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Importance of Iron Complexation for Fenton-Mediated Hydroxyl Radical Production at Circumneutral pH

Christopher J. Miller[1], *Andrew L. Rose*[1,2] *and T. David Waite*[1]*

[1] School of Civil and Environmental Engineering, UNSW Australia, Sydney, NSW, Australia, [2] Southern Cross GeoScience, Southern Cross University, Lismore, NSW, Australia

The reaction between Fe(II) and H_2O_2 to yield hydroxyl radicals ($HO^\bullet$), the Fenton reaction, is of interest due to its role in trace metal and natural organic matter biogeochemistry, its utility in water treatment and its role in oxidative cell degradation and associated human disease. There is significant dispute over whether $HO^\bullet$, the most reactive of the so-called reactive oxygen species (ROS), is formed in this reaction, particularly under circumneutral conditions relevant to natural systems. In this work we have studied the oxidation kinetics of Fe(II) complexed by L = citrate, ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) and also measured $HO^\bullet$ production using phthalhydrazide as a probe compound at pH 8.2. A kinetic model has been developed and utilized to confirm that $HO^\bullet$ is the sole product of the Fe(II)L-H_2O_2 reaction for L = EDTA and DTPA. Quantitative $HO^\bullet$ production also appears likely for L = citrate, although uncertainties with the speciation of Fe(II)-citrate complexes as well as difficulties in modeling the oxidation kinetics of these complexes has prevented a definitive conclusion. In the absence of ligands at circumneutral pH, inorganic Fe(II) reacts with H_2O_2 to yield a species other than $HO^\bullet$, contrary to the well-established production of $HO^\bullet$ from inorganic Fe(II) at low pH. Our results suggest that at high pH Fe(II) must be complexed for $HO^\bullet$ production to occur.

Keywords: reactive oxygen species, hydroxyl radical, Fenton, EDTA, DTPA, citrate

Edited by:
Colleen Hansel,
Woods Hole Oceanographic Institution, USA

Reviewed by:
William P. Inskeep,
Montana State University, USA
William Sunda,
National Oceanic and Atmospheric Administration, USA

Correspondence:
T. David Waite
d.waite@unsw.edu.au

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INTRODUCTION

The reaction between Fe(II) and H_2O_2 (the Fenton reaction) occurs naturally in aquatic environments (White et al., 2003), biological systems (Backa et al., 1993; Winterbourn, 1995; Jomova et al., 2012; Lee et al., 2012), and is also employed in water treatment applications (von Sonntag, 2008). Although ubiquitous and an important source of reactive oxygen species (ROS), if $HO^\bullet$ is indeed produced, the identity of the intermediate formed in this reaction has long been the subject of debate (Goldstein et al., 1993; MacFaul et al., 1998; Walling, 1998; Goldstein and Meyerstein, 1999; Dunford, 2002; Rachmilovich-Calis et al., 2009a; Remucal Keenan and Sedlak, 2011). It is generally accepted that $HO^\bullet$ is formed under acidic conditions and in the absence of ligands. However, an alternative mechanism invoking higher valent Fe complexes such as Fe(IV), first proposed by Bray and Gorin (1932), is also consistent with many findings (Kremer, 1999). Under circumneutral conditions the mechanism is particularly controversial (Remucal Keenan and Sedlak, 2011), with recent studies now mostly in agreement that at higher pH the product of the Fenton reaction with inorganic Fe(II) shifts to a species other than $HO^\bullet$, possibly to a high-valent

Fe species such as Fe(IV) (Hug and Leupin, 2003; Keenan and Sedlak, 2008; Bataineh et al., 2012; Lee et al., 2013). The underlying cause of this is not clear. The presence of organic ligands that are important to the speciation of Fe(II) in natural waters (Roy and Wells, 2011; Hopwood et al., 2015), as well as those employed to solubilize Fe under circumneutral conditions in industrial practices, complicates this further.

For reasons which will be discussed later, we have examined the ligands citrate, ethylenediaminetetraacetic acid (EDTA), and diethylenetriaminepentaacetic acid (DTPA) in this study. Despite extensive study, it is still not clear whether or not $HO^•$ is formed when the Fe(II) complexes of these ligands are oxidized by H_2O_2. Previous investigations have used a wide array of probe compounds to examine the nature of the oxidative intermediate formed in this reaction. By consideration of these methodologies used in this earlier work, particularly their limitations, it has been possible to design a study to avoid these issues and more-effectively isolate the Fe(II)L-H_2O_2 reaction. For example, Rahhal and Richter (1988, 1989) compared the impact of *t*-BuOH and MeOH upon the reaction stoichiometry and kinetics when Fe(II)/Fe(III)DTPA solutions reacted with H_2O_2 or were exposed to radiolytically produced $HO^•$ and concluded that $HO^•$ could not be the reactive intermediate. Similar work however by Rush and Koppenol (1987) (with L = EDTA and DTPA) employing a wider range of scavengers was generally consistent with $HO^•$ formation (although *t*-BuOH and benzoate scavengers yielded contradictory results), in direct contrast to their own earlier work which suggested that $HO^•$ was not formed by peroxidation of Fe(II)EDTA (Rush and Koppenol, 1986). Luzzatto et al. (1995) later examined the Fe(II)EDTA-H_2O_2 system using a β-elimination based assay with alcohol probe compounds, concluding that Fe(II)EDTA reacts with H_2O_2 to yield $HO^•$. All these studies were undertaken in deoxygenated solutions at relatively high Fe concentrations (10^{-5}–10^{-3} M), under which conditions the relatively poorly studied reactions between organic radical intermediates (whatever their mode of formation) and FeL species potentially become important. Although $^•CH_2COH(CH_3)CH_3$ radicals formed from the reaction of $HO^•$ and *t*-BuOH were shown to be unreactive toward Fe-DTPA complexes (Rahhal and Richter, 1989) and unable to reduce Fe(III)EDTA (Rush and Koppenol, 1987), Croft et al. (1992) suggested that they could oxidize Fe(II)EDTA. Fe(III)EDTA has also been shown to oxidize $CO_2^{•-}$ radicals (Rush and Koppenol, 1987). Most carbon-centered organic radicals also typically react rapidly with O_2. It is possible that incomplete O_2-exclusion further complicated the analysis of the earlier work, as even very small amounts of O_2 will lead to competition between O_2 and Fe species for the organic radicals (Rush and Koppenol, 1987). It would therefore seem likely that the complications introduced from the poorly-studied reactions between Fe and the carbon-centered radicals may be the reason for the discrepancies in these previous studies.

The nature of the intermediate formed has also been proposed to be dependent upon the initial reactant concentrations. Yamazaki and Piette (1990, 1991) studied the Fe(II)L-H_2O_2 (L = EDTA, DTPA) reaction under oxic conditions by DMPO spin trapping concluding that Fe(II)DTPA produces only $HO^•$. They demonstrated that $HO^•$ formation was quantitative at low Fe(II) concentrations ($<10^{-6}$ M). However, competitive scavenging with *t*-BuOH was not consistent with $HO^•$ formation, in agreement with similar observations by Rahhal and Richter (1988). Based upon observation of an increase in the total trapped spin when ethanol (EtOH) was added to the system, they also suggested that Fe(II)EDTA peroxidation forms another oxidative intermediate in addition to $HO^•$. This concentration dependence however could also conceivably be caused by competition between DMPO and Fe-L complexes for the oxidant.

Croft et al. (1992) undertook a detailed ESR study of the Fe(II)EDTA/H_2O_2 system by monitoring organic radicals formed from substrate oxidation. With carefully controlled studies they demonstrated that anomalous results from scavenging experiments could be explained if oxidation/reduction of organic radicals by Fe(III)L/Fe(II)L and H_2O_2 was incorporated into the modeling. Previous anomalous results in the Fe(II)EDTA/H_2O_2 system with *t*-BuOH and EtOH as scavengers were thus reconciled when reduction of *t*-BuOH and β-EtOH radicals by Fe(II)EDTA and oxidation of α-EtOH radicals by Fe(III)EDTA was invoked. Often contradictory results have been rationalized by insufficient consideration of intermediate organic radical species, which may further oxidize Fe(II) or reduce Fe(III), depending upon the nature of the radical thus formed (Croft et al., 1992; Kosaka et al., 1992; Yurkova et al., 1999).

