largest of the eleven-lake system on the Catawba and Wateree rivers.\(^{154}\) Lake Wateree provides a dependable water supply for the Lugoff-Elgin Water Authority and the City of Camden, South Carolina. It is also a prime destination for primary and secondary contact recreation.\(^{154}\)
M. wollei field samples were collected from lake Wateree in November 2019 and February 2020. After collection, the samples were kept in an ice bucket. Upon return to the laboratory, the samples were washed with interstitial water (also collected at the same location) and extraneous plant/animal material was removed. Approximately 50 g hand-dried fresh material was kept for enzyme isolation.

4.2.3 Preparation of enzyme extract.

Approximately 50 grams of hand-dried fresh algae was transferred into a blender with 200 mL DI water, and blend continuously for 1 min. 100 mL extraction buffer solution were added into the blended algae, and resulted slurry were transferred into a beaker and sonicated for 30 mins at ambient temperature. Extraction buffer solution composed of 50 mM tris-HCl, 10% w/w sucrose, 2 mM dithiothreitol, 4 mM EDTA, 20% v/v Bugbuster® HT protein extraction reagent, and the pH of final solution was adjusted to 7.6 with ammonium hydroxide (1 M). Cheese fabric was used to squeeze liquid out of solid algae. And resulted liquid was centrifuged twice (4000 rpm, 25 min, 2°C), to get rid of any debris and dirt. Supernatant collected as the crude extract that contained the enzyme and protein, was then brought to 50% saturation by gradually adding solid ammonium sulfate solid into the stirring solution that was kept cold in an ice bath. Afterwards the mixture was centrifuged to precipitate and collect the protein (4000 rpm, 25 min, 2°C). Suspended the protein pellet in a minimum volume using tris-HCl buffer (100 mM, pH 7.5). Protein solution was then transferred into a dialysis tubing (Ward’s science, allow passage of particles up to 14,000 Da), and was dialyzed against the tris-HCl buffer (100 mM, pH 7.5) at 10°C overnight, with changing buffer solution twice. Samples were then stored in -80°C freezer until future analysis.
4.2.4 Enzyme and protein assays

Each raw and purified sample was assayed for peroxidase activities using a modified protocol. Transfer 2.90 mL ABTS substrate (9.1 mM, prepared in 100 mM potassium phosphate buffer at pH 5.0), 0.05 mL H$_2$O$_2$ (0.3%, w/w) into a cuvette. After 10 mins equilibration, 0.05 mL sample was added and immediately mix by inversion and record the increase in $A_{405\ nm}$ for 3 mins. A blank was taken with 0.05 mL phosphate buffer (50 mM, pH 7.5) instead of adding sample. Obtained $\Delta A_{405\ nm}$/min for the linear rate for both test and blank and subtract the blank from the test. Resulted value was used to calculate the unit activity of sample enzyme by using the extinction coefficient of oxidized ABTS at 36.8 mM$^{-1}$ cm$^{-1}$. One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of ABTS$^{+\ +}$ formed per min at room temperature.

Following the extraction step, protein content was determined with the Bradford assay, in which the transmissivity of a mixture of protein co-extracted with the Bradford Dye (Bio-Rad) Protein Reagent was measured with a UV-Vis spectrometer at 595 nm. These values were then compared with a standard curve generated using bovine serum albumin.

4.2.5 Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used as previously described, to assess the composition of enzymes as well as estimation of molecular mass. Briefly, after dilution of the algal enzyme suspension with sample buffer, the mixture was loaded into a well in gel electrophoretic system, and electrophoresis was done at 150 V and 1.5 mA for 60 min. For visualization, the proteinaceous samples
were stained with 0.1% v/v Coomassie brilliant blue solution, 10% acetic acid, and 50% methanol for 1 h.

