Novel Approaches to Investigate The Oxidation Rate Of Fe(II) And The Role Of Fe(II)/Fe(III) Cycling On The Maintenance Of Reactive Oxygen Species In Aquatic Systems

Justin Maurice Copeland

University of South Carolina

Follow this and additional works at: http://scholarcommons.sc.edu/etd

Part of the Chemistry Commons

Recommended Citation


This Open Access Dissertation is brought to you for free and open access by Scholar Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact SCHOLARC@mailbox.sc.edu.
NOVEL APPROACHES TO INVESTIGATE THE OXIDATION RATE OF Fe(II) AND THE ROLE OF Fe(II)/Fe(III) CYCLING ON THE MAINTENANCE OF REACTIVE OXYGEN SPECIES IN AQUATIC SYSTEMS

by

Justin Maurice Copeland

Bachelor of Science
Northwestern State University of Louisiana, 2011

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Chemistry

College of Arts and Sciences
University of South Carolina

2016

Accepted by:

Timothy Shaw, Major Professor
S. Michael Angel, Committee Member
John Ferry, Committee Member
Ken Shimizu, Committee Member
Alicia Wilson, Committee Member
Cheryl L. Addy, Vice Provost and Dean of The Graduate School
DEDICATION

This dissertation is dedicated to my family, who have been a constant source of love and support throughout my time here at USC. For my mother, whose victory over breast cancer showed me that a mix of faith and a little bit of stubbornness can overcome any obstacle life throws at you; my father, who always provided an ear to listen to whatever was going on in my life and was there to give much needed advice and encouragement; my sister, who I should probably call more but am still more proud of than words can convey; and to Zeynep “Maus” Bektas, who never stopped calling me a nerd, but also never doubted that I could make it to a PhD.
ACKNOWLEDGEMENTS

First, I want to thank God for all He has done and is still doing in my life. This dissertation would not have been possible without the guidance and patience of my advisor, Dr. Tim Shaw. While he gave me plenty of freedom to design and conduct my experiments (and never once complained about the music volume), he never allowed me to move on from difficult projects, and in doing so taught me that the hardest scientific questions are only answered by those persistent enough to stay motivated. Dr. John Ferry was invaluable for the kinetics portion of this work. I am also deeply thankful to Dr. Ken Shimizu and Ping Li for their help in synthesizing the acridinium ester, as well as my committee.

I want to thank my tennis partners and fellow Shaw/Ferry group members who made all the late nights and long field campaigns a memorable experience, including Dewamunnage-Muditha Dias, Rebekkah Lively, Carrie Milliken, Shane Meng, Benson Solomon, and Joy Ihekweazu. I also want to thank the many friends I’ve made here at USC for making sure I actually left the lab every now and then: Patrick Barnett, Wayne O’Brien, David Arenivar, Stephanie DeJong, Audrey Duke, Brianna Cassidy, Zhenyu Lu, Bryan Nichols, Shawna Tazik, Alicia Fessler, Tony Cortese and more that I consider myself extremely lucky to have had the opportunity to hang out with. Finally, I want to thank everyone outside of USC who encouraged me to pursue graduate studies and supported me throughout: Dr. Darrell Fry, Dr. April French, Dr. Carol Chin, Dr. Heather Desaire, Dennis Gibson II, and of course, Kimberley Curell.
ABSTRACT

The biogeochemical cycling of iron, oxygen, and organic carbon are inextricably linked through the intermediacy of Reactive Oxygen Species (ROS) brought about by the autoxidation of ferrous iron (Fe(II)). This dissertation presents laboratory and field-based studies to investigate the kinetics of Fe(II) oxidation and its role in the production of ROS at oxic/anoxic interfaces. The net oxidation of Fe(II) in natural waters is a complex process consisting of alternating cycles of Fe(II) oxidation and Fe(III) reduction processes that are ultimately terminated via precipitation as insoluble Fe(III) (oxy)hydroxides. This complicates kinetic measurements of individual reaction steps in the process.

A critical hypothesis examined by this work is that the rate for the direct reaction of Fe(II) with dioxygen (O$_2$) is comparable in magnitude to the reverse reaction of Fe(III) with O$_2^-$ ($10^8$ M$^{-1}$s$^{-1}$). Competitive kinetic assays against a series of Fe(II)-binding ligands determined the bimolecular rate constant for Fe(II) oxidation to be at least 5.3(±0.2)$\times10^4$ M$^{-1}$s$^{-1}$, with evidence pointing towards a true range of $10^7$ – $10^9$ M$^{-1}$s$^{-1}$. A multivariate experimental design was employed to evaluate the impact of factors that change the net rate and magnitude of ROS production during the oxidation of Fe(II). Laboratory-based measurements were validated in the field by measuring ROS production in an iron- and NOM-rich estuarine system. Two chemiluminescence-based methods (Acridinium Ester, MCLA) were modified for the real-time measurement of ROS in the presence of high levels
of Fe(II) and Fe(III). Recommendations for reduction of artifacts arising from Fe-mediated catalysis and method-associated interferences (i.e. precipitation of Ca/Mg-hydroxides) are reported. Results suggest that Fe(II) catalytically initiates the production and consumption of ROS in estuarine systems, with strong evidence for Fe(II) oxidation as a primary source for ROS under aphotic conditions. Removal of Fe(II) from the system leads to the rapid depletion of ROS, suggesting that Fe(II) is not a significant sink for ROS with reference to microbially-mediated decay or reactions with organic scavengers.
# TABLE OF CONTENTS

### DEDICATION

iii

### ACKNOWLEDGEMENTS

iv

### ABSTRACT

v

### LIST OF TABLES

ix

### LIST OF FIGURES

x

### LIST OF ABBREVIATIONS

xvi

### CHAPTER 1: REEVALUATION OF THE RATE CONSTANT FOR THE AUTOXIDATION OF Fe(II) USING A COMPETITIVE KINETICS APPROACH

1

1.1 ABSTRACT .......................................................... 2

1.2 INTRODUCTION .................................................. 3

1.3 EXPERIMENTAL .................................................. 5

1.4 RESULTS ............................................................ 10

1.5 DISCUSSION .......................................................... 14

1.6 CONCLUSIONS .................................................... 21

### CHAPTER 2: METHOD OPTIMIZATION FOR DETERMINATION OF REACTIVE OXYGEN SPECIES IN COMPLEX AQUATIC MATRICES

70

2.1 ABSTRACT .......................................................... 71

2.2 INTRODUCTION .................................................. 72

2.3 EXPERIMENTAL .................................................. 75

2.4 RESULTS AND DISCUSSION .................................................. 79


## 2.5 Conclusions

<table>
<thead>
<tr>
<th>Chapter 3: Field Measurements of Biogeochemically Produced Hydrogen Peroxide in Estuarine Sediments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Abstract</td>
</tr>
<tr>
<td>3.2 Introduction</td>
</tr>
<tr>
<td>3.3 Experimental</td>
</tr>
<tr>
<td>3.4 Results</td>
</tr>
<tr>
<td>3.5 Discussion</td>
</tr>
<tr>
<td>3.6 Conclusions</td>
</tr>
<tr>
<td>REFERENCES</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1.1 Design points for the five-factor central composite design used in all experiments

Table 1.2 Experimental conditions & corresponding maximum H₂O₂ yield for each pH condition

Table 1.3 Parameter estimates and F-tests for the quadratic model fitted to the data (pH 7.0)

Table 1.4 Parameter estimates and F-tests for the quadratic model fitted to the data (pH 7.5)

Table 1.5 Parameter estimates and F-tests for the quadratic model fitted to the data (pH 8.0)

Table 1.6 ANOVA for the response surface generated by the quadratic model fitted to the data (pH 7.0)

Table 1.7 ANOVA for the response surface generated by the quadratic model fitted to the data (pH 7.5)

Table 1.8 ANOVA for the response surface generated by the quadratic model fitted to the data (pH 8.0)

Table 3.1 Experimental decay rate vs predicted rates based on a 15 µM Fe(II) spike (2013)

Table 3.2 Experimental decay rate vs predicted rates based on a 15 µM Fe(II) spike (2014)
LIST OF FIGURES

Figure 1.1 General schematic for the application of competition kinetics to a mixed system containing a target species (X) and two potential reactants (R₁ and R₂) .......... 23

Figure 1.2 Competitive formation of the Fe(phen)_3^{2+} and FeFz₃ complex under anoxic conditions .......................................................... 25

Figure 1.3 Competitive formation of the Fe(phen)_3^{2+} and FeFz₃ complex under oxic conditions .......................................................... 26

Figure 1.4 Effect of pH on formation of the FeFz₃ complex with increasing Ferrozine concentration ......................................................... 27

Figure 1.5 Increasing levels of phosphate led to a decrease in the formation of the Fe(phen)_3^{2+} complex, with higher concentrations becoming more competitive .......... 28

Figure 1.6 Increasing levels of phosphate led to a decrease in the formation of the FeFz₃ complex, with higher concentrations becoming more competitive ................ 29

Figure 1.7 Competition plot between DTPA (variable) and Ferrozine (250 µM) for complexation of Fe(II) under anoxic and oxic conditions (pH 7.0) ......................... 30

Figure 1.8 Competition plot between DTPA (variable) and Ferrozine (250 µM) for complexation of Fe(II) under anoxic and oxic conditions (pH 7.5) ......................... 31

Figure 1.9 Competition plot between DTPA (variable) and Ferrozine (250 µM) for complexation of Fe(II) under anoxic and oxic conditions (pH 8.0) ......................... 32

Figure 1.10 Competition plot between DTPA (variable) and 1,10-phenanthroline (250 µM) for complexation of Fe(II) under anoxic and oxic conditions (pH 7.0) .............. 33

Figure 1.11 Competition plot between DTPA (variable) and 1,10-phenanthroline (250 µM) for complexation of Fe(II) under anoxic and oxic conditions (pH 7.5) .............. 34

Figure 1.12 Competition plot between DTPA (variable) and 1,10-phenanthroline (250 µM) for complexation of Fe(II) under anoxic and oxic conditions (pH 8.0) .............. 35

Figure 1.13 QA/QC – Stability of FeFz₃ complex in the presence of DTPA .................. 36
Figure 1.14 Competition plot between Ferrozine (variable) and DTPA (30 µM) for complexation of Fe(II) under oxic conditions .................................................................37

Figure 1.15 Production of O$_2^-$ following the spike addition of Fe(II) in the presence of increasing DTPA....................................................................................................................38

Figure 1.16 Proposed structure for the Fe(II)DTPA complex .............................................46

Figure 1.17 Calculated rates for Fe(II) oxidation as determined by competition between Ferrozine and dioxygen ........................................................................................................47

Figure 1.18 Calculated rates for the forward reaction as determined by competition between Phenanthroline and O$_2$ for Fe(II) in the presence of phosphate. [Phen] = 50 – 200 µM......................................................................................................................................48

Figure 1.19 Calculated rates for the forward reaction as determined by competition between Ferrozine and O$_2$ for Fe(II) in the presence of phosphate. [Fz] = 50 – 200 µM..49

Figure 1.20 Calculated rates for the forward reaction as determined by competition between Ferrozine and O$_2$ for Fe(II) in the presence of phosphate. [Fz] = 1 – 9 mM.....50

Figure 1.21 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with Ferrozine (pH 7.0).................................................................51

Figure 1.22 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with Ferrozine (pH 7.5).................................................................52

Figure 1.23 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with Ferrozine (pH 8.0).................................................................53

Figure 1.24 Effect of pH on competitive kinetics of Fe(II)DTPA formation under oxic conditions (Fz variable, DTPA constant).................................................................................................54

Figure 1.25 Comparison of predicted vs measured formation of FeFz$_3$ complex in solutions containing different ratios of Fz and DTPA ..................................................................................55

Figure 1.26 Structural comparison of the polyaminocarboxylate ligands EDTA and DTPA .................................................................................................................................56

Figure 1.27 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with 1,10-phenanthroline (pH 7.0)..................................................57

Figure 1.28 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with 1,10-phenanthroline (pH 7.5)..................................................58
Figure 1.29 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with 1,10-phenanthroline (pH 8.0)....59

Figure 1.30 Speciation diagram for DTPA at different aqueous pH conditions............60

Figure 1.31 Speciation diagram for hexaaquoiron(II) at different aqueous pH conditions..................................................................................................................61

Figure 1.32 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with deprotonated 1,10-phenanthroline (pH 7.0)....62

Figure 1.33 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with deprotonated 1,10-phenanthroline (pH 7.5)....63

Figure 1.34 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with deprotonated 1,10-phenanthroline (pH 8.0)....64

Figure 1.35 Speciation diagram for 1,10-phenanthroline at different aqueous pH conditions..................................................................................................................65

Figure 1.36 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with monoprotonated 1,10-phenanthroline (pH 7.0) ....66

Figure 1.37 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with monoprotonated 1,10-phenanthroline (pH 7.5) ....67

Figure 1.38 Calculated complexation rates for Fe(II)DTPA under anoxic and oxic conditions based on competition with monoprotonated 1,10-phenanthroline (pH 8.0) ....68

Figure 1.39 Competition plot for the generation of O$_2^-$ arising from the oxidation of Fe(II) in the presence of DTPA..................................................................................................................69

Figure 2.1 Flow diagram for a typical setup employing the acridinium ester-based FIA-CL method .................................................................89

Figure 2.2 Dependence of DTPA concentration to maintain adequate light transmittance after pH adjustment..................................................................................................................90

Figure 2.3 Matrix effects on the availability of DTPA to chelate Fe(II). No DTPA........91

Figure 2.4 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 5 µM.................................................................................................................................92

Figure 2.5 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 50 µM.................................................................................................................................93
Figure 2.6 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 500 µM .......................................................... 94

Figure 2.7 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 5 mM .......................................................... 95

Figure 2.8 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 50 mM ......................................................... 96

Figure 2.9 Matrix effects on the availability of SRNOM to chelate Fe(II). [SRNOM] = 16 mg C/L ..................................................... 97

Figure 2.10 HCl titration of 15 µM Fe(II)DTPA in the presence of 1 mM 1,10-phenanthroline ..................................................... 98

Figure 2.11 Absorbance spectrum of 15 µM [Fe(phen)_3]^{2+}, 15 µM [Fe(phen)_3]^{3+}, and 18 MΩ (blank) ........................................ 99

Figure 2.12 Maximum H_2O_2 produced via Fe(II) oxidation upon mixing with carbonate buffer .............................................. 100

Figure 2.13 Inhibition of Fe-mediated generation of H_2O_2 in the presence of varying DTPA ................................................... 101

Figure 2.14 “Pseudobase” formed by the reaction of acridinium ester with hydroxide ion ......................................................... 102

Figure 2.15 Concentration-dependent signal of varying acridinium ester in constant H_2O_2 (500 nM) .......................................... 103

Figure 2.16 Concentration-dependent signal of varying acridinium ester in constant H_2O_2 (50 nM) ........................................ 104

Figure 2.17 Quenching of acridinium ester chemiluminescence in the presence of particulate matter ........................................ 105

Figure 2.18 Comparison of reported Fe and DTPA used in literature vs current study ......................................................... 106

Figure 3.1 Mechanistic diagram illustrating the generation of ROS from reduced Fe in estuarine systems ................................... 121

Figure 3.2 Synthesis of the acridinium ester ............................................................................................................................ 122

Figure 3.3 ^1H NMR spectrum of the synthesized acridinium ester ......................................................................................... 123

Figure 3.4 ^13C NMR spectrum of the synthesized acridinium ester ..................................................................................... 124
Figure 3.5 Flow chart detailing the analytical sequence for continuous flow determination of H₂O₂........................................................................................................................................125

Figure 3.6 Representative H₂O₂ calibration curve......................................................................................................................................................126

Figure 3.7 Extent of tidal variation on sampling location during high tide and low tide, Folly Beach, SC ...........................................................................................................................................................................127

Figure 3.8 Physicochemical composition of surface water at the sampling site including salinity, pH, temperature, TOC, and tidal variation (July 7th-8th, 2013)........................................................................................................128

Figure 3.9 Physicochemical composition of surface water at the sampling site including dissolved oxygen, Fe(II), H₂O₂, ²²⁴Ra, and tidal variation (July 7th-8th, 2013)........................................................................................................129

Figure 3.10 Physicochemical composition of surface water at the sampling site including salinity, pH, temperature, pH, and tidal variation (July 30th-31st, 2014) ........................................................................................................130

Figure 3.11 Physicochemical composition of surface water at the sampling site including dissolved oxygen, Fe(II), H₂O₂, O₂⁻, and tidal variation (July 30th-31st, 2014) ..................................................................................................................131

Figure 3.12 A plot of H₂O₂ vs O₂⁻.................................................................................................................................................................................................132

Figure 3.13 A plot of Fe(II) vs O₂⁻.................................................................................................................................................................................................133

Figure 3.14 A plot of Fe(II) vs H₂O₂ .................................................................................................................................................................................................134

Figure 3.15 Fe(II) and H₂O₂ decay following injection of 15 µM Fe(II) to one liter creek water (4:57 PM, 2013) ........................................................................................................................................................................135

Figure 3.16 Fe(II) and H₂O₂ decay following injection of 15 µM Fe(II) to one liter creek water (6:27 PM, 2013) ........................................................................................................................................................................136

Figure 3.17 Fe(II) and H₂O₂ decay following injection of 15 µM Fe(II) to one liter creek water (7:58 PM, 2013) ........................................................................................................................................................................137

Figure 3.18 Fe(II) and H₂O₂ decay following injection of 15 µM Fe(II) to one liter creek water (12:50 AM, 2013) ........................................................................................................................................................................138

Figure 3.19 First order decay of Fe(II) following injection into one liter creek water........................................................................................................139

Figure 3.20 Fe(II) vs H₂O₂ following 15 µM Fe(II) spike (4:57 PM, 2013) ........................................................................................................................................................................140

Figure 3.21 Fe(II) vs H₂O₂ following 15 µM Fe(II) spike (6:27 PM, 2013) ........................................................................................................................................................................141

Figure 3.22 Fe(II) vs H₂O₂ following 15 µM Fe(II) spike (7:58 PM, 2013) ........................................................................................................................................................................142
Figure 3.23 Fe(II) vs H$_2$O$_2$ following 15 µM Fe(II) spike (12:50 AM, 2013) ..........143

Figure 3.24 H$_2$O$_2$ decay rates following injection of 1 µM H$_2$O$_2$ spike to one liter creek water (2013) ..............................................................................................................................................144