In comparison to the Fe(II)-EDTA/DTPA system, the Fe(II)-citrate system has been studied comparatively little. Gutteridge (1991) provided evidence that citrate is able to reduce Fe(III), with the Fe(II)-citrate thus formed able to produce $HO^•$, although the mechanism was not examined nor was quantitative production confirmed (note that the reduction process is not significant at the concentrations and time scales used in this work). Studies on the photo-reduction of Fe(III)-citrate in the presence of H_2O_2 also support the formation of $HO^•$, although details of reaction rates were not examined (Zepp et al., 1992). Vile et al. (1987) directly studied the reaction between H_2O_2 and Fe(II)-citrate (Fe(II)cit) and suggested that an oxidant whose properties are consistent with $HO^•$ is formed based upon formation of similar thiobarbituric acid reactive substances (TBARS) to those formed during deoxyribose degradation with Fe(II)EDTA, which was considered to produce $HO^•$ based upon the impact of various scavengers. Separate experiments measuring CO_2 formation during formate oxidation demonstrated that Fe(II)cit peroxidation produces 60% less $HO^•$ than Fe(II)EDTA peroxidation. The extent of deoxyribose degradation by Fe(II)cit was similar to that observed with Fe(II)EDTA, whereas inorganic Fe(II) produced ∼3 to 4-fold more TBARS, suggesting that inorganic Fe(II) may produce $HO^•$. Results from this work should be considered with some caution in light of more recent evidence regarding the inherent complexity of interpreting results from the TBARS method (Rachmilovich-Calis et al., 2009b).

From these previous studies it is clear that there is significant disagreement as to the reactive intermediate formed in these systems and that detailed consideration of the fate of any organic radicals formed must be considered in any quantitative analysis, as highlighted by Masarwa et al. (2005). By consideration of the fate of these radical species it is conceivable that a kinetic model may be constructed that could adequately describe the concentration profiles of Fe(II)/Fe(III), as well as any production of $HO^{\bullet}$, should it indeed be formed. The main drawback with such an approach is the paucity of studies into the reactivity of carbon-centered radicals with Fe, especially when complexed by organic ligands. However, most carbon-centered radicals react at near diffusion-controlled rates with O_2 to yield organoperoxyl radicals, with some of these organoperoxyl radicals subsequently rapidly eliminating $O_2^{\bullet -}$ (von Sonntag et al., 1997), with the reactions of $O_2^{\bullet -}$ and Fe-complexes thoroughly investigated. Therefore, provided Fe concentrations are significantly lower than O_2 concentrations, and the organoperoxyl radicals that are formed are of the type that rapidly eliminate $O_2^{\bullet -}$, any potential reaction between Fe and these carbon-centered radicals will be insignificant. Under such conditions organic radicals are effectively converted to $O_2^{\bullet -}$, a species with well-known reactivity with Fe and Fe-complexes. This study has been designed around these requirements to allow for a definitive conclusion to be reached, and to avoid the complications that have plagued earlier work in this area.

In the present study the oxidation of inorganic Fe(II) and Fe(II)L by H_2O_2 was examined under oxic conditions at low micromolar concentrations of Fe(II), combining measurements of Fe(II) oxidation kinetics, $HO^{\bullet}$ production kinetics, and competition between well-defined competitive $HO^{\bullet}$ scavengers and the $HO^{\bullet}$ trapping agent phthalhydrazide (Phth). Kinetic modeling was then used to quantitatively examine whether $HO^{\bullet}$ was the key intermediate formed. By employing oxic conditions with relatively low concentrations of FeL and H_2O_2, the fate of intermediate organic radicals was determined by well-defined O_2-mediated pathways, resulting in minimal interference to the Fe(II)L-H_2O_2 system under study. This approach, combined with quantitative kinetic modeling incorporating the fate and further interaction of intermediate organic radical species, has allowed the Fe(II)L-H_2O_2 reaction to be effectively isolated and studied. A critical aspect of this approach is being able to reasonably predict the actual fate of any oxidizing radical formed, as well as the kinetics and mechanism of Fe(II) oxidation [and potentially Fe(III) reduction] in the presence of the ligand. For this reason, well-defined synthetic ligands (L = citrate, EDTA and DTPA) for which the relevant chemistry is reasonably well-understood have been employed in place of the poorly characterized ligands that are typical of marine, estuarine and freshwater systems. A low ionic strength has been used in this study to prevent introduction of redox-active contaminants from the salt solutions, as well as to simplify the kinetic and thermodynamic treatment of the Fe-ligand interaction which can be greatly complicated by high concentrations of cations such as Mg^{2+} and Ca^{2+} (Fujii et al., 2008; Miller et al., 2012).

MATERIALS AND METHODS

Chemicals

Solutions were prepared in 18 MΩ.cm Milli-Q water (MQ) obtained from a Millipore Milli-Q system. Glassware and plasticware were soaked for 3 days in 0.5 M HCl. Reagents were of analytical reagent grade unless noted otherwise. EDTA and DTPA were added from stock solutions adjusted to pH ≈ 7.2 while citrate was added to solutions as solid disodium hydrogen citrate sesquihydrate ($C_6H_6Na_2O_7{\cdot}1.5H_2O$). H_2O_2 stock solutions were prepared by dilution of a 30.7% w/v H_2O_2 solution (Riedel-de Haën, stabilized with 40 mg/L dipicolinic acid) and standardized spectrophotometrically using $\varepsilon_{250} = 22.7\ M^{-1}{\cdot}cm^{-1}$ (Morgan et al., 1988). Fe(II) stock solutions were prepared daily from $Fe(II)SO_4{\cdot}7H_2O$ (4 mM) and adjusted to pH 4 with HCl. 1 M Na_2CO_3 buffer was prepared by dissolving Na_2CO_3 in MQ and allowing it to stand for several days. It was then filtered to remove precipitates (0.22 μm Millipore Millex-GV PVDF membrane) and then adjusted to pH 11 with 32% HCl. $K_5Cu(HIO_6)_2$ (Cu(III)) was synthesized as described previously (Miller et al., 2011). For scavenging experiments, appropriate amounts of sodium formate (Sigma-Aldrich, 99+% ACS Reagent grade) or 2-methyl-propan-2-ol (*t*-BuOH, Ajax Analytical Reagent grade) were added to the standard reaction matrix, which consisted of 10 mM NaCl and 2 mM $NaHCO_3$. When performing $HO^{\bullet}$ production experiments, 0.55 mM phthalhydrazide (Phth, 99%, Aldrich) was also added to the standard matrix. In all experiments the pH of the matrix was adjusted to 8.20 ± 0.05 with HCl and/or NaOH, which remained relatively constant (±0.05 pH units) during an experiment. All reactions were studied under conditions minimizing exposure to room lighting through use of brown glass vessels and shielding with aluminum foil.

Fe(II) Oxidation Experiments

The kinetics of Fe(II)cit oxidation by O_2 and H_2O_2 were examined by monitoring the loss of Fe(II) using luminol chemiluminescence (Rose and Waite, 2001; Miller et al., 2009) with $[Fe(II)]_0 = 0.2$, 0.6, 1.2, and 2 μM, where the subscript 0 denotes initial concentration. Fe(II)L oxidation kinetics (L = EDTA or DTPA; $[Fe(II)]_0 = 2$ μM) were investigated by following Fe(III)L formation spectrophotometrically at 260 nm [$\varepsilon \approx 7600\ M^{-1}{\cdot}cm^{-1}$ for both complexes, background corrected at 500 nm, with the molar absorbance coefficient determined here consistent with the values determined by Hill-Cottingham (1955) after accounting for the pH dependence]. In all cases, inorganic Fe(II) was added to a matrix containing an oxidant and ligand. Although explicitly considered during kinetic modeling, initial competition between Fe(II)-complex formation and oxidation of inorganic Fe(II) was expected to be minimal. This was confirmed by calculations using known rate constants for complex formation and inorganic Fe(II) oxidation for L = EDTA or citrate, and validated experimentally for L = DTPA as described in Supplementary Material Section 1. In all cases, no Fe(III) precipitation was expected to occur in the final solution on short timescales as the solubility product for amorphous ferric oxide (AFO) was not exceeded (see Supplementary Material Section 2 for calculations; in the absence of ligand AFO precipitation occurs rapidly at 2 μM Fe(III) at pH 8.2).

Phth was not present when monitoring Fe(II) oxidation as both Phth and Phth-OH absorb strongly in the UV (interfering with the Fe(III)EDTA and Fe(III)DTPA absorbance) and also due to the formation of chemiluminescent Phth-OH (which interferes with luminol chemiluminescence).

