

EXPLORING THE UTILITY OF FENTON PROCESSES IN WASTEWATER TREATMENT

By

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Abstract

For over 120 years, Fenton processes (the reaction between an iron catalyst and hydrogen peroxide) have been extensively studied owing to their relevance in both environmental and biological systems. However, understanding their underlying mechanisms has remained a challenge to researchers. The goal of the present work was to gain a better mechanistic understanding of Fenton reactions to provide invaluable knowledge to the research community. With a better understanding of these processes, researchers may be able to develop new approaches to address prior issues associated with using Fenton techniques. For example, altering Fenton processes (*i.e.*, changing the concentration of reagents or type of catalyst) may influence the quantity and types of reaction products generated. This could improve remediation efforts.

Contrary to the standard paradigm, this thesis has determined that the main reaction product generated in Fenton processes catalyzed by iron magnetic nanoparticles (Fe MNP) and the iron-based protein hemoglobin (Hb) was singlet oxygen ($^1\text{O}_2$). $^1\text{O}_2$ is a highly energetic form of molecular oxygen (O_2) with the capacity to oxidize various organic compounds in environmental and biological matrices. Apart from mechanistic studies, I was motivated to assess the effectiveness of Fenton processes in the degradation of organic compounds within local wastewater effluent samples from Boat Harbour, located in Pictou County, Nova Scotia. Boat Harbour is a former tidal estuary located near the Pictou Landing First Nation community in Nova Scotia, which treated bleached kraft mill effluent from a nearby pulp mill for over 50 years. The Fe MNP-catalyzed Fenton process was unsuccessful at degrading the model organic dye methyl orange within the Boat Harbour wastewater effluent matrix; however, the Hb-catalyzed Fenton process was successful for this application. This process has the potential to provide a green and cost-effective wastewater treatment strategy. In the future, I would like to combine Fenton processes with other advanced treatment methods, such as electrochemical oxidation or photocatalysis; this may produce a synergistic effect and improve wastewater remediation. In this research, I have shown that Fenton processes can be used to generate $^1\text{O}_2$ in a relatively safe and inexpensive manner. I then showed that $^1\text{O}_2$ can oxidize organic pollutants in a complicated real-world milieu containing many recalcitrant compounds that could interfere with remediation efforts. This novel discovery could be employed for a variety of important applications, such as industrial synthesis reactions, chemotherapy, biofilm removal, or wastewater remediation.

List of Abbreviations and Symbols Used

ABDA.....	9,10-Anthracenediyl-bis (methylene)dimalonic acid
ABTS.....	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AOPs.....	Advanced Oxidation Processes
APIs.....	Active Pharmaceutical Ingredients
A.U.....	Arbitrary Units
BHETF.....	Boat Harbour Effluent Treatment Facility
BKME.....	Bleached Kraft Mill Effluent
BOD.....	Biochemical Oxygen Demand
CCME.....	Canadian Council of Ministers of the Environment
CDOM.....	Coloured Dissolved Organic Matter
dA/dt.....	Change in Absorbance per Unit Time
dF/dt.....	Change in Fluorescence per Unit Time
DABCO.....	1,4-Diazabicyclo[2.2.2]octane
DBB.....	o-Dibenzoylbenzene
DMSO.....	Dimethyl Sulfoxide
DOC.....	Dissolved Organic Carbon
DPIBF.....	1,3-Diphenylisobenzofuran
EPA.....	Environmental Protection Agency
EPR.....	Electron Paramagnetic Resonance
FAO.....	Food and Agricultural Organization

Fe MNP.....	Iron Magnetic Nanoparticles
GC-MS.....	Gas Chromatography-Mass Spectrometry
Hb.....	Hemoglobin
HCl.....	Hydrochloric Acid
OH•.....	Hydroxyl Radical
ICP-MS.....	Inductively Coupled Plasma Mass Spectrometry
ISQGs.....	Interim Sediment Quality Guidelines
kWh.....	Kilowatt hour
MES.....	2-(N-morpholino)ethanesulfonic acid
μM.....	Micromolar (10 ⁻⁶)
MΩ.....	Milliohm (10 ⁻³)
NaN ₃	Sodium Azide
NRC.....	National Research Council
OPD.....	o-Phenylenediamine
PAHs.....	Polycyclic Aromatic Hydrocarbons
PELs.....	Probable Effect Levels
PLFN.....	Pictou Landing First Nation
ROS.....	Reactive Oxygen Species
¹ O ₂	Singlet Oxygen
O ₂ ⁻	Superoxide Anion
TBA.....	tert-Butyl alcohol

TEMP-OH.....	4-Hydroxy-2,2,6,6-tetramethylpiperidine
TMB.....	3,3',5,5'-Tetramethylbenzidine
TOC.....	Total Organic Carbon
TSS.....	Total Suspended Solids
UV-vis.....	Ultraviolet-visible
XTT.....	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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Chapter 1 Introduction

1.1 Problem

The United Nations estimates in their World Water Development Report (UN-Water, 2018) that there are currently over 2 billion people with reduced access to safe drinking water; this is expected to increase to 5 billion by 2050 with global population growth. Unprecedented population increases, urbanization, and industrialization has contributed to the pollution of the world's freshwater supply (Akpor *et al.*, 2014; Juma *et al.*, 2014; Liyanage & Yamada, 2017; Pawar & Gawande, 2015; Rajasulochana & Preethy, 2016). The improper disposal and ineffective treatment of wastewater effluent poses ecological and human health risks (Chaudhary *et al.*, 2020; Eichinger & Walker, 2020; Hoffman *et al.*, 2019); this is evident in Nova Scotia with the current Boat Harbour remediation project (Government of Nova Scotia, 2018a).

Boat Harbour is a former tidal estuary located near the Pictou Landing First Nation (PLFN) community, which has received bleached kraft mill effluent from a nearby pulp mill for over 50 years. Boat Harbour was once used as a site for traditional food and medicine gathering and various recreational activities. However, the accumulation of hazardous pollutants including metals and metalloids, polycyclic aromatic hydrocarbons (PAHs) and dioxins and furans in Boat Harbour has eliminated these activities that were an important part of the culture of the PLFN community (Government of Nova Scotia, 2018a, 2018b). The province of Nova Scotia is committed to cleaning up the legacy of contamination at Boat Harbour and restoring it to its pre-1967 condition as a tidal estuary with an estimated cost exceeding \$292 million CAD (Eichinger & Walker, 2020). Boat Harbour will be detailed further **in Chapter 2**. Recalcitrant organic compounds (*e.g.*, contaminants) found in Boat Harbour wastewater, cannot be completely

degraded with conventional physical¹, chemical², and/or biological³ treatment methods (Ameta *et al.*, 2018; Matavos-Aramyan & Moussavi, 2017). However, advanced oxidation processes (AOPs) including Fenton ($\text{H}_2\text{O}_2 + \text{Fe}^{2+}$) and photo-Fenton ($\text{UV}/\text{H}_2\text{O}_2 + \text{Fe}^{2+}$) processes, have been effective at degrading these organics (Bermúdez *et al.*, 2021; Collivignarelli *et al.*, 2017; Matavos-Aramyan & Moussavi, 2017). AOPs are thought to generate hydroxyl radicals ($\text{OH}\cdot$), which are strong non-selective oxidants that can attack and degrade organic compounds, leading to their mineralization to carbon dioxide and water, which is the ultimate goal for wastewater treatment (Krishnan *et al.*, 2017; Munter, 2001). Fenton and photo-Fenton processes are also advantageous because they use inexpensive materials (*e.g.*, iron, hydrogen peroxide, and sunlight) (Bokare & Choi, 2014; Matavos-Aramyan & Moussavi, 2017). The ubiquity of iron and hydrogen peroxide within environmental and biological systems also results in the natural occurrence of Fenton processes in these matrices. Therefore, it is important to gain a fundamental understanding of these processes due to their potential impacts. For example, Fenton processes may occur naturally within wastewater samples, which could impact remediation processes. The Fenton process may provide a green and cost-effective solution for

¹ Physical treatment processes - Involve the physical separation of solid particles from wastewater *via* filtration, sedimentation, or screening (Toprak, 2006).

²Chemical treatment processes - Involve using chemical reactions to improve water quality (*i.e.*, alters or degrades the contaminants within the water sample) *via* chlorination, ozonation, or coagulation (Toprak, 2006).

³ Biological treatment processes - Use microorganisms to metabolically degrade compounds within water samples (Toprak, 2006).

these processes without leaving a persistent environmental footprint. In this thesis, I explored Fenton processes and their utility for wastewater remediation.

1.2 Research Objectives

In this thesis, I attempted to provide a better understanding of the Fenton process, its underlying mechanisms, and its effectiveness for the remediation of organic pollutants within Boat Harbour wastewater. With a better understanding of these mechanisms, proper treatment strategies can be developed. For example, reaction products generated in these systems may be better at degrading certain contaminants, but not others. These systems could then be modified accordingly to potentially improve past issues associated with this type of treatment. To accomplish this, I developed the following research questions:

(1) Identify the specific compounds produced during the Fenton process responsible for the oxidation of organic compounds. This will be tested with different catalysts (*i.e.*, iron magnetic nanoparticles) and different ligands (*i.e.*, hemoglobin). It has long been assumed that $\text{OH}\cdot$ is the main compound produced during Fenton processes; however, other reactive oxygen species (ROS) (*e.g.*, singlet oxygen [$^1\text{O}_2$] and superoxide [O_2^-]) may play a larger role in these processes than previously thought. The quantity and types of ROS generated will influence the effectiveness of the Fenton process.

(2) The impact of various catalysts⁴ and ligands⁵ on the effectiveness of Fenton processes will also be explored under differing reaction conditions (*i.e.*, varied concentrations and varied

⁴ Catalysts are substances that increase the rates of chemical reactions.

⁵ Ligands are ions or molecules that bind to metal atoms to form coordination complexes with distinct chemical properties (*e.g.*, chemical reactivity).

pH values). When reaction conditions change, the mechanisms of Fenton processes can change dramatically, which is why these processes can be so difficult for researchers to comprehend.

(3) Fenton processes will be employed to degrade synthetic dyes (*i.e.*, methyl orange) within Boat Harbour wastewater effluent. This will determine how effective Fenton processes are at degrading organic micropollutants within a complex wastewater matrix. This matrix contains many contaminants generated from the pulp and paper process, which could interfere with the Fenton process. The knowledge gained from this research will help inform environmental management decisions and determine the feasibility of Fenton processes for wastewater remediation and other applications. If the ROS generated in these Fenton processes can be used to degrade compounds within the complex Boat Harbour effluent, it is very likely that this strategy could be used effectively to treat similarly or less polluted bodies of water around the world.

1.3 Hypotheses

The effectiveness of Fenton processes depends on reaction conditions, including the concentration of reagents (Fe^{2+} and H_2O_2), pH, reaction time, temperature, the initial concentration of contaminants, and the presence and concentration of ligands (Barbusinski, 2009). Under different reaction conditions, the reaction mechanisms will be altered (*i.e.*, different oxidizing species will be produced and at different rates), which will influence the effectiveness of these processes at oxidizing various organic compounds. Fenton processes have been accelerated in the presence of ligands; this has occurred in both environmental and biological settings (Salgado *et al.*, 2013). In environmental settings, these processes can lead to the degradation of wastewater pollutants or naturally occurring organics. In biological settings

the Fenton process can lead to the degradation of biomolecules (*i.e.*, lipids, proteins, and DNA). The Fenton process has been implicated in the aging process and various diseases, including cancer, cardiovascular disease, and neurological disorders (*e.g.*, Alzheimer's and Parkinson's disease) (Barbusinski, 2009; Prousek, 2007; Que *et al.*, 2008).

As the concentration of the Fenton reagents (Fe^{2+} and H_2O_2) increase, the rate of the Fenton reaction should also increase. With concentration, there will be a commensurate increase in the number of collisions between reactant molecules, increasing the formation of products such as Fe^{3+} , $\text{OH}\cdot$, or other ROS (*e.g.*, singlet oxygen [$^1\text{O}_2$], superoxide [O_2^-], or hydroperoxyl radicals [$\text{OOH}\cdot$]) which should oxidize organic compounds they contact. Consequently, increasing the production of ROS should increase the rate of the Fenton process. However, the oxidative power of the specific ROS generated may not be sufficient to oxidize specific recalcitrant organics. Therefore, additional treatment beyond Fenton reactions may be necessary for remediation projects. Fenton reactions may still be a good starting point for remediation projects because they are low-cost and can still remove organics that are resistant to conventional treatment methods; however, it may be more effective to use Fenton reactions in combination with other advanced treatment methods (*e.g.*, electrochemical oxidation). As mentioned earlier, the complexity of the wastewater matrix may also contribute to the success or failure of the remediation effort. This is an important point of consideration for the Boat Harbour wastewater treatment project, which is a very complex milieu.

Fenton processes should be most effective under acidic conditions (pH 3-5) as under these conditions, $\text{OH}\cdot$ are more active in the degradation of organic compounds (Duesterberg *et al.*, 2008). Insoluble iron oxide complexes (*aka* sludges) form as the pH increases past 5; these

complexes are not effective catalysts for Fenton processes (Brienza & Katsoyiannis, 2017; Salgado *et al.*, 2013). This pH dependence can be inconvenient as treated water should be at or near neutral pH. However, ligands and different catalysts (other than traditional iron) may increase the effectiveness of the Fenton process at higher pH values (Salgado *et al.*, 2013), which could prevent the need for neutralization agents (this will be discussed further in **Chapter 2**).

1.4 Methodological Overview

1.4.1 Materials

Chemicals used in all experiments were purchased from reputable chemical suppliers, including Sigma-Aldrich (Oakville, ON, Canada) and Thermo-Fisher (Waltham, MA, USA). Nanopure water (18.2 M Ω) was collected daily from a Barnstead Nanopure System to prepare solutions and experimental samples.

1.4.2 Equipment

UV-vis absorbance and fluorescence spectroscopy data were collected using a Tecan Infinite M1000 Pro microplate reader. Electron paramagnetic resonance (EPR) data was collected using a Bruker microEPR instrument. An infrared spectrometer (StellarNet Inc) was used to measure specific ROS produced in the system. Centrifuge tubes were used to prepare dye samples and chemical reagents. Polypropylene containers were used to collect water samples from Boat Harbour. Personal protective equipment was worn whenever onsite at Boat Harbour, including rubber gloves, steel toe work boots, safety glasses, and safety helmets (**Figure 1**).



Figure 1. Group members wore protective equipment when onsite at Boat Harbour. Geotube® filtrate was collected when onsite.

1.4.3 Trapping Experiments

The quantity and types of ROS generated by Fenton processes determines the effectiveness of these approaches at oxidizing organic compounds. To determine the specific quantities of different ROS produced within Fenton reactions, specific chemical trapping agents were required. Trapping agents will react selectively with specific ROS, which allows for accurate quantification. The trapping agent coumarin reacts selectively with $\text{OH}\cdot$ to produce umbelliferone (**Figure 2**), which will give off a fluorescence signal; this can be detected by setting the excitation and emission wavelengths on the microplate reader to 325 nm and 452 nm, respectively (Louit *et al.*, 2005). The reaction was prepared in a 200 μL volume within a

Nunclon 96-well flat bottom black microplate. This sample was continuously scanned at a regular time interval to quantify $\text{OH}\cdot$ production and determine the rate of $\text{OH}\cdot$ generation. Scans took place every 5 min over a 30-min period. Higher fluorescence signals corresponded to a greater production of $\text{OH}\cdot$.



Figure 2. Coumarin reacts with $\text{OH}\cdot$ to produce umbelliferone, which can be quantified by fluorescence measurements (Nosaka *et al.*, 2011).

Trapping agent 2,3-Bis(2-methoxy-4-nitro-5-sulfohenyl)-2H-tetrazolium-5-carboxanilide (XTT) was used to quantify the production of superoxide anion ($\text{O}_2^{\cdot-}$) as this compound is selectively reduced by $\text{O}_2^{\cdot-}$ to produce a soluble formazan (**Figure 3**), an orange product with peak UV-vis absorbance at 470 nm (Li *et al.*, 2012). The reaction was prepared in a 200 μL volume within a Costar 96-well flat bottom transparent microplate. This sample was also continuously scanned every 5 min over a 30-min period to quantify $\text{O}_2^{\cdot-}$ production and determine the rate of $\text{O}_2^{\cdot-}$ generation. Larger absorbance values corresponded to a larger production of $\text{O}_2^{\cdot-}$.

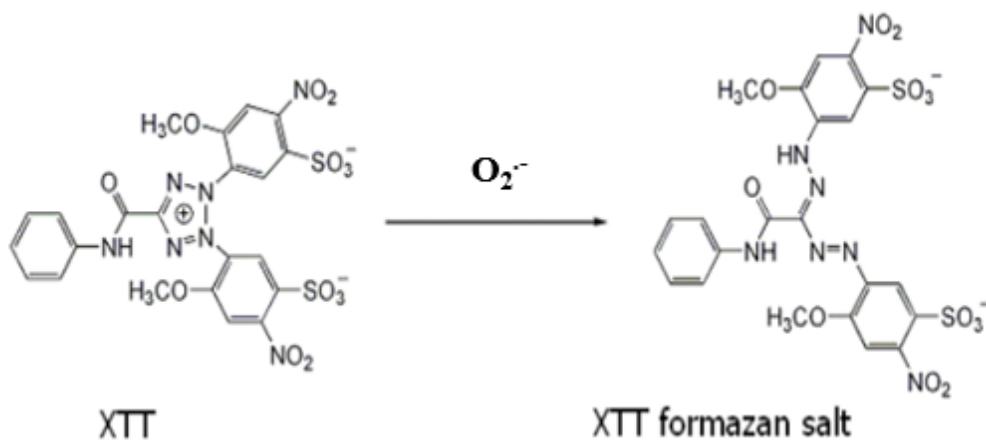


Figure 3. XTT reacts with O_2^- to form XTT formazan, which can be quantified by absorbance measurements (adapted from Sidorova *et al.*, 2009).

Additional specific trapping experiments were performed to quantify the generation of singlet oxygen ($^1\text{O}_2$) and they will be detailed in the relevant chapters.

1.4.4 Electron Paramagnetic Resonance (EPR) Analysis

EPR is a technique that identifies specific ROS produced during Fenton processes. The unpaired electrons of the different ROS will have a unique spin characteristic that can be detected using specific spin trapping chemicals. Distinct EPR signals are produced when specific oxidants are present within the sample. In this project, the chemical TEMP-OH (250 mM) was used as a spin trapping chemical. Depending on the type of ROS present within the sample, TEMP-OH will produce a specific identifiable signal (*e.g.*, $^1\text{O}_2$ can react with TEMP-OH to form TEMPO-OH [Figure 4]) (Carrier *et al.*, 2018; Wang *et al.*, 2015). 200 μL volumes for each reaction were prepared in EPR capillary tubes and scanned in the EPR instrument before and after treatment to determine the types of ROS produced during Fenton processes. This volume was comprised of the spin-trapping chemical (TEMP-OH), H_2O_2 , and the relevant catalyst or ligand at varying concentrations.

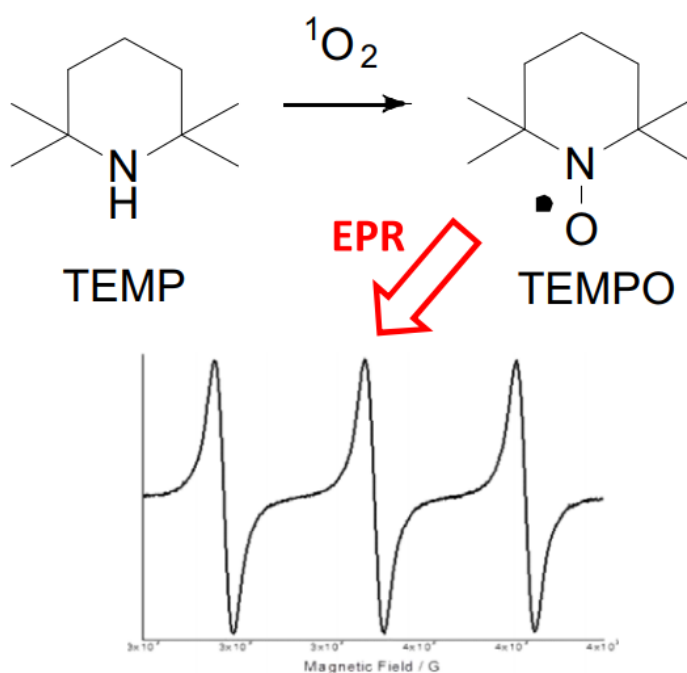


Figure 4. Singlet oxygen peaks that form during the production of TEMPO (these same peaks form during the production of TEMPO-OH) measured using EPR. (Nardi *et al.*, 2014).

1.4.5 Methyl Orange Degradation in Boat Harbour Wastewater Effluent

The commercial dye methyl orange was used as a model contaminant to assess the effectiveness of Fenton reactions at degrading organic compounds present within Boat Harbour wastewater effluent. Geotube® effluent collected from the Boat Harbour site was diluted 10-fold and used as the solvent for these experiments (**Figure 5**) (the Geotube® filtration process will be detailed in **Chapter 2**). Methyl orange was prepared in nanopure water before being transferred into the Geotube® effluent matrix. The methyl orange was subjected to either the Fe MNP- or Hb-catalyzed Fenton reaction and the UV-vis absorbance of the dye was measured at 464 nm before and after treatment. A greater reduction in UV-vis absorbance corresponds to a more effective treatment process. Methyl orange was used as its degradation can be easily quantified, which will help determine the effectiveness that Fenton processes may have at degrading organic

micropollutants in complex wastewater matrices. This is important because recalcitrant compounds may persist following the conventional wastewater treatment process (this will be detailed in **Chapter 2**).