4.2.6 Substrate specificity

For determination of the substrate specificity of the partially purified *M. wolleti* peroxidases, besides ABTS, four other well-known peroxidase substrates: guaiacol, pyrogallol, aminoantipyrine-phenol, pyrocatechol-aniline were used as reducing substrates, during spectrophotometrically determination with H₂O₂. When studying substrate specificity of peroxidase, the activity was measured all under the same pH condition (using 100 mM phosphate buffer, pH 5.0) with optimum concentration of substrates and hydrogen peroxide (shown in Fig. 4.3). The changes in absorbance were read for 3 min using a spectrophotometer. The following wavelengths and extinction coefficients were used in the assays: ε₄₇₀ nm = 26.6 mM⁻¹ cm⁻¹ for guaiacol, ε₄₂₀ nm = 12 mM⁻¹ cm⁻¹ for pyrogallol, ε₅₁₀ nm = 6.58 mM⁻¹ cm⁻¹ for aminoantipyrine-phenol, ε₅₁₀ nm = 6.58 mM⁻¹ cm⁻¹ for pyrocatechol-aniline. One unit of activity is defined as the amount of peroxidase that oxidizes 1 µmol of substrate per min under standard conditions.

4.2.7 pH optima of peroxidase tested by spectrophotometric microplate ABTS and guaiacol assays

In order to adapt the classical cuvette methods to the microplate reader, the volume of solutions and samples were reduced proportionally. The total volume of the mixture was reduced from 3.0 mL to 300 uL for both the ABTS assay and guaiacol assay. A flat bottom glass microplate with 96 wells was used. Absorbance readings were made at 25°C with a Molecular Devices™ SpectraMax M5 Multi-mode Microplate Reader.
The reaction mixture, composed of 5 µL of *M. wollei* enzyme in an appropriate buffer of desired pH and 290 µL of ABTS substrate (9.1 mM) solution at the same pH, was equilibrated for 1 min at room temperature in 96-well plate. The enzyme was assayed using 10 µL of 100 mM H₂O₂ to oxidize the ABTS to ABTS⁺⁺ and monitoring the absorbance increase at 405 nm. Guaiacol substrate was also tested for pH stability. The reaction mixture composed of 50 µL of *M. wollei* enzyme in an appropriate buffer of desired pH and 205 µL of guaiacol substrate (18 mM) solution at the same pH. The enzyme was assayed using 45 µL of 20 mM H₂O₂ and monitoring the absorbance increase at 470 nm. The assay was carried out at 25°C using buffers such as sodium acetate (pH 4.0-5.5), sodium phosphate (pH 6.0-7.5) and Tris (pH 8.0-9.5), all at molarities of 50 mM. Assay was performed at every pH without addition of enzyme and used as control.

The absorbance readings obtained with the microplate methods need to be corrected to correspond to the readings obtained from the cuvette methods because of differences in the light pathlength. According to Siguemoto and Gut, the liquid pathlength in a microplate well can be determined from absorption readings of the water present in the mixture at 1000 nm. The wavelength of 900 nm was used as a reference to eliminate the background absorbance because of the low absorption of water at this wavelength. The correction factor for pathlength can be defined as in Equation 1:

\[
K = \frac{Abs_{1000}^C - Abs_{900}^C}{Abs_{1000}^M - Abs_{900}^M}
\]  

(1)

Where Abs is the absorbance and superscript C and M correspond to cuvette and microplate well readings, respectively. Microplate absorbance reading Abs^M need to be corrected as in Equation 2 to correspond to a cuvette reading with a 1 cm pathlength.
Abs = K \cdot \text{Abs}^M \tag{2}

Cuvette and microplate readings were made at 1000 and 900 nm, 25°C, for the corresponding assay buffers in the total volume of the mixture. For determining K, the numbers of replicates were three for both the cuvette and microplate readings, and the error associated with K were used in error propagation step later to calculate error for the absorbance correspond to cuvette.