Fe(II) Oxidation in the Presence of Formate

The oxidation of formate by HO• initially yields $CO_2^{\bullet-}$, which reacts with O_2 with a rate constant of 2×10^9 $M^{-1}{\cdot}s^{-1}$ to yield $O_2^{\bullet-}$ and CO_2 (Buxton et al., 1976). As such, adding an excess of formate quantitatively converts all HO• that is produced to $O_2^{\bullet-}$ and CO_2. Under the standard reaction conditions used here, excess ligand effectively competes with Fe(II) for HO•, forming ligand-derived organic radicals that are potentially able to influence Fe(II) oxidation kinetics. As the reactivity of these organic radicals is unknown, Fe(II) oxidation experiments were conducted with addition of sodium formate ($[\text{formate}]_0 = 50$ mM for L = EDTA and DTPA and $[\text{formate}]_0 = 3$ mM for L = citrate) at formate concentrations sufficiently high for quantitative scavenging of HO•, i.e., such that the reaction of HO• with excess ligand is rendered insignificant. When formate is present at these concentrations, all HO• will be converted to $O_2^{\bullet-}$; if addition of formate does not change the reaction kinetics then this implies that excess ligand, the primary sink for HO• in the absence of formate, also reacts with HO• in a similar fashion as formate to ultimately yield $O_2^{\bullet-}$, validating the assumption that the reaction of ligand with HO• can be simplified to a reaction that produces $O_2^{\bullet-}$ along with some form of oxidized ligand.

HO• Production Experiments

Experiments were performed at 22 ± 2°C in vessels shielded from light. Experiments were initiated by addition of Fe(II) (2 μM) from an acidic stock (4 mM $Fe^{II}SO_4{\cdot}7H_2O$ in 0.1 mM HCl) to a vigorously stirred solution containing H_2O_2 (0–50 μM), Phth (0.55 mM), and either no ligand, 1 mM citrate, 10 μM EDTA or 10 μM DTPA. HO• formation was monitored using the phthalhydrazide method (Miller et al., 2011). Briefly, HO• reacts with Phth to form a hydroxy-derivative (Phth-OH) that accumulates in solution. Phth-OH is subsequently quantified using chemiluminescence detection during oxidation in an FeLume flowcell (Waterville Analytical) employing $Cu(III)/H_2O_2$ as an oxidant in 0.5 M Na_2CO_3 buffer at pH 11. The term "Phth-OH" is used to represent the total amount of HO• that has reacted with Phth, with the actual analyte, 5-hydroxy-2,3-dihydrophthalazine-1,4-dione (5-HO-Phth), formed in approximately 20% yield (Miller et al., 2011).

Impact of *t*-BuOH upon Phth-Trappable HO• Production

HO• production experiments were repeated in the presence of a range of *t*-BuOH concentrations (2, 5, 20, or 100 mM) with 0.55 mM Phth present in each case to examine whether competition between Phth and *t*-BuOH was consistent with the known behavior of HO•, thus supporting or refuting HO• formation. Experiments were performed with 5 μM H_2O_2 for L = EDTA, 50 μM H_2O_2 for L = DTPA, and 10 μM H_2O_2 for L = citrate.

Kinetic Modeling

Experimental results were initially evaluated in the context of a basic kinetic model for Fe(II) oxidation in the presence of L, O_2, and H_2O_2, which we denote as the "Fe(II)L model," that was constructed using known reactions (**Table 1**). All rate constants were either taken directly from the literature or calculated from literature values, except for rate constants for the oxidation of Fe(II)L by O_2 and the oxidation of Fe(II)L by H_2O_2, which were treated as variable parameters to be determined by fitting of the model to the experimental data. It was assumed in the Fe(II)L model that the reaction of H_2O_2 with Fe(II) (either inorganic or organically complexed) yielded HO•; this was treated as a null hypothesis that was tested by attempting to fit the model to the experimental data. It was also assumed that, under the oxygenated experimental conditions employed, the reaction of HO• with L resulted in the oxidation of L to a compound L_{ox} that did not undergo further reaction, with concomitant formation of $O_2^{\bullet-}$; this is expected for L = EDTA based on the mechanism established by Höbel and von Sonntag (1998) and hypothesized to also be the case for L = DTPA or citrate. This assumption was tested by examining Fe(II)L oxidation in the presence of formate as described earlier. For experiments conducted in the presence of formate, the additional reactions in **Table 2** were also included in the kinetic model.

Kinetic model simulations were performed using Presto-Kinetics V 3.28.3 (Wulkow, 2004) and Kintecus V 4.1 (Ianni, 2003). All model fitting was performed using the routines available in Presto unless specified otherwise, in which case parameters were manually adjusted and the quality of fit assessed qualitatively using Kintecus simulations.

RESULTS AND DISCUSSION

HO• Production by Inorganic Fe(II)

When Fe(II) was oxidized by H_2O_2 in the absence of ligands, Phth-OH concentrations were always below the detection limit of 15 nM, implying no significant production of HO• occurred. This is despite H_2O_2 being the dominant oxidant of Fe(II) under these conditions (King and Farlow, 2000; González-Dávila et al., 2005; Santana-Casiano et al., 2006; Pham and Waite, 2008; Miller et al., 2009), with ≈1 μM Phth-OH expected to form in this process if HO• was indeed the product of the Fe(II)-H_2O_2 reaction. Control experiments confirmed that inorganic Fe(III) did not impact the recovery of 5-HO-Phth nor interfere with any other aspect of the method, confirming the result and suggesting that under the conditions of this study the Fe(II)-H_2O_2 reaction, although the dominant oxidation path for Fe(II), does not yield HO•. This result further supports the notion that a pH threshold exists above which the peroxidation of Fe(II) produces a species other than HO•, the nature of which cannot be determined from this work, although likely to be some form of Fe(IV) compound (Hug and Leupin, 2003; Keenan and Sedlak, 2008; Bataineh et al., 2012; Lee et al., 2013).

Kinetics of Fe(II) Oxidation and HO• Production by Fe(II)EDTA and Fe(II)DTPA

The oxidation kinetics of Fe(II)EDTA and Fe(II)DTPA were both well-described by the Fe(II)L model in **Table 1** (see **Figure 1**).

TABLE 1 | "Fe(II)L" kinetic model for Fe(II)L oxidation.

Reaction	Rate constant ($M^{-1}\ s^{-1}$ unless stated otherwise)			Rxn #
	Citrate	EDTA	DTPA	
OXIDATION OF Fe(II)				
$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\bullet -}$	46.5[a]	–[b]	–	1
$Fe(II) + O_2^{\bullet -} \rightarrow Fe(III) + H_2O_2$	2.2×10^7[c]	–	–	2
$Fe(II) + H_2O_2 \rightarrow Fe(III) + ?$	1.58×10^5[d]	–	–	3
COMPLEXATION OF Fe(II)/Fe(III)				
$Fe(II) + L \rightleftharpoons Fe(II)L$	$k_f = 2.1 \times 10^6$ $k_r = 72.8\ s^{-1}$[e]	$k_f = 2.1 \times 10^6$ $k_r = 1.2 \times 10^{-3}\ s^{-1}$[f]	0[g]	4
$Fe(III) + L \rightarrow Fe(III)L$	3.18×10^6[e]	3.18×10^6[h]		5
OXIDATION OF Fe(II)L				
$Fe(II)L + O_2 \rightarrow Fe(III)L + O_2^{\bullet -}$	3.1[i]	45[i]	0.095[i]	6
$Fe(II)L + O_2^{\bullet -} \rightarrow Fe(III)L + H_2O_2$	2×10^6[e]	2×10^6[j]	2×10^7[k]	7
$Fe(II)L + H_2O_2 \rightarrow Fe(III)L + HO^\bullet + OH^-$	4.0×10^3[i]	3.2×10^3[i]	99[i]	8
$Fe(II)L + HO^\bullet \rightarrow Fe(III)L + OH^-$	1.2×10^8[l]	5×10^9[m]	1×10^{10}[n]	9
$Fe(III)L + O_2^{\bullet -} \rightarrow Fe(II)L + O_2$	800[o]	6×10^4[j]	0[p]	10
$Fe(III)L + HO^\bullet \rightarrow Fe(III)L_{Ox} + O_2^{\bullet -}$[q]	1.2×10^8[k]	5.2×10^8[r]	5×10^9[s]	11
Phth-MEDIATED TRAPPING				
$Phth + HO^\bullet \rightarrow Phth\text{-}OH + O_2^{\bullet -}$	5.3×10^9[t]	–	–	12
$Phth\text{-}OH + HO^\bullet \rightarrow Phth_{Ox} + O_2^{\bullet -}$	5.3×10^9[u]	–	–	13
OTHER OXYGEN RADICAL REACTIONS				
$L + HO^\bullet \rightarrow L_{Ox} + O_2^{\bullet -}$	3.2×10^8[v]	2×10^9[w]	5.2×10^9[w]	14
$HO^\bullet + H_2O_2 \rightarrow HO_2^\bullet + H_2O$	3.2×10^7[x]	–	–	15
$HO^\bullet + HO^\bullet \rightarrow H_2O_2$	5.5×10^9[y]			16
$O_2^{\bullet -} + HO^\bullet \rightarrow O_2 + OH^-$	1.01×10^{10}[z]	–	–	17
$HO_2^\bullet \rightleftharpoons O_2^{\bullet -} + H^+$	$k_f = 1.14 \times 10^6\ s^{-1}$, $k_r = 7.2 \times 10^{10}$[aa]	–	–	18
$HO_2^\bullet + HO_2^\bullet \rightarrow H_2O_2 + O_2$	8.3×10^5[z]	–	–	19
$HO_2^\bullet + O_2^{\bullet -} \rightarrow H_2O_2 + O_2$	9.7×10^7[z]	–	–	20