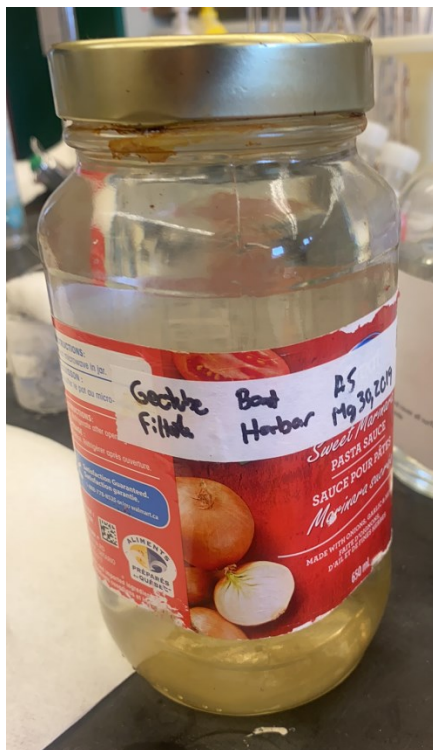


Figure 5. Geotube® effluent was collected from the Boat Harbour site. This was diluted 10-fold with nanopure water for methyl orange degradation experiments.

1.5 Thesis Layout

This thesis will consist of five chapters. In Chapter 1, I have provided an introduction and discussed why I have decided to research Fenton processes. I have also specified the research objectives for this thesis.

Chapter 2 will serve as a literature review highlighting the importance of water and effective wastewater remediation. I will discuss the history of Boat Harbour and the current state of the remediation project, as well as wastewater characteristics and conventional wastewater

treatment techniques. AOPs and Fenton processes will also be discussed in greater detail. Finally, ligands and singlet oxygen ($^1\text{O}_2$) will be discussed because of their importance to this project.

Chapter 3 will focus on my research, which utilized iron magnetic nanoparticles (Fe MNPs) as a catalyst for the Fenton reaction. I will highlight the importance of Fe MNPs in biological and environmental settings, the specific ROS generated within the system, and its applicability for wastewater remediation projects.

Chapter 4 will focus on my research, which utilized hemoglobin (an iron-porphyrin ligand complex) for the Fenton reaction. Similar to chapter 3, this chapter will showcase the relevance of hemoglobin in biological and environmental settings, ROS generation in Hb-catalyzed Fenton reactions, and its potential for wastewater treatment.

In Chapter 5, I will summarize the whole project and my main observations. I will also provide conclusions and recommendations for future research projects.

Chapter 2 Literature Review

2.1 Context - Importance of Water and Effective Remediation

Water is essential for life. It serves as the medium for all metabolic reactions, allowing our bodies to function properly by regulating body temperature, lubricating and protecting joints and tissues, and eliminating waste products via urination, defecation, and perspiration (Mayo Clinic, 2017). As the global population increases, demand for water will also increase. With increasing population are commensurate demands for water used in industrial processes including chemical, food, and paper production (Centers for Disease Control and Prevention, 2016). A report by Parry *et al.*, (2007) discusses how climate change will influence water demand and water quality. In certain regions, an increased frequency of heavy precipitation events and runoff may cause pathogens to enter the freshwater supply (Gleason & Fagliano, 2017). In other regions (*e.g.*, the American Southwest), longer and more severe periods of drought may reduce the water supply (National Oceanic and Atmospheric Administration, 2013). A warmer climate is also problematic because humans need more water during warmer periods to prevent dehydration (Mayo Clinic, 2018). The increased demand for water and the reduced quality and supply of water illustrates the need for effective wastewater remediation.

2.2 Types of Wastewater

There are three categories of wastewater: municipal, agricultural, and industrial. Municipal wastewater is comprised of sanitary sewage from homes and businesses, along with stormwater that drains from roofs, roads, and soil-covered land (Statistics Canada, 2015). Municipal wastewater also contains pharmaceuticals excreted from individuals, as does the sludge applied to agricultural land as a low-cost nutrient amendment. Pharmaceuticals pose risks

to human health and aquatic ecosystems as they are designed to be bioactive at very low concentrations and are resistant to conventional wastewater treatment methods (Kostich *et al.*, 2014; Srain *et al.*, 2021). Agricultural and industrial wastewater typically contains recalcitrant compounds (*e.g.*, PAHs) that are also resistant to conventional wastewater treatment technology; the remediation of this type of wastewater is expensive (Jeworski & Heinzle, 2000). A risk of increasing industrialization is the greater release of recalcitrant compounds into the environment (Babuponnusami & Muthukumar, 2014). It is important that these compounds are completely mineralized to CO₂ and H₂O because during the treatment process, reactive intermediates may form that are more toxic than the original organic compounds (Gulyas, 1997). For example, *o*- and *p*-benzoquinone are oxidized intermediates of the organic compound phenol that are more toxic than phenol itself (Inchaurreondo *et al.*, 2014). The chlorine bleaching of wood pulp can also produce very toxic chlorinated compounds (*e.g.*, dioxins and furans) that are often discharged back into the environment because of their resistance to established treatment methods (Environment Canada, 1991; Hoffman *et al.*, 2019). Following successful treatment, water should be at a quality (above Canadian Environmental Quality Guidelines) sufficient for release back into the environment. This will reduce health risks to humans and environmental ecosystems. Advanced oxidation processes (AOPs) (discussed in **Chapter 1**) have been effective at treating industrial wastewater effluent and toxic recalcitrant compounds (Collivignarelli *et al.* 2017; Matavos-Aramyan & Moussavi, 2017).

2.3 Boat Harbour

The former tidal estuary Boat Harbour is an important part of the culture of the Pictou Landing First Nation (PLFN) community. Referred to as A'Se'k, Boat Harbour was traditionally

used for food and medicine gathering and various recreational activities; however, the accumulation of hazardous pollutants in the impounded estuary following the addition of pulp mill effluent eliminated these traditional activities (Government of Nova Scotia, 2018b; Hoffman *et al.*, 2017; Quanz *et al.*, 2021a, 2021b). The historical rationale underlying these changes can be traced to 1964, when the government of Nova Scotia committed to growing the struggling eastern mainland rural economy by committing to a deal with a pulp company, allowing for the construction and operation of a pulp mill (Baxter, 2017; Quanz, 2019). During business negotiations, Boat Harbour was chosen as the location for the new mill to release its effluent. The PLFN chief and council raised concerns about the resulting odours and were taken to a similar mill in New Brunswick that was odourless to alleviate concerns; however, it was later discovered that the New Brunswick mill was not operating during that time (Baxter, 2017, Castleden *et al.*, 2016; Quanz, 2019).

Beginning in 1967, bleached kraft mill effluent (BKME) flowed from the pulp mill located at Abercrombie Point, near Pictou, Nova Scotia and accumulated in Boat Harbour (Government of Nova Scotia, 2018b). The Boat Harbour Effluent Treatment Facility (BHETF) (**Figure 6**); was constructed in 1972 to treat pulp mill effluent (Eichinger & Walker, 2020). BKME from the mill flowed through an underground pipe under East River, passing through Pictou Landing before entering one of two settling basins, allowing for sedimentation over a 12-h period (Eichinger & Walker, 2020; Hoffman *et al.*, 2015). The effluent was then transferred to an aeration basin for initial treatment (**Figure 7**); at this stage, the BOD of the waste was reduced (Government of Nova Scotia, 2018b). Following this treatment, the water entered into Boat Harbour where it remained for approximately three weeks to allow contaminants to settle to the

bottom of the water column before being eventually released into the Northumberland Strait over a dam structure (Government of Nova Scotia, 2018b). In addition to this, Boat Harbour accumulated wastewater from a chlor-alkali plant that operated from 1971 to 1992; this plant was responsible for producing chemicals for the pulp making process (*i.e.*, chlorine and sodium hydroxide) (Eichinger & Walker, 2020; Quanz, 2019; Quanz *et al.*, 2021a, 2021b).



Figure 6. Map of Boat Harbour Effluent Treatment Facility (BHETF) and its surrounding area (Hoffman *et al.*, 2017). Pictou Landings First Nation (PLFN), whose physical proximity and loss of traditional use of the Boat Harbour former tidal estuary are of primary concern to issues of water treatment, lies between the Northumberland Strait and the BHETF.



Figure 7. Aeration Stabilization Basin Located at Boat Harbour (Baxter, 2019).

The *Boat Harbour Act* (2015) was passed, at least in part, as a response to 47 million litres of raw pulp mill effluent leaking onto a sacred First Nations burial ground at Indian Cross Point following a pipe rupture in 2014 (Baxter, 2017; Castleden *et al.*, 2016; Hoffman *et al.*, 2017; Quanz, 2019; Quanz *et al.*, 2021a, 2021b). The province of Nova Scotia is committed to cleaning up the legacy of contamination at Boat Harbour and restoring it to its pre-1967 condition as a tidal estuary re-connected to the Northumberland Strait with an estimated cost exceeding \$292 million CAD (Eichinger & Walker, 2020).

There have been reports that the volume of unconsolidated sediment accumulated within Boat Harbour and surrounding wetlands over 50 years is approximately 577,000 m³ (Alimohammadi *et al.*, 2019; Alimohammadi *et al.*, 2020; Eichinger & Walker, 2020; Quanz *et al.*, 2021a, 2021b). Sediment analysis has revealed the presence of various contaminants including metals (As, Cd, Cr, Cu, Hg, Pb, and Zn) along with persistent organic contaminants (polycyclic aromatic hydrocarbons [PAHs], dioxins and furans); the concentration of some of these contaminants have been determined to be above toxic threshold concentrations (Hoffman *et al.*, 2017; 2019; Quanz *et al.*, 2021a). Treating residual Boat Harbour contaminated sediments is challenging, with suggestions these materials need to be dredged, dewatered, and properly disposed of to ensure successful remediation (Alimohammadi *et al.*, 2019; GHD, 2018). Geotextile dewatering was employed as a process to dewater contaminated Boat Harbour sediment on a pilot scale (**Figure 8**), to reduce the volume of the unconsolidated sediments and treat associated porewater prior to release (Alimohammadi *et al.*, 2019; Alimohammadi *et al.*, 2020; Tackley, 2019). This technique utilizes polymers to agglomerate suspended materials to enhance the effectiveness of filtration and consolidate the material. The geotextile fabric, which is configured as a tube (Geotube®) can also store sediment over the short-term while encouraging more complete de-watering prior to transport and disposal. The porewater intimately associated with contaminated sediments, as it leaches through the Geotube® fabrics, retains some persistent inorganic and organic pollutants, requiring advanced treatment. Adesida (2020), observed this and used an electrochemical filtration system to further treat Boat Harbour Geotube® filtrate. It is possible that Fenton processes may also provide a cost-effective solution to degrade persistent contaminants within Boat Harbour Geotube® leachate.



Figure 8. Pilot Geotube® de-watering system located just outside the Boat Harbour Aeration Stabilization Basin.

2.4 Kraft Process

The pulp and paper industry requires a significant amount of energy. For example, approximately 25% of the total industrial energy consumed in Canada is from the pulp and paper sector (Bonhivers & Stuart, 2013). The most common pulping process is known as the Kraft process, which was employed by the Abercrombie Point mill. Wood fibre, water, chemicals, and heat are the necessary ingredients required to make pulp⁶. The first step of the process involves

⁶ Pulp is a soft wet fibrous material formed from the breakdown of wood. It is the main intermediate material used to develop a variety of paper products (Bonhivers & Stuart, 2013).

the breakdown of lignin⁷ in wood chips to isolate individual cellulose and hemicellulose fibres (*aka* delignification) (Bonhivers & Stuart, 2013; Catalyst Paper, 2021; Murray, 1992). This process begins within a digester tank where sodium hydroxide and sodium sulfide (*aka* white liquor solution) are mixed with the wood chips under high temperature and pressure conditions (Costa *et al.*, 2009; Environmental Protection Agency [EPA], 1995; Quanz, 2019). Following this process, the mixture is transferred to an atmospheric tank known as a blow tank. In the blow tank, individual fibres become separated from the wood as a result of depressurization (Catalyst Paper, 2021; Quanz, 2019). During the washing stage, residual chemicals or dissolved lignin in the mixture are removed; unseparated wood fibres and other debris are also removed by screening or centrifugation (Catalyst Paper, 2021; EPA, 1995). The pulp mixture then undergoes the bleaching process. This process is used to make paper products white in colour.

Hypochlorite, chlorine dioxide, and alkali treatments are typically used during this process (Murray, 1992; Quanz, 2019). The use of chlorine compounds can lead to the formation of dioxins and furans (Hoffman *et al.*, 2019; Solomon, 1996). However, chlorine compounds are still used today because it helps to create a desirable bright white paper product. Before creating the paper, the pulp mixture enters a storage tank to dry and be pressed. Sheet formers are then used to convert the pulp mixture into sheets, which are dried and cooled before being bundled and shipped (Catalyst Paper, 2021; Costa *et al.*, 2009) although the exact process will differ depending on the mill, given some mills produce unbleached pulp for paper towels, or varying degrees of bleaching to achieve various kappa numbers for whiter hygienic or paper products.

⁷ Lignin is a strong organic polymer that acts as a glue to hold the wood fibres together. This makes them rigid and rot-resistant (Catalyst Paper, 2021).

Regardless, the pulp and paper industry generates large amounts of wastewater requiring treatment (Gopal *et al.*, 2019). 80 million litres of wastewater were previously discharged daily from the Abercrombie Point pulp mill in Nova Scotia (Campbell, 2019).

2.5 Characterizing Wastewater Samples

To characterize the quality of wastewater effluent, various parameters are often assessed, including colour, total suspended solids, salinity, pH, total organic carbon, and biochemical oxygen demand (Food and Agricultural Organization [FAO], 1992.; Jeworski & Heinzle, 2000). Analytical techniques, such as Gas Chromatography-Mass Spectrometry (GC-MS) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) are also important for assessing the specific contaminants present within effluents. Understanding each mill's processes and effluent characteristics is critical as it will provide valuable information about the most appropriate treatment method to be employed.

2.5.1 Odours

One of the major public concerns surrounding wastewater effluent is odours that can arise during the treatment process; the province of Manitoba even has guidelines in place to limit odour emissions to 5 D/T (dilutions/threshold) (Fries, 2016). This measurement refers to the amount of dilutions required to make an odour “non-detectable;” this can be measured with a device known as an olfactometer (St. Croix Sensory, Inc., 2021). Many of the odours from effluent come from hydrogen sulfide (H₂S), other sulfides, and volatile compounds (Dincer & Muezzinoglu, 2008). The intensity of an odour may also increase depending on weather conditions, *e.g.*, on hot or windy days (Howe, 2016). The solution to this problem may include confining the effluent into airtight tanks or adding chemicals to react with the odour-causing

compounds (Howe, 2016). People have complained about getting headaches as a result of the odours coming from Boat Harbour (Baxter, 2017).

2.5.2 Colour

One of the first characteristics of wastewater that is assessed is its colour. Typically treated/clean effluent will be clear and untreated/dirty effluent will be brownish. During wastewater treatment research, the degradation of coloured dye compounds can provide insight into the effectiveness of a wastewater treatment method. This degradation can be measured by assessing the ultraviolet-visible (UV-vis) absorbance of a dye sample pre- and post-treatment.

Dye samples will have a peak UV-absorption measurement at a specific wavelength (λ). For example, the peak absorbance wavelengths of the commonly used dyes methylene blue and methyl orange are 668 nm and 464 nm, respectively. Before treatment these samples would be bright blue and orange and have large absorbance values within their respective wavelengths. Post-treatment, the samples should become more transparent depending on the effectiveness of the process, more effective processes resulting in a greater reduction in the absorbance values. It should be noted that decolouration (reduced absorbance) may not necessarily result in a large degradation of contaminants in the samples. Methods such as TOC analysis, BOD analysis, GC-MS analysis, and ICP-MS analysis may provide a better representation of the effectiveness of a specific treatment method (discussed in more detail later).

2.5.3 Total Suspended Solids (TSS)

Total suspended solids (TSS) represent any particle larger than 2 μm in a wastewater sample; (1) inorganic compounds, including sand, silt, and clay, (2) microorganisms, including bacteria and algae, and (3) organic compounds, including material from decaying plants and

animals comprise the majority of TSS found in wastewater effluent (Fondriest Environmental, Inc., 2014a). The Canadian Council of Ministers of the Environment (CCME) set the water quality limit for TSS in effluent at 25 mg/L (CCME, 2009). Many of these suspended solids will settle to the bottom of a water body over time (Fondriest Environmental, Inc., 2014a). Cloudy or opaque water turbidity may be attributable to microorganisms, inorganic (*e.g.*, clay or silt) or organic compounds. UV-absorption measurements may be taken to assess the turbidity/optical density of a wastewater sample pre- and post-treatment, which specifically measure contributions from coloured dissolved organic matter (CDOM) or the humic stain, the coloured product formed from the release of tannins by decaying plant material (Fondriest Environmental, Inc., 2014a). Pathogenic microorganisms may pose the most potential risks out of all TSS; these may be removed with chemical treatment (*e.g.*, chlorination) or UV irradiation (Al-Gheethi *et al.*, 2018). Other suspended solids should be removed during various stages of the conventional wastewater treatment process.

2.5.4 Salinity

The total concentration of salt within a wastewater sample refers to its salinity. Dissolved mineral salts may include calcium, magnesium, chloride, sodium, sulfate, and potassium (City of Farmington, 2013). Since salt particles are dissolved, it is difficult to remove them during the water treatment process (City of Farmington, 2013). High salt concentrations have also been shown to reduce the efficiency of biological wastewater treatment processes (Wu *et al.*, 2008).

If effluent with a high salt concentration is applied to the land, it will increase the osmotic pressure of the soil and make it harder for plants to obtain the water they need from the soil; this may reduce the growth and survival of plants and result in significant economic consequences

(FAO, 1992). Salt ions that are harmless to humans may have a severe impact on plants (FAO, 1992). Wastewater samples with high salt concentrations will have high electrical conductivities due to the high concentration of ions able to conduct electrical flow (FAO, 1992.; Fondriest Environmental, Inc., 2014b). A salinity meter can be used to assess the electrical conductivity of a water sample and ultimately its salt concentration. Physical separation techniques may be effective at reducing salt concentrations in wastewater, but it is currently an expensive procedure (Chen *et al.*, 2018).

2.5.5 pH

pH refers to how acidic (pH below 7) or basic (pH above 7) a solution is. It is important to consider pH, as either strongly acidic or caustic effluents may be hazardous to aquatic ecosystems and alter the solubility, reactivity, or toxicity of specific chemicals present (Fondriest Environmental, Inc., 2014c). Carbon dioxide (CO₂) plays a large role in determining the acidity of water; the levels of CO₂ may be altered naturally or because of human-induced influences (Fondriest Environmental, Inc., 2014c). The pH of the effluent may also impact the effectiveness of the wastewater treatment process (Theobald, 2012) so various chemicals may be added to adjust the pH of the sample. However, it is difficult to say what additional effects may result from the addition of these chemicals and their overall impact on treatment costs.

2.5.6 Total Organic Carbon (TOC)

Total organic carbon refers to the total concentration of carbon (excluding simple carbon oxides [*e.g.*, CO and CO₂] and cyanides) in a wastewater sample. Samples are injected into a TOC analyzer and the instrument quantifies the total TOC present within the sample. The first step of the TOC process is acidifying the sample to pH 2 with hydrochloric acid, and then adding

nitrogen or helium to remove any CO₂ from the sample (Hocking, 2005). A small volume of this sample will then be injected into the analyzer where any water or volatile organics are vaporized at 200°C. Following this, volatilized organic compounds are oxidized to CO₂ and then converted to methane in the presence of hydrogen; this methane is then quantified using a flame ionization detector to give the TOC measurement (Hocking, 2005). Following successful treatment, the TOC present within the samples should decrease depending on the effectiveness of the treatment, the most effective processes yielding the greatest TOC reduction. It should be noted that this process does not give information regarding the types of organic compounds present within the sample; this could be assessed using GC-MS.

2.5.7 Biochemical Oxygen Demand (BOD)

The biochemical oxygen demand refers to the amount of oxygen required by aerobic bacteria to breakdown the organics present within a wastewater sample. Similar to TOC, more effective treatment processes will be better able to reduce BOD levels of samples. BOD is environmentally significant because higher BOD levels means that oxygen will be more rapidly depleted from the water body (EPA, 2012). Therefore, BOD can be determined by taking a water sample and incubating it for 5 d, then quantifying the remaining dissolved oxygen in a water sample with a dissolved oxygen meter or *via* titration techniques (EPA, 2012). Samples with a high BOD should have low amounts of residual dissolved oxygen. Similar to TOC, this measurement provides no information regarding the types of organics present in the sample, which needs to be assessed by GC-MS.

2.5.8 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is a technique that can be used to identify the specific organic compounds present within wastewater samples. Samples get placed into GC-MS capillary tubes for analysis. Organic compounds have specific peaks that allow them to be identified using GC-MS. The strength of this signal (*i.e.*, height of the peak) corresponds to the abundance of that compound within the sample. Effective remediation will involve reducing or completely eliminating the intensity of these signals. However, new peaks may form following treatment indicating the formation of intermediates, which may even be more toxic than the parent compounds (Quispe *et al.*, 2018).

2.5.9 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

ICP-MS is a technique to identify specific metals present within wastewater samples, such as mercury, arsenic, or cadmium which pose potential human health and aquatic ecosystem risks at Boat Harbour. Similar to GC-MS, metals in wastewater samples will have specific peaks that can be measured by ICP-MS. In Canada, different metals have different maximum acceptable concentrations allowable in drinking water (CARO Analytical Services, 2019). Common techniques to remove metals from wastewater effluent include precipitation, adsorption, and filtration (Hargreaves *et al.*, 2018). These metals may then be collected from the contaminated site for disposal at an approved facility (*e.g.*, at a landfill site). Effective remediation will involve completely eliminating metals from wastewater or more realistically, reducing them to concentrations below established quality guidelines.

2.6 Conventional Wastewater Treatment

In Canada, industrial wastewater effluent must be treated to reduce contaminant values below specific federal and provincial water quality guidelines prior to environmental release. Wastewater discharge regulations are industry specific, with pulp and paper, petroleum, electrical generating stations, iron and steel production, chemical manufacturing, and mining operations all adhering to specific regulations and monitoring regimes (Government of Ontario, 2019). This approach is prudent as different industrial processes will produce different contaminants posing unique threats to ecosystem health, so are regulated accordingly. CCME guidelines are in place to assess the risk of contaminants to biological organisms through interim sediment quality guidelines (ISQGs) and probable effect levels (PELs). When contaminant concentrations in the sediment are below ISQGs they rarely impact biological organisms. When concentrations are between ISQGs and PELs they sometimes impact organisms. And when concentrations are greater than PEL they often impact organisms (CCME, 1999; Walker *et al.*, 2015). For example, dioxins and furans produced during the pulp and paper process, have an ISQG value of 0.85 pg/g and a PEL value of 21.5 pg/g (CCME, 2001; Hoffman *et al.*, 2019; Quanz *et al.*, 2021a). In Boat Harbour, many dioxins and furans exceeded PEL thresholds, which could be disastrous to local ecosystems (Eichinger & Walker, 2020; Hoffman *et al.*, 2019; Quanz *et al.*, 2021a).