Figure 3.25 H$_2$O$_2$ decay rates following injection of 1 µM H$_2$O$_2$ spike to one liter creek water (2014) ..............................................................................................................................................145

Figure 3.26 Effect of DTPA addition after Fe(II) spike on H$_2$O$_2$ activity .................146
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDOM</td>
<td>Chromophoric Dissolved Organic Matter</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FIA</td>
<td>Flow Injection Analysis</td>
</tr>
<tr>
<td>FZ</td>
<td>Ferrozine</td>
</tr>
<tr>
<td>HO·</td>
<td>Hydroxyl Radical</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HEPES</td>
<td>Flow Injection Analysis</td>
</tr>
<tr>
<td>k$_{Fe(II)DTPA}$</td>
<td>Complexation Rate Constant for Fe(II) with DTPA</td>
</tr>
<tr>
<td>k$_{Fe(II)Fz}$</td>
<td>Complexation Rate Constant for Fe(II) with Ferrozine</td>
</tr>
<tr>
<td>k$_{Fe(II)Phen}$</td>
<td>Complexation Rate Constant for Fe(II) with 1,10-phenanthroline</td>
</tr>
<tr>
<td>NOM</td>
<td>Natural Organic Matter</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide Anion Radical</td>
</tr>
<tr>
<td>Phen</td>
<td>1,10-phenanthroline</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>R·</td>
<td>Carbon-Centered Radical</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SGD</td>
<td>Submarine Groundwater Discharge</td>
</tr>
<tr>
<td>TOC</td>
<td>Total Organic Carbon</td>
</tr>
</tbody>
</table>
CHAPTER 1

Reevaluation of the Rate Constant for the Autoxidation of Fe(II) Using a Competitive Kinetics Approach
1.1 Abstract

The rapid redox transformations of Fe(II)/Fe(III) species during the net oxidation of Fe(II) make determination of the forward rate constant difficult. Historically, direct measurements of the oxidation rate under varying conditions have been conducted by monitoring the loss of Fe(II) using colorimetric Fe(II)-binding ligands. However, the utility of these approaches are limited in that they do not account for the rapid Fe(II)/Fe(III) cycling during the net oxidation process. There is a discrepancy between reported oxidation rates arising from these studies ($k = 13 \text{ M}^{-1}\text{s}^{-1}$) and established rate constants for the superoxide-mediated reduction of Fe(III) ($k = 10^8 \text{ M}^{-1}\text{s}^{-1}$). Here, we report the results of a series of competitive kinetics experiments to determine the rate of this reaction. Rates calculated from competition of Ferrozine and dioxygen for Fe(II) yielded rate constants between $61 - 390 \text{ M}^{-1}\text{s}^{-1}$ for the pH range 6.70 – 8.45, highlighting the limitation of direct observations in systems where complexation can compete with rapid redox reactions. Competitive kinetic assays against a series of Fe(II)-binding ligands determined the bimolecular rate constant for Fe(II) oxidation to be at least $5.3(\pm 0.2) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, with evidence pointing towards a true range of $10^7 – 10^9 \text{ M}^{-1}\text{s}^{-1}$. The complexation rate for the reaction of Fe(II) with DTPA was calculated to be $4.9(\pm 0.9) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and found to inhibit the Fe-catalyzed generation of ROS. The proposed mechanism for Fe(II) oxidation (as competition) was validated using a multivariate approach by studying the Fe-catalyzed generation of ROS in the presence of species capable of altering the forward or back reaction. Factors favoring the reverse reaction led to prolonged Fe(II)/Fe(III) cycling and lower observed ROS yields, while factors favoring the removal of Fe(III) through precipitation exhibited shorter net oxidation rates and higher ROS yields.
1.2 Introduction

The net oxidation of Fe(II) in natural waters is a complex process consisting of alternating cycles of Fe(II) oxidation and Fe(III) reduction processes that are ultimately terminated via precipitation as insoluble Fe(III) (oxy)hydroxides\textsuperscript{1-6}. In addition to controlling the bioavailability of Fe(II) for regulation of primary production,\textsuperscript{7} this process serves as an integral link between the biogeochemical cycles of oxygen, carbon, and sulfur through the generation of Reactive Oxygen Species (ROS). The ROS generated by this process, including superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO·), and carbon centered radicals (R·), are all capable of acting as secondary oxidants and reductants, thereby constituting a catalytic mechanism for the cyclic redox transformation of aqueous iron species.\textsuperscript{2, 8, 9} A proposed mechanism for the Fe-mediated generation of ROS is depicted in reactions 1-3.

\begin{align*}
\text{Fe}^{II} + \text{O}_2 & \rightleftharpoons \text{Fe}^{III} + \cdot \text{O}_2^- \\
\text{Fe}^{II} + \cdot \text{O}_2^- + 2\text{H}^+ & \rightleftharpoons \text{Fe}^{III} + \text{H}_2\text{O}_2 \\
\text{Fe}^{II} + \text{H}_2\text{O}_2 & \rightleftharpoons \text{Fe}^{III} + \text{HO}· + \text{OH}^- \\
\end{align*}

While the vital role of iron oxidation reactions towards generation of ROS in aquatic systems has gained recognition in recent years, the kinetics for the reaction of Fe(II) with dioxygen are not fully understood. In particular, there is a discrepancy between published bimolecular rate constants for the autoxidation of Fe(II). While the forward reaction (Rxn 1) has a published rate constant of 13 M$^{-1}$s$^{-1}$,\textsuperscript{10} the reverse reaction has a published rate constant on the order 10$^{8}$ M$^{-1}$s$^{-1}$.\textsuperscript{8, 10} Based on kinetics alone, this would indicate that Fe(II) should be the kinetically stable species in solution, as opposed to
Fe(III). In reality, the opposite is true, suggesting the presence of an unexplored aspect of iron oxidation in aqueous systems. The kinetics of this process have historically been studied by measuring the loss of Fe(II) using an Fe(II)-binding indicator ligand, such as Ferrozine (Fz) or 1,10-phenanthroline (Phen).\textsuperscript{11,12} However, Burns \textit{et al.} reported that iron may cycle between ferrous and ferric oxidation states anywhere between 10 – 2200 times.\textsuperscript{8} The rapid electron exchange implied by Burns’ work suggests that previous studies may be underestimating the rate of Rxn 1 by not differentiating between the forward kinetics of Rxn 1 and the net rate for transformation of Fe(II) to Fe(III).

We hypothesize that the net oxidation of Fe(II) is a path-dependent function consisting of two competing, kinetically-fast reactions. This would imply that the forward reaction must be comparable in magnitude to the back reaction. As a test of the stated hypothesis, the kinetics of the forward reaction were investigated using a competitive kinetics approach.

According to competition kinetics, in a mixed system where two reactants (R\textsubscript{1} and R\textsubscript{2}) compete for reaction with a target species (X) (Figure 1.1), the fraction of X going on to form a particular product (P\textsubscript{1} or P\textsubscript{2}) is given by Eqn 1. This expression is derived from the individual rate law expressions for each reaction.

\[
\text{Fraction}_{X \rightarrow P_1} = \frac{k_1 [X][R_1]}{k_1 [X][R_1] + k_2 [X][R_2]} 
\]  

(1)

The competition kinetics method is a well-known technique for studying rapid electron-transfer reactions \((k \approx \text{diffusion limited})\), particularly those involving highly reactive species where direct measurement of the analyte or product is impractical/impossible, such as carbonate radical (\textbullet\text{CO}_3^{2-}),\textsuperscript{13-15} hydroxyl radical (HO\textbullet),\textsuperscript{16-18} singlet oxygen (\textsuperscript{1}O\textsubscript{2}),\textsuperscript{19-22} solvated electrons (e\textsubscript{aq}{}),\textsuperscript{23-25} and others.\textsuperscript{26-28} The reliable derivation of rate constants via
competitive kinetics is dependent upon proper manipulation of experimental conditions such that reactions other than those involving a reactive species with the target molecule or reference compound are eliminated or minimized. The overwhelming majority of competitive studies avoid such interferences by using pulse radiolytic generation mechanisms, whereby relatively pure solutions are bombarded by a pulse of ionizing radiation to form the reactive species. However, this is impractical for the study of Haber-Weiss and/or Fenton-based mechanisms which can introduce a host of potential side reactions due to the redox cycling of Fe(II) and Fe(III). In this study, the kinetics of Fe(II) oxidation were explored using a series of competitive kinetics experiments designed to isolate the forward reaction from the reverse reaction by the use of Fe(II)-binding ligands. When feasible, experiments were conducted both in the presence (air-saturated) and absence of O₂ to isolate oxidation reactions and kinetics.

1.3 Experimental Materials

Sodium hydroxide, sodium bicarbonate, sodium carbonate, and sodium phosphate monobasic were obtained from Fisher Scientific. Ferrozine reagent (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate), 1,10-phenanthroline, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), catalase from bovine liver, diethylenetriaminepentaacetic acid (DTPA), and H₂O₂ (30%) were obtained from Sigma Aldrich. Iron(II) chloride (anhydrous, 99.5%) was obtained from Alfa Aesar. Ammonium acetate, hydrochloric acid, and 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo-[1,2-a]pyrazine-3-one (MCLA) were obtained from VWR. Unless otherwise noted, reagents were used as received without purification, and all solutions were prepared in 18 MΩ water. All solutions used for H₂O₂ analysis were prepared in water that
had been amended with catalase (3 mg/L) and allowed to sit overnight. Catalase-amended water was boiled for approximately one hour before analysis to render the enzyme inert and give H₂O₂-free water. All glassware was cleaned in a muffle furnace and allowed to soak overnight in a 10% HCl solution, followed by rinsing with 18 MΩ water prior to use.

**Procedures**

**Competitive Kinetics: Complexation vs Oxidation**

A series of pH-adjusted HEPES-buffered solutions (25 mM HEPES) were prepared ranging from 6.70 – 8.5. This range encompasses the published effective range for HEPES (6.80 – 8.20) and provided adequate buffering capacity for all experiments. All solutions were amended with a colorimetric Fe(II)-binding ligand, either Ferrozine or 1,10-phenanthroline, to reach a concentration of 60 µM ligand. Competition experiments were performed both in the presence and absence of dioxygen, hereafter referred to as oxic (e.g. air-saturated) and anoxic, respectively. In a system containing O₂ and some Fe(II)-binding ligand, the fate of Fe(II) can be described by Rxn 4 and Rxn 5, where L is either Ferrozine or 1,10-phenanthroline. The complexation of Fe(II) with Ferrozine (k_{Fe(II)Fz} = 3.08 × 10^{11} \text{M}^{-3}\text{s}^{-1}) and 1,10-phenanthroline (k_{Fe(II)Phen} = 2.25 × 10^{17} \text{M}^{-3}\text{s}^{-1}) are well-known, kinetically fast processes yielding stable, colored products.\textsuperscript{12, 29, 30} Since the products of Rxn 5 are colorless, the fate of Fe(II) between the two reaction pathways can be monitored easily by the development, or absence, of the FeFz\textsubscript{3} or Fe(phen)\textsubscript{3}^{2+} complexes.

\[
\text{Fe(II)+3L} \rightarrow \text{FeL}_3 \quad (4)
\]

\[
\text{Fe(II)+O}_2 \rightleftharpoons \text{Fe(III)+O}_2 \quad (5)
\]
Oxic experiments were performed using air-saturated 18 MΩ. Anoxic experiments were conducted inside a N₂-filled glovebox. All pH adjusted solutions for anoxic experiments were sparged for a minimum of 12-hours with N₂ prior to being transferred to a N₂-filled glovebox. In order to prevent oxidation, preparation of Fe(II) stock solutions differed depending on anoxic or oxic experimental conditions. Preparation of Fe(II) stock solutions for oxic conditions consisted of boiling 18 MΩ for one hour, followed by cooling to room temperature under a gentle stream of N₂. Solid FeCl₂ (anhydrous) stock was added to the room temperature, deoxygenated 18 MΩ and the solution was continuously sparged with N₂ for the duration of the experiment. For anoxic experiments, 18 MΩ was boiled for one hour, cooled while being sparged with N₂, and acidified to pH 3 before being transferred to a glovebox. Addition of solid FeCl₂ stock (anhydrous) to acidified 18 MΩ occurred inside the glovebox.

Experiments were initiated by the spike addition of anoxic Fe(II) stock solution to reach a concentration of 15 µM in the indicator-amended HEPES solutions. Solution preparation for anoxic experiments was performed manually, while oxic experiments utilized a J-Kem scientific solution-handling robot. All experiments were run in triplicate (n = 3). Samples were analyzed spectrophotometrically for formation of the FeFz₃ (λₘₐₓ = 562 nm, ε = 27,900 M⁻¹cm⁻¹) or Fe(phen)₃²⁺ (λₘₐₓ = 510 nm, ε = 11,100 M⁻¹cm⁻¹) complex.

**Competitive Kinetics: Complexation vs Oxidation in the Presence of Phosphate**

To investigate the effect of the back reaction on the calculated forward reaction, competition experiments were conducted in the presence of increasing levels of a known Fe(III) scavenger, phosphate. pH adjusted phosphate solutions (pH 8.50, [PO₄³⁻] = 5 – 250 mM) were amended with varying levels of either Fz or Phen ([Ligand] = 50, 75, 100,
or 200 µM. Experiments were conducted under air-saturated conditions (measured [O₂] = 260 µM) and initiated by the introduction of Fe(II) to reach a concentration of 15 µM. All experiments were performed in triplicate (n = 3) and the concentration of each metal-ligand complex was determined spectrophotometrically as described above.

**Competitive Kinetics: Colorimetric Ligands vs DTPA**

While the kinetics for complexation of Fe(II) with Ferrozine are well-known, formation rates of the Fe(II)DTPA complex have yet to be determined. To determine the complexation rate of Fe(II) with DTPA, competition experiments were conducted between DTPA vs Fz, as well as DTPA vs Phen. Since the Fe(II)DTPA complex is colorless, the kinetics for the system could be calculated in the same manner as before (i.e. development or absence of colored product). Care was taken to ensure that reaction conditions were identical for both conditions, such that the only reactions for Fe(II) to undergo would be complexation with Ferrozine (Rxn 6) or with DTPA (Rxn 7).

\[
\text{Fe(II)+3Fz} \rightarrow \text{FeFz}_3 \quad (6)
\]

\[
\text{Fe(II)+DTPA} \rightarrow \text{FeDTPA} \quad (7)
\]

Experiments were performed both in the presence and absence of dioxygen, hereafter referred to as oxic and anoxic, respectively. Oxic experiments were performed using air-saturated 18 MΩ. Anoxic experiments were conducted inside a N₂-filled glovebox. All pH adjusted solutions for anoxic experiments were subjected to a minimum 12-hour sparge with N₂ prior to being transferred to the glovebox. Fe(II) stock solutions were prepared as detailed previously, such that solutions for anoxic experiments were acidified to pH 3 before introduction into the glovebox, while Fe(II) stock solutions for oxic experiments were constantly sparged with a steady flow of N₂. 25 mM HEPES buffer
was used for pH maintenance. Initiation of each experiment occurred via introduction of Fe(II) to a concentration of 15 µM. The concentration of FeFz3 complex was quantified spectrophotometrically within 15 minutes of Fe(II) addition. QA/QC experiments indicated that the FeFz3 complex was stable in the presence of DTPA during this time period. All experiments were performed under dark conditions. As a test to the validity of using the competition kinetics method, this experiment was repeated with reverse conditions, such that DTPA was kept constant at 30 µM and Ferrozine was varied from 70 – 900 µM.

**Competitive Kinetics: DTPA vs Oxidation**

Sample solutions consisting of 18 MΩ water that had been amended with varying levels of stock DTPA solution ([DTPA] = 25 µM – 320 mM) were prepared in clean, acid-washed glassware. O2 was sampled continuously using the MCLA method via a commercially available flow-injection analysis (FIA) setup (FeLume, Waterville Analytical). Immediately after data acquisition on the FeLume software was initiated, anoxic Fe(II) stock solution was spiked into the DTPA-amended solutions to reach a concentration of 15 µM. O2 was monitored continuously following the spike addition of Fe(II). Chemiluminescent data (n = 1000 data points) were averaged and converted to O2 concentrations via calibration curves obtained prior to sampling. Fe(II) was measured at regular intervals by transferring aliquots of sample solution into separate vials preloaded with Ferrozine for spectrophotometric analysis.
Factors Affecting Fe-Catalyzed ROS Formation

A 3-factor central composite experimental design was employed to interrogate the relationship between Fe(III), carbonate (CO$_3^{2-}$-$\text{Total}$), and phosphate (PO$_4^{3-}$) on the rate of Fe(II) oxidation and consequent generation of H$_2$O$_2$. Each factor was varied across five levels, as dictated by Design Expert software (Table 1.1). For interrogation of parameter space, the design required six replicate measurements at the center point, and three replicate measurements for all other conditions, leading to 20 experimental conditions and a total of 48 individual experiments. Since pH could be used as an independent factor, the overall matrix was run at pH 7.0, 7.5, and 8.0. The stock solutions of CO$_3^{2-}$ and PO$_4^{3-}$ were pH-adjusted prior to the desired value for each matrix, and pH measurements before and after each experiment stayed within 0.1 pH unit. Each experiment was initiated by the simultaneous introduction of an (anoxic) Fe(II) stock solution and an Fe(III) stock solution. Fe(II) and H$_2$O$_2$ were measured using the Ferrozine and acridinium ester technique, respectively.

1.4 Results
Competitive Kinetics: Complexation vs Oxidation

Experiments conducted under anoxic conditions showed 100% formation of the FeFz$_3$ and Fe(phen)$_3^{2+}$ complexes throughout the entire pH range studied (Fig. 1.2). Experiments conducted under oxic conditions saw inhibited formation of each complex at higher pH conditions, consistent with studies reporting pH-dependence on the oxidation rate of Fe(II) (Fig. 1.3). Dioxygen competed for Fe(II) against Fz at pH 7.84 – 8.45, effectively inhibiting the formation of the FeFz$_3$ complex to 50%. Some competition was observed against Phen, but only at the higher pH regime of 8.14 – 8.45, with a 20% decrease in formation of the Fe(phen)$_3^{2+}$ complex. This result is consistent with the
differences in complexation rates for the two ligands. The competition between Fz and O₂ was further tested using higher concentrations of ligand at pH conditions where dioxygen competition was significant. The observed concentration dependence serves as validation for the use of the competition kinetics method for this system (Fig. 1.4).