[a]*Miller et al. (2009).* [b]*"–"indicates that the same rate constant was used for all ligands.* [c]*Calculated using speciation model of King and Farlow (2000), ionic strength corrections performed using Davies Equation ($A = 1.17$, $b = 0.3$, $\ln \gamma = -AZ_i^2(I^{1/2}/(1+I^{1/2}) - bI)$ and rate constants from Santana-Casiano et al. (2005), $I = 0$.* [d]*Rate doubled to reflect pseudo-first order rate constant from Miller et al. (2009); the stoichiometry is not explicitly considered to prevent speculation, although in this work it is not critical since H_2O_2 is typically in excess.* [e]*Assumed to proceed at same rate as for EDTA with reverse rate constant calculated using the apparent overall formation constant of Fe(II)cit of $\log_{10}(K/M^{-1}) = 4.46$ determined using speciation modeling (see Supplementary Material Section 3).* [f]*Taken from Fujii et al. (2011).* [g]*Complexation not explicitly modeled as experimentally shown to be rapid.* [h]*Fujii et al. (2008).* [i]*Fitted to data.* [j]*Determined by analysis of literature values, see discussion in text and Supplementary Material.* [k]*Butler and Halliwell (1982).* [l]*Taken from Zepp et al. (1992) for Fe(III)cit; Fe(II)cit assumed to react at a similar rate.* [m]*Lati and Meyerstein (1978).* [n]*Rahhal and Richter (1989) demonstrated a ratio of 2:1 for rate constants for reactions between $HO^\bullet$ and Fe(II)DTPA/Fe(III)DTPA, rate constant for Fe(III)DTPA from Cabelli et al. (1989).* [o]*Garg et al. (2007) give 800 $M^{-1}s^{-1}$, Rose and Waite (2005) give 9×10^{4}· $M^{-1}s^{-1}$; see text for discussion.* [p]*Reaction considered slow ($<10^4\ M^{-1}s^{-1}$, Buettner et al. (1983)) and as such was not included.* [q]*Nature of reaction is not established, especially at the low Fe(III)L concentrations used here. $HO^\bullet$ initially oxidizes ligand by H-abstraction, followed by either further oxidation by intra-molecular electron transfer (to yield $Fe(II)L_{Ox}$) or inter-molecular electron transfer from Fe(III)L to yield $Fe(II)L + Fe(III)L_{Ox}$ (Kundu and Matsuura, 1975; Rahhal and Richter, 1989). Alternatively O_2 may oxidize this radical in a similar fashion to uncomplexed EDTA (Höbel and von Sonntag, 1998) to yield $Fe(III)L_{Ox} + O_2^{\bullet -}$. At the low concentrations of Fe(III)L used in this work, either the O_2-mediated or intramolecular process are considered most likely, with the O_2-mediated pathway adopted for modeling.* [r]*Kundu and Matsuura (1975).* [s]*Cabelli et al. (1989).* [t]*Schiller et al. (1999), note that the reaction of the intermediate cyclohexadienyl-type radical with O_2 to yield $O_2^{\bullet -}$ via the Dorfman et al. (1962) mechanism is implicitly included.* [u]*assuming same rate constant as for reaction between $HO^\bullet$ and Phth, although likely faster.* [v]*Zepp et al. (1992) with the assumption that the initial organic radical formed is rapidly oxidized by O_2 to yield $O_2^{\bullet -}$, verified as discussed in text.* [w]*Cabelli et al. (1989) for DTPA, Lati and Meyerstein (1978) for EDTA. It is assumed that the initial organic radical is subsequently oxidized to a diamagnetic species by O_2, forming $O_2^{\bullet -}$, with the work of Höbel and von Sonntag (1998) strongly suggesting this is the case for EDTA.* [x]*Yu (2004).* [y]*Buxton et al. (1988).* [z]*Bielski et al. (1985).* [aa]*Calculated using published value of reverse rate constant and $pK_a = 4.8$.*

The kinetics of Fe(II)L oxidation (L = EDTA and DTPA) were unchanged in the presence of formate, supporting the formation of $O_2^{\bullet -}$ during ligand oxidation by $HO^\bullet$, as anticipated (Höbel and von Sonntag, 1998). It should be noted that bridged dimeric species of the form such as $Fe(III)L(O_2^{2-})Fe(III)L$, proposed to occur during O_2-mediated oxidation by Seibig and Van Eldik (1997) for L = EDTA are not considered in the kinetic model as they are unimportant at the low Fe concentrations used here despite their demonstrated importance at millimolar concentrations of Fe(II) (Zang and Van Eldik, 1990b). The rate constants for O_2- and H_2O_2-mediated oxidation of Fe(II)L (L = EDTA, DTPA) found here are generally consistent with previously determined values under similar conditions (see Supplementary Material Sections 4 and 5 for details). For both oxidants, the oxidation of Fe(II)DTPA was an order of magnitude slower than that of Fe(II)EDTA, despite the similar nature of these ligands, which is consistent with previous results (Graf et al., 1984). The absence of a readily-exchangeable bound water molecule in the Fe(II)DTPA complex (Zang and Van Eldik, 1990a) could explain the much slower oxidation kinetics observed for this complex if oxidation of Fe(II)L (L = EDTA, DTPA) by O_2 and H_2O_2 was an inner sphere process; in this case, with the initial binding of the oxidant to Fe(II)L being the rate-limiting step, exchange of a bound H_2O molecule for an oxidant molecule should presumably proceed at a much greater rate and with less steric hindrance than would be observed when oxidant binding first requires breaking of an Fe(II)-chelate bond (Graf et al., 1984). Summers et al. (2008) have demonstrated the importance of the water-loss rate to the related superoxide-mediated reduction of Fe(III)EDTA and Fe(III)DTPA.

For both Fe(II)EDTA and Fe(II)DTPA, significant Phth-trappable $HO^\bullet$ production was observed. However, the interaction of the Fe-EDTA and Fe-DTPA complexes with $O_2^{\bullet-}$ (which is formed from the reaction of O_2 with Fe(II)L and also during oxidation of the hydroxycyclohexadienyl-radical resulting from the reaction of $HO^\bullet$ and Phth) complicates the kinetic modeling analysis. Although EDTA and DTPA are structurally quite similar, their Fe complexes exhibit markedly different reactivity with $O_2^{\bullet-}$, likely due to the presence (EDTA) or absence (DTPA) of a readily exchangeable bound water molecule (Summers et al., 2008). Fe(III)DTPA is essentially unreactive with $O_2^{\bullet-}$, with reported rate constants of $<10^4$ $M^{-1}s^{-1}$ (Buettner et al., 1983) and $\approx 10^3$ $M^{-1}s^{-1}$ (Bolann et al., 1992), whereas Fe(II)DTPA is readily oxidized, with $k_7 = 2 \times 10^7$ $M^{-1}s^{-1}$ (Butler and Halliwell, 1982), such that $O_2^{\bullet-}$ acts only as an oxidant and Fe redox cycling does not occur. In contrast, Fe(II)EDTA is readily oxidized by $O_2^{\bullet-}$, with $k_7 \approx 2–8 \times 10^6$ $M^{-1}s^{-1}$ (Ilan and Czapski, 1977; Bull et al., 1982, 1983), with $k_7 = 2 \times 10^6$ $M^{-1}s^{-1}$ appropriate under the conditions of this study (see Supplementary Material Section 6 for further discussion). Unlike DTPA, Fe(III) complexed by EDTA is readily reduced by $O_2^{\bullet-}$, $k_{10} = 10^3–10^7$ $M^{-1}s^{-1}$ (Bull et al., 1983). The wide range of reported values for the reduction rate constant is due to significant media/ionic strength effects (due to changes in the thermodynamics of the speciation and because the reaction involves two like-charged ions with $z = -1$) and also a pH effect as only the $[Fe^{III}(EDTA)(H_2O)]^-$ form is reactive with $O_2^{\bullet-}$; the hydrolyzed complex $[Fe^{III}(EDTA)(OH)]^{2-}$ is essentially unreactive, with the pK_a for $[Fe^{III}(EDTA)(H_2O)]^- \approx 7.6$ (Bull et al., 1983). As the current work was conducted at pH 8.2, near the pK_a of Fe(III)EDTA, it is difficult to obtain a reliable literature estimate for the reduction rate constant. From a compilation of literature rate constants (discussed further in the Supplementary Material Section 6), a value for k_{10} of between 2×10^4 and 6×10^5 $M^{-1}s^{-1}$ is considered appropriate. Using the kinetic model it was shown that significant redox cycling of Fe occurs when k_{10} is at the upper end of this range, but not at the lower end. This Fe redox cycling results in elevated Fe(II) concentrations at later stages of the reaction; although this has negligible impact upon the kinetics of Fe(II) oxidation, when H_2O_2 is present in excess of Fe, this continual flux of Fe(II)EDTA results in ongoing $HO^\bullet$ production even after the oxidation of Fe(II)EDTA appears to be complete (see Supplementary Figures 4, 5 in the Supplementary Material). Such an ongoing production of $HO^\bullet$ is not supported by the data, suggesting the value for k_{10} appropriate to the conditions of study is at the lower end of the possible range.