Industrial wastewater effluent can undergo varying levels of treatment including preliminary, primary, secondary, and tertiary treatment processes to remove potentially hazardous contaminants, varying with effluent toxicity, receiving environment characteristics

and dilution capacity, and regulatory regime. Advanced Oxidation Processes (AOPs) can be employed, if necessary, to remove recalcitrant compounds following conventional treatment.

2.6.1 Preliminary Treatment

In preliminary wastewater treatment, bar screens are often used to filter out large objects and non-degradable materials. These large objects can interfere with the treatment process and cause damage to the equipment and pumps of the wastewater treatment plant (EPA, 2003; Toprak, 2006). Large non-degradable materials may include: paper, plastics, clothing, and metallic compounds (EPA, 2003) although larger objects such as ropes and even shovels have been reported (Ken Oakes, *personal communication*, June 20, 2021). Grit chambers and mesh screens are also used to filter waste; this process helps eliminate sand-like particles that could interfere with the treatment process (National Research Council [NRC], 1996). After the removal of larger material, which is landfilled, the effluent moves on to the primary treatment process. Combined sewer systems are typical of older neighbourhoods in urban areas and were designed to collect municipal and industrial wastewater, but also urban stormwater runoff in the same pipes (EPA, 2013) which then transport all wastewater to the treatment plant. Combined sewer overflows (CSOs) may occur during periods of heavy rain or snowmelt if the volume of wastewater exceeds the capacity of the treatment system, with excess water discharged into nearby lakes, rivers, streams, and other bodies of water without undergoing treatment. This problem may become more significant as a result of climate change and an increase in the number of storm events (Levy *et al.*, 2018; Gleason & Fagliano, 2017).

2.6.2 Primary Treatment

Primary wastewater treatment is a physical process. During this process, effluent moves through primary tanks very slowly to allow heavier solids to settle to the bottom of the tank; this process leads to the formation of a sludge layer, which will be pumped to a digester (NRC, 1996; Toprak, 2006). Lighter compounds, including fats, oils, and grease can move to the top and create a scum layer, which can be removed and further treated along with the sludge (Saravanane *et al.*, 2014).

Coagulants (*e.g.*, iron or aluminum salts) may also be added during the primary treatment process as their positive charges attract negatively charged compounds in wastewater effluent causing the particles to coagulate. The coagulate (or floc) can be filtered out or it can settle to the bottom of the primary treatment tank and be removed with the sludge layer for further treatment (Safe Drinking Water Foundation, 2017).

2.6.3 Secondary Treatment

Secondary wastewater treatment is a biological process. Effluent moves into an aeration tank where aerobic microorganisms degrade the organics in the effluent to form CO₂ and other products (NRC, 1996). Following this process, microorganisms can combine to form a biomass floc, which can be collected. This may be known as biological sludge or return activated sludge, which may return back to the aeration tank to begin the secondary treatment process again (NRC, 1996; Toprak, 2006). A trickling filter is often used during secondary treatment. This is a fixed rock or gravel bed where wastewater effluent is periodically added by a sprinkler and aerobic microorganisms embedded in the filter degrade organics in the wastewater (Tilley *et al.*, 2014).

2.6.4 Tertiary Treatment

In tertiary wastewater treatment, any remaining particles get filtered out using a sand filter and then the remaining effluent undergoes disinfection. Chlorine is often added into the wastewater to kill microorganisms, similar to swimming pools, but at a much higher concentration. Residual chlorine may be toxic to receiving environment organisms, so it is often removed with sodium bisulphite prior to discharging into the environment (Napa Sanitation, 2021). Irradiation with ultraviolet light or ozonation are also frequently used for disinfection (NRC, 1996; Toprak, 2006).

It should be noted that there is great variability in the types of wastewater treatment processes employed from region to region. The characteristics of the effluent can also provide information regarding the type of treatment that should be employed.

2.7 Fenton and Photo-Fenton Processes

The Fenton reagent is a solution first developed by the British chemist H.J.H Fenton and described in his seminal paper *Oxidation of tartaric acid in presence of iron* (Fenton, 1894). This solution consists of hydrogen peroxide (H_2O_2) and a ferrous iron (Fe^{2+}) catalyst. Equations (1) and (2) comprise the traditional Fenton process (Barb *et al.*, 1951; Barbusinski, 2009). The oxidation⁸ of Fe^{2+} by H_2O_2 leads to the production of ferric iron (Fe^{3+}), a hydroxyl radical ($\text{OH}\bullet$), and a hydroxide ion (OH^-) (Equation [1]). H_2O_2 can also reduce Fe^{3+} to Fe^{2+} , a hydroperoxyl radical ($\text{OOH}\bullet$) and a proton (H^+) (Equation [2]). The regeneration of Fe^{2+} is necessary for the continuous production of $\text{OH}\bullet$, which can oxidize organics within wastewater. However, this

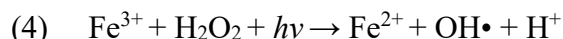
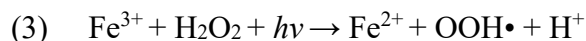
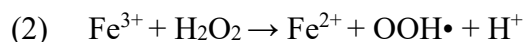
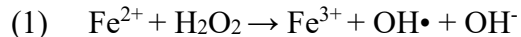
⁸ Oxidation - The removal of an electron from an atom or molecule.

reduction⁹ reaction is very slow, which limits the production of OH• in the Fenton process, which is the radical required for the oxidation of organic compounds (Pham *et al.*, 2009). Thus, the effectiveness of the Fenton process is limited by Fe³⁺ reduction (Equation [2]).

The photo-Fenton process is also described by equations (1) and (2), however, this process uses a UV light energy source for the photoreduction of Fe³⁺ (Equations [3] and [4]) (Ameta *et al.*, 2018) making the photo-Fenton process more effective than the traditional Fenton-process for the oxidation of organics (Ameta *et al.*, 2018). A major drawback of Fenton and photo-Fenton processes is that they are only effective under acidic conditions. Under neutral or alkaline conditions, iron oxide complexes form that are not effective catalysts for Fenton processes (Brienza & Katsoyiannis, 2017; Salgado *et al.*, 2013). The use of UV light in the photo-Fenton process may also interfere with nitrogen-containing compounds in wastewater, which could lead to the production of hazardous nitrogen species (Brienza & Katsoyiannis, 2017). Photo-Fenton experiments using UV-lamps or solar simulators are also more expensive than the standard Fenton process (Hansson *et al.*, 2012) for areas receiving limited sunlight (Babuponnusami & Muthukumar, 2014). However, other areas closer to the equator could employ photo-Fenton processes in water treatment applications year-round. Babuponnusami & Muthukumar (2014) provide a detailed review of wastewater treatment utilizing Fenton and photo-Fenton processes and their respective success rates (briefly, both processes were effective at degrading specific organics, including phenol (Yalfani *et al.*, 2009; Martínez *et al.*, 2005) and catechol (Lofrano *et al.*, 2009).

⁹ Reduction - The addition of an electron to an atom or molecule.

Equations



2.7.1 Ligands and the Fenton and Photo-Fenton Processes

Salgado *et al.* (2013) details specific ligands that have increased the effectiveness of the Fenton process, *e.g.*, vitamin C (Kang *et al.*, 2012) and EDTA (Šnyrychová *et al.*, 2006). Oxalate increased the effectiveness of the photo-Fenton process (Aplin *et al.*, 2001; Xiao *et al.*, 2014). The formation of ligand-metal complexes will influence the reaction mechanisms of the Fenton and photo-Fenton processes (*i.e.*, different ROS can be produced). A study by Manenti *et al.* (2015) showed the effectiveness of using iron-ligand complexes to treat textile wastewater samples. In this study, oxalate was the most effective ligand for the degradation of organic dyes within water samples. The effectiveness of this treatment was measured by comparing the dissolved organic carbon (DOC)¹⁰ in the samples pre- and post-treatment. More research needs to be performed assessing the influence of ligands on Fenton processes because it is a relatively unexplored area of study that is of significant importance in biological and environmental systems.

Previous research by the Zhang research group has shown that chloride can be used as a ligand to accelerate the copper-based Fenton-like processes (Shan *et al.*, 2016). Chloride can

¹⁰ Dissolved Organic Carbon (DOC) - measures the total concentration of carbon dissolved within a water sample.

bind with copper (I) and (II) to form a ligand-metal complex. The formation of these complexes will stabilize the metals and accelerate the metal-catalyzed decomposition of H_2O_2 by lowering the activation energy¹¹ of this reaction (Perez-Benito, 2001). A follow-up study by Carrier *et al.* (2018) showed that chloride accelerated the copper-Fenton process because of the increased production of singlet oxygen ($^1\text{O}_2$). This study showed that $^1\text{O}_2$ is the main ROS produced in the copper-based Fenton-like process. Another study by Carrier *et al.* (2019) showed that $^1\text{O}_2$ is the main ROS produced in the traditional iron-based Fenton reaction; however, chloride did not have an effect on the reaction rate. These studies contradict the previous assumptions that $\text{OH}\cdot$ was the main ROS produced. $\text{OH}\cdot$ is considered the most effective and important oxidant for wastewater treatment (Krishnan *et al.*, 2017) and Buxton (1988) provides rate constants for over 3500 reactions of $\text{OH}\cdot$ with other molecules. Other ROS can be produced in the iron-Fenton process and have been measured, including $^1\text{O}_2$ (Agnéz-Lima *et al.*, 2012) and superoxide anion (O_2^-) (Waite, 2002), which may oxidize organic pollutants. Despite evidence other ROS were produced, $\text{OH}\cdot$ was assumed to be the main ROS produced in the iron-Fenton system although these assertions are controversial and strongly debated (Barbusinski, 2009). Clarifying ROS produced under Fenton systems are difficult to quantify due to their short lifetimes (in the millisecond range) and further complicated under different reaction conditions, including when ligands are present. It is in the light of these constraints that researchers continue to struggle to understand the details of Fenton processes, first reported over 120 years ago. However, it is important to optimize Fenton processes for the effective remediation of wastewater pollutants,

¹¹ Activation Energy - The minimum amount of energy required for a reaction to occur between chemical reagents.

and gain a mechanistic understanding of their resultant ROS because of their implications in the environment and within biological systems, *e.g.*, ROS-mediated disease states (Prousek, 2007). The knowledge generated from these mechanistic studies could also help researchers improve approaches utilizing Fenton techniques, which may have been unsuccessful previously.

2.7.1.1 Types of Ligands

The type of ligand that is present in solution will also influence the Fenton process. Certain ligands are more likely to complex with either Fe(II) but the majority form complexes with Fe(III) due to its larger charge density and stronger bonds (*e.g.*, deferiprone will only form a complex with Fe(III)) (Liu *et al.*, 2002).

Organic and inorganic ligands can impact the products of the Fenton process. Different ROS can be formed in the presence of different ligands, *e.g.*, carbon-centered radicals can be formed when organic ligands are present and chlorine radicals can be formed when chloride is in the system. These radicals could potentially increase the efficiency of the Fenton process for oxidizing certain compounds (pro-oxidants) or decrease the efficiency (antioxidants) (Pignatello *et al.*, 2006; Salgado *et al.*, 2013). In Salgado *et al.* (2013), it is reported that certain ligands can be an antioxidant or prooxidant depending on the reaction conditions (*e.g.*, catecholamines and vitamin C). This information is important in both environmental contexts (*e.g.*, wastewater treatment) and biological contexts (*e.g.*, developing effective medications). There are several ligands that can participate in Fenton reactions and much more research needs to be conducted to gain a better understanding of these systems.

2.7.2 pH Effect

In traditional Fenton Systems the optimum pH is approximately 3, which is dependent on the speciation of Fe (II) and Fe (III) (Pignatello *et al.*, 2006; Salgado *et al.*, 2013). In the absence of ligands, under acidic conditions Fe(II) forms a complex with water to form the hexaquo ion $(\text{Fe}[\text{H}_2\text{O}]_6)^{2+}$ and this is typically referred to as free Fe^{2+} , which is effective at reacting with H_2O_2 . As the pH increases beyond 3, iron (III) hydroxides (*aka* sludge) tend to form, which will precipitate out of solution and reduce the efficiency of the process (Pignatello *et al.*, 2006; Salgado *et al.*, 2013). Ligands help to stabilize the metals over a wide pH range, which increases the solubility of the metals and improves the efficiency of the Fenton reaction (Fischbacher *et al.*, 2017; Sun & Pignatello, 1992). However, at highly basic pH values (*e.g.*, $\text{pH} > 9$), ligands can completely chelate the metal and prevent it from reacting in the Fenton process (Salgado *et al.*, 2018).

2.8 Singlet Oxygen ($^1\text{O}_2$)

Singlet oxygen ($^1\text{O}_2$) is a high energy form of molecular oxygen (O_2). $^1\text{O}_2$ is more reactive than O_2 and can therefore oxidize a larger number of organic compounds (*e.g.*, $^1\text{O}_2$ may be used to degrade dioxins and furans) (Zeinali *et al.*, 2019). However, it is not as strong an oxidant as $\text{OH}\cdot$ (Krumova & Cosa, 2016). $^1\text{O}_2$ is commonly formed through the use of photosensitizers, *e.g.*, Rose Bengal (DeRosa & Crutchley, 2002). This process can be exploited for cancer treatment as photodynamic therapy where photo-generated $^1\text{O}_2$ reacts with and kills tumor cells (Liang *et al.*, 2012). $^1\text{O}_2$ can also be used in industrial settings for the synthesis of specific compounds (*e.g.*, ascaridole - a compound found in different drugs) (Choi & Jung, 2016;

Günther *et al.*, 1944; Wasserman & Ives, 1981) or for the removal of biofilms (Wang *et al.*, 2017).

$^1\text{O}_2$ exists naturally in the environment and is formed primarily from photosensitizing compounds (Larson & Marley, 1999). It will form at a greater rate in water bodies containing large amounts of photosensitizers (*i.e.*, coloured bodies of water) and during periods with increased sunlight (*i.e.*, the summer) (Zepp *et al.*, 1977). Therefore, wastewater containing photosensitizing compounds may naturally generate $^1\text{O}_2$, which can have implications on the treatment process.

Our research group has shown ways to generate $^1\text{O}_2$ (Carrier *et al.*, 2018; Carrier *et al.*, 2019) using Fenton processes. It is also possible that other Fenton catalysts can produce it and other ligands may accelerate its production. Due to its important role in a variety of different disciplines, a better understanding of $^1\text{O}_2$ and how it is generated is essential. It would also be useful to see which catalysts are the most effective.

2.9 Additional Advanced Oxidation Processes (AOPs) for Wastewater Treatment

2.9.1 Electrochemical Oxidation

Electrochemical oxidation is another type of AOP that could be used to help degrade recalcitrant compounds in wastewater. In this process, ROS are generated through electrochemical means (Asghar *et al.*, 2015; Cruz-González *et al.*, 2012). For example, in electro-Fenton reactions, H_2O_2 can be generated at the cathode *via* oxygen reduction. This H_2O_2 can then react with a Fenton catalyst to generate ROS. The generation of H_2O_2 in this system is more cost-effective than traditional Fenton processes that require the purchase and subsequent transport of H_2O_2 . Relatively inexpensive electrodes, *e.g.*, carbon felt can also be used with great

efficiency (Brillas *et al.*, 2009). In this system, Fe^{2+} is also continuously regenerated on the cathode, which should increase the rate of the Fenton process and the rate of ROS generation. Many factors influence this process, including, the surface area of the electrodes, pH, temperature, and the initial concentration of the catalyst (Brillas *et al.*, 2009).

2.9.2 Photocatalysis

Photocatalysis is also another promising wastewater treatment technology. During this process, light-energy can excite a semi-conductor (*e.g.*, titanium dioxide) to produce an electron-hole pair, which can undergo further reactions to produce ROS (Ameta *et al.*, 2003). This type of treatment is relatively inexpensive and easy to learn, making it deployable in rural communities and developing countries without the need for rigorous education and training, which may not be readily available.

2.10 Mixtures

The complexity of wastewater should not be underestimated, nor the dynamic composition of industrial effluents that often change seasonally with climate, or over shorter or longer timeframes due to influent characteristics or industrial or municipal processes. When performing analysis on wastewater samples, the presence of other chemicals may interfere with the quantification of specific contaminants (Snyder, 2019). The combinatory (synergistic) effect of different chemicals together may also make the current water quality guidelines inadequate. For example, many different estrogens may bind to one common receptor and result in negative consequences even though the concentration of each of those specific contaminants is below water quality guidelines (Snyder, 2019).

It is important to note that certain chemicals may not have even been tested before they are put on the market, which makes it hard to say what effect they will have on the water samples or how they will interact with other chemicals (Snyder, 2019). This is concerning because the risks to human health and the environment may not be known. It is also possible that certain chemicals will inhibit the decomposition of others (Ken Oakes, *personal communication*, June 20, 2021). In the future, more research needs to be performed on matrix effects especially with the rising issue of pharmaceuticals and active pharmaceutical ingredients (APIs) in wastewater effluent (Kostich *et al.*, 2014).

Chapter 3 Singlet Oxygen Generation in Iron Magnetic Nanoparticle-Catalyzed Fenton Reactions

Role of the co-authors:

- David Oakley, MES candidate who performed the research, collected and analyzed experimental data, and wrote this chapter about this ongoing research project.
- Dr. Saher Hamid, Dr. Andrew Carrier, and Dr. Collins Nganou assisted with scientific concepts, experimental design, data collection, writing guidance, and general advice.
- Dr. Ken Oakes, Dr. Tony Walker, and Dr. Xu Zhang served as the co-supervisors of David Oakley who assisted with scientific concepts, provided experimental advice, project expectations, editing, and general advice

3.0 Abstract

Iron magnetic nanoparticles (Fe MNPs), including magnetite (Fe_3O_4) can be employed as Fenton catalysts. Fe MNP-catalyzed Fenton reaction is more effective at lower pH values as more Fe^{2+} and Fe^{3+} ions are released from nanoparticles *via* hydrolysis to participate in Fenton reactions. Although generation of reactive oxygen species (ROS) by Fe MNPs may be useful in the degradation of various organic compounds, the specific ROS generated by these processes was previously unclear. In this investigation, I was motivated to determine the main ROS produced in Fe MNP-catalyzed Fenton reactions. The evidence demonstrates that the main ROS generated is singlet oxygen ($^1\text{O}_2$) which contrasts previous studies suggesting the main ROS generated was hydroxyl radical ($\text{OH}\cdot$). The primacy of $^1\text{O}_2$ rather than ($\text{OH}\cdot$) as the main ROS in Fe MNP-catalyzed Fenton reactions could have important biological implications due to its more moderate reactivity and longevity, making this radical more suitable for applications such as cancer treatment. This information is critical to all researchers who employ these processes. Iron nanoparticles are also present within the natural environment and their presence, and ability to produce ROS, could impact a variety of ecosystems. The low cost of Fe MNP-generated ROS could serve as a viable green alternative for wastewater remediation projects. Experiments were performed to assess the effectiveness of Fe MNP-catalyzed Fenton reactions in degrading the commercial dye methyl orange within wastewater effluent collected from Boat Harbour, a legacy contaminated site in Nova Scotia; however, these experiments were unsuccessful. In the future, modifying the reaction conditions or using this process in combination with another treatment method (*e.g.*, electrochemical oxidation) may yield better results. Nevertheless, this research provided a better understanding of a naturally occurring process which could be employed for a variety of applications requiring generation of $^1\text{O}_2$.

3.1 Introduction

Iron magnetic nanoparticles (Fe MNPs) are versatile compounds that can be used for a variety of applications, including cancer diagnostics (Yoo *et al.*, 2011), water treatment (Gutierrez *et al.*, 2017), and biofilm removal (Gao *et al.*, 2014). Fe MNPs are often utilized because of their stability, large surface area, and low toxicity (Natarajan *et al.*, 2019). Fe MNPs contain a mixture of Fe²⁺ and Fe³⁺ ions, both of which can participate in reactions, including the Fenton reaction. Iron nanoparticles are also found in the natural environment; therefore, a better understanding of their chemical properties and how they react with naturally occurring compounds could help guide environmental management decisions (von der Heyden *et al.*, 2019). For example, these reactions and the generation of reactive oxygen species (ROS) may negatively impact organisms living in specific ecosystems (Kieber *et al.*, 2003).

It has been previously assumed that hydroxyl radicals (OH•) were the main ROS produced in Fe MNP-catalyzed Fenton reactions (Moura *et al.*, 2005). My group's previous work has shown that singlet oxygen (¹O₂) is the main ROS generated in the traditional iron-based Fenton reaction (Carrier *et al.*, 2019). Therefore, I hypothesized it would also be the main ROS generated in this system, which similarly employs an iron-based catalyst. The mechanism for the Fe MNP-catalyzed Fenton reaction should be analogous to the mechanism for the traditional iron-based Fenton process as the Fe²⁺ and Fe³⁺ ions in the nanoparticles should react with H₂O₂ to generate the same ROS. It is important to confirm this because of the prevalence of Fe MNPs in a variety of biological and environmental settings. Fe MNPs may also be more effective catalysts than traditional iron, which may improve their utility depending on the application. The aim of this study was to determine the specific ROS generated in the Fe MNP-catalyzed Fenton

process. In this chapter, evidence will be presented demonstrating $^1\text{O}_2$ is the main ROS generated in Fe MNP-catalyzed Fenton reactions.

The rate of the Fe MNP-catalyzed Fenton reaction should be higher at lower pH values as Fe ions will be more reactive *via* the formation of more reactive iron-ligand complexes (**Detailed in Chapter 2**). This should result in greater ROS generation at low pH values (Carrier *et al.*, 2019). At lower pH values, a larger proportion of Fe ions should also be released from the nanoparticles to react with H_2O_2 in the solution *via* hydrolysis¹² and generate ROS (Huang *et al.*, 2016). Aside from the varied pH experiments, all additional experiments were performed at pH 5.5. In this investigation, evidence will be presented that shows that the rate of Fe MNP-catalyzed Fenton reactions and the generation of ROS is greater at low pH values.