4.2.8 Halogenation activity tested for *M. wollei* peroxidase

Taurine can react with HOBr and HOCl to form taurine monobromamine (TauBr) and taurine monochloramine (TauCl) respectively. For determination of enzyme halogenation activity, we quantified the formation of TauBr catalyzed by *M. wollei* peroxidase, along with HRP and marine-derived *Lyngbya* spp enzyme by mass spectrometry. In detail, 0.5 units enzyme were incubated with 5 mM taurine, 500 µM H₂O₂, 50 mM KBr in 50 mM phosphate buffer, pH 7.5, at room temperature for 10 min. Measurements were performed by injecting samples into a Waters (Milford, MA, USA) Acquity UPLC® coupled with a Xevo triple quadrupole mass spectrometer (MS/MS) equipped with an electrospray ionization sources (ESI). Mobile phases consisted of H₂O/MeOH with 10 mM Ammonium acetate buffer. Monitoring the MRM transition under ES+ mode, with parent (m/z) 204.0209 to daughter (m/z) 122.0190 with collision energy 16 V and cone voltage 22 V. Quantification method was generated by IntelliStart detecting a TauBr standard solution produced by mixing NaOCl : KBr : Taurine = 1 :1.5 : 15 ratio of moles. TauBr concentration was calibrated by direct absorbance by UV-Vis spectroscopy (ε₂₈₈ nm, TauBr = 430 M⁻¹ cm⁻¹).
4.2.9 Peroxidase activity as a function of \( \text{H}_2\text{O}_2 \) cone and algaecide

The effect of initial hydrogen peroxide concentration on the peroxidase activity of \( M. \text{wollei} \) peroxidase was investigated at pH 5.0 and 25°C with different concentration of hydrogen peroxide. Sodium percarbonate based algaecide had also been investigated with the same diluted concentration of corresponding to hydrogen peroxide on the peroxidase activity. The absorbance was measured at 405 nm with 8.65 mM ABTS used as reducing substrate.

4.3 Results and Discussion

4.3.1 Enzyme activity in terms of algae biomass

We compared the enzyme activity from two sampling trips between November and February and calculated the total enzyme and protein for 50 grams of hand-dried clean algae. Results show that the protein and enzyme are consistent between two samples from two time points (Table 4.1). At around 10 mg of protein/g of algae and 1.40 units of enzyme/g of algae. This is particularly interesting because it shows the enzyme during the winter season with not growing and sinking to the bottom of the lake, the enzyme still retains its activity and act as antioxidant to protect the algae from oxidative damage.

<table>
<thead>
<tr>
<th>Sampling Date</th>
<th>Vol. (mL)</th>
<th>Enzyme (U/mL)</th>
<th>Protein (mg/mL)</th>
<th>Specific activity (Units/mg)</th>
<th>Total Enzyme (U)</th>
<th>Total Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov 22\textsuperscript{nd}, 2019</td>
<td>167</td>
<td>0.42</td>
<td>3.03</td>
<td>0.14</td>
<td>70.87</td>
<td>506.50</td>
</tr>
<tr>
<td>Feb 4\textsuperscript{th}, 2020</td>
<td>260</td>
<td>0.27</td>
<td>1.73</td>
<td>0.16</td>
<td>70.90</td>
<td>449.41</td>
</tr>
</tbody>
</table>

Note: Measurements were independent and performed under the same condition with protocols described in methods.
Crude extract enzyme sample were then concentrated using ammonium sulfate cut method by adding salt to 50% saturation and precipitate the protein pellet slowly for later experiment. Recovery varies from 50-70% for total enzyme activity.

4.3.2 Molecular weight estimation by electrophoresis

The SDS-PAGE analysis of the partially purified peroxidase revealed three protein band, suggesting that the partially purified peroxidase enzyme may consisted of multiple polypeptide chains with molecular weights of 14, 100 and 135 kDa. Horseradish peroxidase has a molecular weight known within the range of 40 - 44 kDa, the marine-derived Lyngbya spp enzyme has 86 KDa. Our results indicate that the freshwater *M. wolleii* peroxidases have two enzymes that have higher molecular weight compare to marine-derived *Lyngbya* spp enzyme as previously reported, and one low molecular weight enzyme at 14 kDa. However, nothing were found close to horseradish peroxidase, indicate that the two are significant different enzymes.