TABLE 2 | Additional reactions in the presence of formate.

Reaction	Rate constant	Rxn #
$HCOOH \rightleftharpoons HCOO^- + H^+$	$k_f = 1.78 \times 10^6$ s^{-1} $k_r = 10^{10}$ $M^{-1}s^{-1}$[a]	21
$HO^\bullet + HCOOH \rightarrow CO_2 + HO_2^\bullet$	3.1×10^8 $M^{-1}s^{-1}$[b]	22
$HO^\bullet + HCOO^- \rightarrow CO_2 + O_2^{\bullet-}$	3.2×10^9 $M^{-1}s^{-1}$[b]	23

[a] *Rate constant of 1010 $M^{-1}s^{-1}$ assumed for reaction with H+; dissociation rate constant calculated using pKa = 3.75 from Duesterberg et al. (2005).* [b] *Rate constants taken from Kwan and Voelker (2002), implicitly includes the rapid further reaction of the initially formed $CO_2^{\bullet-}$ with O_2 to yield CO_2 and $HO_2^\bullet/O_2^{\bullet-}$, which is the only significant pathway under the conditions of this study.*

It is clear that the behavior of the system, especially with regards to the kinetics and magnitude of $HO^\bullet$ production, is strongly influenced by the interaction of Fe-EDTA complexes with $O_2^{\bullet-}$. The sensitivity of the system to the rate constant for reduction of Fe(III)EDTA by $O_2^{\bullet-}$ is not evident when examining

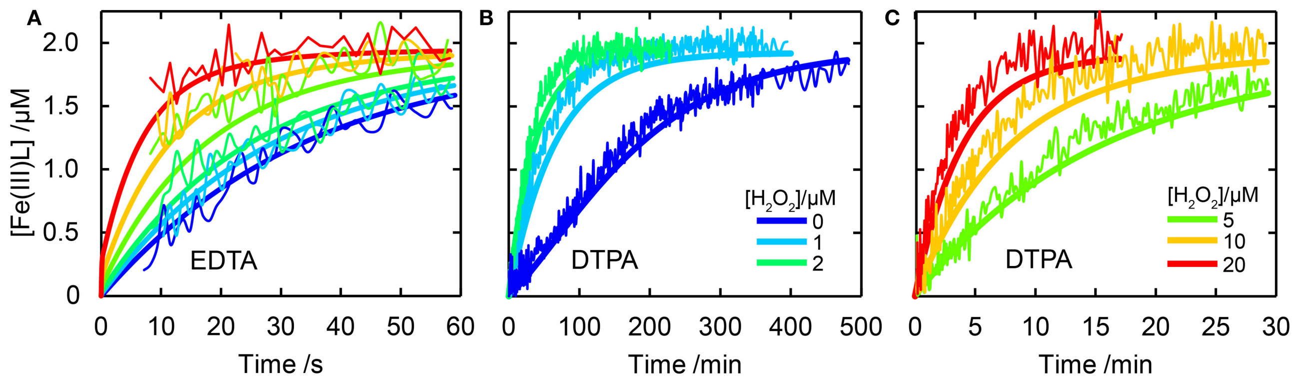

FIGURE 1 | Comparison of experimental data to model results for Fe(II)EDTA and Fe(III)EDTA oxidation studies. In all cases thin lines are experimental data and thick lines model output. Experimental conditions were $[Fe(II)]_0 = 2$ μM and [EDTA or DTPA] = 10 μM. Panel **(A)** shows results for EDTA for $[H_2O_2] = 0$ (blue), 1, 2, 5, 10, and 20 μM (red). Panel **(B,C)** show results for DTPA with $[H_2O_2]$ as labeled.

Fe(II) oxidation kinetics alone, and highlights the need to carefully consider the fate of the reactive intermediates. Due to the sensitivity of the model to the magnitude of k_{10}, several values of this rate constant were trialed within the plausible bounds determined from previous studies, and the magnitude of the O_2- and H_2O_2-mediated oxidation rate constants then fitted to the data. From this process a value for k_{10} of 6×10^4 $M^{-1}s^{-1}$ was found to both adequately describe the data and lie within the range expected from previous studies. After accounting for the complications introduced by reactions involving $O_2^{\bullet -}$, both the Fe-EDTA and Fe-DTPA systems were well described by a kinetic model that proposed HO$^\bullet$ as the sole-product of the reaction between H_2O_2 and Fe(II)L (L = EDTA, DTPA), as can be seen in **Figures 2**, **3**. The ability to describe all the experimental results using the kinetic model developed demonstrates that there is no need to invoke higher-valent Fe species when L = EDTA or DTPA, and the hypothesis that quantitative formation of HO$^\bullet$ occurs in this system is not inconsistent with the observed behavior.

Experiments with the addition of *t*-BuOH (0, 2, 5, 20, 100 mM), which competes with Phth (0.55 mM) for HO$^\bullet$ via a well-described mechanism (Rxn 12 in **Table 1**; see Supplementary Material Section 7 for the reactions of *t*-BuOH), were used to validate that HO$^\bullet$ was the species trapped by Phth. The final Phth-OH concentration was determined from both the experimental data and model predictions, then the impact of *t*-BuOH assessed by fitting a standard competition equation to the data:

$$[\text{Phth} - \text{OH}] = \frac{[\text{Phth} - \text{OH}]_\infty}{1 + R\frac{[t - \text{BuOH}]}{[\text{Phth}]}} \tag{1}$$

where [Phth-OH]$_\infty$ is the final Phth-OH concentration in the absence of *t*-BuOH and *R* is the ratio of the rate constants for reaction of HO$^\bullet$ with *t*-BuOH and Phth, i.e., *k*(HO$^\bullet$, *t*-BuOH)/*k*(HO$^\bullet$, Phth). The value of *R* was calculated for both the experimental data and also the kinetic model prediction; agreement between both values provides additional evidence for HO$^\bullet$ production. For L = EDTA, the experimental data yielded $R = 0.117 \pm 0.003$, in good agreement with $R = 0.107 \pm 0.001$ obtained from fitting the competition equation to kinetic model output. For L = DTPA the experimental data yielded $R = 0.073 \pm 0.007$ which is also in good agreement with $R = 0.089 \pm 0.002$ obtained from the kinetic model output (in all cases *R* is given as best fit value ±one standard error from the curve fitting procedure, which likely underestimates the true uncertainty). For each ligand, the kinetic model output value of *R* differed from the value of 0.11 predicted from literature constants (Buxton et al., 1988; Schiller et al., 1999) due to the more complicated interactions present in the kinetic models compared to the assumptions made in the simplified competition equation; however, in both cases, the results are consistent with HO$^\bullet$ as the only source of Phth-OH in this system. While Rahhal and Richter (1988) have previously asserted that the product of the Fe(II)DTPA-H_2O_2 reaction is completely unreactive with *t*-BuOH, our results clearly demonstrate that the product of this reaction does react with *t*-BuOH in a manner quantitatively consistent with the product being HO$^\bullet$.