3.2 Materials and Methods

3.2.1 Reagents

Commercially available Fe-magnetic nanoparticles (Fe_3O_4) were purchased from US Research Nanomaterials (Houston, TX, USA). MES buffer, 3,3',5,5'-tetramethylbenzidine (TMB), o-phenylenediamine (OPD), 2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT), coumarin, sodium azide (NaN_3), beta-carotene, tert-butyl alcohol (TBA), dimethyl sulfoxide (DMSO), 1,3 diphenylisobenzofuran (DPIBF), 4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMP-OH), and methyl orange were purchased from Sigma Aldrich (Oakville, ON, Canada). Hydrogen peroxide was obtained from Thermo Fisher Scientific (Waltham, MA, USA). A Barnstead Nanopure water system (Thermo Scientific, Waltham, MA, USA) was used to collect fresh nanopure water ($18.2 \text{ M}\Omega \text{ cm}$) daily; this was used as the main

¹² Hydrolysis – Breakdown of a compound through a chemical reaction with water.

solvent for all experiments with the exception being the methyl orange degradation experiments. These experiments used Boat Harbour Geotube® filtrate as the solvent. Geotube® effluent was collected from the Boat Harbour site. All experiments were performed at pH 5.5 in 1 mM MES buffer aside from the methodically varied pH experiments and the methyl orange degradation experiment, which took place at pH 3. The pH of the Geotube® filtrate was adjusted using hydrochloric acid (HCl).

3.2.2 Equipment

Absorbance and fluorescence experiments were performed using a Tecan Infinite M1000 Pro microplate reader (Männedorf, Switzerland). All experiments were performed at 25°C. Costar 96-well flat transparent polystyrene microplates (Corning, NY, USA) were used for all absorbance experiments. Nunclon 96-well flat black polystyrene microplates (Thermo Fisher, Waltham, MA, USA) were used for all fluorescence experiments.

3.2.3 TMB Oxidation and OPD oxidation

TMB oxidation was used as a colorimetric assay to assess the rate of the Fe MNP-catalyzed Fenton reaction. TMB is oxidized non-selectively to form a blue product known as ox-TMB, which can be quantified photometrically at 652 nm (Shan *et al.*, 2016). The concentrations of Fe MNP and H₂O₂ were adjusted accordingly to properly assess the concentration-dependent nature of the reaction. Sodium azide, beta-carotene, coumarin, TBA, and XTT were used as ROS scavengers. These compounds will selectively react with a specific ROS (*e.g.*, sodium azide reacts selectively with ¹O₂); this identifies the specific ROS produced in the Fe MNP-catalyzed Fenton reaction. For example, if TMB oxidation decreases with increasing concentrations of sodium azide, this means ¹O₂ is produced in the Fe-MNP catalyzed Fenton reaction and

responsible for TMB oxidation. The sodium azide reacts with $^1\text{O}_2$, which means it is unavailable to oxidize TMB. If the reaction rate for TMB oxidation stays the same with increasing concentrations of sodium azide, then $^1\text{O}_2$ is not produced in the system, but rather a different ROS was produced and responsible for TMB oxidation.

OPD oxidation was also used as a colorimetric assay to assess the rate of the Fe MNP-catalyzed Fenton reaction. OPD is oxidized non-selectively to form a yellow product known as ox-OPD, which can be quantified photometrically at 452 nm (Carrier *et al.*, 2019). The same chemical scavengers used in the TMB experiments were used in the OPD experiments.

3.2.4 DPIBF Conversion

1,3-Diphenylisobenzofuran (DPIBF) is a fluorescent compound that will react selectively with $^1\text{O}_2$ to form an endoperoxide¹³, which is subsequently converted to o-dibenzoylbenzene (DBB) (Carloni *et al.*, 1993; Carrier *et al.*, 2019; Krieg, 1993). The formation of DBB will reduce the fluorescence intensity of the DPIBF probe; greater reductions in fluorescence intensity correspond to higher levels of $^1\text{O}_2$. The excitation and emission wavelengths for DPIBF were set at 410 nm and 455 nm, respectively.

3.2.5 Coumarin Oxidation

Coumarin will react with $\text{OH}\cdot$ to form umbelliferone, which is a fluorescent product that can be measured at excitation and emission wavelengths of 325 and 452 nm, respectively (Carrier *et al.*, 2019; Manevich, 1997; Leandri *et al.*, 2019). This experiment will measure the amount of $\text{OH}\cdot$ produced in the Fe MNP-catalyzed Fenton reaction. Higher fluorescence intensity values correspond to higher levels of $\text{OH}\cdot$.

¹³ endoperoxide – A bond between two oxygen atoms within a larger compound.

3.2.6 XTT Reduction

XTT is selectively reduced by the superoxide radical ($O_2^{\cdot-}$) to form an orange coloured formazan, which can be quantified photometrically at an absorbance of 470 nm (Carrier *et al.*, 2019; Sutherland & Learmoth, 1997). This experiment will measure the amount of $O_2^{\cdot-}$ generated in the Fe MNP-catalyzed Fenton reaction. Higher absorbance values correspond to higher levels of $O_2^{\cdot-}$.

3.2.7 Methyl Orange Degradation

The commercial dye methyl orange was used as a model contaminant to assess the effectiveness of the Fe MNP-catalyzed Fenton reaction at degrading organic compounds present within Boat Harbour wastewater effluent. Geotube® effluent that was collected at the Boat Harbour site was diluted 10-fold and used as the solvent for these experiments (**Figure 5**). The pH of the diluted Geotube® effluent was adjusted to pH 3 with HCl. The UV-vis absorbance of the dye was measured at 464 nm before and after treatment. The reaction between H_2O_2 and Fe_3O_4 ran 15 min in a microcentrifuge tube prior to centrifugation. Centrifugation will allow the nanoparticles to settle to the bottom of the tube, so that the UV-vis absorbance of the methyl orange (supernatant) can be quantified.

3.3 Results and Discussion

3.3.1 Singlet Oxygen (1O_2) Generation

The highly selective chemical probe 1,3-diphenylisobenzofuran (DPIBF) was used to assess the generation of singlet oxygen (1O_2). 1O_2 quencher molecules, *i.e.*, sodium azide and beta carotene were also used to confirm that 1O_2 is the main ROS produced in the Fe MNP catalyzed Fenton reaction. TMB and OPD colorimetric assays were first employed to assess the

rate of total ROS generation in the Fe MNP-catalyzed Fenton reaction, which increased with respect to the concentration of Fe MNP or H₂O₂ (Figure 9). TMB and OPD oxidation decreased with increasing concentrations of sodium azide and beta carotene (Figure 10). This suggests that ¹O₂ is a major product formed in the Fe MNP-catalyzed Fenton reaction. These results are similar to the results achieved when traditional iron was used as a Fenton catalyst (Carrier *et al.*, 2019).

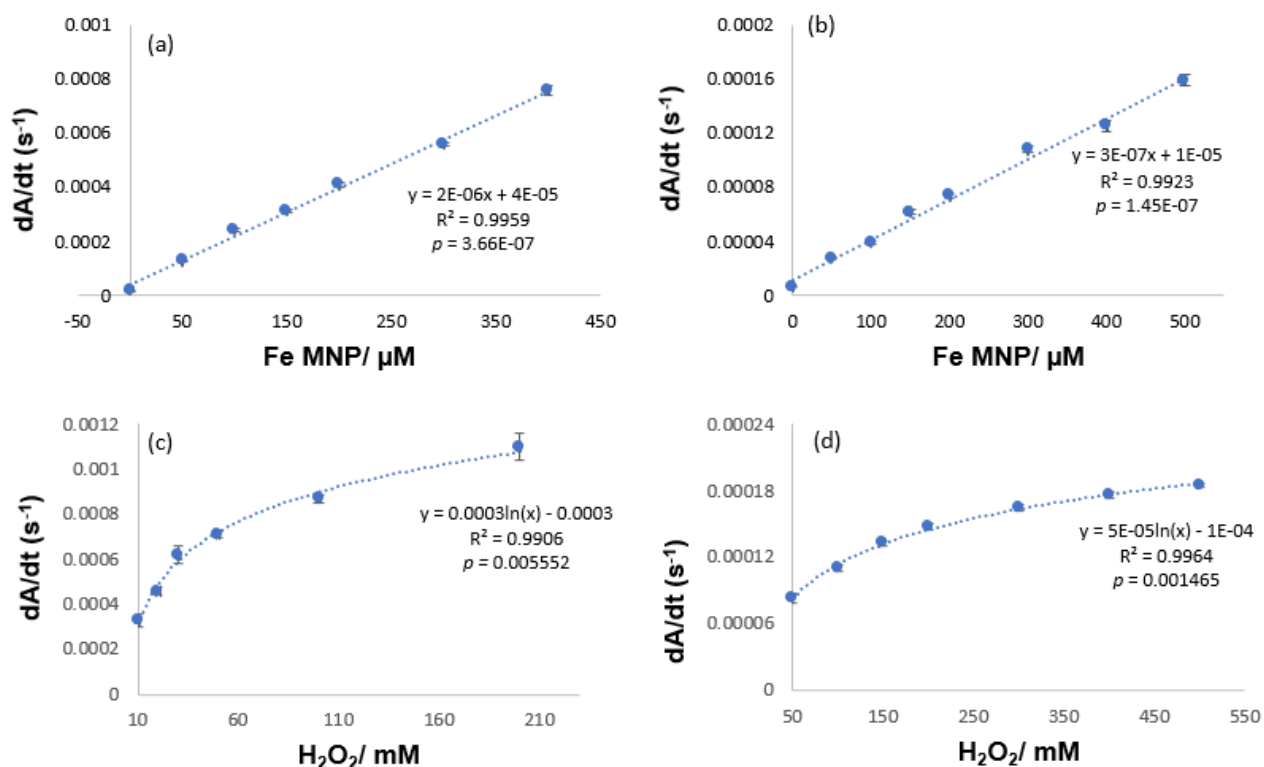


Figure 9. TMB (a and c) and OPD (b and d) oxidation with varying concentrations of Fe MNP (a-b) and H₂O₂ (c-d). [Fe MNP] = 200 μ M for TMB oxidation (c) and 500 μ M for OPD oxidation (d), [MES pH 5.5] = 1 mM, [TMB and OPD] = 500 μ M, [H₂O₂] = 200 mM for TMB oxidation (a) and 50 mM for OPD oxidation (b).

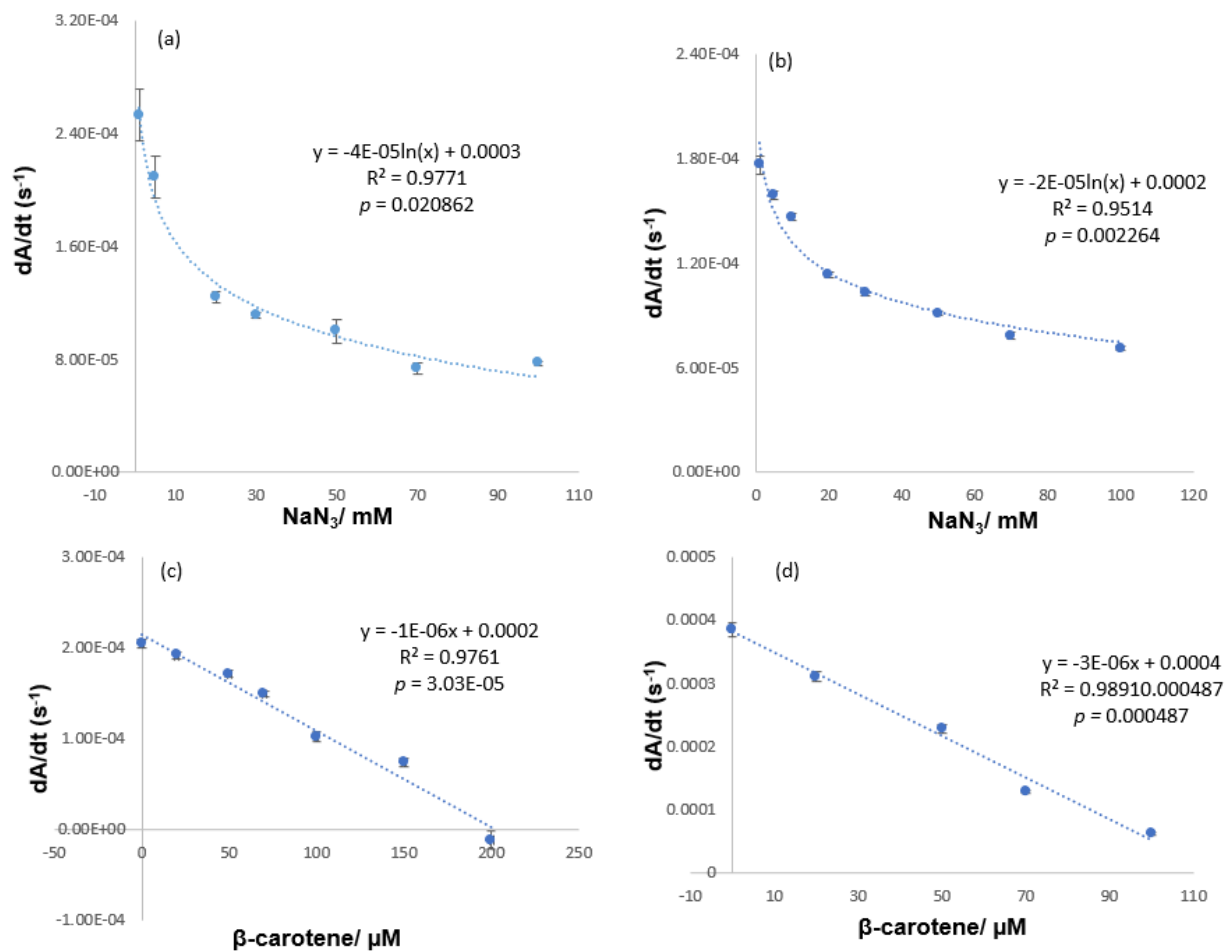


Figure 10. TMB (a and c) and OPD (b and d) oxidation with varying concentrations of sodium azide (a-b) and β -carotene (c-d). $[\text{Fe MNP}] = 200 \mu\text{M}$ for TMB oxidation (a and c) and $500 \mu\text{M}$ for OPD oxidation (b and d), $[\text{MES pH } 5.5] = 1 \text{ mM}$, $[\text{TMB and OPD}] = 500 \mu\text{M}$, $[\text{H}_2\text{O}_2] = 200 \text{ mM}$ for TMB oxidation (a and c) and 500 mM for OPD oxidation (b and d).

DPIBF conversion experiments were performed to assess the rate of $^1\text{O}_2$ formation. The conversion of DPIBF to DBB is facilitated by $^1\text{O}_2$, which corresponds to a decrease in the fluorescence intensity of DPIBF. There was a slight decrease in DPIBF fluorescence with Fe MNP alone; however, there is a dramatic decrease when H_2O_2 was added to the system (**Figure 11**), further demonstrating the Fe MNP-catalyzed Fenton reaction generates $^1\text{O}_2$.

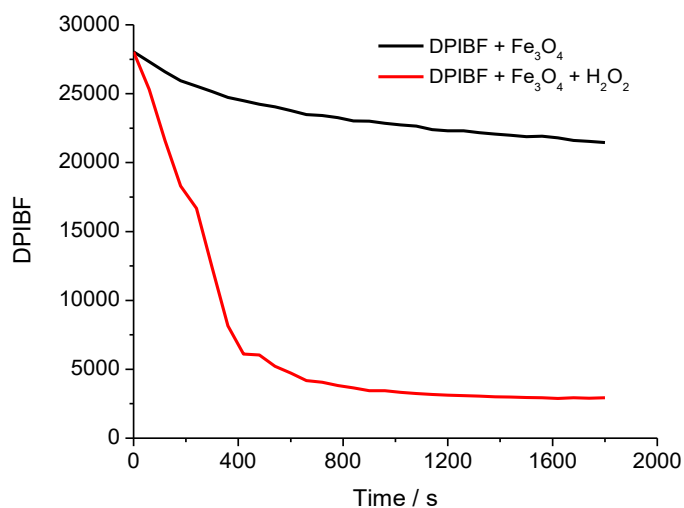


Figure 11. DPIBF conversion. [Fe MNP] = 200 μ M, [MES pH 5.5] = 1 mM, [DPIBF] = 1 mM, [H₂O₂] = 200 mM.

3.3.2 Hydroxyl Radical (OH•) and Superoxide Anion (O₂⁻) Generation

The reaction rate for both TMB and OPD oxidation assays were independent of the presence of the OH• radical scavenger TBA (**Figure 12**), suggesting no significant production of OH•. Coumarin was used as a highly selective probe to assess the generation of hydroxyl radicals (OH•) within the system. Similarly, the highly selective probe XTT was used to assess the generation of superoxide anion (O₂⁻). With increasing concentrations of Fe MNP and H₂O₂, the rate of coumarin oxidation increased (**Figure 13**), indicating some generation of OH•. However, OH• are not likely to be the main ROS generated in this system as previously assumed since both TMB and OPD oxidation assays were not inhibited in the presence of the OH• scavenger TBA, whereas the reaction was inhibited in the presence of ¹O₂ scavengers.

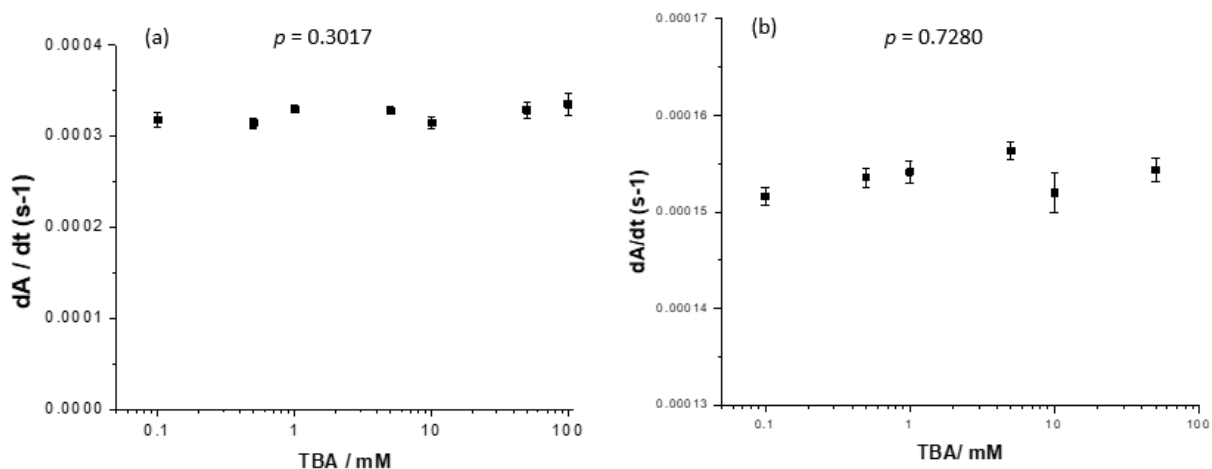


Figure 12. TMB (a) and OPD (b) oxidation with varying concentrations of tert-butyl alcohol. [Fe MNP] = 200 μM for TMB oxidation (a) and 500 μM for OPD oxidation (b), [MES pH 5.5] = 1 mM, [TMB and OPD] = 500 μM , [H_2O_2] = 200 mM for TMB oxidation (a) and 500 mM for OPD oxidation (b).

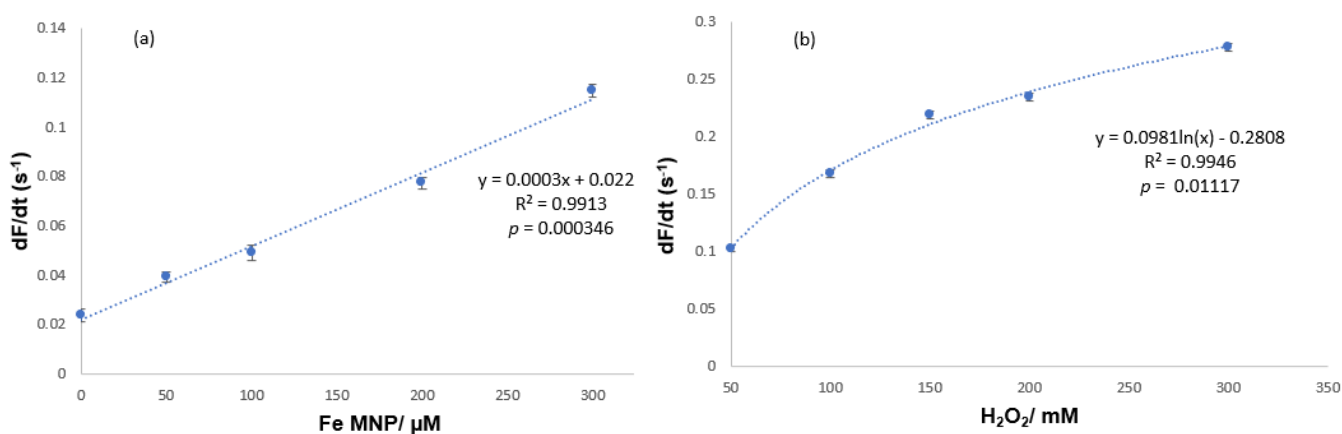


Figure 13. Coumarin oxidation with varying concentrations of Fe MNP (a) and H_2O_2 (b). Fe MNP = 200 μM for (a), [MES pH 5.5] = 1 mM, [coumarin] = 100 μM , [H_2O_2] = 200 mM for (b).

The rate of XTT reduction was very small at pH 5.5; however, it increased with increasing pH values (**Figure 14**). There are two possible explanations for this: (1) O_2^- is a stronger reducing agent at higher pH values and (2) O_2^- is more likely to be displaced from the iron it was bound to at higher pH values (Armstrong *et al.*, 2013; Carrier *et al.*, 2019).