![Figure 4.1 Photographic representation of the SDS-PAGE of partially purified peroxidase. Peroxidase obtained after (1) salt-cut step and comparing to (2) standard proteins.](image)
4.3.3 Substrate specificity

The data on substrate specificity of the enzyme are summarized in Table 4.2. The enzyme revealed highest activity against ABTS assay; with the second only tested 36.6 ± 2.3% of the enzyme activity with pyrocatechol/aniline assay. Results suggested that the better substrate for all three enzymes was ABTS. The marine-derived *Lyngbya* spp peroxidase (LP) activity was measured against two assays, pyrogallol and pyrocatechol/aniline. Both showed a non-linear hyperbolic profile (which the absorbance increased rapidly in the beginning and slight slowed down was observed later), indicated that pyrogallol and pyrocatechol/aniline substrates are not suitable for measuring LP activity.

Table 4.2 Substrate specificity for *M. wollei* peroxidases, HRP and marine-derived *Lyngbya* spp peroxidase (LP)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Relative Activity</th>
<th>M. wollei Peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>H2O2</td>
<td>HRP</td>
</tr>
<tr>
<td>ABTS</td>
<td>8.65</td>
<td>3.28</td>
<td>100.0 ± 0.9</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>15</td>
<td>3</td>
<td>33.3 ± 1.2</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>42</td>
<td>8.9</td>
<td>31.4 ± 0.4</td>
</tr>
<tr>
<td>4-Aminopyrine/phenol</td>
<td>120.4</td>
<td>1</td>
<td>23.9 ± 0.7</td>
</tr>
<tr>
<td>Pyrocatechol/aniline</td>
<td>76.5</td>
<td>1</td>
<td>84.2 ± 6.2</td>
</tr>
</tbody>
</table>

Note: Measurements were done at pH 5.0. Substrates were added to the assay at concentration as indicated. Data are given as mean ± SD with replicate measurement n=3.

Fluorescence is used for measuring the activity of all three enzymes using amplex red technique. Peroxidases are well-known to be the catalyzing agent for amplex reacting with H2O2 and form a fluorescence product resorufin, thus can be detected by a fluorescence detector. Here, we compared the sensitivity of amplex red technique used three enzymes under the same conditions. Results showed that the HRP is the most efficient on catalyzing the formation of resorufin, and the marine-derived *Lyngbya* spp enzyme only
has one-third of sensitivity comparing to the HRP (Fig. 4.2). Amplex red was used to measure the *M. wollei* peroxidases activity, although its sensitivity is relatively lower compare to other enzymes, the linear correlation ($r^2 = 0.997$) sill proves that Amplex red can be a quantifying probe to measure the *M. wollei* peroxidases activity. Since fluorescence techniques are very sensitive due to the minimization of the interference, such as occurring in absorbance technique, the amplex red was tested and confirmed can be a detection method to measure the *M. wollei* peroxidases activity.
Enzyme activity were calibrated with ABTS assay. Sensitivity for amplex red detection on three enzymes: HRP (A) > LP (B) > *M. wolfei* peroxidase (C).
4.3.4 Optimum pH