Kinetics of Fe(II) Oxidation and HO$^\bullet$ Production by Fe(II)Citrate

The apparent rate of H_2O_2-mediated Fe(II)-citrate oxidation varied with Fe(II) concentration. This dependence could not be reproduced by the Fe(II)L model in **Table 1**, which fitted the data poorly (**Figure 4**). Attempts to fit this model simultaneously to the Fe(II) oxidation and HO$^\bullet$ production data were also unsuccessful. Due to the inability of the Fe(II)L model to reproduce the Fe(II) concentration dependence, and given that HO$^\bullet$ production experiments were only conducted with [Fe(II)]$_0$ = 2 μM, a second model scenario was examined in which the kinetic model in **Table 1** was fitted to the HO$^\bullet$ production data (which was conducted with [Fe(II)]$_0$ = 2 μM) and also only to the oxidation data where [Fe(II)]$_0$ = 2 μM. This fitting scenario, which we denote as the "Fe(II)L-2 μM" model, also yielded poor

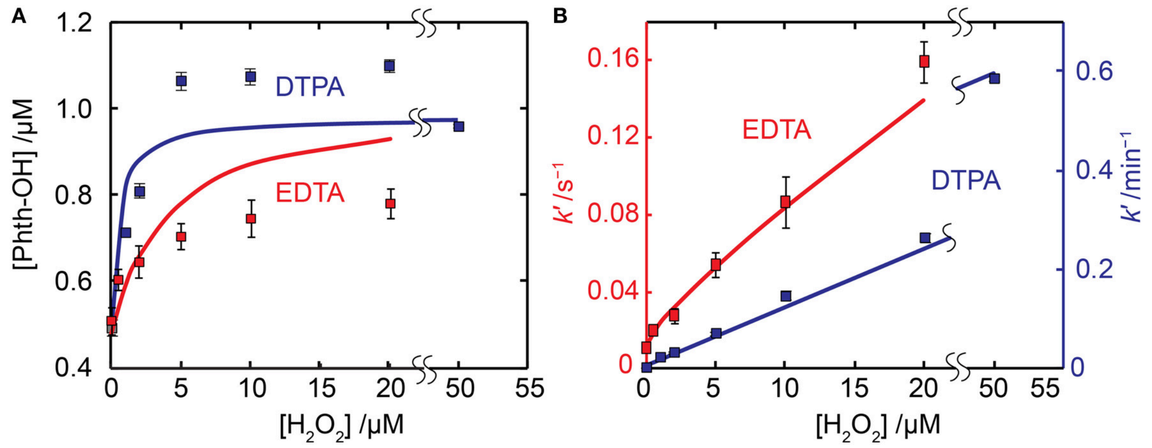

FIGURE 2 | Comparison of experimental and model results for the magnitude (A) and pseudo-first order rate constant (B) of Phth-OH production for L = EDTA and DTPA. Experimental data are shown as symbols in all panels with the model results shown as solid lines. In all cases [Fe(II)]$_0$ = 2 μM and [L] = 10 μM.

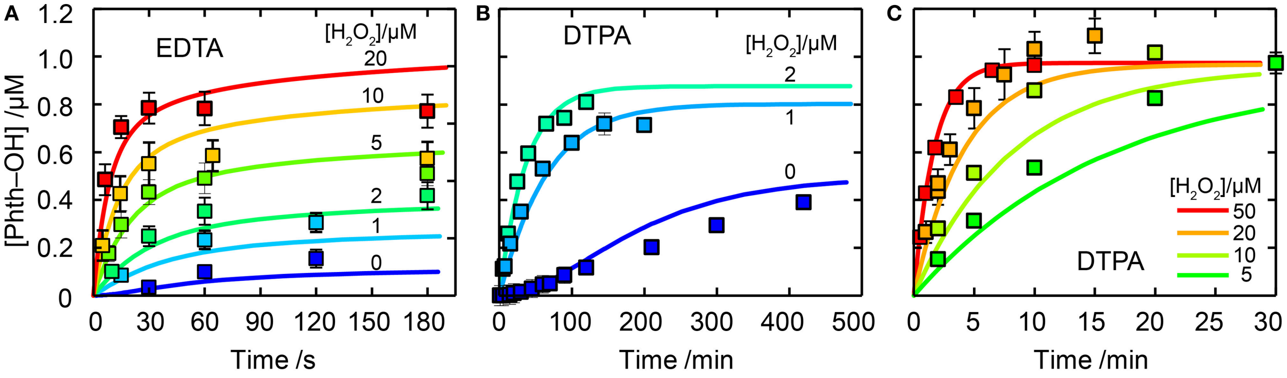

FIGURE 3 | Phth-OH production by Fe(II)EDTA (A) and Fe(II)DTPA (B,C). Experimental data are shown as symbols with error bars the standard deviation of three replicate experiments. Kinetic model results are shown as solid lines (with $k_{10} = 6 \times 10^4$ M^{-1}s^{-1} for EDTA). In all cases [Fe(II)] = 2 μM, [L] = 10 μM and [H_2O_2] is as labeled.

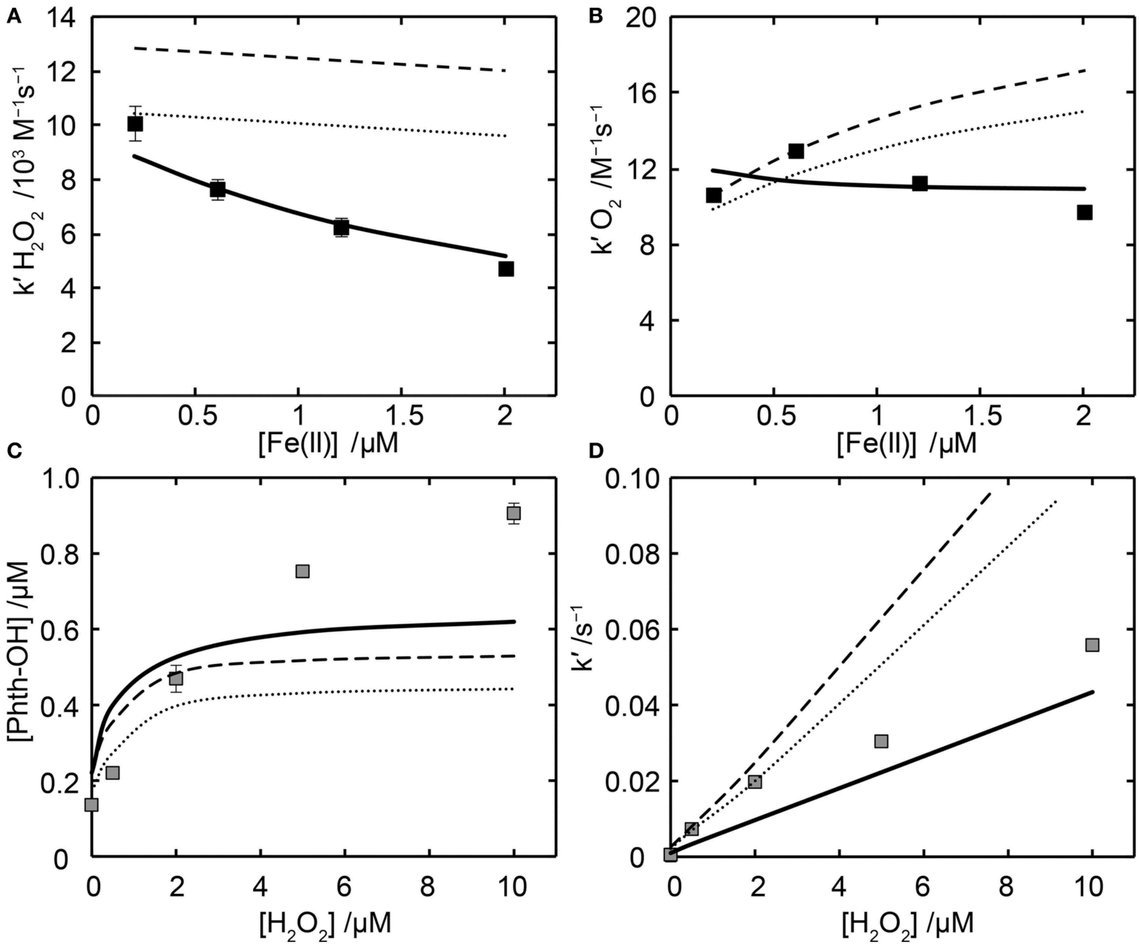

FIGURE 4 | Kinetics of Fe(II) oxidation and resulting Phth-OH production in the presence of citrate. Panels **(A,B)** show the apparent rate constants for Fe(II)-citrate oxidation by **(A)** H_2O_2 and **(B)** O_2 as a function of initial Fe(II) concentration ([cit] = 1 mM in all cases). Symbols are derived from linear fits of experimental pseudo-first order oxidation rate constants as a function of [H_2O_2] **(A)** or from the pseudo-first order rate constant divided by [O_2] **(B)**. Panels **(C,D)** show HO• production, with panel **(C)** showing the magnitude of total Phth-OH production when $[Fe(II)]_0 = 2$ μM and panel **(D)** showing the kinetics of Phth-OH formation (the pseudo-first order rate constant of Phth-OH production). In all panels model predictions are indicated by lines, with the Fe(II)L model shown as a dashed line, the Fe(II)L-2 μM model as a dotted line and the Fe(II)$_2$L model as a solid line.

results. The failure of the Fe(II)L model, which only considers one form of Fe(II)-cit species, is not surprising, as the most likely reason for a concentration dependence of the oxidation rate is the formation of higher-order complexes, which will lead to speciation being a function of the Fe(II) concentration as well as the Fe(II):cit ratio.