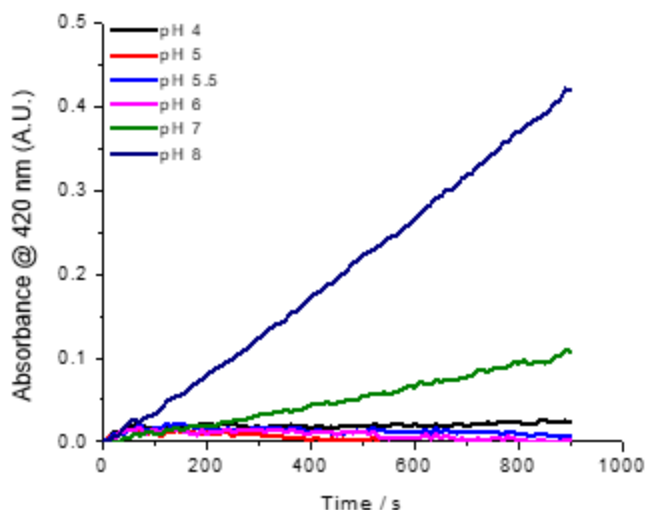


Figure 14. XTT reduction at varied pH values. [Fe MNP] = 200 μ M, [MES at all pH values] = 1 mM, [DPIBF] = 1 mM, [H₂O₂] = 200 mM.

3.3.3 pH Effects

The rate of the Fe MNP-catalyzed Fenton reaction for both TMB and OPD oxidation assays were higher at lower pH values (**Figure 15**), supporting the hypothesis that more ROS are generated at lower pH values. Coumarin oxidation also increased at lower pH values, which indicates that more OH• are generated under acidic conditions (**Figure 16**). OH• is also a stronger oxidant under acidic pH conditions (Duesterberg *et al.*, 2008). It is likely that OH• is an intermediate in the formation of ¹O₂ (Carrier *et al.*, 2019; Moffett & Zika, 1987). XTT reduction was greater at higher pH values; however, the O₂⁻ is still likely produced in higher amounts at lower pH values. O₂⁻ is undetectable at lower pH values because at these values it is not a great reducing agent and is less likely to be displaced from iron atoms (Armstrong *et al.*, 2013; Carrier *et al.*, 2019). O₂⁻ is another likely intermediate produced in the formation of ¹O₂ (Carrier *et al.*, 2019; Moffett & Zika, 1987). Unreactive iron hydroxide complexes also form as the pH increases, which are unable to act as effective Fenton catalysts; these insoluble complexes may

precipitate out of the solution or adsorb on MNP surfaces (Brienza & Katsoyiannis, 2017; Salgado *et al.*, 2013). The lifetime of H₂O₂ also increases at low pH, which would improve its effectiveness (Jung *et al.*, 2008).

Figure 17 shows that more iron ions leach into solution at lower pH values (with a few exceptions). In this experiment, Fe MNPs were placed in MES buffer at different pH values and centrifuged. The supernatant was then used as the catalyst for the oxidation of TMB *via* the Fenton reaction. More leaching (*i.e.*, release of iron ions from the nanoparticles *via* hydrolysis) can influence the rate of the Fenton process because it will allow compounds to be oxidized that have a low affinity to the nanoparticle surface (Lee *et al.*, 2013; Scheres Firak *et al.*, 2018). However, iron ions on the surface of the nanoparticles will also be affected by pH (*i.e.*, form different iron-ligand complexes, such as hydroxide), which will influence surface ROS generation *via* the Fenton process. The Fe MNP-catalyzed Fenton process was more effective at lower pH values, which supports this hypothesis.

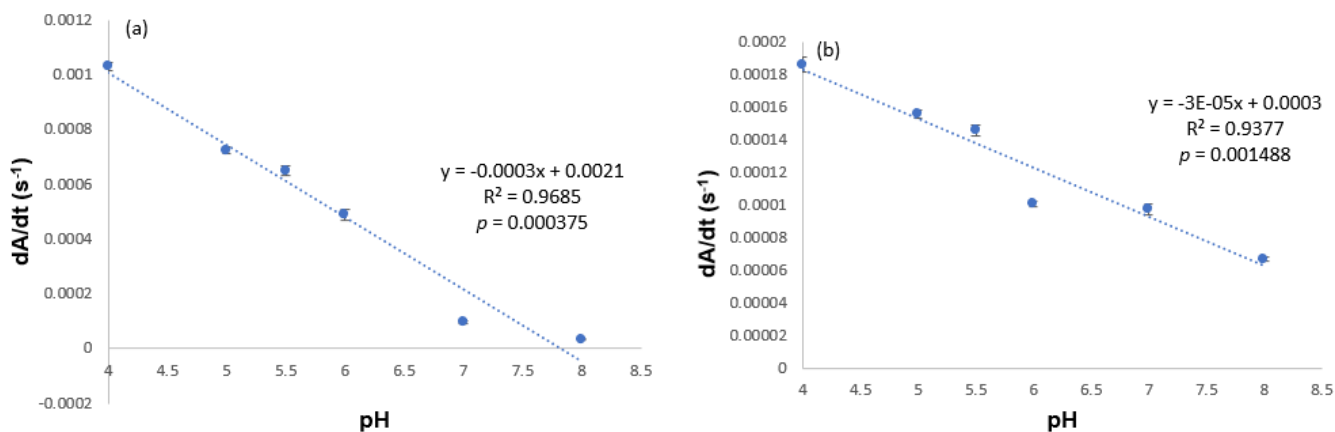


Figure 15. TMB (a) and OPD (b) oxidation with varying pH values. [Fe MNP] = 200 μ M for TMB oxidation (a) and 500 μ M for OPD oxidation (b), [MES at all pH values] = 1 mM, [TMB and OPD] = 500 μ M, [H₂O₂] = 200 mM for TMB oxidation (a) and 500 mM for OPD oxidation (b).

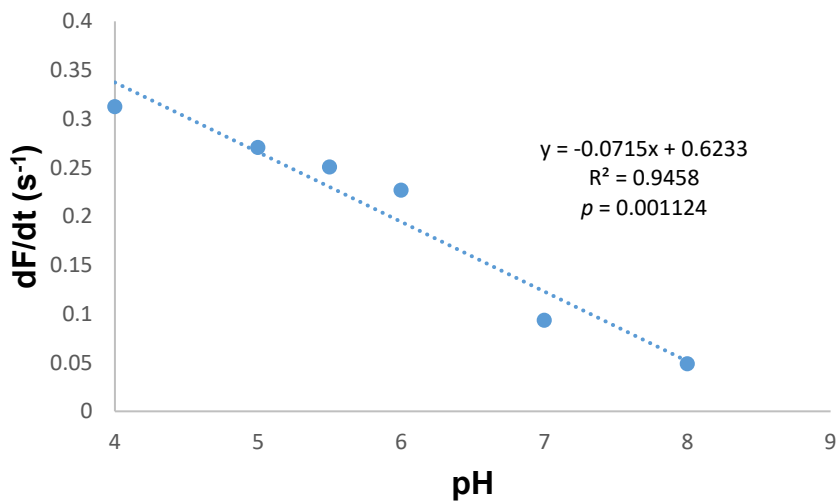


Figure 16. Coumarin oxidation at varied pH values. [Fe MNP] = 200 μ M, [MES at all pH values] = 1 mM, [coumarin] = 100 μ M, [H₂O₂] = 200 mM.

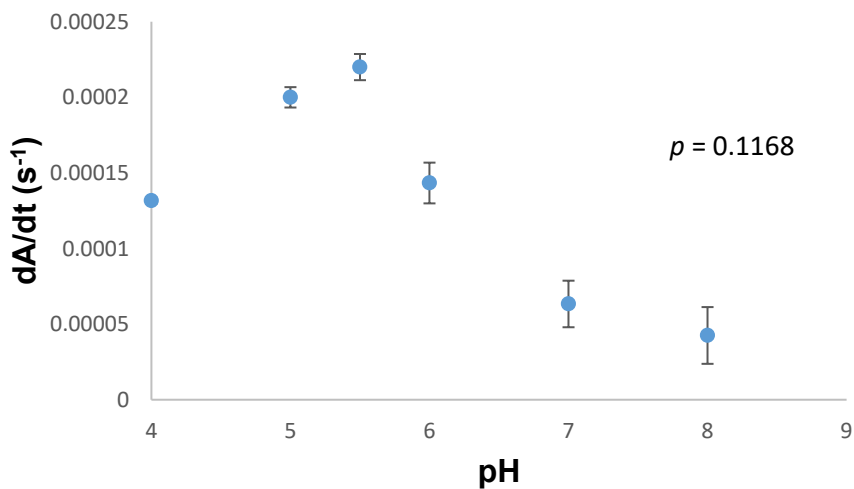


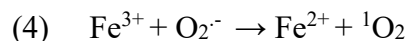
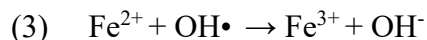
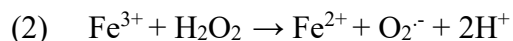
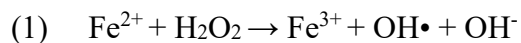
Figure 17. Leaching test for TMB oxidation at varied pH values. [Fe MNP] = 200 μ M, [MES at all pH values] = 1 mM, [TMB] = 500 μ M, [H₂O₂] = 200 mM.

3.4 Proposed Mechanism

Iron ions can be released by iron magnetic nanoparticles *via* hydrolysis and participate in the Fenton reaction by reacting with H₂O₂ (Huang *et al.*, 2016). Iron ions on the surface of the nanoparticles will also participate in Fenton reactions. The main ROS generated during this process is ¹O₂. The generation of OH• and O₂^{•-} were also observed in this investigation. Both ROS serve as reaction intermediates. The mechanism for the Fe MNP-catalyzed Fenton reaction should be analogous with the mechanism for the tradition iron Fenton system, since the iron ions released from the nanoparticles will react with H₂O₂ in the same manner. In the future, research should be performed to assess the surface chemistry of the iron nanoparticles and determine how that influences the reaction mechanism; however, the result should still indicate that ¹O₂ is the main ROS produced.

The mechanism proposed for the Fe MNP-catalyzed Fenton reaction (**Figure 18**) is consistent with the mechanism proposed by Moffett & Zika (1987); however, in their paper they claimed the formation of oxygen when it should be ¹O₂ (Eq 4) (Carrier *et al.*, 2019).

Equations



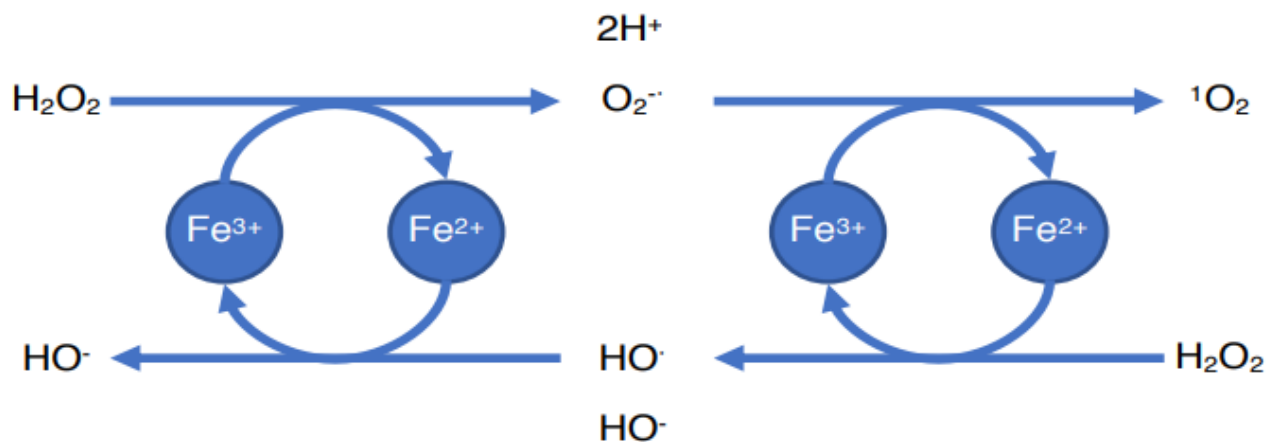


Figure 18. The proposed mechanism for the Fe MNP-catalyzed Fenton reaction has been shown to be analogous to the mechanism for the traditional iron-based Fenton reaction (Carrier *et al.*, 2019).

3.5 Methyl Orange Degradation Result

The trial experiments for methyl orange degradation were unsuccessful (samples stayed orange) with the Fe MNP catalyst (**Figure 19**). These experiments were performed at pH 3 (the pH value most likely to achieve a positive result) and were still unsuccessful. The complicated Boat Harbour wastewater matrix will contain many interfering compounds that could prevent the degradation of methyl orange. These compounds could bind with the catalyst and prevent the initiation of the Fenton reaction, or they could scavenge the ROS generated in the system. An experiment was also performed that showed that the Fe MNP did not adsorb methyl orange to its surface (**Figure 20**). If methyl orange did adsorb to the Fe MNPs, the absorbance would decrease with increasing concentrations of Fe MNPs. If this process succeeded, any reduction in UV-vis absorbance would be due to the Fenton reaction degrading the methyl orange. The goal of this research is to ultimately achieve complete mineralization and degrade pollutants into CO_2 and

H₂O. If methyl orange simply adsorbed to the nanoparticles, the pollutants would be removed from solution, but would require further processing for mineralization.

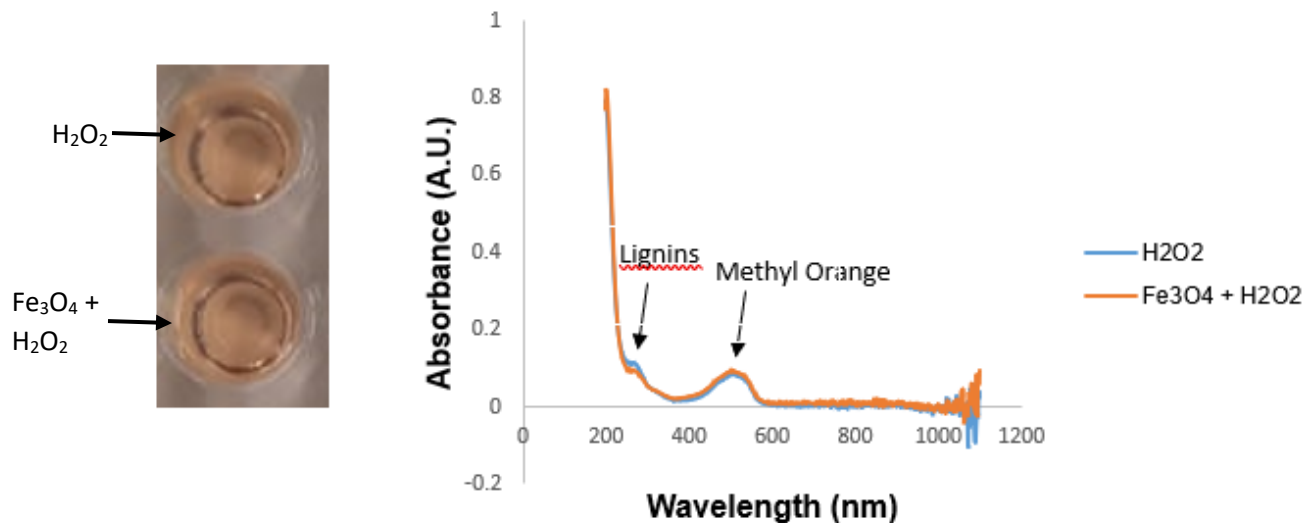


Figure 19. The Fe MNP-catalyzed Fenton reaction was unsuccessful at degrading methyl orange within Boat Harbour Geotube® effluent that was diluted 10-fold and adjusted to pH 3 using hydrochloric acid (HCl). The reaction was performed with and without Fe MNP. [Fe MNP] = 200 μ M, [Methyl Orange] = 10 μ M, [H₂O₂] = 1 mM.

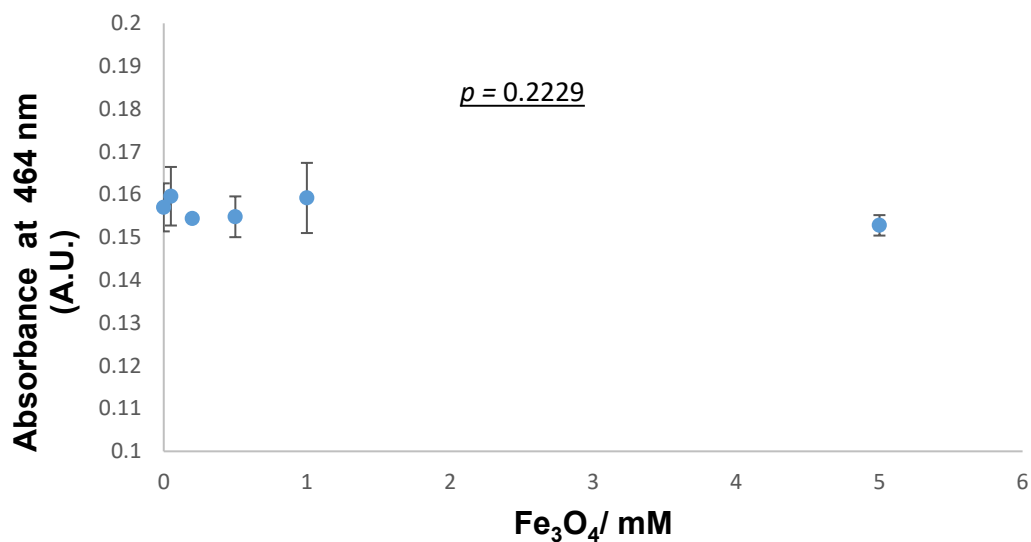


Figure 20. Methyl orange does not adsorb to iron magnetic nanoparticles. [Fe MNP] = varied, [Methyl Orange] = 10 μ M.

3.6 Research Limitations

This research faced certain limitations, which need to be highlighted. EPR and IR spectroscopy were attempted to further quantify the ROS produced, however, these failed due to the properties of the Fe magnetic nanoparticles. These experiments need to be performed at low temperatures, which could not be completed with my lab setup (Collins Nganou, *personal communication*, December 15, 2020). In the future, it would be valuable to obtain this information.

This investigation also did not assess the surface chemistry of the iron nanoparticles, which could be essential in determining the underlying mechanisms of the Fenton process. To get a proper sense of these chemical properties, outside analysis would be required, which was beyond the research budget. The XTT experiment is also limited in accurately assessing the production of $O_2^{\cdot-}$ in the system. This research was also performed at pH 5.5, which may not be

reflective of how these reactions proceed in different environmental settings. The chemical properties of the commercial nanoparticles can also differ slightly from nanoparticles within the natural environment. In the environment, there would be many uncontrollable variables, which could influence these reactions. However, based on the data collected from this investigation, I can confidently predict that $^1\text{O}_2$ will be the main ROS produced in the system.

3.7 Viability for Environmental-Based Research

The economic viability of a research project needs to be considered prior to implementation. One goal of this research was to assess whether the Fe MNP-catalyzed Fenton reactions could provide a cost-effective solution for the remediation of organic micropollutants within the Boat Harbour wastewater matrix. The attraction of using Fe MNP-catalyzed Fenton reaction is that it may provide an environmentally friendly and inexpensive water treatment strategy. Iron-magnetic nanoparticles were purchased commercially from US Research Nanomaterials, Inc (Houston, TX, USA) for \$89 USD for 100 g. Hydrogen peroxide (30% v/v) was purchased from Sigma Aldrich (Oakville, ON, Canada) for a price of \$107 CAD for 500 mL. To perform this research on a larger scale and at a lower cost, iron nanoparticles could be synthesized from mine wastewater, readily available locally from Cape Breton mines (Razanamahandry *et al.*, 2020). However, based on my experimental results, the use of Fe MNPs may be ineffective if released iron ions are quickly bound by organics within complex matrices, such as Boat Harbour effluent. Although the nanoparticles adsorb some pollutants from the wastewater, thereby cleaning the matrix they are deployed in, the adsorbed contaminants are not mineralized. Adsorbed contaminants would have to be dealt with at a later time, using a secondary approach, functionally negating any theoretical advantages of this system. In a bigger

context, although the Fe MNP-catalyzed Fenton reaction was unsuccessful at degrading methyl orange within Boat Harbour effluent, important knowledge was generated, which will help all researchers who employ these processes. Fenton reactions within the natural environment could be generating significant amounts of singlet oxygen, which could be harmful to certain organisms due to its greater longevity and commensurate dispersal from point of generation than OH•, the presumed primary ROS produced. A more viable wastewater treatment strategy is the Hb-catalyzed Fenton process, which will be detailed in **Chapter 4**.

3.8 Conclusion

I have presented evidence in this investigation that shows $^1\text{O}_2$ is the main ROS generated in Fe MNP-catalyzed Fenton reactions, contrasting the previous assumption that OH• was the main ROS. The Fe MNP-catalyzed Fenton reaction was unable to successfully degrade methyl orange, which is likely the result of the iron ions binding to the organics present within the Boat Harbour effluent.

The mechanism associated with the Fe MNP-catalyzed Fenton reaction is complex; however, understanding it is vital to applying this technology for medicine and water treatment among other applications. A better understanding of these processes will improve current Fenton research approaches. Therefore, I was motivated to determine the main ROS associated with the Fe MNP-catalyzed Fenton reaction. My results demonstrate that $^1\text{O}_2$ was the main ROS, which supports the original hypothesis and is consistent with other recent studies in my lab (Carrier *et al.*, 2018; Carrier *et al.*, 2019). OH• and O_2^- were also produced in the system as minor constituents, and it is possible other ROS were produced, beyond those specifically evaluated.

Additional research should be performed to better assess the surface chemistry of Fe MNPs as this may influence the reaction mechanism and affect ROS generation. However, data collected in this study is consistent with $^1\text{O}_2$ as the main ROS produced in this system. In different environments (*e.g.*, different pH values or presence of different interfering chemicals/ligands), the mechanism of the Fenton process may also change, which makes this a very complex research subject that needs to be examined exhaustively when considering research application.

The Fe MNP-catalyzed Fenton reaction was unable to degrade methyl orange within Boat Harbour effluent. This suggests that this process is unlikely to degrade organic micropollutants in complex wastewater matrices. This experiment was also performed at pH 3, which is neither ideal nor cost effective as it would require adjusting the pH before and after treatment with out-sourced chemicals, but this pH regime is also the optimal condition to assess water treatment efficacy of the system. In the future, it may be worthwhile to combine the Fe MNP-catalyzed Fenton reaction with additional advanced treatment techniques. Regardless, the generation of $^1\text{O}_2$ in this system is a significant research discovery that has many real-world implications.