Lake water that contain HABs are often accompanied by a pH fluctuation throughout the day. HABs generally consume oxygen and generate CO₂ as a result of respiration that occurred in the lake overnight. Then CO₂ dissolved in water and forms a mild acid, thus lowering the pH of lake water. As sun rises, plants and algae start photosynthesis thereby consuming CO₂ and causing the pH to rise (more basic) as day progresses. Dense algal blooms can be a significant pH driver in this process. From our experimental results (Fig. 4.3 A), *M. wollei* peroxidase showed optimum activity at different level for two assays, pH 4.0 for ABTS and pH 9.5 for guaiacol assay. A rapid decrease in activity tested by ABTS was found on the basic side of its optimum pH. Guaiacol method shows the second optimum activity at different pH value around 5.5 with lower activity. Similar optimum pH for *M. wollei* peroxidase at 4.0 was observed for peroxidase from marine-derived *Lyngbya* spp. using ABTS assay. However, using guaiacol assay shows different optimum result at pH 4.5. These suggests that the *M. wollei* enzymes and marine derived *Lyngbya* enzymes have different pH optimum value, thus they are not identical enzymes. Horseradish peroxidase was also tested here as a reference (Fig. 4.3 B).

If we link the guaiacol tested enzyme activity under different pH with the lake pH range that were reported throughout a year, we found that the lake can reach basic pH at around 9.3 in the daytime, and drop to the lowest as pH 5.5 at night time. This superimposed with the enzyme activity we tested here for their optimum pH value regarding to the highest activity, shows that the lake algae enzyme may developed an
Adaptation function regarding to surrounding pH in order to keep the enzyme activity in a degree that can protect the algae from oxidative stress.

Figure 4.3 Peroxidase activity of *M. wollei* peroxidase (A) and HRP (B) in the pH range 4.0 to 9.5. Substrate concentration: 8.65 mM ABTS, 15 mM guaiacol. Activity is given in percentage of maximum activity (100%) at respective pH-optimum, with 100% corresponding to 0.23 mU/mL (ABTS) and 0.01 mU/mg (guaiacol) for algal enzyme, and 0.38 mU/mL (ABTS) and 0.37 mU/mL (guaiacol) for HRP.

We also tested the halogenation effect of three enzymes, results come back as only marine-derived *Lyngbya* spp. peroxidase has positive effect on forming halogenated compound that are induced by generated hypohalous acid. Algal enzyme and HRP have no
halogenation ability. This result showed the difference in functionality between *M. wollei* peroxidases and marine-derived *Lyngbya spp.* peroxidase.

### 4.3.5 Effect of H<sub>2</sub>O<sub>2</sub> and algaecide on peroxidase activity

The effect of initial hydrogen peroxide concentration on the peroxidase activity was investigated at the pH 5.1 and 25°C with the same enzyme concentration. The maximum peroxidase activity was observed at the initial hydrogen peroxide concentration of about 2 mM, followed by the decline as the concentration increases (Fig. 4.4). Similar pattern of H<sub>2</sub>O<sub>2</sub> inhibition is detected in case of algaecide, however, the algaecide showed higher peroxidase activity compare to H<sub>2</sub>O<sub>2</sub> at higher peroxide concentration. But still lower than the optimum 2 mM hydrogen peroxide concentration. This is due to the higher concentration of H<sub>2</sub>O<sub>2</sub> can oxidize the peroxidase compound II into inactive compound III, instead of at normal peroxide concentration the peroxidase compound II will be reduced to native peroxidase. Overall, result shows the optimum hydrogen peroxide concentration to activate 2.64 unit/L of *M. wollei* peroxidase is at 2.0 mM.
Figure 4.4 Effect of \( H_2O_2 \) concentration on peroxidase activity. Activity measurement were calculated at different \( H_2O_2 \) concentration in the range of 0.2 – 33.3 mM with ABTS as substrate. All reactors were performed in controlled pH at 5.1 ± 0.1 (pH confirmed at T=final) using 100 mM phosphate buffer.