None-the-less, several possible explanations were considered for the poor performance of this model. The possibility of organic radicals reducing Fe(III)L was discarded since the rate constant for reaction between carbon-centered radicals and O_2, which is typically near 2×10^9 $M^{-1}s^{-1}$ (von Sonntag et al., 1997), is likely much greater than that for the reaction of carbon centered radicals with Fe(III)cit; reported rate constants for reduction of inorganic Fe(III) by hydroxyalkyl radicals are (1–6) $\times 10^8$ $M^{-1}s^{-1}$ (Neta et al., 1996). Formate addition experiments further supported the assumption that O_2 was the main sink for carbon-centered radicals, as its presence did not alter the Fe(II) oxidation results. The reaction of organic radicals with O_2 is likely to ultimately yield $O_2^{\bullet -}$, which may oxidize or reduce iron. Garg et al. (2007) reported a rate constant for $O_2^{\bullet -}$-mediated reduction of Fe(III)cit of 800 $M^{-1}s^{-1}$, which is likely to be applicable under the current conditions based upon the kinetic model presented in their work. Assuming that the rate constant for $O_2^{\bullet -}$-mediated oxidation of Fe(II)cit is similar to that of Fe(II)EDTA (2×10^6 $M^{-1}s^{-1}$), $O_2^{\bullet -}$ will be a net oxidant. This reasoning was supported by the HO$^\bullet$ trapping experiments, in which a limiting Phth-OH concentration of $\approx$1 μM (which is half the initial Fe(II) concentration) was obtained at high [H_2O_2]. This limit occurs because $O_2^{\bullet -}$ formed during the HO$^\bullet$-Phth reaction oxidizes Fe(II)cit, hence limiting the amount of Fe(II)cit that is able to react with H_2O_2. If $O_2^{\bullet -}$ was also able to effectively reduce Fe(III)cit complexes, such an effect would not be observed, and enhanced Phth-OH production would be expected as a result of this additional flux of Fe(II)cit. This conclusion is consistent with the results of Sutton (1985) who employed similar logic to formate degradation studies. Even if a higher rate constant for $O_2^{\bullet -}$-mediated reduction was used in the modeling [e.g., 9×10^4 $M^{-1}s^{-1}$ from Rose and Waite (2005) which was determined at lower citrate:Fe ratios than those used here] it was still not possible to replicate the trends observed. As such, it can be deduced that reactions of $O_2^{\bullet -}$ are unable to account for the apparent Fe(II)cit concentration dependence.

The trends observed can be reproduced if the existence of a di-iron citrate species of the form $Fe(II)_2L$ is proposed with differing oxidation kinetics to Fe(II)L [termed the "$Fe(II)_2L$" model, which includes the additional reactions shown in **Table 3**]. The oxidation data could only poorly constrain parameters in the $Fe(II)_2L$ model, with Presto unable to converge to a unique solution. As such, an analytical expression was used to fit the $Fe(II)_2L$ complexation constants (K_4 and K_{24}) to the data (see Supplementary Material Section 8 for details; note that the related formation constants, $\beta_{M,\,L}$ are also referred to, with $\beta_{1,\,1} = K_4$ and $\beta_{2,\,1} = K_4K_{24}$). Provided that log $K_4 > 4$ and log $K_{24} \approx$ log $K_4 + 3.1$, it was possible to find values for the Fe(II)L oxidation rate constant that fitted the data well. The physical basis for this lower bound on K_4 being that small amounts of inorganic Fe(II) have low impact, so as long as the majority of Fe(II) is in complexed form, the results will be similar. In all cases a good fit could only be achieved if it was assumed that $Fe(II)_2L$ was comparatively unreactive (i.e., apparent H_2O_2-mediated oxidation rate constant $<10^3$ $M^{-1}s^{-1}$, with a value of 1 $M^{-1}s^{-1}$ adopted for modeling). A value of log $K_4 = 4.75$ was determined from fitting to the HO$^\bullet$ production data, with log K_{24} then fixed from the analytical modeling process. As is apparent in **Figures 4A,B**, only the $Fe(II)_2L$ model was able to provide a good fit to the Fe(II)cit oxidation data under all conditions. The $Fe(II)_2L$ species is speculative and does not accord with the speciation model of Königsberger et al. (2000), with published formation constants of similar species from other proposed models e.g., $Fe(II)_2(cit)_2(OH)_2^{4-}$ from Amico et al. (1979), also inconsistent with those required to reproduce the data here. Inclusion of the proposed species is considered justified however due to the general uncertainty surrounding Fe(II)-citrate speciation at the high pH of this work, and that inclusion of this species was able to describe the data well, while all other possibilities considered failed to do so. This issue is explored further in Supplementary Material Section 9.

TABLE 3 | Additional reactions used in the $Fe(II)_2L$ model.

Reaction	Rate constant ($M^{-1}s^{-1}$)	Rxn #
$Fe(II)L + Fe(II) \rightleftharpoons Fe(II)_2L$	$k_f = 10^{10}$, $k_r = 128$ s^{-1}[a]	24
$Fe(II)_2L + O_2 \rightarrow Fe(II) + Fe(III)L + O_2^{\bullet -}$[b]	3.9[c]	25
$Fe(II)_2L + O_2^{\bullet -} \rightarrow Fe(II) + Fe(III)L + H_2O_2$[b]	3×10^6[d]	26
$Fe(II)_2L + H_2O_2 \rightarrow Fe(II) + Fe(III)L + HO^\bullet + OH^-$[b]	1[e]	27
$Fe(II)_2L + HO^\bullet \rightarrow Fe(II) + Fe(III)L + OH^-$[b]	1.2×10^8[d]	28

[a] Values for $\log K_4 = 4.75$, $\log K_4K_{24} = 12.6$ and assuming rapid reaction, i.e., k_f for both complexation steps set at $10^{10} M^{-1}s^{-1}$, see text for further discussion. [b] Each oxidant assumed to oxidize one molecule of iron only, with remaining Fe(II) re-equilibrating with the Fe(II) pool. [c] As fitted to data by fitting the O_2 oxidation data using Presto and manually adjusting k_8 until good agreement between apparent H_2O_2-mediated oxidation rates achieved. [d] Same rate constant assumed as for Fe(II)L. [e] Good fits achieved so long as $Fe(II)_2L$ is comparatively unreactive, see text for discussion.

Peroxidation of Fe(II) in the presence of citrate resulted in substantial HO$^\bullet$ production. The apparent rate constant for reaction with H_2O_2 of $(4.9 \pm 0.4) \times 10^3$ $M^{-1}s^{-1}$ was determined from the Phth-OH production data (assuming quantitative formation of HO$^\bullet$); this compares to an apparent rate constant of $(4.7 \pm 0.2) \times 10^3$ $M^{-1}s^{-1}$ for the H_2O_2-mediated oxidation of Fe(II)cit when $[Fe(II)]_0 = 2$ μM (i.e., under comparable conditions to the HO$^\bullet$ production experiments). The close correspondence between these rate constants provides evidence that the production of HO$^\bullet$ is closely coupled to the reaction of H_2O_2 with Fe(II)cit, and that it is likely that HO$^\bullet$ is the sole product of this reaction. Competition experiments with *t*-BuOH supported the conclusion that HO$^\bullet$ is the only species trapped by Phth in the Fe(II)-citrate system, with experiments yielding a value of $R = 0.088 \pm 0.004$, in good agreement with predicted values of $R = 0.078$, 0.085, and 0.088 for the $Fe(II)_2L$ model, the Fe(II)L model, and the Fe(II)L-2 μM model, respectively.