Chapter 4 Singlet Oxygen Generation in Hemoglobin-Catalyzed Fenton Reactions

Role of the co-authors:

- David Oakley, MES candidate who performed the research, collected and analyzed experimental data, and wrote this chapter about this ongoing research project.
- Dr. Andrew Carrier and Dr. Collins Nganou assisted with scientific concepts, provided experimental advice, writing guidance, and general advice.
- Amanda Nicholson and Minh Ngoc Pham assisted with data collection and general advice
- Dr. Ken Oakes, Dr. Tony Walker, and Dr. Xu Zhang served as committee members of David Oakley who assisted with scientific concepts, provided experimental advice, project expectations, editing, and general advice.

4.0 Abstract

Hemoglobin (Hb), a critical respiratory molecule of vertebrates and many invertebrates, can act as a catalyst in Fenton reactions. The generation of reactive oxygen species (ROS) *via* Hb-catalyzed Fenton reactions can lead to oxidative stress and the development of various pathological conditions but can also be exploited to degrade organic pollutants within wastewater. However, the exact mechanism associated with this process has remained ambiguous. In this investigation, I was motivated to determine the main ROS generated in Hb-catalyzed Fenton processes. Herein, I demonstrate that singlet oxygen ($^1\text{O}_2$) is the main ROS produced in Hb-catalyzed Fenton reactions, as opposed to previously reported hydroxyl radicals ($\text{OH}\cdot$). The reaction between Hb and hydrogen peroxide (H_2O_2), leads to formation of transient iron (IV)-oxo (ferryl) species, which catalyze $^1\text{O}_2$ production. $^1\text{O}_2$ has been shown to damage various biomolecules (*e.g.*, DNA), which may cause disease, so understanding Hb-catalyzed Fenton reactions could help prevent disease in medical applications and by extension a tool to assess ecosystem health. Therefore, this knowledge would be vital to researchers in these fields. Experiments were performed to assess whether Hb-catalyzed Fenton reactions could degrade the organic dye methyl orange within Boat Harbour wastewater effluent. These experiments were successful even with low concentrations of H_2O_2 (0.5 mM) and Hb (500 nM). Results show that this process can be useful for degradation of organic micropollutants in complex real-world wastewater matrices (*i.e.*, Boat Harbour effluent). Notably, this process is also effective at neutral pHs, eliminating the need to adjust pH before and after treatment, as typically required for the traditional Fenton process, which incurs costs. Hb-catalyzed Fenton reactions have potential for heightened efficiency if used in combination with another treatment (*e.g.*, photocatalysis). $^1\text{O}_2$ generated from this process can also be used in many other biological, environmental, or industrial applications.

4.1 Introduction

Hemoglobin (Hb) is respiratory protein formed when organic ligands known as porphyrins bind to iron to co-operatively deliver oxygen to the tissues of vertebrates and some invertebrates. Hb can react with hydrogen peroxide (H_2O_2), naturally produced by the mitochondrial electron transport chain to form ferrylhemoglobin (ferrylHb) and other transient iron (IV)-oxo (ferryl) compounds (Alayash *et al.*, 2001; Khaket & Ahmad, 2011). These compounds can catalyze the generation of reactive oxygen species (ROS) in a Fenton-like manner. The production of excessive amounts of ROS by Hb-catalyzed Fenton reactions can lead to pathological conditions associated with oxidative stress (Reeder & Wilson, 2005; Vallelian *et al.*, 2008). For example, this reaction has been implicated in tissue injuries of the central nervous system (Rifkind *et al.*, 2015; Sadrzadeh *et al.*, 1987). The protein haptoglobin is produced in our bodies to bind free Hb and limit Hb-catalyzed oxidative damage (Cooper *et al.*, 2013; Garland *et al.*, 2020). Hemoglobin is also present within the natural environment and is found within a variety of different vertebrate and invertebrate species; therefore, this process can also impact many organisms in different ecosystems and may influence bioremediation processes (Hardison, 2012; Lascelles, 1964; Lesage *et al.*, 1993).

Hb-catalyzed Fenton processes are reported to be similar to the reactions of peroxidase enzymes (Chapsal *et al.*, 1986; Liu *et al.*, 2012; Pérez-Prior *et al.*, 2012), which have been used for the treatment of wastewater effluent (Dalal & Gupta, 2007; Na & Lee, 2017), but they are much more expensive than Hb, which can be inexpensively obtained from abattoirs. Therefore, researchers have employed the Hb-catalyzed Fenton reaction for the remediation of wastewater pollutants because it may provide an effective green and low-cost treatment solution (Chapsal *et*

al., 1986; Liu *et al.*, 2012; Pérez-Prior *et al.*, 2012). This process has been effective at degrading potentially hazardous organic pollutants, including PAHs (Laveille *et al.* 2010; Liu *et al.*, 2012), phenolics (Chapsal *et al.*, 1986; Pérez-Prior *et al.*, 2012), and anilines (Liu *et al.*, 2012).

The generation of hydroxyl radicals (OH•) has been previously reported in Hb-catalyzed Fenton reactions (Sadrazadeh *et al.*, 1984; Van Dyke & Saltman, 1996). However, there have also been reports, suggesting the generation of other ‘reactive species’ rather than OH• (Buehler *et al.*, 2009; Puppo & Halliwell, 1988; Reeder, 2010; Vlasova, 2018) including singlet oxygen (¹O₂) (Kikugawa *et al.*, 1983; Ortiz de Montellano & Catalano, 1985) In Ortiz de Montellano & Catalano (1985), the oxidation of styrene *via* the Hb-catalyzed Fenton reaction was slightly inhibited by the ¹O₂ scavenger DABCO. Kikugawa *et al.* (1983) also observed ¹O₂ generation in the Hb-catalyzed Fenton reaction by the selective oxidation of diphenylfuran *via* ¹O₂. However, these researchers did not report ¹O₂ as being the main ROS generated in the Hb-catalyzed Fenton system. Previous research has shown that ¹O₂ can be the main ROS generated in both iron- (Carrier *et al.*, 2019) and iron magnetic nanoparticle-catalyzed (**Chapter 3**) Fenton reactions. Therefore, I hypothesized that ¹O₂ would also be the main ROS produced in the Hb-catalyzed Fenton reaction. It is possible that ¹O₂ is the main ROS responsible for disease states associated with Hb-catalyzed Fenton reactions and the main ROS responsible for degrading wastewater pollutants when this process is implemented. Therefore, in this research I was motivated to determine the specific ROS generated in the Hb-catalyzed Fenton process. With a better understanding of this process, effective medicines and/or treatment(s) could be designed and implemented. Previous applications (*e.g.*, wastewater treatment) that may have utilized this technique could also be improved if researchers understood the process better (*i.e.*, determine

how to maximize ROS generation). This study also assessed the effectiveness of this process at degrading organic micropollutants within Boat Harbour effluent. Herein, I will provide evidence that $^1\text{O}_2$ is the main ROS produced in Hb-catalyzed Fenton reactions, which was able to degrade the dye methyl orange in Boat Harbour Geotube® effluent.

4.2 Materials and Methods

4.2.1 Reagents

Hemoglobin from bovine blood (methemoglobin), iron (III) chloride (99.9+%), MES buffer, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), beta-carotene, sodium azide, tert-butyl alcohol (TBA), coumarin, 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), 2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT), dimethyl sulfoxide (DMSO), deferiprone, methyl orange and 4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMP-OH) were purchased from Sigma Aldrich (Oakville, ON, Canada). Deferiprone is used to chelate any free iron that may be in the system, thus preventing the possibility of free iron from participating in the Fenton reaction (Hider & Hoffbrand, 2018; Sohn *et al.*, 2008). Deferiprone was incubated with hemoglobin for 15 min before the Fenton experiments to allow any free iron to bind with the molecule. Hydrogen peroxide (30%) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Nanopure Water (18.2 MΩ cm) was collected daily from a Barnstead Nanopure water system (Thermo Scientific, Waltham, MA, USA); this was used the main solvent for all experiments except for the methyl orange degradation experiments, which used Boat Harbour Geotube® filtrate as the solvent. The Boat Harbour Geotube® filtrate was collected onsite. All experiments were performed at pH 7 in 1

mM MES buffer except for the methyl orange degradation experiments, which used Boat Harbour wastewater effluent filtrates.

4.2.2 Equipment

A Tecan Infinite M1000 Pro microplate reader (Männedorf, Switzerland) was used for absorbance and fluorescence experiments. All experiments were performed at 25 °C. Costar 96-well flat transparent polystyrene microplates (Corning, NY, USA) were used for all absorbance experiments. Nunclon 96-well flat black polystyrene microplates (Thermo Fisher, Waltham, MA, USA) were used for all fluorescence experiments. Electron paramagnetic resonance (EPR) data was collected using a Bruker microEPR instrument. A photoluminescence experiment was performed using an infrared spectrometer (StellarNet Inc) to measure the specific ROS generated in the system. Differential pulse spectrometry was performed using a CorrTest two-channel electrochemical workstation to identify transient iron (iv) produced in the system.

4.2.3 ABTS Oxidation

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) oxidation was used as a colorimetric assay to assess the rate of the Hb-catalyzed Fenton reaction. ABTS is oxidized non-selectively to form an ABTS radical cation ($\text{ABTS}^{\cdot+}$), which is a blue-green chromogen suitable for quantification photometrically at 420 nm (Katerji *et al.*, 2019). Varying concentrations of Hb and H_2O_2 were used as necessary to assess the concentration-dependent nature of the reaction. Sodium azide, beta-carotene, coumarin, TBA, and XTT were used as ROS scavengers. They will selectively react with a corresponding ROS, identifying specific ROS produced in the Hb-catalyzed Fenton reaction.

4.2.4 ABDA Oxidation

ABDA is a fluorescent probe that reacts with $^1\text{O}_2$ to form an endoperoxide. This reduces the fluorescence intensity of the probe measured at excitation and emission wavelengths of 380 and 407 nm, respectively (Ke *et al.*, 2014; Qian *et al.*, 2009). Greater reductions in fluorescence intensity correspond to higher levels of $^1\text{O}_2$. Varying concentrations of Hb and H_2O_2 were used as necessary to assess the concentration-dependent nature of the reaction. TBA and sodium azide were also used in respective ABDA oxidation experiments as ROS scavengers.

4.2.5 Coumarin Oxidation and XTT Reduction

Coumarin oxidation assessed the formation of hydroxyl radicals ($\text{OH}\cdot$) in the Hb-catalyzed Fenton system. Coumarin is selectively oxidized by $\text{OH}\cdot$ to form the fluorescent molecule umbelliferone; greater fluorescence intensity values during experimentation correspond to a greater production of $\text{OH}\cdot$. The procedure for this experiment is detailed in **Chapter 3**.

XTT reduction assessed the formation of superoxide radicals ($\text{O}_2^{\cdot-}$) in the Hb-catalyzed Fenton system. XTT is selectively reduced by $\text{O}_2^{\cdot-}$ to form an orange coloured formazan; greater absorbance values during experimentation correspond to a greater production of $\text{O}_2^{\cdot-}$. The procedure for this experiment is detailed in **Chapter 3**.

4.2.6 Methyl Orange Degradation

The ability of Hb-catalyzed Fenton reactions to degrade organic compounds was assessed by using 10 μM of the commercial dye methyl orange as a model contaminant. Methyl orange was subjected to the Hb-catalyzed Fenton reaction. These experiments took place within 10-fold diluted Geotube® effluent collected from Boat Harbour (**Figure 5**). The procedure for this experiment is detailed in **Chapter 3**.

4.3 Results and Discussion

4.3.1 Singlet Oxygen ($^1\text{O}_2$) Generation

The generation of singlet oxygen ($^1\text{O}_2$) was observed by using selective chemical probes, *i.e.*, ABDA and selective $^1\text{O}_2$ quencher molecules sodium azide and beta carotene. The ABTS colorimetric assay was first used to assess total ROS generation in the Hb-catalyzed Fenton reaction, which increased with increasing concentrations of Hb or H_2O_2 (**Figure 21**). Singlet oxygen scavengers (sodium azide and beta-carotene) inhibited ABTS oxidation (**Figure 22**), demonstrating $^1\text{O}_2$ is responsible for ABTS oxidation. However, azide did not completely inhibit the reaction, even when its concentration was 100 times greater than Hb (**Figure 22**) eliminating the possibility azide was simply poisoning the catalyst and preventing the Fenton Reaction from occurring. This is consistent with a previous investigation that showed that the $^1\text{O}_2$ scavenger DABCO slightly inhibited the Hb-catalyzed Fenton reaction for the oxidation of styrene (Ortiz de Montellano & Catalano, 1985). The DABCO concentration in their experiment was 50 mM relative to 10 μM of Hb, which suggests that $^1\text{O}_2$ was scavenged, as opposed to poisoning the Hb catalyst.

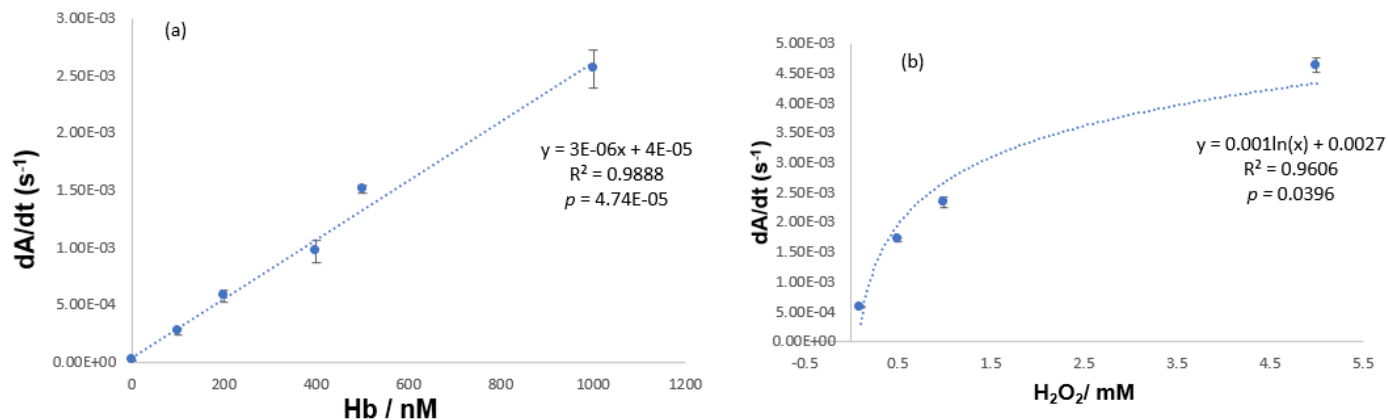


Figure 21. ABTS oxidation with increasing concentrations of Hb (a) and H_2O_2 (b). $[Hb] = 1 \mu M$, $[MES \text{ pH } 7] = 1 \text{ mM}$, $[Deferiprone] = 20 \mu M$, $[ABTS] = 500 \mu M$, $[H_2O_2] = 500 \mu M$.

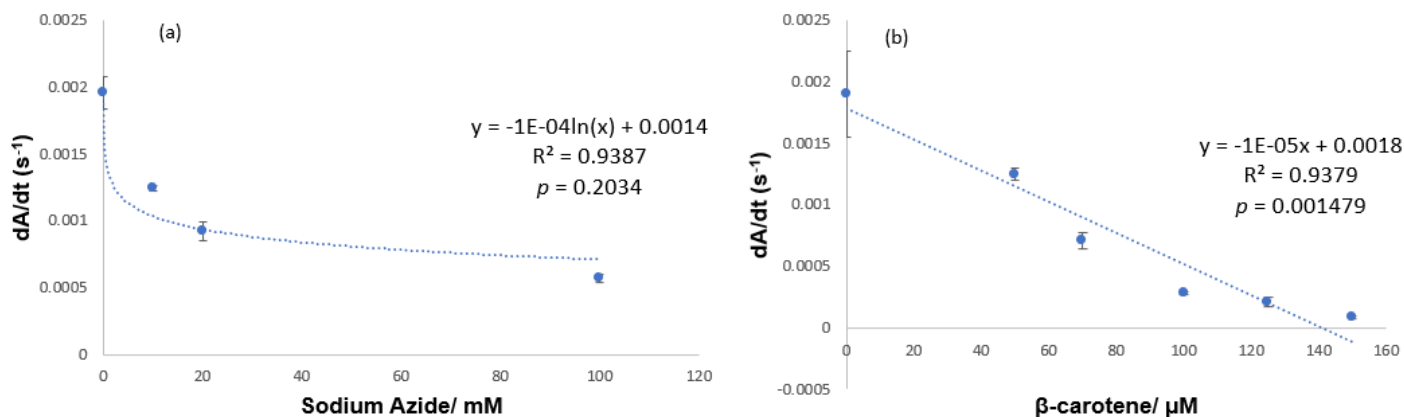


Figure 22. ABTS oxidation with varying concentrations of sodium azide (a) and beta-carotene (b). $[Hb] = 1 \mu M$, $[MES \text{ pH } 7] = 1 \text{ mM}$, $[Deferiprone] = 20 \mu M$, $[ABTS] = 500 \mu M$, $[H_2O_2] = 500 \mu M$.

ABDA oxidation experiments were used to assess the rate of 1O_2 formation. The oxidation of ABDA is facilitated by 1O_2 , producing a decrease in the fluorescence intensity of ABDA. The oxidation of ABDA increased with increasing concentrations of Hb or H_2O_2 (**Figure 23**) but decreased with increasing concentrations of azide (**Figure 24**). Similar to the ABTS result, azide did not completely inhibit the reaction, even at a concentration more than 1000 times greater than Hb. This result demonstrates ABDA is oxidized by 1O_2 .

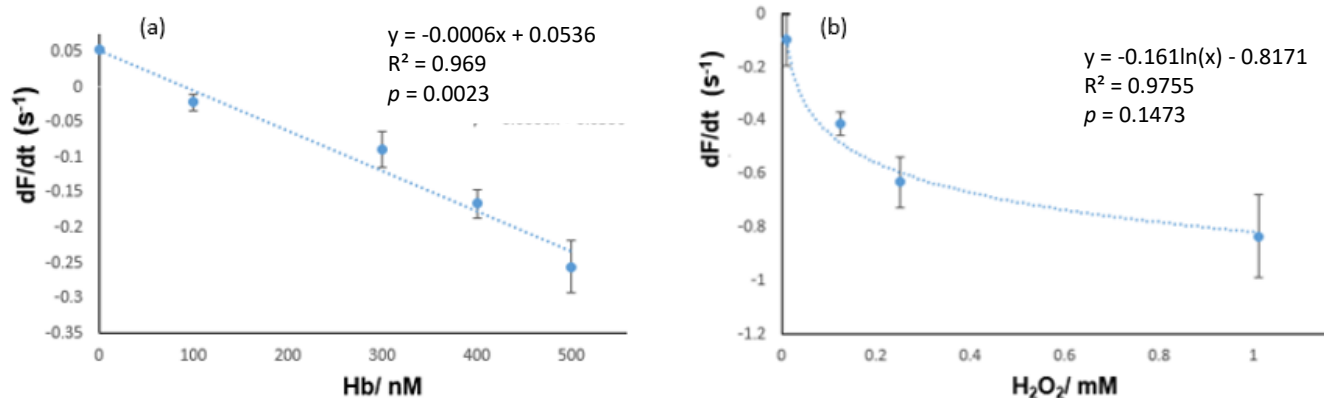


Figure 23. ABDA transformation with varying concentrations of Hb (a) and H_2O_2 (b). $[Hb] = 1 \mu M$, $[MES \text{ pH } 7] = 1 \text{ mM}$, $[Deferiprone] = 20 \mu M$, $[ABDA] = 250 \text{ nM}$, and $[H_2O_2] = 0.5 \text{ mM}$.

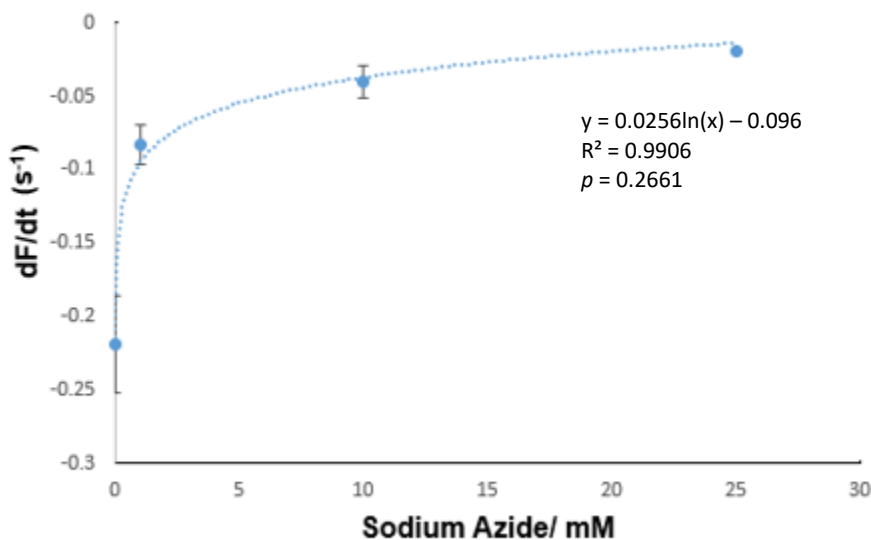


Figure 24. ABDA transformation with varying concentrations sodium azide. $[Hb] = 1 \mu M$, $[MES \text{ pH } 7] = 1 \text{ mM}$, $[Deferiprone] = 20 \mu M$, $[ABDA] = 250 \text{ nM}$, and $[H_2O_2] = 0.5 \text{ mM}$.

Further evidence for 1O_2 generation was obtained by performing a photoluminescence experiment; the peak produced at 1270 nm corresponds to 1O_2 (**Figure 25**). The EPR result also

shows the formation of TEMPO-OH, which indicates the production of singlet oxygen (**Figure 26**).