4.3.6 Peroxide based algaecide amount estimated to effectively treat \( M. \ wollei \) algae in Lake Wateree

Estimation has been made in order to calculate the effective amount of peroxide-based algaecide were needed to treat the lake HABs. Lake Wateree contains about 10 million kg of algae, generated from rough estimation, were presented in 5% lake area. Based on the results (Table 4.3), we have the minimum concentration of \( H_2O_2 \) needed to be at those affected areas of lake at approximately 19 ppm to effectively ease the antioxidant enzyme generated by algae and kill the algae. Table 4.4 shows the recommendation for applying at max and min level for two types of algaecide, 30% Hydrogen Peroxide solution, and sodium percarbonate solid. General recommendation is to apply the algaecide to areas that has algae occupation, however, due to the water mixing
between main lake body and algae occupied portion, some of the algaecide will lost during
the treatment. Max value were estimated based on the equilibrium of the whole lake, which
is close but still maintain the safe level for most fish (19.7 ppm). It’s very important to
use both the algal density and the activity of algal peroxidase per gram of algae, measured
from the specific condition that are presenting prior to the application, to scale laboratory
results to an in situ treatment that could improve the efficiency. This predication can help
with decreasing the chance of applying an ineffective concentration and maintaining the
safe level for non-targeted organism.

Table 4.3 Estimation of H$_2$O$_2$ level needed in the algae area to activate the peroxidase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated lake Vol. (L)\textsuperscript{165}</td>
<td>$3.82 \times 10^{11}$</td>
</tr>
<tr>
<td>Estimated algae coverage percent (%)</td>
<td>5</td>
</tr>
<tr>
<td>Vol. of lake that contains algae (L)</td>
<td>$1.91 \times 10^{10}$</td>
</tr>
<tr>
<td>Estimated total algal mass (g)</td>
<td>$1.00 \times 10^{10}$</td>
</tr>
<tr>
<td>Algal density (mg/L)</td>
<td>0.52</td>
</tr>
<tr>
<td>Peroxidase activity (unit/g of algae)</td>
<td>1.40</td>
</tr>
<tr>
<td>Millimoles of H$_2$O$_2$ treated per unit of peroxidase (mmol)</td>
<td>0.76</td>
</tr>
<tr>
<td>Peroxidase activity in algae area (unit/L)</td>
<td>0.73</td>
</tr>
<tr>
<td>Conc. H$_2$O$_2$ needed in the algae area (mM)</td>
<td>0.56</td>
</tr>
<tr>
<td>Conc. H$_2$O$_2$ needed in the algae area (ppm)</td>
<td>18.88</td>
</tr>
</tbody>
</table>

Table 4.4 Estimation of amount of algaecide for effectively treating \textit{M. wollel} in Lake Wateree, SC

<table>
<thead>
<tr>
<th></th>
<th>Apply to the whole lake (Max.)*</th>
<th>Apply to part of affected area (Min.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol. 30% H$_2$O$_2$ needed (Gallon)</td>
<td>$5.60 \times 10^6$</td>
<td>$2.80 \times 10^5$</td>
</tr>
<tr>
<td>Amount of Algaecide (Na$_2$CO$_3$\textsuperscript{1.5} \times$H$_2$O$_2$) needed (millions of KG)</td>
<td>22.2</td>
<td>1.11</td>
</tr>
</tbody>
</table>

*Note: Maximum represents assumption of the whole lake reaches equilibration with the hydrogen peroxide concentration at 18.88 ppm. Minimum represents assumption that the treatment is only applied to affected areas where no mixing happening to the whole lake.
4.4 Conclusion

As harmful algal infestations, such as *M. wollei*, continue to increase and pose significant economic, ecological and human health risks, a better understanding of effective management options is needed. This research provides the first report of the peroxidase in *M. wollei* from Lake Wateree. Extraction and partial purification of peroxidases were performed and resulted peroxidases were studied in terms of pH optima, substrate specificity and the activity over the range of hydrogen peroxide. Most importantly, data from the experiment in this thesis increase our fundamental understanding of peroxide-based algaecide and algal antioxidant enzyme relationship, and provide more accurate predication to support the effective and ecologically safe use of peroxide-based algaecide in drinking water resource management.
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