**Competitive Kinetics: Complexation vs Oxidation in the Presence of Phosphate**

The presence of increasing levels of phosphate led to a decrease in the formation of both the Fe(phen)₃²⁺ (Fig. 1.5) and FeFz₃ (Fig. 1.6) complexes. While formation of the Fe(phen)₃²⁺ complex decreased with increasing PO₄³⁻, it remained above detection limit even at 250 mM PO₄³⁻. Formation of the FeFz₃ complex, on the other hand, fell below detection limit at equal concentrations (Fz = 50 – 200 µM). However, experiments where Fz was present above 1 mM exhibited competition similar to the Phen experiments (Fig 1.5 – 1.6). These results are consistent with Burns’ finding that elevated levels of phosphate rapidly scavenge Fe(III) from engaging in further redox cycling.¹

**Colorimetric Ligands vs DTPA**

DTPA competed with both Fz and Phen for available Fe(II) under both oxic and anoxic conditions (Fig. 1.7 - 1.12). The colored complexes were stable in the presence of DTPA within our experimental timeframe encompassing the initial formation of complex and measurement via UV-Vis (Fig. 1.13). No significant difference was observed between oxic and anoxic experiments at each pH condition, suggesting that Fe(II) oxidation was not significant in the presence of two Fe(II) binding ligands. No evidence was observed for pH dependence in the competition of DTPA vs Fz However, pH effects were apparent in the competition of DTPA vs Phen as the DTPA-mediated inhibition of Fe(phen)₃²⁺ formation seemed to increase with pH.
QA/QC experiments where Fz was varied and DTPA held constant showed similar behavior (Figure 1.14). Based off of the minimal differences observed between previous anoxic and oxic conditions, this experiment was conducted only under oxic conditions. Again, no pH effects were observed when DTPA was pitted against Fz.

**Competitive Kinetics: DTPA vs Oxidation**

Generation of $O_2^-$ following the addition of Fe(II) was inversely proportional to the concentration of DTPA (Fig. 1.15), with the exception of experiments conducted in the absence of DTPA, where $O_2^-$ was likely low due to reactions with unchelated iron species. These results show that complete inhibition of Fe(II) oxidation, experimentally defined by $O_2^-$ generation at or below detection limit, was only achieved when DTPA was present in large excess with respect to Fe(II). No Fe(II) was detectable in aliquots that were transferred into vials preloaded with Fz. While preceding experiments showed competition between DTPA and Fz for Fe(II), this experiment differed in that samples from which aliquots were pulled had already been exposed to DTPA. The Fe(II)DTPA complex has a stability constant of 16.0 (as log K) (Fig 1.16)\(^{,31}\) making it unlikely that Fe(II) already bound to DTPA would be mobilized for complexation with Fz.

**Factors Affecting Fe-Catalyzed ROS Formation**

The relationship between the dependent variable, generation of $H_2O_2$, and the independent variables $x_1$ ([Fe(III)]), $x_2$ ($CO_3^{2-}_{\text{Total}}$), and $x_3$ ($PO_4^{3-}$) was interrogated by fitting a full quadratic expression to the data, such that:

$$[H_2O_2] = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$  \hspace{1cm} (2)

Where $\beta_0$ is a constant offset term (intercept); $\beta_1$, $\beta_2$, and $\beta_3$ are estimates of the linear effects of each variable to the outcome ($H_2O_2$ generation); $\beta_{11}$, $\beta_{22}$, $\beta_{33}$ are estimates...
accounting for curvature in the linear quadratic model from each variable; and $\beta_{12}$, $\beta_{13}$, $\beta_{23}$ are estimates for effects of variable-variable interaction. All $\beta$ values were calculated for a quadratic model using Design Expert 7 software and used to determine the relative impact of each variable and variable interaction to the outcome ($H_2O_2$ generation). A comprehensive list of model $\beta$ values, as well as ANOVA results for the response surface and final equations expresses in both coded and uncoded factors are depicted in Table(s) 1.2-1.8.

Simplification of the quadratic expression for each pH condition, using only those terms deemed significant at the 95% confidence level yields Eqn 3-5.

\[
[H_2O_2](pH=7.0) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2
\]  

(3)

\[
[H_2O_2](pH=7.5) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{12} x_1 x_2
\]  

(4)

\[
[H_2O_2](pH=8.0) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{12} x_1 x_2
\]  

(5)

The model indicated that, of those terms deemed significant, Fe(III), Fe(III)-CO$_3^{2-}$, and Fe(III)-PO$_4^{3-}$ all had negative impacts on the generation of $H_2O_2$, while CO$_3^{2-}$, PO$_4^{3-}$, (Fe(III))$^2$, (CO$_3^{2-}$)$^2$, and (PO$_4^{3-}$)$^2$ all showed positive impacts. The sum of squares for each factor indicated that regardless of pH, the presence of Fe(III) accounted for between 40 – 50% of the model, with CO$_3^{2-}$ accounting for 5 – 18%, and PO$_4^{3-}$ accounting for 12-28%. The interaction between Fe(III)-PO$_4^{3-}$ was only found to be significant at pH 7.0, with the interaction between Fe(III)-CO$_3^{2-}$ rising to prominence at pH 7.5 and 8.0. Comparison of the predictive capacity for $H_2O_2$ generation and net Fe(II) oxidation rate favored the former, as evidenced by $R^2$ values for pH 7.0, 7.5 and 8.0 ($H_2O_2$ – 0.9731, 0.9239, 0.9297; Fe(II) – 0.6095, 0.6287, 0.8001). This suggests that measurement of oxidation products
such as H₂O₂ may serve as more reliable and sensitive indicators for determining the kinetics of Fe(II) oxidation.

1.5 Discussion

The kinetics of the reaction of Fe(II) with O₂ were investigated under the hypothesis that it is kinetically competitive with the O₂⁻-mediated reduction of Fe(III) (k ~ 10⁸ M⁻¹s⁻¹). According to the competition kinetics method, a system where dioxygen competes with a bidentate ligand (L = Ferrozine, 1,10-phenanthroline, etc) for reaction with Fe(II) can be described by Eqn 6.

\[
\text{Fraction}_{\text{Fe(II)} \rightarrow \text{FeL}^3} = \frac{k_{\text{Fe-L}} [\text{Fe(II)}]_0 [L]^3}{k_{\text{Fe-L}} [\text{Fe(II)}]_0 [L]^3 + k_{\text{Fe-O2}} [\text{Fe(II)}]_0 [O_2]}
\]

(6)

For each condition where a significant difference in formation of the FeFz₃ complex between oxic and anoxic experiments was observed, an estimate for the rate of Fe(II) oxidation was calculated (Fig. 1.17). Averaged Fe(II) autoxidation rates ranged from 61 to 390 M⁻¹s⁻¹ when compared against Ferrozine. No ROS measurements were taken for these experiments. However, given proposed mechanisms for Haber-Weiss reactions, it is likely that significant production of ROS took place. If this were the case, then the possibility of rapid Fe(II)/Fe(III) cycling cannot be ruled out and no differentiation can be made between the forward reaction and the net oxidation rate of Fe(II). This experiment highlights the limitations inherent in the determination of Fe(II) oxidation kinetics.

For the accurate measurement of the kinetics for the forward reaction, experiments were conducted in the presence of the Fe(III) scavenger, phosphate, to block the back reaction. Calculated rates based on Phen competition showed little variation 10⁸ – 10⁹ M⁻¹s⁻¹ (Fig. 1.18) while those calculated from Fz competition varied from 10⁴ – 10⁷ M⁻¹s⁻¹ (Fig. 1.19 - 1.20). The convergence of the Fz rates with those calculated by the Phen-based
competition suggests that the oxidation rate for Fe(II) is within the range $10^7 - 10^9$ M$^{-1}$s$^{-1}$.

These results highlight the need for kinetic studies to account for the back reaction and make efforts to isolate it.

The autoxidation of Fe(II) leads to the generation of two species, Fe(III) and the short-lived superoxide anion radical ($O_2^-$). Competition studies focusing on the generation of $O_2^-$ may offer a more sensitive approach for determining the oxidation rate of Fe(II). The reaction of Fe(II) with DTPA results in formation of a strong, stable complex with no visible color, making it advantageous for chemiluminescent measurements of $O_2^-$. An additional set of competitive kinetics experiments were performed to investigate the rate of Rxn 1 by using formation of $O_2^-$ as the observable for competition of Fe(II) between dioxygen and DTPA.

Prior to this experiment, it was necessary to calculate a rate for the complexation of Fe(II) with DTPA. In the preceding experiment, the concentrations of both dioxygen and the Fe(II) binding ligand were kept constant. In a system where one species is kept constant and the concentration of the second species is varied, Eqn 6 can be algebraically rearranged to Eqn 7.

\[
\frac{1}{\text{Fraction}_{\text{Fe(II)}\rightarrow\text{FeL}_3}} - 1 = \frac{k_{\text{Fe-DTPA}}[\text{DTPA}]}{k_{\text{Fe-Fz}}[L]^3}
\]  

(7)

A plot of the left side of Eqn 6 vs [DTPA]/[L]$^3$ yields a straight line with slope equal to a ratio of the rates, from which the complexation rate of DTPA can be calculated. Competition experiments against Fz yielded Fe(II)DTPA complexation rates of $4.9 \pm 3.9 \times 10^5$ M$^{-1}$s$^{-1}$ (Fig. 1.21 - 1.23). QA/QC experiments where DTPA was held constant and the concentration of Fz was varied yielded complexation rates of $4.9(\pm 0.9) \times 10^5$ M$^{-1}$s$^{-1}$ (1.24). Despite the large error in the preceding rate, the two are in close agreement. As a
further test, the calculated complexation rate for Fe(II)DTPA was used to generate a model to predict the formation of the FeFz3 complex in the presence of varying DTPA and constant Ferrozine. A plot of the measured concentrations of FeFz3 vs predicted concentrations of FeFz3 for pH 7.0 – 8.0 (Fig. 1.25) confirms the validity of the calculated kFe(II)DTPA. To our knowledge, this is the first reported calculation of the bimolecular rate constant for Fe(II)DTPA, so no direct comparisons are available. However, Fujii et al. utilized the method of competitive kinetics to determine the formation rate for Fe(II)EDTA as 2.1(±0.2)×10^6 M^{-1}s^{-1}. Our value for kFe(II)DTPA is approximately an order of magnitude slower, presumably due to increased steric effects/ring strain involved in the ring closure of the larger DTPA as compared to EDTA (Fig. 1.2).

DTPA was observed to compete with Phen for Fe(II) at much higher concentrations than necessary for Fz. Calculated kFe(II)DTPA derived from the DTPA-Phen (Fig. 1.27-1.29) competition exhibited a dependence on pH and were several orders of magnitude higher than those calculated in the DTPA-Ferrozine competition. This deviation is unexpected, as both Fz and Phen are bidentate ligands forming 1:3 complexes with Fe(II). Theoretically, the only difference in competition between DTPA-Fz and DTPA-Phen would be the values for kFe(II)Fz and kFe(II)Phen. This is seemingly backed up by results showing the increase in DTPA necessary to outcompete reaction with Phen as compared to Fz. The increase in calculated kFe(II)DTPA with pH implies ligand speciation may be a significant factor. pH effects arising from DTPA speciation (Fig. 1.30) or Fe(II) speciation (as Fe(H2O)6^{2+}, Fig 1.31) were discounted as no such effects were observed in any of the DTPA-Fz competitions (Fig 1.7 - 1.9, 1.14, 1.21 - 1.23). Competition plots taking relevant concentrations of Phen speciation into account do not seem to resolve the issue. No
significant difference is observed between the total Phen concentration and the deprotonated Phen species (Fig 1.32-1.34), consistent with this being the major species at pH 7.0 and above (Fig 1.35). Phen is often used to bind Fe(II) in highly acidic media, so it may be possible that the relevant species to take into account is the monoprotonated form (HPhen⁺). Calculated $k_{Fe(II)DTPA}$ taking only the monoprotonated phenanthroline species into account yields much lower values on the order of $10^0 - 10^2$ M⁻¹s⁻¹, with a noticeable decrease as pH is increased (Fig. 1.36 - 1.38). While the kinetics of $k_{Fe(II)DTPA}$ have not been measured in previous studies, it has widely been acknowledged to be rapid, suggesting that this calculation is incorrect. This suggests that phenanthroline speciation can be ruled out as the root cause of the difference.

If speciation is not the cause, then the observed difference between DTPA-Fz and DTPA-Phen competitions must be related to the actual mechanism of complexation. Both Fz and Phen are reported to bind Fe(II) by the mechanism illustrated in Eqn 8-10 involving two rapidly established equilibrium steps and a final rate-determining step leading to formation of a stable, colored complex. Dissociation constants for FeFz₃ (4.25×10⁻⁵ s⁻¹) and Fe(phen)₃²⁺ (7.5×10⁻⁵ s⁻¹) are negligibly small with respect to formation, so formation of the complexes are assumed to go to completion.

$$\text{Fe(II)} + \text{L} \rightleftharpoons \text{Fe(II)L} \quad (8)$$

$$\text{Fe(II)L} + \text{L} \rightleftharpoons \text{Fe(II)L}_2 \quad (9)$$

$$\text{Fe(II)L}_2 + \text{L} \rightleftharpoons \frac{k_f}{k_d} \text{Fe(II)L}_3 \quad (10)$$
Some evidence has been reported regarding the relative stability and persistence of intermediate Fe(phen)$^{2+}$ and Fe(phen)$_2^{2+}$ species, with Lee et al. reporting observance of a “straw yellow” color that appeared prior to the characteristic red color of Fe(phen)$_3^{2+}$. No such evidence has been observed for intermediates of the FeFz$_3$ system. Additionally, studies performed in non-aqueous systems have demonstrated deviation from typical bidentate chelation mechanisms, particularly the lack of a ring-closure event. Chelation with multidentate ligands is generally accepted to go through a scheme comprised of first bond-formation followed by closure of the chelate ring around the metal species (with subsequent replacement of solvent molecules). While both of these processes can be potentially rate-limiting, ring closure and solvent exchange in aqueous systems is accepted to be rapid. Brown et al. utilized Fourier-transform NMR to monitor both of these potentially rate-determining steps for a series of multidentate ligands, however they noticed that complexation with Phen occurred in a one-step mechanism with no evidence for a ring-closure event. It has been concluded that the favorable orientation of the N atoms arising from the fused ring structure is responsible for the fast rate constant. The absence of a ring-closure event, as well as the possible stability of intermediate Fe-Phen complexes suggests a higher level of complexity for the Fe(II)-Phen system than the Fe-Fz system. Thus, on the basis of this complexity, as well as agreement between our $k_{\text{Fe(II)DTPA}}$ and $k_{\text{Fe(II)EDTA}}$ derived from Fz-based competition, we utilize the Fz-derived $k_{\text{Fe(II)DTPA}}$ for further calculations.

Using $k_{\text{Fe(II)DTPA}}$ derived from the DTPA-Fz competition, the oxidation rate of Fe(II) was calculated to be $5.3(\pm0.2)\times10^4$ M$^{-1}$s$^{-1}$ using Eqn 8 (simplified algebraically to Eqn 9) (Fig 1.39).
oxidation 2
Fe(II) O2-
oxidation 2 Fe(II) DTPA

\[
\text{Fraction}_{\text{Fe(II)→O}_2} = \frac{k_{\text{oxidation}}}[\text{Fe(II)}]_0 [\text{O}_2] - k_{\text{Fe(II)DTPA}} [\text{Fe(II)}]_0 [\text{DTPA}]
\]  

\[
\left( \frac{1}{\text{Fraction}_{\text{Fe(II)→O}_2}} \right)^{-1} = \frac{k_{\text{Fe(II)DTPA}} [\text{DTPA}]}{k_{\text{oxidation}} [\text{O}_2]}
\]  

There are at least 4 orders of magnitude difference between our maximum calculated rate constant for Fe(II) oxidation ($10^4$ M$^{-1}$s$^{-1}$) and the reverse reaction ($10^8$ M$^{-1}$s$^{-1}$). The highest production of O$_2^-$ observed under our conditions was 7 nM, while O$_2$ was present at ~ 250 µM (saturated). This 5-fold difference in reactant concentrations could explain the discrepancy in kinetics. Assuming a nanomolar level of O$_2^-$ ($10^{-9}$), O$_2$-saturation (250 µM), and a rate constant for Fe(III) reduction of $10^8$ M$^{-1}$s$^{-1}$, then the minimum rate constant necessary for autoxidation to be competitive with the reverse reaction is at least $2.8\times10^3$ M$^{-1}$s$^{-1}$. This is in good agreement with the value obtained from experiments where production of O$_2^-$ was the observable. Speculatively, this calculation is suggestive of a minimum value in order for the forward reaction to be equivalent to the reverse reaction. A rate constant of $2.8\times10^3$ M$^{-1}$s$^{-1}$ would lead to steady-state conditions as the reactions are equally competitive. However, the known instability of Fe(II) in aqueous systems suggests that the actual rate constant is much higher in order to facilitate the observed net oxidation to Fe(III).

The net oxidation of Fe(II) in natural waters is a complex process consisting of alternating cycles of Fe(II) oxidation and Fe(III) reduction processes that is ultimately terminated via precipitation as insoluble Fe(III) (oxy)hydroxides$^{1-6}$. The rate of cyclic Fe(II)/Fe(III) redox transformations is sensitive to environmental conditions (e.g. pH,$^{37}$ temperature,$^{38}$ salinity,$^{39,40}$ solar irradiation$^{41,42}$, etc) as well as the presence of commonly occurring environmental constituents (e.g. bicarbonate/carbonate,$^{43-45}$ phosphate,$^{46}$
nitrate,\textsuperscript{46} halides, sulfate,\textsuperscript{47} organic matter\textsuperscript{10, 48}, etc). Environmental matrices are complex mixtures where these substituents are known to coexist at varying, though predictable, levels. Thus, univariate studies that do not account for both independent effects as well as those arising from interaction of multiple species at once are limited in their predictive capacity. In response to this issue, recent studies employing multivariate experimental approaches have proven to be powerful tools for predicting Fe activity in a variety of systems.\textsuperscript{1, 8, 49, 50} The chief utility of these approaches is that they treat complex environments as a reducible system that can be described in relatively simple terms (i.e. aquatic systems are a mixture of species that vary in consistent ranges). In addition to stronger predictive capacity and mechanistic insight, multivariate models are attractive for studying environmental processes because they allow direct comparison of the impact of factor interactions on the process itself. For example, while halide species, SRNOM and CO$_3^{2-}$ have all been found to affect the oxidation rate of Fe(II), the study performed by Burns \textit{et al.} showed that in a realistic system containing all those species, oxidation is primarily dominated by carbonate interactions.\textsuperscript{8} While multivariate studies have proven to be useful for investigating Fe(II) oxidation in complex systems, results from field studies indicate that ROS do not behave according to stoichiometric predictions. Thus, multivariate models for Fe(II) oxidation kinetics may not necessarily reflect the same predictive capacity for ROS formation.