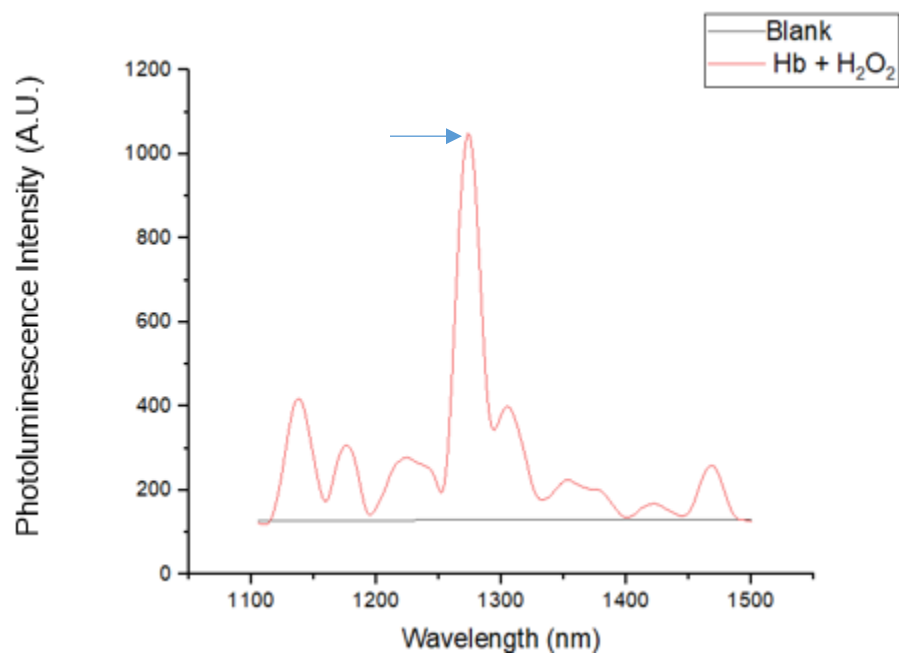


Figure 25. Photo Luminescence result for Hb-Fenton reaction. [Hb] = 50 μ M, [Deferiprone] = 20 μ M, [H₂O₂] = 1 M. Peak at 1270 nm corresponds to ¹O₂.

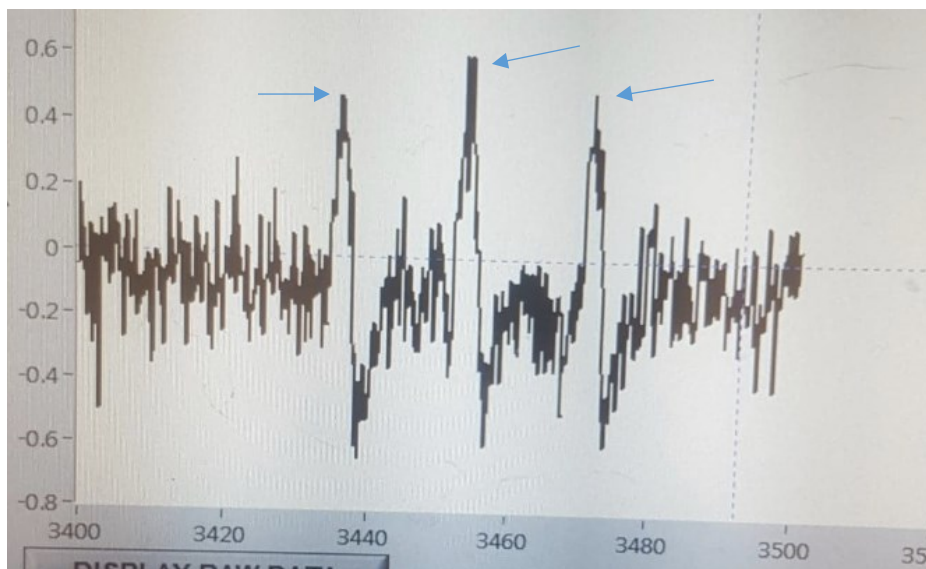


Figure 26. Electron Paramagnetic Resonance (EPR) result for the Hb-Fenton reaction. The peaks correspond to TEMPO-OH, which indicates the generation of $^1\text{O}_2$. $[\text{Hb}] = 5 \mu\text{M}$, $[\text{TEMP-OH}] = 250 \text{ mM}$, $[\text{H}_2\text{O}_2] = 1 \text{ M}$.

4.3.2 Hydroxyl Radical ($\text{OH}\cdot$) and Superoxide Anion ($\text{O}_2^{\cdot-}$) Generation

Hydroxyl radical (TBA) (**Figure 27**) and superoxide anion (XTT) (**Figure 28**), scavengers had no effect on the reaction rate for ABTS oxidation, suggesting no significant contributions from $\text{OH}\cdot$ or $\text{O}_2^{\cdot-}$.

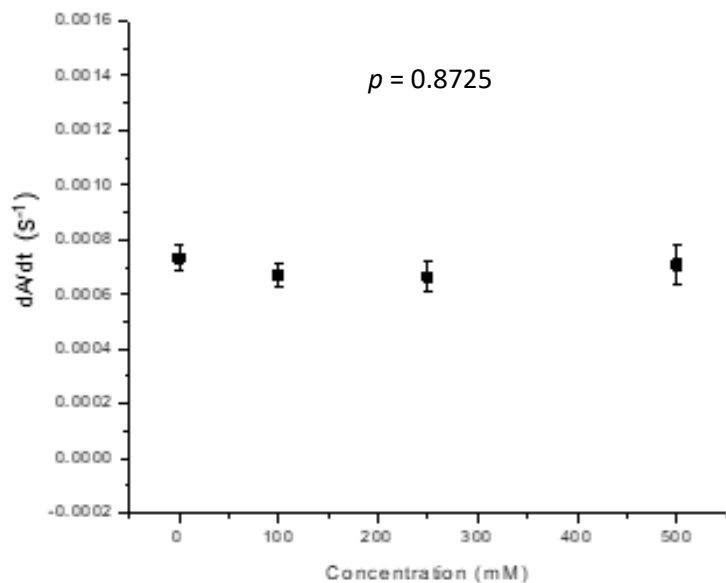


Figure 27. ABTS oxidation with increasing concentrations of TBA. [Hb] = 1 μ M, [MES pH 7] = 1 mM, [Deferiprone] = 20 μ M, [ABTS] = 500 μ M, [H₂O₂] = 500 mM.

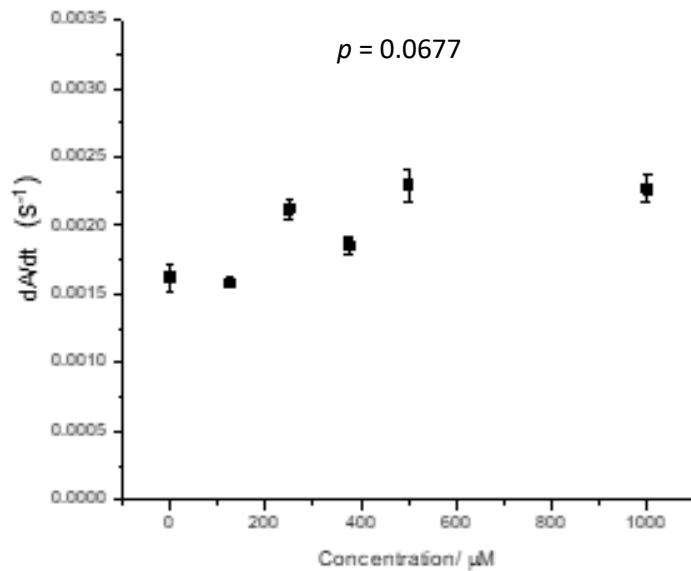


Figure 28. ABTS oxidation with increasing concentrations of XTT. [Hb] = 1 μ M, [MES pH 7] = 1 mM, [Deferiprone] = 20 μ M, [ABTS] = 500 μ M, [H₂O₂] = 500 mM.

Coumarin oxidation and XTT reduction assays were then used to quantify the production of OH• (**Figure 29**) and O₂⁻ (**Figure 30**), respectively. However, neither rate was concentration dependent on Hb or H₂O₂ demonstrating OH• and O₂⁻ are not directly produced by Hb-catalyzed Fenton reactions. However, these results contrast previous reports, which suggested OH• production (Sadrzadeh *et al.*, 1984; Van Dyke & Saltman, 1996) although these prior research methods may not have been highly selective for OH• (*e.g.*, thiourea - a proposed OH• scavenger) inhibited the formaldehyde generation from DMSO (a process requiring OH•) *via* the Hb-catalyzed Fenton reaction (Sadrzadeh *et al.*, 1984). The selectivity of thiourea as a OH• scavenger has been questioned as it may directly react with H₂O₂ and/or bind to metal ions, modifying their reactivity (Puppo & Halliwell, 1988). The selective hydroxylation of salicylate to 2,3- and/or 2,5-dihydroxybenzoate *via* the Hb-catalyzed Fenton reaction has also been reported as evidence for OH• generation (Van Dyke & Saltman, 1996) but this method also has been questioned since salicylate is hydroxylated by other reactive species besides OH•, *e.g.*, peroxyxynitrite (Halliwell & Kaur, 1997). Any OH• produced in this system may stay within the Hb structure and/or react with the protein directly, which would prevent OH• measurement. OH• may also be a transient intermediate present at a very low steady state concentration; this is also possible for O₂⁻.

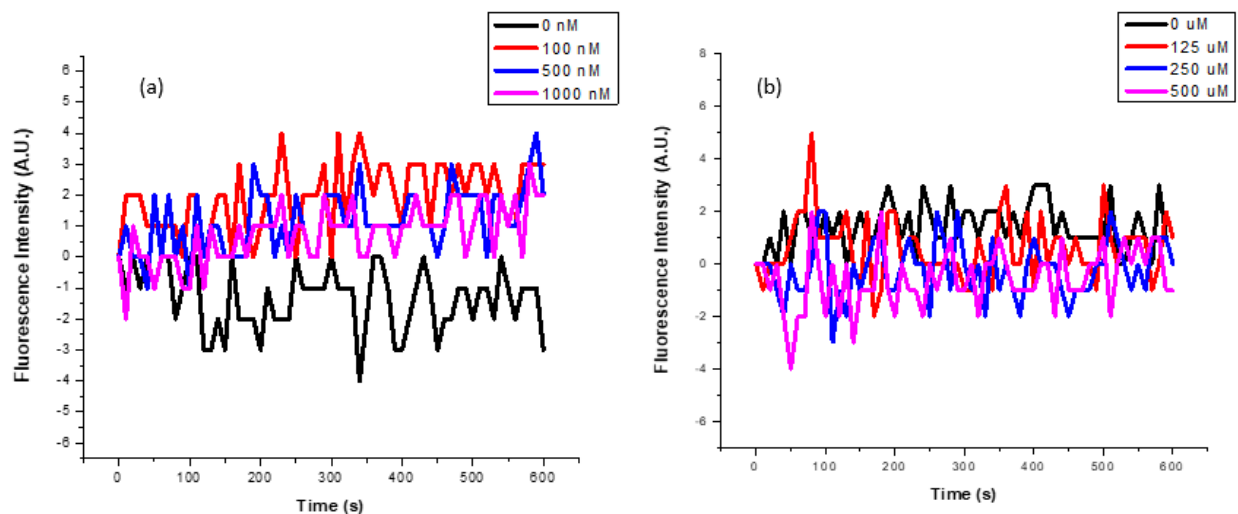


Figure 29. Coumarin oxidation with varying concentrations of Hb (a) and H₂O₂ (b). [Coumarin] = 100 μM, [Hb] = 1 μM, [MES pH 7] = 1 mM, [Deferiprone] = 20 μM, and [H₂O₂] = 0.5 mM.

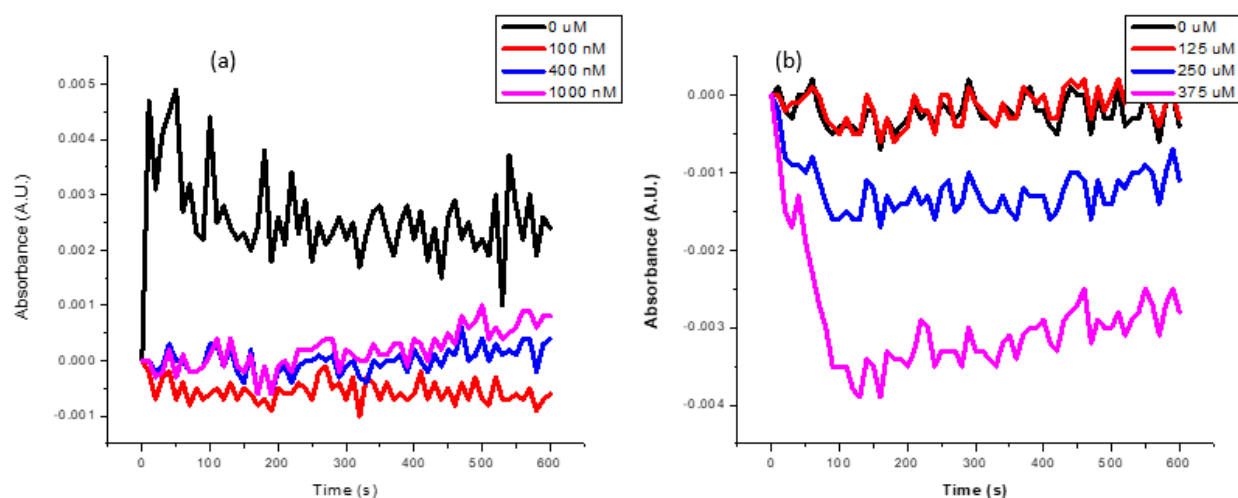


Figure 30. XTT reduction with varying concentrations of Hb (a) and H₂O₂ (b). [XTT] = 500 μM, [Hb] = 1 μM, [MES pH 7] = 1 mM, [Deferiprone] = 20 μM, and [H₂O₂] = 0.5 mM.

4.4 Proposed Mechanism

It has been reported that iron ions released from Hb are responsible for catalyzing Fenton reactions (Puppo & Halliwell, 1988). However, deferiprone is an effective sequestrant of Fe³⁺

and would have greatly reduced these reactions. **Figure 31** shows that when deferiprone is present in the system, the absorbance values for the traditional iron-based Fenton reaction were so low that they were indistinguishable from noise. The same catalyst and reagent concentrations were used in the successful Hb-catalyzed Fenton reaction (**Figure 21**) indicating released iron ions are unlikely to be the main catalysts in these reactions. Hb likely reacts with H_2O_2 to form ferrylHb and other ferryl (Fe [IV]) compounds, as previously described in similar peroxidase enzyme systems (Gumiero *et al.*, 2010; Moody & Raven, 2018). These ferryl compounds (*e.g.* compound I and compound II), can undergo a series of reactions and catalyze the formation of $^1\text{O}_2$ (**Figure 32**). Ferryl species were detected using differential pulse voltammetry (**Figure 33**).

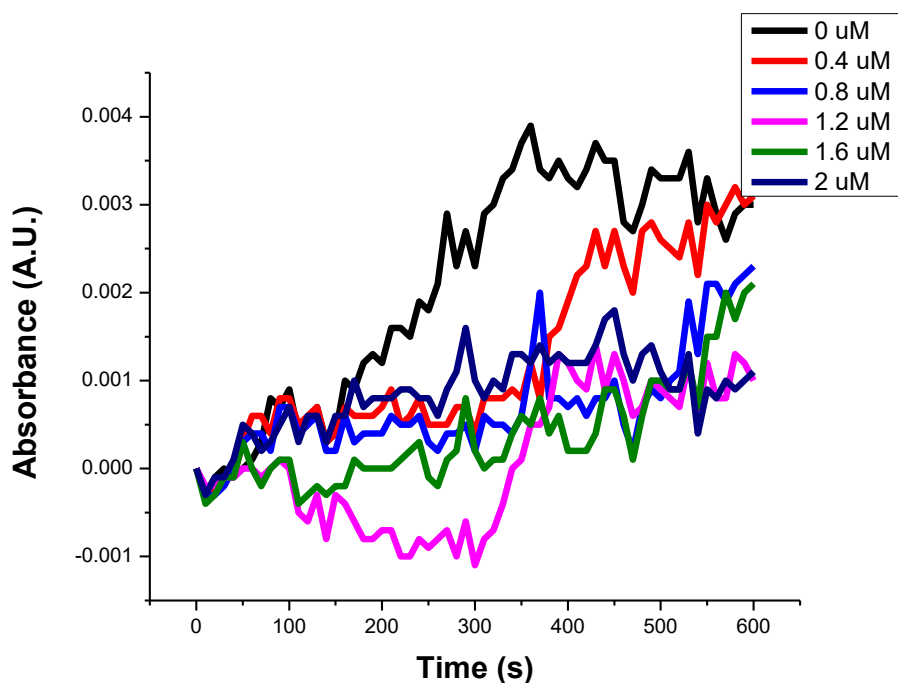


Figure 31. ABTS oxidation with increasing concentrations of FeCl_3 . [MES pH 7] = 1 mM, [Deferiprone] = 20 μM , [ABTS] = 500 μM , [H_2O_2] = 500 μM .

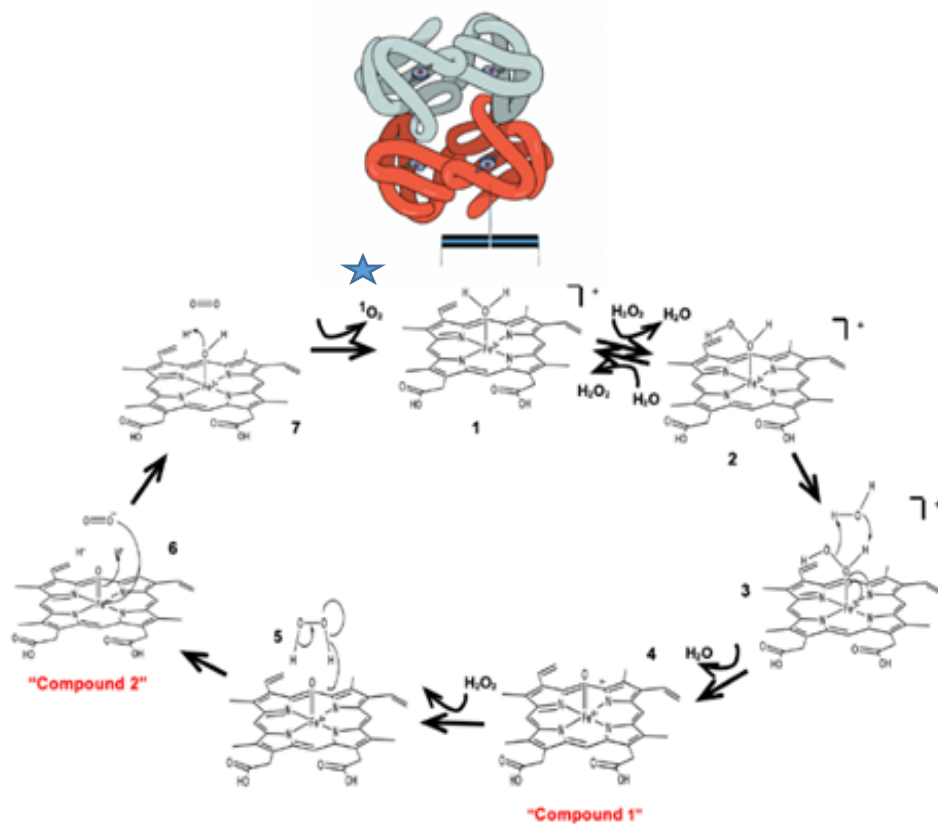


Figure 32. Proposed mechanism for the Hb-catalyzed Fenton reaction (adapted from The Life Scientist, 2020 & Zhang, 2019). The formation of various ferryl compounds eventually leads to the production of $^1\text{O}_2$.

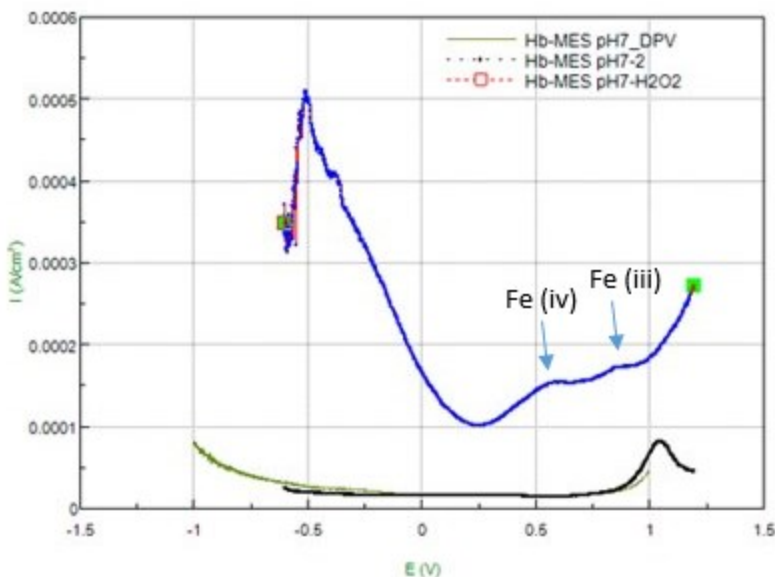


Figure 33. Ferryl species were detected using differential pulse voltammetry. $[\text{Hb}] = 1 \mu\text{M}$, $[\text{MES pH } 7] = 1 \text{ mM}$, and $[\text{H}_2\text{O}_2] = 0.5 \text{ mM}$.

4.5 Methyl Orange Degradation Result

Methyl orange was successfully degraded (solution turned clear) when employing the Hb-catalyzed Fenton reaction as a treatment process during the trial experiments. Methyl orange degradation increased with increasing dilutions of the Boat Harbour Geotube® filtrate (**Figure 34**). This is environmentally relevant to Hb-catalyzed Fenton applications at Boat Harbour since after closure in 2020, any water coming into the system is further diluting the residual wastewater. While there are still toxic organic pollutants present within the Boat Harbour system, the Hb-Fenton reaction could be employed to degrade these compounds. Highly concentrated Geotube® effluent contains more interfering compounds from the interstitial porewaters, preventing degradation of methyl orange. These recalcitrant porewater compounds either scavenge the ROS generated in the system or bind to the catalyst and prevent the reaction from occurring. With increasing concentrations of both Hb and H_2O_2 (**Figure 35**) the

degradation of methyl orange increased (*i.e.*, the absorbance decreased at a faster rate). A further experiment was performed demonstrating iron (II) and iron (III) could not act as catalysts (irrespective of the presence of H₂O₂) to successfully degrade methyl orange (**Figure 36**). Consequently, any methyl orange degradation within the Boat Harbour samples was due to the Hb-catalyzed Fenton reaction.