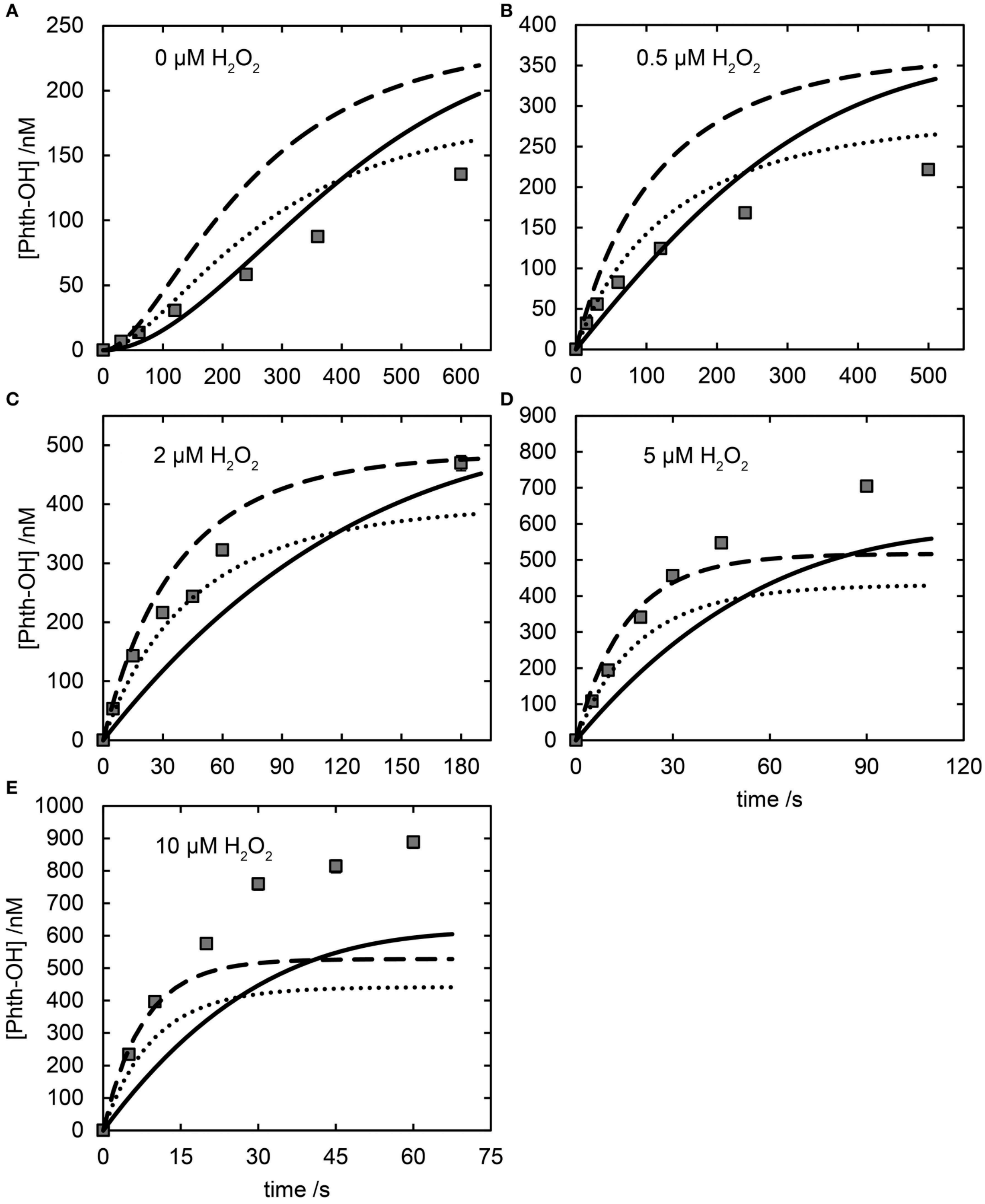

FIGURE 5 | Comparison of experimental data and model results for HO• production by Fe(II)cit when oxidized by H_2O_2 (0–10 μM, panels A–E respectively). In all panels [Fe(II)] = 2 μM, [cit] = 1 mM, experimental data are shown as symbols, the Fe(II)L model as a dashed line, the Fe(II)L-2 μM model as a dotted line and the $Fe(II)_2L$ model as a solid line.

However, the Fe(II)L, Fe(II)L-2μM, and the $Fe(II)_2L$ models all provided a poor fit to the experimental HO• production data (see **Figures 4C,D**, **5**). Although this may suggest that HO• is not the sole product of the Fe(II)cit-H_2O_2 interaction, it is far more likely that the poor fit is due to inadequacies in all the models described and that HO• is still likely the dominant product. As shown above for L = EDTA, without a thorough understanding of the oxidation and reduction kinetics of Fe(II)L/Fe(III)L by O_2, $O_2^{\bullet-}$, and H_2O_2 it is difficult to quantitatively determine whether HO• is the exclusive intermediate from the reaction of Fe(II)L and H_2O_2. It is clear that existing models of Fe(II)-citrate speciation and oxidation are not sufficiently refined to definitively rule out the presence of additional oxidative species, with additional work needed to better understand the Fe(II)-citrate-H_2O_2 system. However, this work strongly supports the notion that HO$^\bullet$, although potentially not the sole oxidative intermediate present, is certainly the dominant species formed.

Marine Implications

In this study we have tested simple model ligands as a proxy for the far more complex natural materials that bind Fe(II) in marine, estuarine and freshwater systems. This simplification is necessary due to the importance of thorough treatment of all potential radical reactions before any quantitative assessment of the yield of HO$^\bullet$ production can be made. Even a ligand as simple as citrate introduces significant challenges in the interpretation of the data, with further work to constrain both the nature of the complexes formed as well as their individual ROS reactivity needed before a definitive conclusion could be drawn.

Although this study has further confirmed that at pH ∼ 8, inorganic Fe(II) does not react with H_2O_2 to give HO$^\bullet$, it is still not clear *why* the nature of the oxidant formed on oxidation of inorganic Fe(II) by H_2O_2 is pH dependent, nor why the organic ligands that we have examined here are able to overcome this limitation. While it has been demonstrated that this same logic seems to apply to terrigeneous NOM (Miller et al., 2013), which would appear to be a good model of estuarine Fe(II) ligands (Hopwood et al., 2015), it is not clear if this is of relevance to oceanic Fe-ligands, which are known to be important controls on Fe(II) oxidation rates in the open ocean (Roy and Wells, 2011). It is also unknown how colloidal Fe(II) associated with organic material may behave (von der Heyden et al., 2014). It is also still unclear indeed what properties a complexing agent must have to promote formation of HO$^\bullet$, however it would seem quite likely that complexed Fe(II) in marine, estuarine and freshwater environments would react with H_2O_2 to yield HO$^\bullet$.

Despite the minimal role of cycling of Fe between the +2 and +3 oxidation states under the conditions of this work, in natural systems this process is potentially more important. As superoxide is now recognized to be produced in a wide variety of natural contexts (Shaked and Rose, 2013), a steady flux of superoxide may be sufficient to continually reduce Fe(III) complexes to the Fe(II) form, which combined with observed H_2O_2 concentrations on the order of tens to hundreds of nanomolar (Kieber and Helz, 1995; Avery et al., 2005) may lead to a continual, albeit slow, production pathway for HO$^\bullet$.

CONCLUSIONS

Under circumneutral conditions and in the presence of organic ligands, Fe(II) is oxidized by H_2O_2 to yield hydroxyl radicals. Competition experiments with *t*-BuOH confirmed that HO$^\bullet$ was indeed the species being measured. Kinetic modeling indicates that the difference in magnitude of HO$^\bullet$ production between ligands can be adequately explained by consideration of all relevant reactions between ROS and Fe(II)L/Fe(III)L without the need to invoke different HO$^\bullet$-production efficiencies for various Fe(II)L complexes. Of particular importance are the reactions of Fe(II)L with O_2, $O_2^{\bullet-}$, and H_2O_2 and the reaction of Fe(III)L with $O_2^{\bullet-}$. For the well-studied ligands EDTA and DTPA there is excellent agreement between model predictions and experimental results, with the poor HO$^\bullet$ production predictions for Fe(II)citrate likely due to an incomplete description of the speciation and oxidation mechanism rather than the production of an alternative oxidant by Fe(II)-citrate complexes. In the absence of organic ligands, no HO$^\bullet$ production could be measured under conditions where H_2O_2 was the dominant oxidant of Fe(II). As such, it is concluded that, under the conditions of this work, inorganic Fe(II) and H_2O_2 react to form an intermediate that is not HO$^\bullet$, the nature of which was unable to be examined. Overall, it is clear that under environmentally relevant conditions the Fenton reaction will only result in HO$^\bullet$ production if Fe(II) is complexed by an organic ligand.

AUTHOR CONTRIBUTIONS

TW initiated the study which was undertaken by CM and supervised by TW and AR. All authors contributed to manuscript preparation.

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SUPPLEMENTARY MATERIAL

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