Based on ANOVA results, each of the chosen factors exhibited significant effects, both individually and cooperatively, on the generation of ROS. While CO$_3^{2-}$ and PO$_4^{3-}$ both showed significantly positive impacts, Fe(III) emerged as the dominant factor with a net negative impact. This suggests that the back reaction plays an important role in regulating
the generation of ROS during the net oxidation of Fe(II). Together, these results point to an intricate mechanism for Fe(II) oxidation and concomitant ROS generation. Following Fe(II) oxidation, an initial burst of \( \text{O}_2^- \) is generated, which in the presence of Fe(III)-precipitating ligands such as \( \text{CO}_3^{2-} \) and \( \text{PO}_4^{3-} \) undergoes dismutation to \( \text{H}_2\text{O}_2 \). However, the presence of significant quantities of amorphous Fe(III) promotes the back reaction (superoxide mediated reduction of Fe(III)), thus initiating Fe(II)/Fe(III) cycling and using up the generated ROS. It is important to note that while Fe(III) may serve to limit the net generation of ROS, it is vital towards prolonging the cycling, and therefore bioavailability) of Fe(II) species in aquatic systems.

1.6 Conclusions

The competitive kinetics method was applied to determine the kinetics of the direct reaction of Fe(II) with dioxygen. This method offers a simple, cost-effective alternative to more expensive stopped-flow analyses. Rapid Fe(II)/Fe(III) cycling limits the effectiveness of direct measurement of Fe(II) since complexation competes with redox reactions. Rate constants derived from measurements not accounting for cycling range from \( 10^1 \) – \( 10^2 \) M\(^{-1}\)s\(^{-1}\), while those derived from measurements accounting for cycling range from \( 10^7 \) – \( 10^9 \) M\(^{-1}\)s\(^{-1}\). This implies that direct colorimetric determination of Fe(II) should only be considered for measurement of the net oxidation rate (including cycling). The measurement of \( \text{O}_2^- \) (a direct oxidation product) is a much more sensitive indicator for the accurate determination of Fe(II) oxidation kinetics. However, care must be taken to use ligands that will not introduce artifacts such as suppression of light signal (i.e. color development) or allow Fe(II) to remain labile (e.g. incomplete sequestration via open coordination sites on the Fe(II) - EDTA).\(^{51}\)
Experiments using the production of ROS are more suitable for determining the complex kinetics of Fe(II) oxidation as they are more sensitive to the cycling of Fe(II)/Fe(III) species, as opposed to direct indicator based assays (Ferrozine). This better sensitivity would explain the discrepancy between our Fe(II)-indicator and ROS-based kinetic calculations.
Figure 1.1 General schematic for the application of competition kinetics to a mixed system containing a target species (X) and two potential reactants (R₁ and R₂).
Table 1.1: Design points for the five-factor central composite design used in all experiments

<table>
<thead>
<tr>
<th>Factor Units</th>
<th>Factor Concentration Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coded Factor Level</td>
<td>-2</td>
</tr>
<tr>
<td>Factor $x_1$ [Fe(III)], µM</td>
<td>1.50</td>
</tr>
<tr>
<td>Factor $x_2$ [CO$_3^{2-}$], mM</td>
<td>0.00</td>
</tr>
<tr>
<td>Factor $x_3$ [PO$_4^{3-}$], mM</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Figure 1.2 Formation of the Fe(phen)$_3^{2+}$ (red) and FeFz$_3$ (blue) complexes (as %) under anoxic conditions. $[\text{Fe(II)}]_0 = 15 \, \mu\text{M}$, $[\text{L}] = 60 \, \mu\text{M}$; pH maintained with 25 mM HEPES buffer.
Figure 1.3 Formation of the Fe(phen)$_3^{2+}$ (red) and FeFz$_3$ (blue) complexes (as %) under oxic conditions. [Fe(II)]$_0$ = 15 µM, [L] = 60 µM; pH maintained with 25 mM HEPES buffer.
Figure 1.4 Effect of pH on formation of the FeFz3 complex with increasing Ferrozine concentration. pH: 7.84 (orange), 7.97 (green), 8.05 (purple), 8.14 (red), 8.28 (blue), and 8.45 (black). [Fe(II)]₀ = 15 μM, 25 mM HEPES buffer
Figure 1.5 Increasing levels of phosphate led to a decrease in the formation of the Fe(phen)$_3$ complex, with higher phenanthroline concentrations becoming more competitive. [Phen] = 50 µM (blue), 75 µM (red), 100 µM (black), 200 µM (green)
Figure 1.6 Increasing levels of phosphate led to a decrease in the formation of the FeFz$_3$ complex, with higher Ferrozine concentrations becoming more competitive. [Fz] = 50 µM (blue), 75 µM (red), 100 µM (black), 200 µM (green), 1 mM (purple), 3 mM (brown), 5 mM (pink), 9 mM (light blue)
Figure 1.7 Competition plot between DTPA (variable) and Ferrozine (250 µM) for complexation of Fe(II) under anoxic (red) and oxic (blue) conditions (pH 7.0, 25 mM HEPES)
Figure 1.8 Competition plot between DTPA (variable) and Ferrozine (250 µM) for complexation of Fe(II) under anoxic (red) and oxic (blue) conditions (pH 7.5, 25 mM HEPES)
Figure 1.9 Competition plot between DTPA (variable) and Ferrozine (250 µM) for complexation of Fe(II) under Anoxic (red) and Oxic (blue) conditions (pH 8.0, 25 mM HEPES)
Figure 1.10 Competition plot between DTPA (variable) and 1,10-phenanthroline (250 µM) for complexation of Fe(II) under anoxic (red) and oxic (blue) conditions (pH 7.0, 25 mM HEPES)
Figure 1.11 Competition plot between DTPA (variable) and 1,10-phenanthroline (250 µM) for complexation of Fe(II) under anoxic (red) and oxic (blue) conditions (pH 7.5, 25 mM HEPES)
Figure 1.12 Competition plot between DTPA (variable) and 1,10-phenanthroline (250 µM) for complexation of Fe(II) under anoxic (red) and oxic (blue) conditions (pH 8.0, 25 mM HEPES)
Figure 1.13 QA/QC - Stability of FeFz₃ complex in the presence of DTPA. Error bars represent 95% CI (n = 3)
Figure 1.14 Competition plot between Ferrozine (60 – 900 μM) and DTPA (30 μM) for complexation of Fe(II) under oxic Conditions (pH = 7.0 (black), 7.5 (red), and 8.0 (blue); 25 mM HEPES)
Figure 1.15 Production of $O_2^-$ following the spike addition of Fe(II) in the presence of increasing DTPA. pH adjusted to 7.0 using HCl and NaOH. $[\text{Fe(II)}]_0 = 15 \mu\text{M}$.
Table 1.2: Experimental conditions & corresponding H$_2$O$_2$ yield for each pH condition

<table>
<thead>
<tr>
<th>Run</th>
<th>[Fe(III)] (µM)</th>
<th>[CO$_3^{2-}$] (mM)</th>
<th>[PO$_4^{3-}$] (mM)</th>
<th>H$_2$O$_2$ yield (nM) (pH 7.0)</th>
<th>RSD%</th>
<th>H$_2$O$_2$ yield (nM) (pH 7.5)</th>
<th>RSD%</th>
<th>H$_2$O$_2$ yield (nM) (pH 8.0)</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.60</td>
<td>2.39</td>
<td>2.39</td>
<td>153</td>
<td>19.4</td>
<td>145</td>
<td>14.7</td>
<td>45</td>
<td>6.3</td>
</tr>
<tr>
<td>2</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>140</td>
<td>15.7</td>
<td>167</td>
<td>12.5</td>
<td>59</td>
<td>23.5</td>
</tr>
<tr>
<td>3</td>
<td>75.75</td>
<td>1.50</td>
<td>1.50</td>
<td>488</td>
<td>15.7</td>
<td>383</td>
<td>11.3</td>
<td>133</td>
<td>31.1</td>
</tr>
<tr>
<td>4</td>
<td>119.90</td>
<td>0.61</td>
<td>0.61</td>
<td>587</td>
<td>12.7</td>
<td>877</td>
<td>10.7</td>
<td>359</td>
<td>17.8</td>
</tr>
<tr>
<td>5</td>
<td>119.90</td>
<td>2.39</td>
<td>0.61</td>
<td>382</td>
<td>1.1</td>
<td>665</td>
<td>8.1</td>
<td>288</td>
<td>10.8</td>
</tr>
<tr>
<td>6</td>
<td>75.75</td>
<td>1.50</td>
<td>1.50</td>
<td>547</td>
<td>14.0</td>
<td>351</td>
<td>12.3</td>
<td>115</td>
<td>36.1</td>
</tr>
<tr>
<td>7</td>
<td>31.60</td>
<td>0.61</td>
<td>2.39</td>
<td>185</td>
<td>15.4</td>
<td>220</td>
<td>17.2</td>
<td>80</td>
<td>14.5</td>
</tr>
<tr>
<td>8</td>
<td>31.60</td>
<td>0.61</td>
<td>0.61</td>
<td>391</td>
<td>17.0</td>
<td>396</td>
<td>7.6</td>
<td>126</td>
<td>9.3</td>
</tr>
<tr>
<td>9</td>
<td>75.75</td>
<td>1.50</td>
<td>1.50</td>
<td>511</td>
<td>15.0</td>
<td>291</td>
<td>14.9</td>
<td>201</td>
<td>20.6</td>
</tr>
<tr>
<td>10</td>
<td>75.75</td>
<td>1.50</td>
<td>1.50</td>
<td>603</td>
<td>12.7</td>
<td>335</td>
<td>12.9</td>
<td>110</td>
<td>37.4</td>
</tr>
<tr>
<td>11</td>
<td>119.90</td>
<td>2.39</td>
<td>2.39</td>
<td>358</td>
<td>7.0</td>
<td>319</td>
<td>12.2</td>
<td>82</td>
<td>6.9</td>
</tr>
<tr>
<td>12</td>
<td>119.90</td>
<td>0.61</td>
<td>2.39</td>
<td>394</td>
<td>15.4</td>
<td>375</td>
<td>10.6</td>
<td>114</td>
<td>7.0</td>
</tr>
<tr>
<td>13</td>
<td>75.75</td>
<td>1.50</td>
<td>0.00</td>
<td>1094</td>
<td>8.8</td>
<td>2255</td>
<td>4.7</td>
<td>269</td>
<td>40.0</td>
</tr>
<tr>
<td>14</td>
<td>75.75</td>
<td>1.50</td>
<td>1.50</td>
<td>698</td>
<td>11.0</td>
<td>414</td>
<td>10.5</td>
<td>92</td>
<td>44.9</td>
</tr>
<tr>
<td>15</td>
<td>31.60</td>
<td>2.39</td>
<td>0.61</td>
<td>201</td>
<td>10.9</td>
<td>157</td>
<td>19.6</td>
<td>62</td>
<td>1.5</td>
</tr>
<tr>
<td>16</td>
<td>75.75</td>
<td>3.00</td>
<td>1.50</td>
<td>320</td>
<td>21.9</td>
<td>307</td>
<td>16.0</td>
<td>74</td>
<td>21.2</td>
</tr>
<tr>
<td>17</td>
<td>75.75</td>
<td>1.50</td>
<td>3.00</td>
<td>291</td>
<td>6.2</td>
<td>294</td>
<td>17.4</td>
<td>77</td>
<td>1.7</td>
</tr>
<tr>
<td>18</td>
<td>75.75</td>
<td>1.50</td>
<td>1.50</td>
<td>609</td>
<td>12.6</td>
<td>381</td>
<td>11.4</td>
<td>88</td>
<td>46.9</td>
</tr>
<tr>
<td>19</td>
<td>150.00</td>
<td>1.50</td>
<td>1.50</td>
<td>507</td>
<td>30.9</td>
<td>619</td>
<td>3.6</td>
<td>161</td>
<td>20.1</td>
</tr>
<tr>
<td>20</td>
<td>75.75</td>
<td>0.00</td>
<td>1.50</td>
<td>450</td>
<td>2.8</td>
<td>540</td>
<td>6.5</td>
<td>136</td>
<td>4.6</td>
</tr>
</tbody>
</table>

*a n=3 for all experiments except the midpoint (n=18)*
Table 1.3: Parameter estimates and F-tests for the quadratic model fitted to the data (pH 7.0)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\beta_x$ Key</th>
<th>Coefficient Estimate ($\times 10^{-3}$)</th>
<th>Standard Error ($\times 10^{-3}$)</th>
<th>F value</th>
<th>Prob $&gt; F$</th>
<th>Sum of Squares ($\times 10^{-6}$)</th>
<th>% Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_0$</td>
<td>Intercept</td>
<td>1.75</td>
<td>1.61E-01</td>
<td>40.15</td>
<td>$&lt; 0.0001$</td>
<td>56.58</td>
<td></td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>[Fe(III)]</td>
<td>-1.37</td>
<td>1.07E-01</td>
<td>163.03</td>
<td>$&lt; 0.0001$</td>
<td>25.53</td>
<td>45.1</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>[CO$_3^{2-}$]</td>
<td>0.46</td>
<td>1.07E-01</td>
<td>18.48</td>
<td>0.0016</td>
<td>2.89</td>
<td>5.1</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>[PO$_4^{3-}$]</td>
<td>0.71</td>
<td>1.07E-01</td>
<td>44.51</td>
<td>$&lt; 0.0001$</td>
<td>6.97</td>
<td>12.3</td>
</tr>
<tr>
<td>$\beta_{11}$</td>
<td>[Fe(III)]$^2$</td>
<td>-0.30</td>
<td>1.40E-01</td>
<td>4.64</td>
<td>0.0567</td>
<td>0.73</td>
<td>1.3</td>
</tr>
<tr>
<td>$\beta_{22}$</td>
<td>[CO$_3^{2-}$]$^2$</td>
<td>-0.43</td>
<td>1.40E-01</td>
<td>9.52</td>
<td>0.0115</td>
<td>1.49</td>
<td>2.6</td>
</tr>
<tr>
<td>$\beta_{33}$</td>
<td>[PO$_4^{3-}$]$^2$</td>
<td>-0.24</td>
<td>1.40E-01</td>
<td>3.02</td>
<td>0.1129</td>
<td>0.47</td>
<td>0.8</td>
</tr>
<tr>
<td>$\beta_{12}$</td>
<td>[Fe(III)]-[CO$_3^{2-}$]</td>
<td>1.09</td>
<td>1.04E-01</td>
<td>108.38</td>
<td>$&lt; 0.0001$</td>
<td>16.97</td>
<td>30.0</td>
</tr>
<tr>
<td>$\beta_{13}$</td>
<td>[Fe(III)]-[PO$_4^{3-}$]</td>
<td>0.41</td>
<td>1.04E-01</td>
<td>15.79</td>
<td>0.0026</td>
<td>2.47</td>
<td>4.4</td>
</tr>
<tr>
<td>$\beta_{23}$</td>
<td>[CO$_3^{2-}$]-[PO$_4^{3-}$]</td>
<td>0.24</td>
<td>1.04E-01</td>
<td>5.25</td>
<td>0.0450</td>
<td>0.82</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Table 1.4: Parameter estimates and F-tests for the quadratic model fitted to the data (pH 7.5)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>βx Key</th>
<th>Coefficient Estimate (x 10^{-3})</th>
<th>Standard Error (x 10^{-3})</th>
<th>F value</th>
<th>Prob &gt; F</th>
<th>Sum of Squares (x 10^{-6})</th>
<th>% Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>β0</td>
<td>Intercept</td>
<td>2.79</td>
<td>0.26</td>
<td>13.49</td>
<td>0.0002</td>
<td>50.23</td>
<td></td>
</tr>
<tr>
<td>β1</td>
<td>[Fe(III)]</td>
<td>-1.41</td>
<td>0.17</td>
<td>65.36</td>
<td>0.0000</td>
<td>27.04</td>
<td>53.8</td>
</tr>
<tr>
<td>β2</td>
<td>[CO_3^{2-}]</td>
<td>0.69</td>
<td>0.17</td>
<td>15.61</td>
<td>0.0027</td>
<td>6.46</td>
<td>12.9</td>
</tr>
<tr>
<td>β3</td>
<td>[PO_4^{3-}]</td>
<td>0.78</td>
<td>0.17</td>
<td>20.15</td>
<td>0.0012</td>
<td>8.34</td>
<td>16.6</td>
</tr>
<tr>
<td>β_{11}</td>
<td>[Fe(III)]^2</td>
<td>-0.67</td>
<td>0.23</td>
<td>8.71</td>
<td>0.0145</td>
<td>3.60</td>
<td>7.2</td>
</tr>
<tr>
<td>β_{22}</td>
<td>[CO_3^{2-}]^2</td>
<td>0.08</td>
<td>0.23</td>
<td>0.12</td>
<td>0.7413</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>β_{33}</td>
<td>[PO_4^{3-}]^2</td>
<td>-0.17</td>
<td>0.23</td>
<td>0.59</td>
<td>0.4597</td>
<td>0.24</td>
<td>0.5</td>
</tr>
<tr>
<td>β_{12}</td>
<td>[Fe(III)]-[CO_3^{2-}]</td>
<td>0.52</td>
<td>0.17</td>
<td>9.52</td>
<td>0.0115</td>
<td>3.94</td>
<td>7.8</td>
</tr>
<tr>
<td>β_{13}</td>
<td>[Fe(III)]-[PO_4^{3-}]</td>
<td>0.08</td>
<td>0.17</td>
<td>0.24</td>
<td>0.6323</td>
<td>0.10</td>
<td>0.2</td>
</tr>
<tr>
<td>β_{23}</td>
<td>[CO_3^{2-}]-[PO_4^{3-}]</td>
<td>-0.14</td>
<td>0.17</td>
<td>0.68</td>
<td>0.4299</td>
<td>0.28</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Table 1.5: Parameter estimates and F-tests for the quadratic model fitted to the data (pH 8.0)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\beta_x$ Key</th>
<th>Coefficient Estimate (x 10^{-3})</th>
<th>Standard Error (x 10^{-3})</th>
<th>F value</th>
<th>Prob &gt; F</th>
<th>Sum of Squares (x 10^6)</th>
<th>% Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_0$</td>
<td>Intercept</td>
<td>8.81</td>
<td>0.76</td>
<td>14.71</td>
<td>0.0001</td>
<td>458.02</td>
<td></td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>[Fe(III)]</td>
<td>-3.74</td>
<td>0.50</td>
<td>55.10</td>
<td>0.0002</td>
<td>190.65</td>
<td>41.6</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>[CO_3^{2-}]</td>
<td>2.46</td>
<td>0.50</td>
<td>23.87</td>
<td>0.0006</td>
<td>82.61</td>
<td>18.0</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>[PO_4^{3-}]</td>
<td>3.07</td>
<td>0.50</td>
<td>37.14</td>
<td>0.0001</td>
<td>128.52</td>
<td>28.1</td>
</tr>
<tr>
<td>$\beta_{11}$</td>
<td>[Fe(III)]^2</td>
<td>-1.80</td>
<td>0.66</td>
<td>7.51</td>
<td>0.0208</td>
<td>25.99</td>
<td>5.7</td>
</tr>
<tr>
<td>$\beta_{22}$</td>
<td>[CO_3^{2-}]^2</td>
<td>0.47</td>
<td>0.66</td>
<td>0.51</td>
<td>0.4910</td>
<td>1.77</td>
<td>0.4</td>
</tr>
<tr>
<td>$\beta_{33}$</td>
<td>[PO_4^{3-}]^2</td>
<td>0.59</td>
<td>0.66</td>
<td>0.81</td>
<td>0.3885</td>
<td>2.81</td>
<td>0.6</td>
</tr>
<tr>
<td>$\beta_{12}$</td>
<td>[Fe(III)]-[CO_3^{2-}]</td>
<td>1.16</td>
<td>0.49</td>
<td>5.64</td>
<td>0.0390</td>
<td>19.51</td>
<td>4.3</td>
</tr>
<tr>
<td>$\beta_{13}$</td>
<td>[Fe(III)]-[PO_4^{3-}]</td>
<td>0.74</td>
<td>0.49</td>
<td>2.26</td>
<td>0.1634</td>
<td>7.83</td>
<td>1.7</td>
</tr>
<tr>
<td>$\beta_{23}$</td>
<td>[CO_3^{2-}]-[PO_4^{3-}]</td>
<td>-0.02</td>
<td>0.49</td>
<td>0.00</td>
<td>0.9673</td>
<td>0.01</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Table 1.6: ANOVA for the response surface generated by the quadratic model fitted to the data (pH 7.0)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>DF</th>
<th>Mean Square</th>
<th>F-Value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>5.66E-05</td>
<td>9</td>
<td>6.29E-06</td>
<td>40.15</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>1.57E-06</td>
<td>10</td>
<td>1.57E-07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>1.31E-06</td>
<td>5</td>
<td>2.61E-07</td>
<td>5.03</td>
<td>0.0505</td>
</tr>
<tr>
<td>Pure Error</td>
<td>2.60E-07</td>
<td>5</td>
<td>5.20E-08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>5.81E-05</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. Dev</td>
<td>3.96E-04</td>
<td></td>
<td>R-Squared</td>
<td>0.9731</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2.94E-03</td>
<td></td>
<td>Adj. R-Squared</td>
<td>0.9488</td>
<td></td>
</tr>
<tr>
<td>C.V. %</td>
<td>13.47</td>
<td></td>
<td>Pred. R-Squared</td>
<td>0.8221</td>
<td></td>
</tr>
<tr>
<td>Press</td>
<td>1.04E-05</td>
<td></td>
<td>Adeq. Precision</td>
<td>21.067</td>
<td>Desire &gt; 4</td>
</tr>
</tbody>
</table>
Table 1.7: ANOVA for the response surface generated by the quadratic model fitted to the data (pH 7.5)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>DF</th>
<th>Mean Square</th>
<th>F-Value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>5.02E-05</td>
<td>9</td>
<td>5.58159E-06</td>
<td>13.49</td>
<td>0.0002</td>
</tr>
<tr>
<td>Residual</td>
<td>4.14E-06</td>
<td>10</td>
<td>4.14E-07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>3.48E-06</td>
<td>5</td>
<td>6.97E-07</td>
<td>5.32</td>
<td>0.0452</td>
</tr>
<tr>
<td>Pure Error</td>
<td>6.54E-07</td>
<td>5</td>
<td>1.31E-07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>5.44E-05</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. Dev</td>
<td>6.43E-04</td>
<td></td>
<td></td>
<td>R-Squared</td>
<td>0.9239</td>
</tr>
<tr>
<td>Mean</td>
<td>3.11E-03</td>
<td></td>
<td></td>
<td>Adj. R-Squared</td>
<td>0.8554</td>
</tr>
<tr>
<td>C.V. %</td>
<td>20.67</td>
<td></td>
<td></td>
<td>Pred. R-Squared</td>
<td>0.4889</td>
</tr>
<tr>
<td>Press</td>
<td>2.78E-05</td>
<td></td>
<td></td>
<td>Adeq. Precision</td>
<td>12.830</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Desire &gt; 4</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.8: ANOVA for the response surface generated by the quadratic model fitted to the data (pH 8.0)