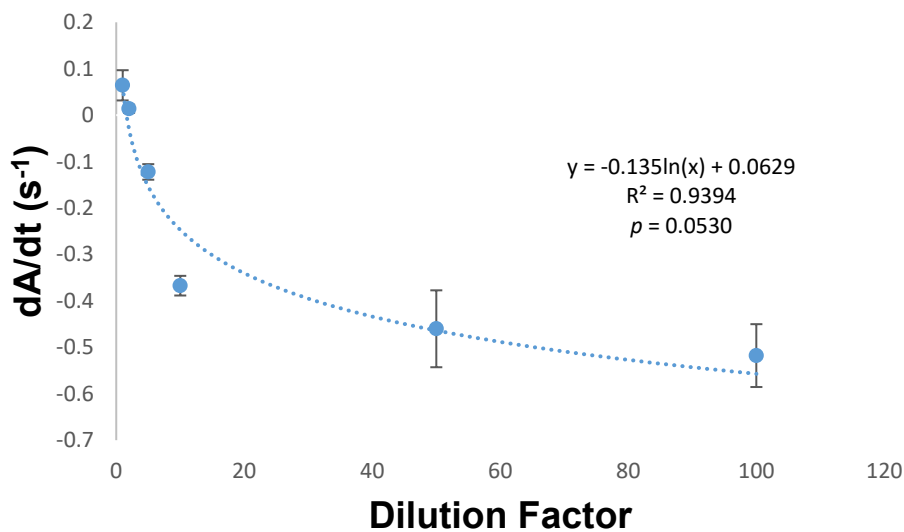


Figure 34. The Hb-catalyzed Fenton Reaction for the degradation of methyl orange within Boat Harbour Geotube® water of various dilution factors. [Hb] = 1 μM, [methyl orange] = 10 μM, [H₂O₂] = 1 mM.

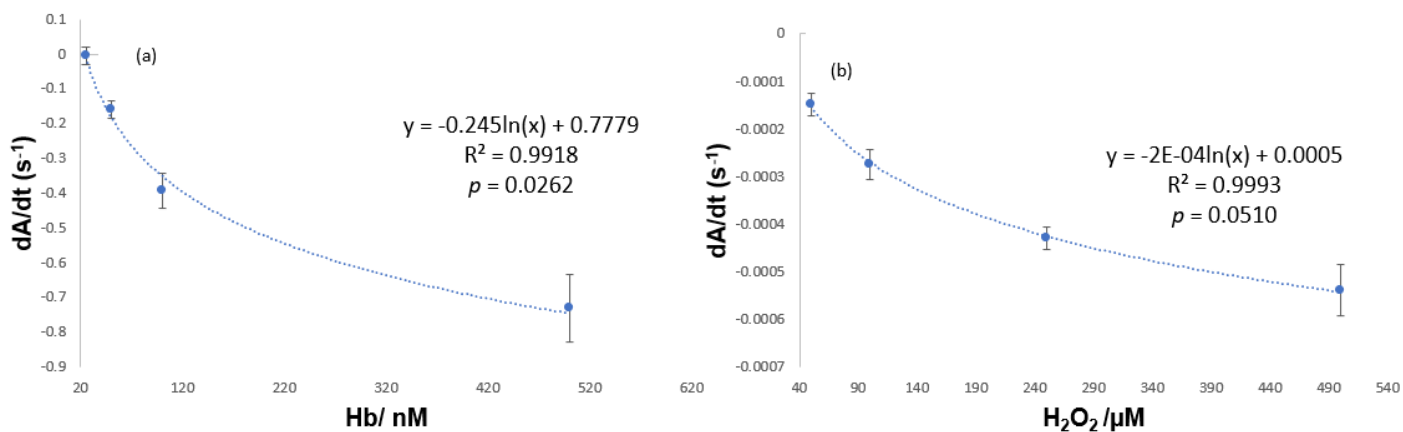


Figure 35. The Hb-catalyzed Fenton reaction for the degradation of methyl orange within Boat Harbour Geotube® water (diluted 10-fold) with varying concentrations of Hb (a) and H_2O_2 (b). $[Hb] = 1 \mu M$, $[methyl\ orange] = 10 \mu M$, $[H_2O_2] = 1 mM$.

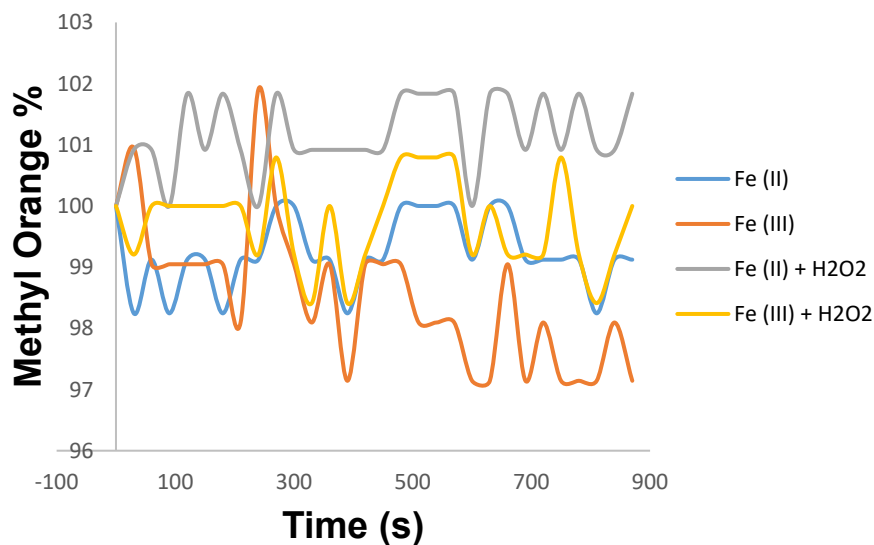


Figure 36. The traditional iron-based Fenton Reaction was unsuccessful at degrading methyl orange present within the Geotube® water (diluted 10-fold). $[Fe] = 4 \mu M$, $[methyl\ orange] = 10 \mu M$, $[H_2O_2] = 1 mM$.

4.6 Research Limitations

This research faced specific limitations. The surface chemistry of the Hb protein was not assessed; this could be essential in determining the underlying mechanisms of the Fenton process, *e.g.*, protein radicals may be produced in this system and have an important impact. Any ROS produced in this system may also stay within the Hb structure and/or react with the protein directly, which would inhibit their measurement. To get a proper sense of these chemical properties, outside analysis would be required, exceeding the research budget. Commercially acquired Hb may also slightly differ from Hb that could be obtained locally (*i.e.*, the purity may differ), which could affect the system. However, based on the data collected in this investigation, I can confidently claim that $^1\text{O}_2$ is the main ROS produced in this system.

4.7 Viability for Environmental-Based Research

The economic viability of a new technology is a critical consideration which could limit or prevent its implementation. One goal of this research was to assess whether the Hb-catalyzed Fenton reaction could provide a cost-effective and green solution for the remediation of organic micropollutants within the complex Boat Harbour wastewater matrix. Hemoglobin (Hb) from bovine blood (methemoglobin) and H_2O_2 were purchased from Sigma Aldrich (Oakville, ON, Canada). Hb cost \$177 CAD for 5 g and H_2O_2 cost \$107 CAD for 500 mL. If this research were to be performed on a larger scale and at a lower cost, Hb could be obtained from waste blood from local abattoirs, while 3% (v/v) peroxide could be obtained from a local Walmart pharmacy for \$3.50/L. Alternatively, the continuous generation and flow of H_2O_2 achieved through electrochemical means would eliminate the need for constant replenishment and keep expenses low. Such a system would incur startup and electrical costs, but that would be preferable to a

design requiring the constant addition of externally sourced H₂O₂. The continuous flow and generation of H₂O₂ could be achieved through electrochemical means. Li *et al.*, (2020) demonstrated that 9.3 kWh of electricity would be required to generate 1 kg of H₂O₂. The current cost of electricity in Nova Scotia using the domestic service tariff rate is approximately 16 ¢/kWh (Nova Scotia Power, 2021). Based on this rate, it would cost \$1.49 CAD to produce 1 kg of H₂O₂. Based on my experimental data (**Figure 37**), it took 0.1 mM (or 0.0001 M) of H₂O₂ (MW= 34.0147 g/mol) to effectively treat Boat Harbour wastewater effluent containing methyl orange with a 10 min contact time. From this data, the mass of H₂O₂ required to treat 1 L of effluent is calculated to be 0.0034 g (0.0000034 kg) based on stoichiometric calculations.¹⁴ Expressed in a more applied context, \$1.49 worth of electricity at current prices would produce 1 kg of H₂O₂ that based on my experimental data could treat 294,118 L of Boat Harbour effluent.¹⁵ The 100% v/v H₂O₂ generated electrochemically should be purer and more reactive than commercially available 30% v/v H₂O₂, improving degradation efficiencies beyond those of the 30% v/v H₂O₂. It should also be noted that the Geotube® effluent was diluted 10-fold during experimentation to approximate the current environmental conditions in Boat Harbour since effluent addition ceased in 2020 after the mill ceased operation. Since that time, Boat Harbour has been diluted with spring meltwater, precipitation, and surface water contributions from creeks and tributaries (Ken Oakes, *personal communication*, June 23, 2021), which could make this calculated value a close estimate of how effective this process would be on a larger scale.

¹⁴ 0.0001 mol/L * 1 L = 0.0001 mol; 0.0001 mol * 34.0147 g/mol = 0.0034 g to treat 1 L of effluent.

¹⁵ 0.0000034 kg = 1 L; 1 kg = x L; x = (1 L * 1 kg)/0.0000034 kg = 294,118 L of effluent, which can be treated by 1 kg of H₂O₂.

Li *et al.*, (2020) used a carbon black-polytetrafluoroethylene electrode for their research (this is very similar to the inexpensive carbon felt electrode previously used in my research group [Adesida, 2020]), which showed that their small surface area electrode could generate approximately 0.225 kg of H₂O₂ during its lifetime, which based on the cost of the electrode (a function of surface area, which could be scaled for industrial applications to increase longevity) was approximately \$0.33 CAD/kg. The lifetime of these carbon electrodes is not ideal; however, it is possible that modifications could enhance their lifetime, *e.g.*, altering the hydrophobicity of the electrode or alternating back and forth whether an electrode is being used as an anode or cathode to prevent water buildup (Li *et al.*, 2020).

For the Hb costs, based on my experimental data (**Figure 38**), it took 500 nM (or 0.0000005 M) of Hb (MW= 64,458 g/mol) to effectively treat the effluent containing methyl orange. From this, 0.032 g (0.000032 kg) of Hb would be required to treat 1 L of effluent¹⁶ which expressed in a more relevant framework means 31,250 L of effluent can be theoretically treated with 1 kg of Hb.¹⁷ The largest quantity of purified bovine Hb (used in this project) that can be purchased from Sigma is 25 g (0.025 kg) for \$614 CAD, which means it would cost \$24,560 CAD to purchase 1 kg worth (excluding a wholesale discount). The cost of using this commercial Hb as a catalyst is clearly not economically viable. If Hb is pursued as a wastewater treatment catalyst on a larger scale, it will need to be sourced from a local abattoir, which as a waste product, could be procured at a low (or no) direct cost. However, processing would incur

¹⁶ 0.0000005 mol/L * 1 L = 0.0000005 mol; 0.0000005 mol * 64,458 g/mol = 0.032 g to treat 1 L of effluent.

¹⁷ 0.000032 kg = 1 L; 1 kg = x L; x = (1 L * 1 kg) / 0.000032 kg = 31,250 L of effluent, which can be treated by 1 kg of Hb.

costs. Centrifugation to isolate RBC-containing Hb from plasma and their subsequent lysis would have a cost. At this point, it is difficult to calculate how efficient this will be on a large scale. However, as purity would not be critical for water treatment applications, this option should be explored as an inexpensive way to obtain Hb. As the concept of adding an iron-based porphyrin from a bovine source to wastewater would be undesirable or objectionable from an aesthetic or ethical perspective in some treatment contexts, adding an electrochemical treatment to remove the added iron for disposal may, in some applications, be deemed necessary.

In the future, a cost-benefit analysis should be performed to properly assess electrochemical approaches that incorporate Fenton techniques for the remediation of wastewater. My lab previously developed an electrochemical filtration system that treated Geotube® effluent collected from Boat Harbour (Adesida, 2020). It is possible that combining the Hb-catalyzed Fenton system with this electrochemical filtration system would create a synergistic effect and improve wastewater remediation efficiency while alleviating concern for the added iron during the treatment process. While this research is preliminary, the future use of singlet oxygen generation *in situ* has promise as a processing step in the degradation of wastewater pollutants.

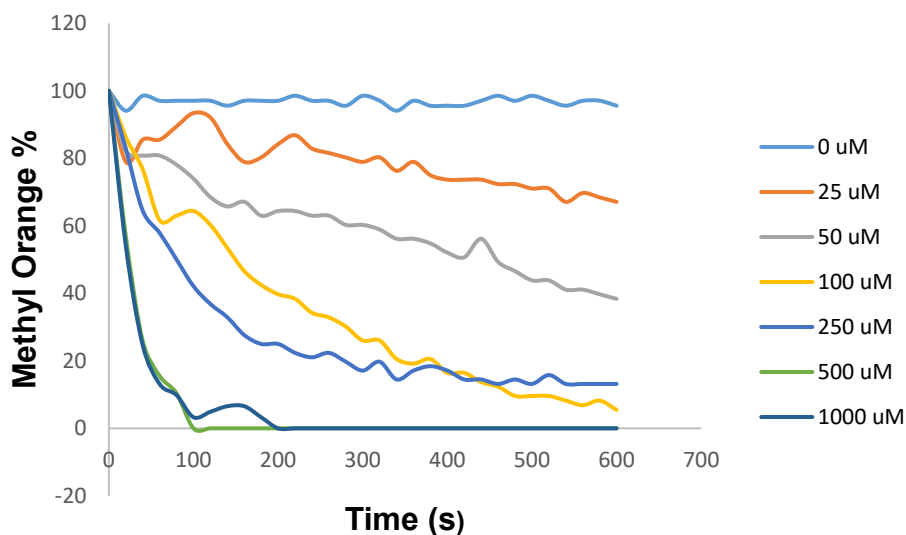


Figure 37. The Hb-catalyzed Fenton reaction for the degradation of methyl orange within Boat Harbour Geotube® water (diluted 10-fold) with varying concentrations of H₂O₂. [Hb] = 1 μM, [methyl orange] = 10 μM. This figure was used to make the treatment cost calculations.

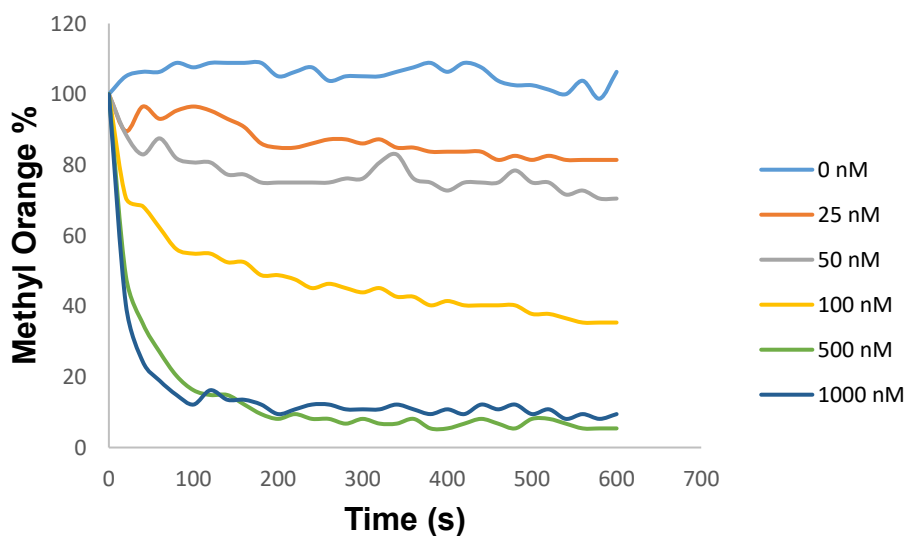


Figure 38. The Hb-catalyzed Fenton reaction for the degradation of methyl orange within Boat Harbour Geotube® water (diluted 10-fold) with varying concentrations of Hb. [H₂O₂] = 1 mM, [methyl orange] = 10 μM. This figure was used to make the treatment cost calculations.

4.8 Conclusion

I have presented evidence that $^1\text{O}_2$ is the main ROS generated in Hb-catalyzed Fenton reactions. $^1\text{O}_2$ plays an important role in biological, environmental, and industrial applications. If free Hb in the body produces $^1\text{O}_2$, medicines and treatment regimens could be developed to specifically combat this. $^1\text{O}_2$ can also be used in wastewater treatment applications to degrade organic micropollutants. In this investigation, $^1\text{O}_2$ degraded methyl orange within Boat Harbour Geotube® samples. The concentrations of reagents required to achieve methyl orange degradation were very low, which would make this process cost-effective. Hb is also environmentally friendly and could be sourced locally. Critically, this process can also occur at neutral pH values, eliminating the need to adjust pH before and after treatment. This knowledge can serve as a guide to all researchers who study these systems and can be built upon to further improve the efficiency of these processes.

Chapter 5 Conclusions and Future Recommendations

5.1 Summary

The aim of my research project was to provide a better understanding of the complex Fenton process, its underlying mechanisms, and its effectiveness for industrial wastewater remediation. This knowledge could be invaluable to environmental and biological researchers. To accomplish this, the following research questions were developed:

(1) Identify the specific compounds produced during the Fenton process, which are responsible for the oxidation of organic compounds. This was tested with different catalysts (*i.e.*, iron magnetic nanoparticles) and different ligands (*i.e.*, hemoglobin). These reagents were chosen because they are environmentally friendly and relatively inexpensive. They also serve as alternatives to traditional iron, which may have certain research limitations.

(2) The impact of various catalysts and ligands on the effectiveness of Fenton processes was explored under different reaction conditions (*i.e.*, varied concentrations and varied pH values). This was in an attempt to better understand how varying these conditions could affect the underlying mechanisms of these reactions (*i.e.*, ROS generation). The quantity and types of ROS generated can significantly impact the effectiveness of the Fenton process.

(3) Fenton processes were employed to degrade synthetic dyes. This was performed to assess whether Fenton processes may be effective at degrading organic pollutants present within the complex real-world milieu, which is Boat Harbour wastewater effluent. Certain pollutants may persist following initial remediation processes (*i.e.*, geotextile filtration) and Fenton processes may provide an effective and inexpensive treatment strategy to remove these contaminants.

The first objective was achieved by using specific chemical trapping agents to unambiguously identify the specific quantities of different ROS produced within these reactions. Trapping agents react in a highly selective manner with specific ROS, which allows for accurate quantification, *i.e.*, coumarin, XTT, DPIBF, and ABDA will each only react with one specific ROS. The second objective was achieved by using a unique catalyst (iron magnetic nanoparticles [Fe MNPs]) and a unique iron-ligand complex (hemoglobin [Hb]) and assessing their rates of reactions with different chemical probes (*i.e.*, TMB, OPD, ABTS). The third objective was achieved by preparing the synthetic dye methyl orange and exposing it to the Fenton treatment within Boat Harbour Geotube® wastewater effluent. I was motivated to determine what is generated in these processes because that could help explain why previous applications may have been unsuccessful. This could also lead to the development of new and more effective approaches.

While this thesis contributed some important mechanistic insight of value for applications as diverse as wastewater treatment and medicine, the demonstration that Fenton processes mainly generate singlet oxygen ($^1\text{O}_2$) rather than hydroxyl radical ($\text{OH}\cdot$) (as was the long-held belief) is probably the most useful and important contribution of this series of experiments. Researchers can now generate and exploit the unique properties of $^1\text{O}_2$, which although is a less powerful oxidant than $\text{OH}\cdot$, possesses an intermediate reactivity and longer half-life perhaps uniquely qualifying it for novel applications. $^1\text{O}_2$ may also be used to degrade dioxins and furans (Zeinali *et al.*, 2019), which is highly relevant to the Boat Harbour remediation project (Eichinger & Walker, 2020; Hoffman *et al.*, 2019; Quanz *et al.*, 2021a). This chapter will focus

on the main research observations, research limitations and setbacks, and future recommendations.

5.2 Main Observations

Previous research argued that hydroxyl radicals ($\text{OH}\cdot$) were the main ROS generated in Fe MNP- (Moura *et al.*, 2005) and Hb-catalyzed (Sadrzadeh *et al.*, 1984; Van Dyke & Saltman, 1996) Fenton processes. However, this research has demonstrated that $^1\text{O}_2$ is the main ROS produced in these systems. The rates of the Fenton processes increased with increasing concentrations of the reagents, *i.e.*, Fe MNP, Hb, and H_2O_2 , and for the Fe MNP-catalyzed Fenton process, with lower pH values. Since most Hb-catalyzed Fenton reactions occur at neutral pH within organisms, their potential use is greater, as acidic conditions are not required to maximize ROS production. The Fe MNP-catalyzed Fenton process was unsuccessful at degrading the organic dye methyl orange; however, methyl orange was readily degraded when Hb was used as the Fenton catalyst. This latter technology can provide a cost-effective and green alternative for wastewater remediation.

5.3 Recommendations and Final Comments

The data collected during this investigation has demonstrated $^1\text{O}_2$ is the main ROS produced in both Fe MNP- and Hb-catalyzed Fenton processes. $^1\text{O}_2$ is an important molecule and can be employed for a variety of applications, *e.g.*, industrial synthesis (Choi & Jung, 2016; Günther *et al.*, 1944; Wasserman & Ives, 1981), biofilm removal (Wang *et al.*, 2017), chemotherapy (Liang *et al.*, 2012), and wastewater remediation (**Chapter 4**). This research presents an inexpensive and relatively safe method for $^1\text{O}_2$ generation.

In the future it would be worth retrying degradation experiments using Fe MNP and Hb-catalyzed Fenton reactions in combination with other advanced treatment methods, such as electrochemical oxidation or photocatalysis. Other catalysts and ligands could also be employed. Hopefully this would have a synergistic effect and improve the efficiency of the Fenton processes for wastewater remediation. This advanced treatment system could be scaled-up to treat polluted bodies of water around the world, including Boat Harbour and help the people of the Pictou Landing First Nations community who are negatively affected by legacy Boat Harbour contaminants in their soon to be remediated tidal estuary.

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