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>DF</th>
<th>Mean Square</th>
<th>F-Value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>4.58E-04</td>
<td>9</td>
<td>5.09E-05</td>
<td>14.71</td>
<td>0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>3.46E-05</td>
<td>10</td>
<td>3.46E-06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>6.65E-06</td>
<td>5</td>
<td>1.33E-06</td>
<td>0.24</td>
<td>0.9295</td>
</tr>
<tr>
<td>Pure Error</td>
<td>2.8E-05</td>
<td>5</td>
<td>5.59E-06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>4.93E-04</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. Dev</td>
<td>1.86E-03</td>
<td></td>
<td></td>
<td></td>
<td>R-Squared 0.9298</td>
</tr>
<tr>
<td>Mean</td>
<td>1.01E-02</td>
<td></td>
<td></td>
<td></td>
<td>Adj. R-Squared 0.8665</td>
</tr>
<tr>
<td>C.V. %</td>
<td>18.42</td>
<td></td>
<td></td>
<td></td>
<td>Pred. R-Squared 0.8156</td>
</tr>
<tr>
<td>Press</td>
<td>9.09E-05</td>
<td></td>
<td></td>
<td></td>
<td>Adeq. Precision 14.085 Desire &gt; 4</td>
</tr>
</tbody>
</table>
Figure 1.16 Proposed structure for the Fe(II)DTPA complex
Figure 1.17 Calculated rates for the forward reaction as determined by competition between Ferrozine (60 µM) and O₂ (250 µM, air-saturated)
Figure 1.18 Calculated rates for the forward reaction as determined by competition between Phenanthroline and O$_2$ (260 µM, air-saturated) for Fe(II) in the presence of phosphate. [Phen] = 50 µM (blue), 75 µM (red), 100 µM (black), 200 µM (green)
Figure 1.19 Calculated rates for the forward reaction as determined by competition between Ferrozine and O$_2$ (260 µM, air-saturated) for Fe(II) in the presence of phosphate. [Phen] = 50 µM (blue), 75 µM (red), 100 µM (black), 200 µM (green)
Figure 1.20 Calculated rates for the forward reaction as determined by competition between Ferrozine and O₂ (260 µM, air-saturated) for Fe(II) in the presence of phosphate. [Phen] = 1 mM (blue), 3 mM (red), 5 mM (black), 9 mM (green)
Figure 1.21 Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions based on competition with 250 µM Fz (pH 7.0, 25 mM HEPES)
Figure 1.22: Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions based on competition with 250 µM Fz (pH 7.5, 25 mM HEPES)
**Figure 1.23** Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions based on competition with 250 µM Ferrozine (pH 8.0, 25 mM HEPES)

$k_{\text{Fe(II)DTPA}} = 2.46 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Anoxic)

$k_{\text{Fe(II)DTPA}} = 2.46 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Oxic)

$y = 8\times10^{-7}x + 0.676$

$R^2 = 0.938$

$y = 8\times10^{-7}x + 0.4139$

$R^2 = 0.9837$
Figure 1.24 Effect of pH on competitive kinetics of Fe(II)DTPA formation under oxic conditions (Ferrozine variable, DTPA constant). pH range was 7.0 (blue), 7.5 (red), and 8.0 (black).
Figure 1.25 Comparison of predicted vs measured formation of FeFz3 complex in solutions containing different ratios of Fz and DTPA. pH = 7.0 (blue), 7.5 (red), and 8.0 (black)
Figure 1.26 Structural comparison of polyaminocarboxylate ligands EDTA and DTPA
Figure 1.2 Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with 250 µM Phen (pH 7.0, 25 mM HEPES)

\[ k_{\text{Fe(II)DTPA}} = 4.19 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \text{ (Anoxic)} \]

\[ k_{\text{Fe(II)DTPA}} = 4.16 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \text{ (Oxic)} \]
Figure 1.28  Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with 250 μM Phen (pH 7.5, 25 mM HEPES)
$k_{Fe(II)DTPA} = 5.29 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$ (Anoxic)

$k_{Fe(II)DTPA} = 4.37 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$ (Oxic)

**Figure 1.29** Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with 250 µM Phen (pH 8.0, 25 mM HEPES)
Figure 1.30 Speciation diagram for DTPA at different aqueous pH conditions. \( \text{H}_5\text{DTPA} \) (red), \( \text{H}_4\text{DTPA}^- \) (orange), \( \text{H}_3\text{DTPA}^{2-} \) (purple), \( \text{H}_2\text{DTPA}^{3-} \) (black), \( \text{HDTPA}^4^- \) (blue), \( \text{DTPA}^5^- \) (green)
Figure 1.31 Speciation diagram for hexaaquoiron(II) ion at different aqueous pH conditions. Fe(H$_2$O)$_6^{2+}$ (red), Fe(H$_2$O)$_5$OH$^+$ (blue)
Figure 1.32 Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with deprotonated Phen (PhenTotal = 250 µM) (pH 7.0, 25 mM HEPES)
Figure 1.33 Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with deprotonated Phen (Phen\textsubscript{Total} = 250 µM) (pH 7.5, 25 mM HEPES)

\[ k_{\text{Fe(II)DTPA}} = 1.36 \times 10^{10} \text{ M}^{-1}\text{s}^{-1} \text{ (Anoxic)} \]
\[ k_{\text{Fe(II)DTPA}} = 2.15 \times 10^{10} \text{ M}^{-1}\text{s}^{-1} \text{ (Oxic)} \]
Figure 1.34 Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with deprotonated Phen (Phen_{Total} = 250 \mu M) (pH 8.0, 25 mM HEPES)
Figure 1.35 Speciation diagram for Phen at different aqueous pH conditions. HPhen$^+$ (red), Phen (blue)
Figure 1.36 Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with monoprotonated Phen (Phen$_{\text{Total}}$ = 250 µM) (pH 7.0, 25 mM HEPES)
Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with monoprotonated phenanthroline (Phen\text{Total} = 250 \mu M) (pH 7.5, 25 mM HEPES)

- $k_{Fe(II)DTDA} = 8.24 \times 10^1 \text{ M}^{-1}\text{s}^{-1}$ (Anoxic)
- $k_{Fe(II)DTDA} = 1.30 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ (Oxic)

**Figure 1.37** Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with monoprotonated phenanthroline (Phen\text{Total} = 250 \mu M) (pH 7.5, 25 mM HEPES)
Calculated complexation rates for Fe(II)DTPA under anoxic (red) and oxic (blue) conditions, based on competition with monoprotonated phenanthroline (Phen\textsubscript{Total} = 250 µM) (pH 8.0, 25 mM HEPES)

\[ \text{[DTPA]/[HPhen]}^3 \]

\[ k_{\text{Fe(II)DTPA}} = 1.00 \times 10^1 \text{ M}^{-1}\text{s}^{-1} \text{ (Anoxic)} \]

\[ k_{\text{Fe(II)DTPA}} = 8.30 \times 10^0 \text{ M}^{-1}\text{s}^{-1} \text{ (Oxic)} \]
Figure 1.39 Competition plot for the generation of $\text{O}_2^-$ arising from oxidation of Fe(II) in the presence of increasing DTPA. $k_{\text{oxidation}} = 5.32 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$
CHAPTER 2

Method Optimization for Determination of Reactive Oxygen Species in Complex Aquatic Matrices
2.1 Abstract

The co-existence of Fe(II), Fe(III), dioxygen (O2), superoxide (O2−) and hydrogen peroxide (H2O2) has been demonstrated in many natural waters, including cloudwater, the photic zone of the water column, and points of anoxic groundwater emergence. These mixtures occur as metastable states, usually poised by the input of fresh material or energy maintaining the components at an apparent steady state. The simultaneous measurement of all five species is complicated by their high rates of reaction. Historically, these systems are interrogated by the addition of fast acting, redox-inert ligands to “freeze” the system in place by locking the metals at a fixed oxidation state. Here, we present a study of the ligand most widely used for this purpose, diethylenetriaminepentaacetic acid (DTPA), and show the necessity of accounting for reaction(s) that may compete with complexation. The effective sequestration of Fe(II) from redox reactions is found to be a function of competition between complexation with the ligand itself and rapid oxidation by available oxidants. Titration studies indicate that complexation with DTPA maintains the initial redox-state of Fe(II), suggesting that under these conditions, previously reported anomalous behavior is likely due to rapid oxidation occurring before complete binding of Fe(II) with DTPA. The utility of including DTPA for acridinium ester-based measurements of ROS in iron-rich, high salinity systems is reported.
2.2 Introduction

The biogeochemical cycling of iron, oxygen, and organic carbon are inextricably linked through the intermediacy of Reactive Oxygen Species (ROS), brought about by the one-electron oxidation of Fe(II). The ROS generated by this process, including superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO·), and carbon centered radicals (R·), are all capable of acting as secondary oxidants and reductants, thereby constituting a catalytic mechanism for the cyclic redox transformation of aqueous Redox-Sensitive Metals (RSMs). While this process is ultimately limited by the precipitation of Fe(III) species, model estimates indicate that cycling between Fe(II) and Fe(III) may occur anywhere from 10-2200 times, depending on environmental conditions. This process can occur wherever reduced Fe(II) species are exposed to oxic conditions, such that it can be observed in biological, environmental, and industrial systems. Laboratory studies have observed sustained levels of high ROS production associated with iron redox cycling.

While ROS production in environmental and industrial systems is considered beneficial in regards to degradation of organic pollutants, these reactive species can be extremely harmful in biological systems. Medical studies have observed a link between ROS-mediated tissue damage and cases of iron overload (hemochromatosis). Iron-induced oxidative damage can be prevented by sequestration of iron such that it is unavailable to react with dioxygen or other biological species. A range of both biological and synthetic chelators have been studied for their ability to chelate excess iron from the body as well as protect tissue from oxidative injury. The effectiveness of these chelators has ranged from partial/complete inhibition to catalysis of redox activity (i.e. oxidative stress). Whereas ligands such as ethylenediaminetetraacetic acid (EDTA) and
nitrilotriacetate (NTA) exhibit pro-oxidant behavior (i.e. induction of oxidative stress via generation and/or reaction with ROS), augmenting the catalytic activity of iron,\(^{68-72}\) ligands such as desferrioxamine (DFO) and diethylenetriaminepentaacetic acid (DTPA) have exhibited suppression and/or inhibition of catalytic activity.\(^{51, 73-77}\) The latter chelators are attractive for their ability to completely enclose the iron molecule, effectively sealing off the coordination sites necessary for redox reactions. While DFO is commonly used in medical studies where cellular membranes must be protected from oxidative damage, there is conflict in the literature as to the pro- or anti-oxidant activity of iron-DTPA complexes. As a polydentate iron chelator, DTPA has exhibited the therapeutic capability to both treat iron overload as well as prevent iron-mediated oxidative stress on par with DFO.\(^{78, 79}\) However, the selectivity of the latter makes it much more ideal for use in complex matrices. Additionally, the ability of DTPA to act as a preservative for ROS in the presence of redox-reactive transition metals has been demonstrated in a number of environmental studies.\(^{80-87}\) This is in stark contrast to its structurally similar analogue (EDTA). Graf, \textit{et al} concluded that the presence (or absence, in the case of EDTA) of an additional coordination site is responsible for protection of the metal from oxidation, thereby rendering it inert to redox reactions.\(^{51}\) However, studies done by Buettner, \textit{et al.} concluded that iron-DTPA complexes are labile with respect to redox reactions, and that the apparent suppression is simply a result of kinetically slow reactions of the complex with oxidants.\(^{73-77}\)
The large number of literature studies reporting contradictory redox activity of iron-DTPA complexes suggests the presence of unexplored factors ultimately contributing to the availability of Fe(II) and Fe(III). Whereas DFO shows complete inhibition of redox reactions, DTPA has only been shown to decrease reactivity, albeit by a large amount.\textsuperscript{74,76,88-91} This suggests an interesting hypothesis: the anti-oxidant activity of iron-DTPA complexes is a kinetically-controlled process, whereby formation of the complex actively competes with O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} for available iron. The presence of additional cations typically present in coastal and marine waters only serves to decrease the likelihood for complexation of Fe with available DTPA due to its non-selectivity.

This work presents a test of the stated hypothesis via the optimization of the chemiluminescent acridinium ester method for H\textsubscript{2}O\textsubscript{2} determination in estuarine systems as a proxy. The acridinium ester method has gained widespread use in environmental analysis, due to its high specificity against organic peroxides, low detection limits, portability, and low instrumentation cost.\textsuperscript{92-95} The ability to measure H\textsubscript{2}O\textsubscript{2} in real-time is essential for environmental studies due to the rapid reaction rate (and thus short residence time) of H\textsubscript{2}O\textsubscript{2} with many species common in natural waters. Despite its advantages over fluorometric and titrimetric methods, acridinium ester-based measurements are complicated by artifacts arising from matrix interferences that have not been fully addressed within the literature. For example, the high pH necessary to initiate the chemiluminescent reaction between H\textsubscript{2}O\textsubscript{2} and the acridinium ester leads to precipitation of calcium (Ca\textsuperscript{2+}) or magnesium (Mg\textsuperscript{2+}) salts in high salinity matrices. This previously unreported phenomena can negatively affect light transmittance (Figure 2.1), leading to erroneous data or instrument failure due to clogging. The effect can be especially pronounced in areas where fresh
surface water and groundwater mix with seawater, leading to variation of salinity conditions. This variability can lead to significant changes in sensitivity between samples. Additionally, the presence of Redox-Sensitive Metals (RSMs) such as iron can act as catalytic interferents by generating or consuming H₂O₂ during analysis. These interferences imply the presence of reliability issues in previous studies using the acridinium ester technique for environmental analyses.

2.3 Experimental Materials

An acridinium ester, 10-methyl-9-(p-formylphenyl)-acridinium carboxylate trifluoromethanesulfonate, was synthesized according to Cooper, et al. 92 Sand, (sea-washed), sodium hydroxide, sodium bicarbonate, sodium carbonate, and sodium phosphate monobasic were obtained from Fisher Scientific. Ferrozine reagent (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p’-disulfonic acid monosodium salt hydrate), catalase from bovine liver, diethylenetriaminepentaacetic acid, and hydrogen peroxide (30%) were obtained from Sigma Aldrich. Suwannee River Natural Organic Matter (SRNOM) was obtained from the International Humic Substances Society (IHSS). Ca-rich Montmorillonite and Na-rich Montmorillonite were obtained from the Clay Sources Repository. Kaolinite was obtained from Fluka. Iron(II) chloride (anhydrous, 99.5%) was obtained from Alfa Aesar. Ammonium acetate and hydrochloric acid were obtained from VWR. Unless otherwise noted, reagents were used as received without purification, and all solutions were prepared in 18 MΩ water. All solutions used for H₂O₂ analysis were prepared in water that had been amended with catalase (3 mg/L) overnight and boiled for one hour to give H₂O₂-free water. All glassware was cleaned in a muffle furnace and
allowed to soak overnight in a 10% hydrochloric acid solution, followed by rinsing with 18 MΩ water prior to use.

**Salinity Effects in the AE Method**

A series of salinity-fixed solutions (0, 10, 20, 30 ppt) were prepared by dilution of Sargasso seawater (35 ppt). These solutions were amended with various concentrations of DTPA (0-100 mM) and loaded into a 96 well plate. Replicate samples (n=3) were pH-adjusted by injection of a carbonate buffer (0.1 Na₂CO₃, pH 12), thereby inducing precipitation. The presence of calcium and/or magnesium precipitates was analyzed by measuring the difference in light absorbance (later converted to transmittance) at the wavelength corresponding to the chemiluminescent emission of the acridinium ester method, 470 nm.

**Ionic Interferences in DTPA-Mediated Fe Sequestration**

A deoxygenated iron(II) chloride stock solution (10 mM) was prepared by boiling 18 MΩ water, sparging with N₂ (99.999%), and acidifying to pH 2 with HCl. Concentration of the iron(II) stock solution was verified via spectrophotometric determination using the Ferrozine method. Varying amounts of stock iron(II) solution were spiked into a reactor containing one of a series of well-mixed salinity-fixed solutions that had been amended with varying concentrations of DTPA (0-50 mM). Immediately after addition of the iron(II) stock solution (t < 10 seconds), aliquots were withdrawn and transferred into a vial preloaded with an excess of Ferrozine reagent. Iron(II) that was not complexed by DTPA, hereafter referred to as “free iron” was quantified spectrophotometrically by measuring the absorbance of the FeFz₃ complex at 562 nm. To investigate the potential role of organic matter as a ligand for iron in these matrices, an additional set of experiments was conducted in the presence of SRNOM (16 mg C/L).
Preservation of Fe(II) Redox State via DTPA Chelation

Two separate solutions were prepared: one containing 1 mM 1,10-phenanthroline and the other containing 1 mM 1,10-phenanthroline and 70 mM DTPA. Neither solution was buffered, however pH was adjusted to between 9.5 – 10.0 with NaOH. A deoxygenated Fe(II) stock solution was spiked into both solutions to reach a final concentration of 15 µM. Following the Fe(II) spike, solutions were titrated with HCl, followed by intermittent transfer of aliquots for spectrophotometric determination of the Ferroin complex (Fe(II)phen₃) at 510 nm.

Inhibition of Fe-Catalyzed ROS Production via DTPA Chelation

Sample solutions consisting of 18 MΩ water that had been amended with varying levels of a stock DTPA solution were prepared in clean, acid-washed glassware. Sampling lines for H₂O₂ and O₂⁻ were placed into individual sample solution and data acquisition was initiated via Waterville Analytical software. Immediately after data acquisition was initiated, 65 µL of iron(II) stock solution (23 mM, as determined spectrophotometrically) was spiked into the DTPA-amended 18 MΩ water (final concentration, 15 µM) and ROS activity was monitored over time. At regular intervals, 1 mL aliquots were transferred into separate vials that had been preloaded with 1 mL of 1 mM Ferrozine for spectrophotometric analysis. A 15 minute experimental timeframe was arbitrarily set, as the solution level after this time became low. Chemiluminescent data was converted to ROS concentration via standard addition calibration curves. A minimum of 1000 data points were averaged and converted to concentration of ROS via calibration curves obtained prior to sampling.
Inhibition of Base-Catalyzed Fe Interferences via DTPA Chelation

Salinity-fixed solutions were amended with 5 mg/L catalase, varying levels of DTPA, and adjusted to pH 7. Continuous-flow analysis was performed on each sample both before and after injection of an Fe(II) stock solution at a final concentration of 25 µM. All lines were flushed with 2 M HCl and 18 MΩ after each sample to avoid carryover. Peristaltic pump speed and PMT settings were identical to those mentioned previously. Data acquisition was delayed until 3.5 minutes after Fe(II) spike to allow catalase to decompose any background H₂O₂ and furthermore until the signal stabilized. A minimum of 200 data points were collected for each sample (2 data points/second), and data was converted to concentration of H₂O₂ via standard addition plots for each salinity condition.

Optimal Concentration for the AE Probe

A 1.7 mM acridinium ester stock solution was prepared in an acidic phosphate buffer (pH 3) and stored in the dark to avoid hydrolysis-mediated degradation. Varying concentrations of acridinium ester were made by dilution of the stock solution into the phosphate buffer. These solutions were analyzed via chemiluminescent detection in a modified flow-injection system, whereby sample was mixed with H₂O₂ (concentration verified by dilution of a stock solution standardized via absorbance at 254 nm) before mixing with a carbonate buffer (pH 12) inside a specially-designed flow cell (Waterville Analytical). Chemiluminescence from the reaction of acridinium ester with H₂O₂ (as hydroperoxyl anion, HOO⁻) was quantified by a Hamamatsu photon multiplier (PMT).
Particulate-Mediated Quenching of AE Chemiluminescence

Aqueous suspensions of each model solid (150 ppm and 300 ppm) were prepared in a phosphate buffer solution. A blank solution consisting of only phosphate buffer was included for comparison sake. An acridinium ester stock solution was spiked to reach a final concentration of 800 µM into 20 mL of each constantly stirred solid suspension. A maximum of 60 seconds were allowed before aliquots were withdrawn. Aliquots were centrifuged to avoid introducing solid matters into the flow setup. Samples were analyzed via chemiluminescent detection as stated previously. The method of standard additions was used to avoid artifacts brought about by matrix effects.

2.4 Results and Discussion
Salinity Effects on the Reduction of Artifacts in the Acridinium Ester Method:

The reliability of the acridinium ester (AE) method in high-salinity waters can become questionable due to precipitation arising from the addition of the pH 11 carbonate buffer necessary to initiate chemiluminescence. To this end, the relationship between precipitate formation and DTPA concentration on the AE chemiluminescence-specific light transmittance was investigated under several salinity conditions (Figure 2.2). Samples to which DTPA was amended with concentrations stoichiometrically equivalent to the Mg²⁺ concentration showed no measurable change in light transmittance, likely due to complexation rendering Mg unavailable for precipitation. While both Ca²⁺ and Mg²⁺ are known to form insoluble hydroxides at high pH, the minor change in light transmittance in the presence of 50 mM DTPA suggests the precipitation of Mg(OH)₂ is the major artifact causing light obstruction in aquatic samples. The identification of magnesium as the primary precipitating salt, either as the carbonate or the hydroxide, is consistent with the
study done by Shaw et al. Conversely, samples in which DTPA was absent or present at insufficient levels showed immediate precipitation of solid upon addition of the buffer. These results indicate that DTPA aids in the reduction of matrix-mediated artifacts and that the concentration of cationic interferents in the matrix must be accounted for when attempting to use the acridinium ester method in aquatic samples. The use of ligand at levels below 50 mM has the potential to introduce uncertainty in field measurements, ranging from instrument failure (i.e. clogging due to uninhibited precipitate accumulation) to erroneous data (i.e. signal suppression due to precipitation-induced optical density as well as catalytic production and/or degradation of ambient ROS due to the presence of uncomplexed redox-active metals).

**Metals that Compete with Fe for DTPA in Waters with High Dissolved Solids**

Polyaminocarboxylate ligands are attractive chelators due to their non-specificity in binding cationic metal species present in solution. If DTPA is to be used to chelate redox-sensitive metals for the analysis of natural waters, then the presence of competing metals must be accounted for in the concentration of DTPA to be used. To investigate the significance of competing ions in the sequestration of Fe(II) by DTPA, a series of salinity-fixed solutions containing variable levels of DTPA were spiked with Fe(II) and aliquots withdrawn into vials preloaded with Ferrozine reagent. Previous research studies utilizing DTPA for the purpose of removing transition metal interferences from seawater matrices have used concentrations ranging from as low as 3.8 µM, up to 50 µM. 

80-82, 84, 97
Within the timeframe of each experiment ($t < 30$ seconds, where $t$ is the time between addition of Fe(II) spike and aliquot withdrawal) complete sequestration of Fe(II) occurred only at concentrations at or above 5 mM (Fig. 2.3-2.8). When DTPA was present at 500 µM, approximately 94-96% of added Fe(II) was complexed, however higher levels of DTPA consistently gave results below the detection limit for the Ferrozine technique, indicating a lower limit for efficient chelation of added Fe(II). There was no significant difference between samples amended with 5 µM DTPA and experiments done in the absence of ligand, suggesting that competition with seawater cations within the matrix are non-trivial. An important distinction between the work presented here and previously published work is the lack of an equilibration time in our study. Heller & Croot reported that for seawater samples amended with 3.8 µM DTPA, equilibration times under 12 hours still showed evidence for catalytic activity brought about by uncomplexed trace metal species. Reported stability constants (as log K) for DTPA complexes with Ca$^{2+}$ (9.3) and Mg$^{2+}$ (10.8) are significantly lower than for Fe$^{2+}$ (16.5) and Fe$^{3+}$ (28.6) suggesting that the time-dependent increase in chelation activity observed by Heller et al. is likely due to slow metal-exchange processes. Our data do not indicate a salinity dependence in the DTPA-mediated complexation of Fe(II). This particular experiment did not exhibit the well-pronounced effects observed in the reduction of precipitation-induced artifacts. In this regard, the utility of higher concentrations of DTPA to mediate salinity effects predominately serves to maintain optical clarity for chemiluminescent measurements.
NOM has been recognized as a naturally-occurring Fe chelator that plays a major role in the transport and mobilization of Fe in aquatic systems. Due to its ubiquity, it is one of the most important iron chelators in aquatic systems, with Rue et al. reporting over 99% of dissolved Fe being complexed by organic ligands. However, the bioavailability of Fe chelated to NOM may be an issue in regards to ROS-mediated reactions. SRNOM was evaluated for its ability to chelate iron as a model for naturally-occurring Fe-binding species. As evidenced by Figure 2.9, SRNOM is not suitable for sequestration of Fe(II), as it not only bound no Fe(II), but in some cases actually released Fe(II) into solution. This suggests that naturally occurring ligands associated with organic matter are not strong ligands and do not completely remove Fe(II) from solution.

**Chelation Inhibits Fe Oxidation by Preserving Redox State**

There is controversy in the literature regarding the lability of Fe-DTPA complexes towards oxidation. While some investigators assert DTPA blocks Fe(II) oxidation, others contend that DTPA accelerates oxidation. To investigate the lability of Fe(II)-DTPA chelates towards oxidation, a solution containing the complex was titrated with HCl in the presence of the Fe(II) chelator 1,10-phenanthroline (Fig. 2.10). The chelating ability of polyaminocarboxylate ligands like DTPA is diminished with lower pH due to protonation of the carboxyl groups. If DTPA blocks oxidation of Fe(II), then titration of the complex in the presence of 1,10-phenanthroline (pKa 4.27) should result in the quantitative formation of the Ferroin complex. If Fe(II)-DTPA complexes are labile with respect to oxidation, then formation of the Ferroin complex should not be observed. As depicted in Figure 2.10, titration of the complex resulted in near complete transformation into the Ferroin complex. It is possible that titration-induced dilution may account for the
difference. The fully protonated DTPA is insoluble in acidic media, and precipitation was observed at pH 1 and below. QA/QC experiments confirmed that under these conditions, there was no significant interference from formation of the Fe(III)-1,10-phenanthroline complex, Fe(phen)_3^{3+} (Figure 2.11), indicating that the observed signal was solely due to the release of Fe(II) from DTPA. This is consistent with reports that formation of the blue Fe(phen)_3^{3+} species does not occur by direct mixture, but by deliberate oxidation of the Fe(phen)_3^{2+} complex.\textsuperscript{104, 105} This is strong evidence supporting the preservative role of DTPA towards Fe species. This implies that the pro-oxidant activity observed by other researchers is likely due to either incomplete chelation of Fe species (due to insufficient DTPA being used) or competition between the ligand and oxidants occurring prior to chelation.

**The Impact of DTPA on Fe(II)-mediated H_2O_2 Production upon Mixing with Buffer**

The high pH buffer necessary to trigger chemiluminescence of the acridinium ester can introduce artifacts into the analysis of H_2O_2 by the rapid oxidation of available Fe(II). However, the presence of a large excess of DTPA should prevent this from occurring by binding Fe(II) into a form unavailable to react with dioxygen, thereby preventing the production of superoxide and ultimately H_2O_2. To investigate the ability of DTPA to prevent artifacts from this process, the H_2O_2 signal was monitored after the introduction of Fe(II) into various salinity-fixed solutions (Figure 2.12). As with previous experiments in this study, high millimolar concentrations showed a much more prominent effect on eliminating artifacts as compared to lower micromolar concentrations. The addition of catalase to the reactor, in addition to the delay period after injection of Fe(II), ensured that H_2O_2 in the sample was essentially zero. Thus, the chemiluminescent signal was only
representative of the \( \text{H}_2\text{O}_2 \) produced by the oxidation of uncomplexed Fe(II) upon mixing with the high pH buffer. Lower levels of DTPA showed little difference, with the exception of the 0 salinity condition, which showed consistent decrease, indicating significant matrix effects in higher salinity samples. The overall trend supports the use of high concentrations of DTPA for the prevention of artifacts brought about by the oxidation of Fe(II) by the buffer under our conditions.

**Required Levels of DTPA to Inhibit Generation of ROS as a Function of the Oxidation of Fe(II)**

Fe(II) has been documented to coexist with \( \text{O}_2 \) and a number of additional ROS in a variety of natural systems. Based on our competitive experiments with \( \text{O}_2 \), we conducted a case study on the DTPA-mediated inhibition of Fe-catalyzed \( \text{H}_2\text{O}_2 \) generation. The yield of \( \text{H}_2\text{O}_2 \) was observed to be inversely related to the concentration of DTPA (Fig. 2.13). These results indicate that under our experimental conditions, the process was not sufficiently inhibited until DTPA was above 160 millimolar. Studies by Burns* et al., reported that even sub-micromolar and lower levels of Fe(II) gave noticeable degradation of probe molecules, presumably via HO· production.\(^8\) Calculated first-order rate constants for DTPA indicate that complete inhibition did not occur until \( k \approx 10^4 \text{ s}^{-1} \), providing evidence that the initial reaction rate for Fe(II) and \( \text{O}_2 \) is much faster than previously reported.
Optimal Concentration for the Chemiluminescent Probe

The composition of estuarine water matrices is highly variable and dynamic, due in part to the semi-diurnal tidal cycling. Prior field campaigns attempted to use the method of standard additions as an attempt to account for the changing matrix; however, slope degradation was always an issue, especially during periods of high-particulate loading. King, et al. made use of probe concentrations ranging from 1 µM up to 10 µM in order to ensure that H$_2$O$_2$ remained the limiting factor in the reaction.\textsuperscript{93} Assuming a 1:1 stoichiometry for Rxn 1-2, estuarine systems with micromolar levels of Fe(II) should consequently yield micromolar levels of O$_2^-$, which in turn would dismutate to give micromolar levels of H$_2$O$_2$. In order to determine an optimal concentration of probe that would give a consistent chemiluminescent response under changing environmental conditions, we investigated the chemiluminescent reaction as a function of H$_2$O$_2$:Probe ratio. The chemiluminescent response at ratios (0-200) showed an increasing linear response. However, ratios of 1500 and above showed stabilization in terms of response. This suggests that we have reached a ratio at which there is sufficient probe present to react with all H$_2$O$_2$ in the sample, as opposed to the low ratios where response is highly dynamic. This is in agreement with Krzymiński et al., who proposed that higher proportions of H$_2$O$_2$ with respect to the probe ultimately results in an increase in the portion of the probe going through a “dark pathway” via reaction with hydroxide ion to form a pseudobase (Figure 2.14), leading to the non-chemiluminescent formation of N-methyl acridone. For detailed review on both chemiluminescent and non-chemiluminescent pathways for acridinium ester, see Krzymiński, et al.\textsuperscript{106,107} While increasing the concentration of the probe does not eliminate the dark pathway, it does increase the likelihood that there will be sufficient probe
present to react with all of the H$_2$O$_2$ in the sample. Thus, the flattening out of the chemiluminescent responses is evidence that, at the very least, increasing proportions of H$_2$O$_2$ are being forced to react with the probe (Fig. 2.15-2.16). This data suggests that measurements performed under a 1000-fold excess with respect to H$_2$O$_2$ of the chemiluminescent acridinium ester probe may be underestimating the true concentration of H$_2$O$_2$ present in their samples.

**Quenching of AE Chemiluminescence in Solutions Containing High Levels of Suspended Solids**

The presence of particulate matter when analyzing water in estuarine systems is often unavoidable, especially during low-tide periods. The transient and reactive nature of ROS necessitates relatively quick analysis times, thus making filtration steps unattractive. Numerous acridinium ester derivatives have been formulated for use as biosensors in a wide range of biomedical assays.\textsuperscript{108-110} To investigate the influence of particulate matter in quenching the chemiluminescent reaction of the acridinium ester in the presence of varying particulate matter loading, we tested the method in the presence of four model sediments: sand, kaolinite, Na-montmorillonite, and Ca-montmorillonite. Comparison of standard addition slopes found no significant difference between the model sediment types, save for the 300 ppm Ca-montmorillonite (Figure 2.17). However, this high loading would be rare in the field, and could likely be overcome by implementing a brief settling period after sample collection to allow particulates to fall to the bottom and withdrawing sample from the top.
2.5 Conclusions

The objective for the present study was to demonstrate the utility in incorporating DTPA into the measurement of H$_2$O$_2$ in iron-rich systems and/or complex matrixes (e.g. estuarine waters, hydrothermal vent waters) by the acridinium ester method. We have found that under conditions similar to those found in estuarine environments (high iron, variable salinity, etc.), the acridinium ester method for H$_2$O$_2$ analysis could be used reliably with the incorporation of DTPA. DTPA was found to form a strong complex with Fe(II) in solution that outcompeted the reaction of Fe(II) with dioxygen at DTPA levels above 50 mM, thereby preventing artifacts in H$_2$O$_2$ measurements. However, this is only the case when DTPA is used in stoichiometric excess with respect to the major seawater cations, Ca$^{2+}$ and Mg$^{2+}$. The suppression of Fe(II) autoxidation by excess DTPA, as well as the stability of ROS in the system suggests that the preservative role of DTPA for ROS in the presence of redox-reactive species is critically dependent on ligand concentration. While previous studies investigating the pro- or anti-oxidative behavior or iron-DTPA complexes typically made use of large excesses of ligand with respect to iron, the use of concentrations below the millimolar range are insufficient to compete with O$_2$-mediated Fe(II) autoxidation. The number of literature studies even approaching this level are far outnumbered by those in the micromolar range (Figure 2.18). Experiments performed at levels similar to published values are in agreement with the variable pro- and anti-oxidant behavior of iron chelates.
The presence of excess DTPA eliminates matrix effects leading to signal suppression and potential equipment failure with respect to the acridinium ester method, and reduces the catalytic effect of trace metals within the timeframe of the sample analysis. The method has been found to give reliable results under the varying salinity conditions and Fe(II) concentrations representative of an estuarine system receiving groundwater discharging through an iron-rich salt marsh. Particulate matter was found to give no significant effect on the method, rather signal suppression was primarily attributed to uncomplexed Mg$^{2+}$ precipitating upon mixing with the buffer. The use of the chemiluminescent acridinium ester probe at a minimum 1000:1 ratio with respect to H$_2$O$_2$ is also recommended in order to ensure accurate quantitative detection of analyte present within the system. The implications of this study will have relevance to research directed at both coastal and oceanic systems, in order to observe the contribution of Fe-mediated geochemical processes to overall ROS production without suffering from matrix issues.
Figure 2.1 Flow diagram for a typical setup employing the acridinium ester-based FIA-CL method. Image at right illustrates the extent of signal suppression brought about by mixing carbonate buffer with high-salinity solutions.
Figure 2.2 Dependence of DTPA concentration to maintain adequate light transmittance after pH adjustment. [DTPA] = 0, 5 μM, 50 μM, 500 μM, 5 mM, and 50 mM. Salinity = 0 ppt (blue), 10 ppt (red), 20 ppt (orange), 30 ppt (purple), 35 ppt (green)
Figure 2.3 Matrix effects on the availability of DTPA to chelate Fe(II). No DTPA. $[\text{Fe(II)}] = 0 \mu\text{M (blue), 30 } \mu\text{M (green), 60 } \mu\text{M (red), 90 } \mu\text{M (purple), 120 } \mu\text{M (black), 150 } \mu\text{M (orange)
Figure 2.4 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 5 µM. [Fe(II)] = 0 µM (blue), 30 µM (green), 60 µM (red), 90 µM (purple), 120 µM (black), 150 µM (orange)
Figure 2.5 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 50 µM. [Fe(II)] = 0 µM (blue), 30 µM (green), 60 µM (red), 90 µM (purple), 120 µM (black), 150 µM (orange)
Figure 2.6 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 500 µM, [Fe(II)] = 0 µM (blue), 30 µM (green), 60 µM (red), 90 µM (purple), 120 µM (black), 150 µM (orange)
Figure 2.7 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 5 mM, [Fe(II)] = 0 µM (blue), 30 µM (green), 60 µM (red), 90 µM (purple), 120 µM (black), 150 µM (orange)
Figure 2.8 Matrix effects on the availability of DTPA to chelate Fe(II). [DTPA] = 50 mM. [Fe(II)] = 0 µM (blue), 30 µM (green), 60 µM (red), 90 µM (purple), 120 µM (black), 150 µM (orange)
Figure 2.9 Matrix effects on the availability of SRNOM to chelate Fe(II). [SRNOM] = 16 mg C/L mM. [Fe(II)] = 0 µM (blue), 30 µM (green), 60 µM (red), 90 µM (purple), 120 µM (black), 150 µM (orange)
**Figure 2.10** HCl Titration of 15 µM Fe(II)DTPA in the presence of 1 mM Phen. Control solution conditions (red): 15µM [Fe(II)], 1 mM Phen. Test solution conditions (blue): 15µM [Fe(II)], 1 mM Phen, 70 mM DTPA
Figure 2.11 Absorbance spectrum of 15 µmol L$^{-1}$ [Fe(phen)$_3$]$^{2+}$ (blue), 15 µmol L$^{-1}$ [Fe(phen)$_3$]$^{3+}$ (red), and 18 MΩ blank. Under the experimental conditions of this study, [Fe(phen)$_3$]$^{3+}$ (red) was indistinguishable from the water blank, indicating no significant interference for detection of [Fe(phen)$_3$]$^{2+}$ (blue).
Figure 2.12 Maximum H$_2$O$_2$ produced via Fe(II) oxidation upon mixing with carbonate buffer (0.1 mol L$^{-1}$ Na$_2$CO$_3$, 20 mmol L$^{-1}$ NaOH), plotted as a function of DTPA at salinity 0 ppt (Ca + Mg = 0 mmol L$^{-1}$; blue), 10 ppt (Ca + Mg $\geq$ 20 mmol L$^{-1}$; red), 20 ppt (Ca + Mg $\geq$ 40 mmol L$^{-1}$; green), 30 ppt (Ca + Mg $\geq$ 60 mmol L$^{-1}$; purple)
Figure 2.13 Inhibition of Fe-mediated generation of H₂O₂ in the presence of varying DTPA (blue) and corresponding 1ˢᵗ order rates for Fe(II)DTPA for rough estimation of Fe(II) oxidation rate (red)
Figure 2.14 “Pseudobase” formed by the reaction of acridinium ester with hydroxide ion
**Figure 2.15** Concentration-dependent signal of varying acridinium ester in constant H$_2$O$_2$ (500 nM)
Figure 2.16 Concentration-dependent signal of varying acridinium ester in constant H$_2$O$_2$ (50 nM)
Figure 2.17 Quenching of acridinium ester chemiluminescence in the presence of particulate matter
Figure 2.18 Comparison of reported Fe and DTPA used in literature (blue) vs current study (red)
CHAPTER 3

Field Measurements of Biogeochemically Produced Hydrogen Peroxide in Estuarine Sediments
3.1 Abstract

The oxidation of reduced transition metals at redox interfaces can produce Reactive Oxygen Species (ROS) in the water column of estuarine systems. The tidally driven efflux of groundwater containing Fe(II) is shown to produce inventories of ROS in estuarine waters in excess of that produced by photochemical reactions. The instantaneous Fe(II) and ROS concentrations in estuarine surface waters are reported for a diurnal time series over high and low tide cycles (2.6 $\mu$M to 77.3 $\mu$M). The Fe(II) concentration in tidal creek waters was highly correlated with a groundwater tracer, $^{224}$Ra. The oxidation of emergent Fe(II) resulted in the formation and persistence of the superoxide anion radical ($O_2^-$), with steady state concentrations of approximately 7.5 to 67.8 nM. $H_2O_2$ was also measured at concentrations varying from 125 nM to 900 nM, appearing to coincide with Fe(II). Exogenous Fe(II) or $H_2O_2$ was added to sampled waters to determine their respective lifetimes in waters from different portions of the tide cycle. Our results are indicative of a mechanism where Fe acts catalytically to produce and maintain high levels of ROS that is supported by the presence of reductants and microbial reactions.
3.2 Introduction

Reactive Oxygen Species (ROS), the collective name for the short-lived partial reduction products of dioxygen (O$_2$), include superoxide (O$_2^-$), hydroperoxyl radical (HOO$^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (HO$^-$). ROS in aquatic systems are typically identified as photoproducts of the photoexcitation of Chromophoric Dissolved Organic Matter (CDOM), nitrate, ligand-metal charge transfer processes of transition metals, or the bandgap excitation of semiconducting metal oxides (e.g. Fe$_2$O$_3$, ZnO, TiO$_2$, etc). Thus, studies of ROS production are often limited to the photic zone of the water column. However, recent studies indicate the existence of inventories of O$_2^-$ and H$_2$O$_2$ arising from aphotic sources in a variety of aquatic systems. The associated ROS production has been attributed to the oxidation of reduced Fe and S species or enzymatic reactions.

Despite decades of study, estimates of the global production of ROS are not well constrained due to the ongoing discovery of new ROS sources. Estimates of global ROS inventories in freshwater and seawater are of the order $10^{-12} - 10^{-9}$ for O$_2^-$, $10^{-11} - 10^{-7}$ M for H$_2$O$_2$, and $10^{-18} - 10^{-15}$ for HO$^-$. However, these data are often reported as steady-state inventories (concentrations) in different systems without estimates of their contribution to ROS production.

One source of ROS production is the oxidation of reduced transition metals, such as Fe$^2$, Mn, Co, and Cu, by atmospheric oxygen. Given the role of these metals in promoting ROS formation (illustrated with Fe(II) oxidation, Rxn 1), the plethora of systems where Fe(II) is introduced into oxic interfaces at high concentrations is tantamount to discovery of new sources of ROS.
Fe^{II} + O_2 ⇌ Fe^{III} + O_2^− \quad \text{Rxn 1}

Estimates by Beck et al. indicate that the emergence of anoxic porewater into oxic overlying waters (e.g. Submarine Groundwater Discharge (SGD)) contributes 19 – 54 millimoles of Fe per meter of shoreline per day (based on a total SGD-associated volume flux of 1.49 m$^3$/m shoreline/day). Global estimates for hydrothermal vent-associated reduced Fe input (as Fe(II) or Fe sulfide phases) range from 7 ×10$^8$ – 9 ×10$^8$ moles/year based on cumulative regional hydrothermal activity. Regardless of origin, the chemistry of Fe(II) in the presence of O$_2$ is predictable, whereby the net oxidation of Fe(II) to Fe(III) leads to the rapid production and consumption of ROS, as depicted in Figure 3.1.

Salt marsh systems make up a significant fraction of coastal environments and are the site of rapid exchange of Fe rich pore water and ground water with surface waters. The assumption that groundwater-derived Fe(II) undergoes oxidation upon emergence into overlying oxic waters suggests that these systems can be a significant source of ROS in coastal environments. This study reports a test of the hypothesis that the tidally driven expulsion of groundwater from anoxic sediments and subsequent exposure of associated reduced transition metals to oxic conditions results in the aphotic production of ROS. The instability of ROS in the presence of transition metals limits the reliability of methods employing sample collection for future laboratory analysis. In this work, a commercially available flow-injection analysis chemiluminescence detector system (FeLume) was employed for the analysis of ROS in real-time.
A sampling station was established overlooking a tidal creek on Sol Legare Island, South Carolina. The partial passage of the chemocline across the sediment surface into open air at low tide and its retreat below the sediment surface at high tide was observed on two separate occasions: July 7-8th, 2013 and July 30th-31st, encompassing periods that included daylight and night time hours. The emergence of high levels of Fe(II) was noted as was a decline in surface oxygen concentrations to less than 20% saturation. Salinity was essentially constant, suggesting the tidal prism at this location was dominated by recycled seawater with no significant freshwater input.

3.3 Experimental Materials

10-methyl-9-(p-formylphenyl)-acridinium carboxylate trifluoromethanesulfonate (acridinium ester) was synthesized according to Cooper, et al and confirmed via NMR (Figure 3.2-3.4). 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p’-disulfonic acid monosodium salt hydrate (Ferrozine); (97%), catalase (bovine liver, ≥10,000 units/mg protein), and hydrogen peroxide (30%) were purchased from Sigma Aldrich. Diethylenetriaminepentaacetic acid (98%) was supplied by Acros Organics. Iron (II) chloride (anhydrous, 99.5%) was obtained from Alfa Aesar. Hydrochloric acid and potassium hydrogen phthalate (KHP) were obtained from VWR. Sodium hydroxide, sodium carbonate, and sodium phosphate monobasic were obtained from Fisher Scientific. Unless otherwise noted, reagents were used as received and all solutions were prepared in 18 MΩ water. All glassware and high-density polyethylene (HDPE) containers were soaked in a 10% HCl bath for 12 hours followed by a thorough rinse with 18 MΩ water. Containers were rinsed several times with ambient water prior to water sample collection.
Sample Collection

Surface water was continuously pumped from the creek via Teflon tubing and a peristaltic pump into a clean beaker for sample collection. Ambient levels for dissolved oxygen (DO), temperature, pH and conductivity were measured via probes (Thermo-Fisher). Samples for Fe(II) analysis were added directly to Ferrozine indicator solution.\textsuperscript{11,184} An acridinium ester technique was used for the determination of H\textsubscript{2}O\textsubscript{2} by monitoring the selective chemiluminescence of acridinium ester (vide infra).\textsuperscript{92, 93, 185} A delayed coincidence counting technique developed by Moore was used for determination of $^{224}$Ra.\textsuperscript{186-189} Total organic carbon in acidified, unfiltered samples was determined spectrophotometrically on a Shimadzu Total Organic Carbon Analyzer (TOC-L) calibrated against freshly prepared KHP standards.

Hydrogen Peroxide Measurements

A flow injection analysis system (Felume, Waterville Analytical) was used for all hydrogen peroxide measurements.\textsuperscript{93,185} The technique was modified to include the in-line addition of 70 mM diethylenetriaminepentaacetic acid (DTPA) to prevent transition-metal catalyzed artifacts, modified from Rose \textit{et al.} (Fig. 3.5).\textsuperscript{84,87} Optimal instrument parameters for the experimental setup were as follows: flow rate = 1.5 mL min\textsuperscript{-1}; PMT voltage = 760 V; integration time = 200 ms; data collection rate = 2 data points s\textsuperscript{-1}. The system was periodically rinsed with 2 M HCl for general instrument cleaning and maintenance. A typical calibration curve is included as Figure 3.6.
Iron and Hydrogen Peroxide Spike Experiments

Spike experiments were conducted coincident with the time series measurements to elucidate timescales of ROS and Fe dynamics as a function of ambient conditions. Fe(II) spikes were added to ambient water to test the null hypothesis that initiation and maintenance of the ROS production/consumption chain is limited by Fe(II) inventory. H$_2$O$_2$ spikes were added to test the null hypothesis that H$_2$O$_2$ consumption proceeds solely through the Fenton reaction with Fe(II). Creek water was withdrawn during field surveys and sufficient Fe(II) or H$_2$O$_2$ added to increase their concentration by 15 µM or 1 µM respectively. The decay of resulting total Fe(II) or H$_2$O$_2$ were monitored over time to obtain the lifetime of these materials at different points in the tide cycle.

3.4 Results
A sampling station was established overlooking a tidal creek on Sol Legare Island, South Carolina (32°41'25.62"N and 79°57'10.73"W). Field campaigns were conducted on July 7th-8th (2013) and July 30th-31st (2014) encompassing partial tidal and diurnal cycles (Figure 3.7). Surface water from the site was monitored for pH, temperature, dissolved oxygen, total organic carbon, salinity, Ra, Fe(II), O$_2^-$ and H$_2$O$_2$ during field campaigns that took place on July 7th-8th, 2013 (Fig 3.8 – 3.9) and July 30th-31st (Fig 3.10-3.11). Sampling typically began in late afternoons and extended into the early morning. Salinity remained relatively constant, at 32.4±0.4 ppt (2013) and 32.6±0.3 ppt (2014), consistent with a marine source for both overlying and sediment pore water. Water temperature varied from 34.7°C – 25.8°C (2013) and 29.8°C – 27.8°C (2014), while pH remained relatively constant at 7.54±0.24 (2013) and 7.42±0.15 (2014). Total Organic Carbon varied from 36 mg/L to 130 mg/L at high and low tide (2013), respectively. No TOC measurements were
performed in 2014. Dissolved oxygen (O₂) varied from a daytime high of 250 µM to 35 
µM at low tide at night (2013) and 250 µM to 58 µM (2014). Surface water composition 
was dominated by incoming seawater at high tide and expelled sedimentary porewater at 
low tide, as indicated by measurements of ²²⁴Ra vs time (Figure 3.17). Fe(II) 
measurements ranged from a low (at high tide) at approximately the method detection limit 
(2 µM) to the highest of 80 µM at low tide, night (2013). Much lower concentrations of 
Fe(II) were observed in 2014, ranging from detection limit to a high of 16 µM (Figure 
3.19). Superoxide was measured (2014 only) and seemed to correlate with H₂O₂ (Fig 3.12), 
but not with Fe(II) (Fig 3.13). Hydrogen peroxide was detected in all samples, with 
instantaneous concentrations ranging from 125 nM to 900 nM (2013), and 40 nM to 352 
nM (2014). No correlation was observed between H₂O₂ and Fe(II) (Fig 3.14).

**Iron(II) Spikes 2013**

The spike addition of Fe(II) resulted in the induction of an ROS reaction chain, 
observed as initial net production of H₂O₂ (Figure 3.15-3.18). This is consistent with the 
Fe(II) oxidation product, O₂⁻, reacting with Fe(II) to form H₂O₂. The initial H₂O₂ 
production period was followed by a sustained period of net consumption for both Fe(II) 
and H₂O₂ (indicated by a peak in H₂O₂ concentration, Figure 3.15-3.18. Calculated ΔFe(II) 
and ΔH₂O₂ indicated relatively slow net consumption rates (Figure 3.19) (t₁/₂ = ~12 
minutes). No clear correlation was observed between Fe(II) and H₂O₂ for each spike 
(Figure 3.20-3.23).
Hydrogen Peroxide Spikes 2013 and 2014

The combination of \( \text{H}_2\text{O}_2 \) inventory (as instantaneous concentration) and consumption rate in natural waters was used to estimate the rate of production necessary to maintain the observed inventory by Fe(II)/Fe(III) cycling. Consumption rates were determined for ambient creek water as \( \text{H}_2\text{O}_2 \) following a spike that yielded a net increase to 1 micromolar \( \text{H}_2\text{O}_2 \) (a spike level similar to the maximum observed ambient level). When \( \text{H}_2\text{O}_2 \) was spiked into reactors containing water collected between the mid and high tide periods (\( 2.6 \mu\text{M} \leq \text{Fe(II)} \leq 10.5 \mu\text{M} \)), \( \text{H}_2\text{O}_2 \) inventories were quite variable, but overall decay kinetics were relatively slow relative to the signal oscillation (\( k = 10^{-4} \text{s}^{-1}\text{L}^{-1} \), Fig 3.24-3.25). It is worth noting that the observed oscillation in the signals only occurred following spike additions, and was not observed for ambient measurement and thus was not considered noise in the measurement technique. A significantly faster net decay was observed during the low tide period in 2013 (water containing \( \sim 50 \mu\text{M Fe(II)} \)) (Figure 3.24). Experiments in 2014 showed similar signal oscillation to that of 2013 with similar overall decay rate (\( 10^{-4} \text{s}^{-1}\text{L}^{-1} \), Fig. 3.25).

The contribution of Fe(II) as a source and/or sink for \( \text{H}_2\text{O}_2 \) was investigated by blocking the production of superoxide and \( \text{H}_2\text{O}_2 \) during one of the Fe(II) spike experiments with the addition of DTPA to the reaction vessel.\(^{51, 84, 101, 102}\) By adding DTPA into the reactor itself (at a much lower concentration than that added in-line), the stability of the ROS inventory could be monitored as the Fe(III) and then Fe(II) were sequestered (according to reported formation constants).\(^{31}\) Previous environmental studies using DTPA to inhibit iron-mediated artifacts used concentrations ranging from as low as 3.8 \( \mu\text{M} \) up to 50\( \mu\text{M} \).\(^{190}\) However, the non-selective chelating ability of DTPA, as well as its historically
recorded formation of strong alkaline earth complexes indicate that effective use of DTPA to sequester Fe must take competition with other metals into account.\textsuperscript{191,192} As shown in Figure 3.26, following the addition of Fe(II), H\textsubscript{2}O\textsubscript{2} signal increased and then rapidly decayed to near ambient levels. Following the introduction of \textasciitilde 350 \mu M DTPA, a brief increase was immediately followed by a rapid decrease in H\textsubscript{2}O\textsubscript{2} signal to near background. The data indicates that the chelation of Fe species by DTPA leads to a rapid net loss of the H\textsubscript{2}O\textsubscript{2} inventory. The maintenance of the ROS inventory observed under ambient conditions in the presence of the ambient ROS scavengers implies significant Fe mediated ROS production. The scavengers may be organic, inorganic, or biotic, however specific identification of these scavengers is beyond the scope of this investigation.

3.5 Discussion

The time series results are consistent with varying mixtures of oxygen rich surface water (primarily coastal seawater) and sediment pore water containing species associated with reducing conditions (e.g. Fe(II) or sulfide species). Pore water can be expelled from the sediments during a falling tide by gravitational drainage or surface-tension driven release from compressible storage. The resultant exposure of this water to the atmosphere leads to rapid reaction of Fe(II) with O\textsubscript{2} and consequent production of ROS. Figure 3.18 illustrates the emergence of a concentrated pulse of Fe(II) into overlying surface waters coincident with an increase in \textsuperscript{224}Ra activity with the falling tide. Fe(II) reached a maximum of approximately 80 \mu M and remained above 10 \mu M for several hours. The correlation between \textsuperscript{224}Ra activity and Fe(II) concentration was not observed during the daylight sampling period.
The system was assessed for its condition with regards to being stoichiometric vs catalytic for ROS production via Fe(II) oxidation by the spiked addition of feedstocks to ambient creek water. First order Fe(II) net consumption rates were calculated from ΔFe(II) data to one half-life (10 minutes) and compared to theoretical Fe(II) consumption rates (as oxidation) calculated for ambient solution conditions (after Burns et al.\textsuperscript{29}). Comparison of observed versus calculated rates were used to estimate the number of Fe(II)/Fe(III) cycles per hour in ambient creek water (Table 3.1 and Table 3.2) after Burns et al.\textsuperscript{8}.

The number of Fe(II)/Fe(III) cycles induced by each Fe(II) spike ranged from 14 to 65 indicating that 7% or less of the spiked Fe(II) was immediately lost to the system through oxidation to insoluble oxides. The remaining 93% of Fe(II) that underwent oxidation to Fe(III) was regenerated back to Fe(II), indicating a significant inventory of ambient species capable of reducing Fe(III). The amount of Fe(II) being spiked was well below the ambient O\textsubscript{2} concentrations and solutions were exposed to air, so calculated rates were not limited by oxygen availability. The assumed 2:1 stoichiometry of moles Fe(II) consumed vs moles H\textsubscript{2}O\textsubscript{2} produced, should yield production of 7.5 μmoles of H\textsubscript{2}O\textsubscript{2} per liter for the 15 μmole/liter Fe(II) spike. This was not the case, as calculated H\textsubscript{2}O\textsubscript{2} production following the Fe(II) spike ranged from 7 to 10 millimoles per liter per hour (based on published rate constants and instantaneous Fe(II), O\textsuperscript{2−}, and H\textsubscript{2}O\textsubscript{2}) suggesting a series of reactions initiated by the Fe(II) spike but sustained by other ambient electron donors.
For a system at steady state (i.e. ROS production equal to consumption), there was an expectation that spiked materials would rapidly be consumed and return to “background” levels. However, in most cases, spikes yielded ROS production in excess of the added reductive equivalents. Based on the consumption rates and the number of Fe(II) cycles, the total estimated excess H$_2$O$_2$ production (over background) following spike additions was up to 4 mM H$_2$O$_2$. Comparison of the bimolecular rate constants for the production of H$_2$O$_2$ and degradation via the Fenton reaction ($10^7$ M$^{-1}$s$^{-1}$ vs $10^4$ M$^{-1}$s$^{-1}$)$^2$, suggests that Fe(II) was not the primary ROS sink during the low tide conditions, consistent with the results of the DTPA addition experiment.

Direct measurement of HO· in these systems is difficult, as addition of the high levels of probe molecule necessary to quantitatively measure HO· would effectively distort the system and likely form bicarbonate/carbonate radicals.$^{194-196}$ Based on ΔFe(II) and ΔH$_2$O$_2$ from the Fe(II) spike experiments and k$_{Fenton}$ of $10^4$ M$^{-1}$s$^{-1}$,$^{193}$ estimated production of HO· ranged from 0.3 – 1.1 millimoles per liter per hour. It is difficult to separate out the specific contribution of ROS or natural reductants such as sulfide towards the promotion of Fe(II)/Fe(III) cycling due to the dynamic nature of the system being studied. The results of this study present strong evidence that the introduction of Fe(II) into oxic systems serves to catalytically initiate and sustain the rapid production and maintenance of ROS, respectively, in aquatic systems. The data is consistent with a system where the redox cycling of Fe(II)/Fe(III) acts catalytically towards the production of a chain of ROS in carbon-rich systems.
This process reflects a mechanism of coupling microbial production of reduced species (e.g. Fe(II), sulfide species) to abiotic ROS production. As the DTPA spike experiments show, microbially-mediated degradation of ROS is rapid, which is ultimately beneficial given the highly toxic and even mutagenic levels of ROS being generated by Fe(II) oxidation alone. Elucidation of the relationship between Fe(II) oxidation and microbial processes and the establishment of an optimal level of ROS will require further study. This study focused on estuarine locations due to their ecological importance as areas of primary production, nutrient transport, burial/recycling of organic carbon, as well as providing habitats for countless wildlife species. However, the process investigated in this study is ubiquitous for any system where anoxic Fe(II) is introduced to oxic waters (i.e. hydrothermal vents, upwelling of deep sea water, sediment disturbance events in permeable sediments, etc).

3.6 Conclusions

Under the environmental conditions studied here, aphotic mechanisms of ROS production associated with Fe(II) oxidation have been shown to be capable of surpassing photochemical mechanisms in both magnitude of ROS produced as well as contribution to net redox reactivity of the system. This does not serve to diminish the contribution of known photochemical mechanisms to ROS production. Rather, results suggest that the emergence of reduced species such as Fe(II) at the ground water/surface water interface can initiate production of significant quantities of ROS. While this study focused on estuarine systems, the process investigated is ubiquitous for any system where anoxic Fe(II) is introduced to oxic waters (i.e. hydrothermal vents, upwelling of deep sea water, sediment disturbance events in permeable sediments, etc).
Geochemical production of ROS may be a potentially significant factor in the mineralization of organic matter. Terrestrial organic carbon has been shown to undergo rapid oxidative losses upon passing through coastal boundaries. Coastal systems have been implicated to be of great importance in the mineralization of terrestrial organic matter, with estimated contributions of 50-80% of the global mineralization budget. The sustained levels of Fe(II) and H₂O₂ observed during extended periods of time suggest that these systems are capable of supporting Fenton chemistry. While no direct measurements of HO⁻ were attempted in this study, the high ambient concentrations of total organic carbon observed could be a likely sink for HO⁻ generated by Fenton chemistry. An alternative sink could also be HCO₃⁻/CO₃²⁻ species, which are known to reduce the efficiency of HO⁻ towards oxidation of organics through competing nonproductive reactions (i.e. formation of the less reactive carbonate radical, ·CO₃²⁻). While beyond the scope of this investigation, the efficiency of the proposed mechanism towards mineralization of organic carbon in natural aquatic systems requires more intensive study.
Figure 3.1 Dissolved Fe(III) is readily reduced to generate an inventory of Fe(II) which rapidly oxidizes upon exposure to surface waters, initiating rapid Fe(II)/Fe(III) cycling during the net oxidation of Fe to a solid, redox-inactive form (FeP\(_{(s)}\)). Abbreviations: FeRB – Fe-reducing bacteria, SRB – Sulfate-reducing bacteria, L\(_{x}\) – water-soluble ligand, P – precipitating ligand.
**Figure 3.2** Synthesis of the acridinium ester
Figure 3.3 $^1$H NMR spectrum for synthesized Acridinium Ester. (400 MHz, d$_6$-Acetone) δ 10.16 (s, 1H), 9.09 (d, $J = 9.3$ Hz, 2H), 8.80 (dd, $J = 8.6$, 0.8 Hz, 2H), 8.71 – 8.64 (m, 2H), 8.32 – 8.25 (m, 2H), 8.24 – 8.19 (m, 2H), 8.03 – 7.94 (m, 2H), 5.26 (s, 3H)
Figure 3.4 $^{13}$C NMR spectrum for synthesized Acridinium Ester. (100 MHz, d6-Acetone) δ 191.89, 163.89, 154.96, 148.47, 143.47, 140.57, 136.65, 132.30, 130.82, 128.77, 123.87, 123.62, 120.60, 40.58)
Continuous-Flow Sample Monitoring

Sample → Mix with 70 mM DTPA pH 6.3 → Mix with 1 mM Acridinium Ester pH 3.0 → Mix with 100 mM Carbonate Buffer pH 12.0 → Chemiluminescent Detection via PMT (Continuous)

**Figure 3.5** Flow chart detailing the analytical sequence for both continuous-flow H₂O₂ samples
Figure 3.6 Representative calibration curve for H$_2$O$_2$ analysis

\[ y = 1485.3x + 115448 \]
\[ R^2 = 0.9924 \]
Figure 3.7 Extent of tidal variation on sampling location during high tide (left) and low tide (right)
Figure 3.8. Physicochemical composition of surface water at the sampling site including salinity, pH, temperature (water), TOC, and tidal variation (Folly Beach, SC - July 7th-8th, 2013)
Figure 3.9 Physicochemical composition of surface water at the sampling site including dissolved oxygen, Fe(II), H$_2$O$_2$, $^{224}$Ra, and tidal variation (Folly Beach, SC - July 7$^{th}$-8$^{th}$, 2013)
Figure 3.10 Physicochemical composition of surface water at the sampling site including salinity, temperature (water), pH, and tidal variation (Folly Beach, SC - July 30th-31st, 2014)
Figure 3.11 Physicochemical composition of surface water at the sampling site including dissolved oxygen, Fe(II), H2O2, O2⁻, and tidal variation (Folly Beach, SC - July 30th-31st, 2014)
Figure 3.12 A plot of $\text{H}_2\text{O}_2$ vs $\text{O}_2^-$
**Figure 3.13** A plot of Fe(II) vs O$_2^-$
Figure 3.14 A plot of H$_2$O$_2$ vs Fe(II)

$y = 0.6332x - 0.6257$

$R^2 = 0.115$
Figure 3.15 Fe(II) and H$_2$O$_2$ decay following injection of 15 µM Fe(II) to one liter creek water (4:57 PM, 2013). Gap in H$_2$O$_2$ due to accidental displacement of sampling line.
Figure 3.16 Fe(II) and $\text{H}_2\text{O}_2$ decay following injection of 15 $\mu$M Fe(II) to one liter creek water (6:27 PM, 2013)
Figure 3.17 Fe(II) and H$_2$O$_2$ decay following injection of 15 µM Fe(II) to one liter creek water (7:58 PM, 2013)
Figure 3.18 Fe(II) and H$_2$O$_2$ decay following injection of 15 μM Fe(II) to one liter creek water (7:58 PM, 2013)
Figure 3.19 First order decay of Fe(II) following injection into one liter creek water
Figure 3.20 Fe(II) vs H₂O₂ following 15 μM Fe(II) spike (4:57 PM, 2013)
Figure 3.21 Fe(II) vs H₂O₂ following 15 µM Fe(II) spike (6:27 PM, 2013)
Figure 3.22 Fe(II) vs H₂O₂ following 15 µM Fe(II) spike (7:58 PM, 2013)
Figure 3.23 Fe(II) vs $\text{H}_2\text{O}_2$ following 15 $\mu$M Fe(II) spike (12:50 AM, 2013)

$y = 61.521x - 321.3$

$R^2 = 0.3573$
Figure 3.24 $\text{H}_2\text{O}_2$ decay following injection of 1 $\mu$M $\text{H}_2\text{O}_2$ to one liter creek water (2013)
Figure 3.25 $\text{H}_2\text{O}_2$ decay following injection of 1 µM $\text{H}_2\text{O}_2$ to one liter creek water (2014)
Figure 3.26 Effect of DTPA addition following an Fe(II) spike on H₂O₂ decay
Table 3.1: Experimental decay rates compared against predicted rates based on a 15 µM Fe(II) spike into one liter of creek water

<table>
<thead>
<tr>
<th>Time of Fe(II) Spike July 7th-8th, 2013</th>
<th>pH</th>
<th>[Cl(^-)] (mM)</th>
<th>[CO(_3^{2-})] (mM)</th>
<th>[Fe(II)](_0) (µM)</th>
<th>(k_{\text{theoretical}}), ((s^{-1}) \times 10^{-3})</th>
<th>(k_{\text{experimental}}), ((s^{-1}) \times 10^{-3})</th>
<th>Number of Fe(II)/Fe(III) Cycles per Liter per Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:57 PM</td>
<td>7.75</td>
<td>512(^a)</td>
<td>2.2(^b)</td>
<td>6.1(^c)</td>
<td>7.8</td>
<td>0.26</td>
<td>31</td>
</tr>
<tr>
<td>6:27 PM</td>
<td>7.75</td>
<td>504(^a)</td>
<td>2.2(^b)</td>
<td>8.9(^c)</td>
<td>7.9</td>
<td>0.13</td>
<td>60</td>
</tr>
<tr>
<td>7:58 PM</td>
<td>7.75</td>
<td>503(^a)</td>
<td>2.2(^b)</td>
<td>3.2(^c)</td>
<td>7.9</td>
<td>0.58</td>
<td>14</td>
</tr>
<tr>
<td>12:50 AM</td>
<td>7.44</td>
<td>512(^a)</td>
<td>2.2(^b)</td>
<td>19(^c)</td>
<td>5.8</td>
<td>0.21</td>
<td>28</td>
</tr>
</tbody>
</table>

\(^a\) Calculated via Salinity
\(^b\) Estimate based on typical seawater concentrations
\(^c\) Denotes pre-spike concentration
Table 3.2: Experimental decay rates compared against predicted rates based on a $15 \, \mu\text{M}$ Fe(II) spike into one liter of creek water

<table>
<thead>
<tr>
<th>Time of Fe(II) Spike</th>
<th>July 31st, 2014</th>
<th>pH</th>
<th>[Cl\textsuperscript{−}] (mM)</th>
<th>[CO\textsubscript{3}\textsuperscript{2−}] (mM)</th>
<th>[Fe(II)]\textsubscript{0} (µM)</th>
<th>$k_{\text{theoretical}}$, $\left(s^{-1}\right) \times 10^{-3}$</th>
<th>$k_{\text{experimental}}$, $\left(s^{-1}\right) \times 10^{-3}$</th>
<th>Number of Fe(II)/Fe(III) Cycles per Liter per Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:22 AM</td>
<td>7.47</td>
<td>507</td>
<td>2.2\textsuperscript{b}</td>
<td>6.2\textsuperscript{c}</td>
<td>6.0</td>
<td>0.80</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>4:39 AM</td>
<td>7.47</td>
<td>507</td>
<td>2.2\textsuperscript{b}</td>
<td>8.9\textsuperscript{c}</td>
<td>6.8</td>
<td>0.72</td>
<td></td>
<td>65</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Calculated via Salinity
\textsuperscript{b} Estimate based on typical seawater concentrations
\textsuperscript{c} Denotes pre-spike concentration
REFERENCES


106. Krzymiński, K.; Ożóg, A.; Malecha, P.; Roshal, A. D.; Wróblewska, A.; Zadykowicz, B.; Błażejowski, J., Chemiluminogenic Features of 10-Methyl-9-(phenoxy carbonyl)acridinium Trifluoromethanesulfonates Alkyl Substituted at the


