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Organic exudates promote Fe(II) oxidation in Fe limited cultures of *Trichodesmium erythraeum*

Hanieh T. Farid[1*], Kai G. Schulz[2] and Andrew L. Rose[1, 3]

[1]Southern Cross Geoscience, Southern Cross University, Lismore 2480, Australia

[*]Corresponding author email address: hanieh.farid@scu.edu.au

[2]Centre for Coastal Biogeochemistry, School of Environment, Science and Engineering, Southern Cross University, Lismore NSW, 2480, Australia

[3]School of Environment, Science and Engineering, Southern Cross University, Lismore, NSW, 2480, Australia

Abstract.

A luminol chemiluminescence method was employed to study the oxidation kinetics of Fe(II) in both the absence and the presence of organic exudates released by the marine cyanobacterium *Trichodesmium erythraeum*. The apparent Fe(II) oxidation rate constant was studied for batch cultures grown with varying Fe' concentrations of 0.05-0.29 and 1.44-2.03 nmol L^{-1} at pH ranges from 8.1-8.6, corresponding to the change in pH in the cultures during the entire growth cycle. Fe(II) oxidation was accelerated when cells were growing exponentially and gradually decreased towards the stationary phase, consistent with the presence of organic exudates. The best fit of the kinetic model to the data also demonstrated clear differences in apparent Fe(II) oxidation rate constants during different growth phases. However, no significant difference was observed in oxidation rate constants between the two Fe' treatments. These findings suggest that *Trichodesmium* releases organic compounds into the extracellular environment that influence Fe redox chemistry, potentially affecting Fe bioavailability, and that the nature of the Fe(II) complexes formed is not influenced by Fe limitation of the organism's growth.

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1 Introduction

While photosynthetic algae are considered to be the main source of dissolved organic matter (DOM) in the ocean, the production rate and its molecular composition can vary, depending on the cellular growth phase (Myklestad, 2000). In exponentially-growing cells the composition of DOM exudates shifts from proteins towards carbohydrates when approaching nutrient limitation and reaching the stationary phase (Myklestad, 2000). An increase in cellular DOM exudation rate with decreasing nutrient availability is an important metabolic strategy to dissipate excess light energy during nutrient starvation (Myklestad, 2000). The release of DOM has been also suggested as a strategy to regulate the speciation, bioavailability and toxicity of trace metals in the external milieu (Jones, 1998;Moffett and Zafiriou, 1990).

Iron (Fe) is an essential trace metal and micronutrient for all phytoplankton and thought to limit primary production in up to 40 % of the oceans (Falkowski et al., 1998). Fe limitation negatively impacts photosynthetic and respiratory performance, and enzymatic processes in all phytoplankton (Geider 1999). Diazotrophic cyanobacteria suffer additional consequences from Fe limitation, which negatively influences their N_2 fixation activity (Berman-Frank et al., 2003). Marine bacteria are known to release strong iron-binding ligands (e.g. siderophores, which are low-molecular-weight Fe binding molecules specifically excreted by organisms for Fe acquisition) and/or weak iron-binding ligands (e.g. polysaccharides) under Fe-deficient conditions (Ito and Butler, 2005;Sohm et al., 2011). However, to the best of our knowledge, no systematic study has yet been performed on how cyanobacterial exudates may influence Fe chemistry during the various growth phases.

In marine surface waters, Fe exists in two oxidation states (Fe(II) and Fe(III)), and is mostly (up to 99%) complexed with organic ligands (L) (Hutchins et al., 1999;Nolting et al., 1998;Völker and Wolf-Gladrow, 1999). While unchelated forms of Fe, and especially unchelated forms of Fe(III) (typically denoted Fe(III)´), are assumed to constitute the most bioavailable pool in oxygenated seawater, dissociation of organic Fe complexes (FeL) is usually a precursor step in Fe supply to microorganisms (Fujii et al., 2010a). While Fe(III) typically forms relatively strong complexes with organic compounds, Fe(II)L complexes are typically much more labile, and thereby constitute a more bioavailable pool under the same physicochemical conditions (Morel et al., 2008;Shaked et al., 2005). However, the slightly alkaline and oxygenated conditions typical of seawater, Fe(II) is rapidly oxidised to the more dominant and thermodynamically stable Fe(III). While some studies have found Fe(III) reduction to be a prerequisite for Fe acquisition by microorganisms (Fujii et al., 2010b;Morel et al., 2008;Rose et al., 2005;Salmon et al., 2006;Shaked et al., 2005), oxidation of Fe(II) to Fe(III) has been also reported in a few eukaryotic microorganisms prior to uptake (Garg et al., 2007;Maldonado et al., 2006). This poses challenges for understanding the role of Fe(II) in the Fe nutrition of phytoplankton, as the ultimate source of Fe for phytoplankton uptake is still subject to debate.

Trichodesmium erythraeum, a globally significant diazotrophic cyanobacterium, has shown an intriguing variation in Fe acquisition mechanisms, depending upon the potentially available Fe species (Roe and Barbeau, 2014;Rubin et al., 2011). For instance, Roe et al. (2012) reported a higher propensity for the cultured strain IMS101 to acquire inorganic Fe (including both Fe(II) and Fe(III)) and Fe(III) that was weakly bound to organic ligands than Fe bound in Fe(III)-

siderophore complexes. This finding was in accordance with previous reports on the existence of some homologous genes to an ABC-type Fe^{3+} transporter within the genome in the absence of membrane receptor proteins for Fe(III)-siderophore complexes (Chappell and Webb, 2010;Webb et al., 2001). Roe and Barbeau (2014) also found a higher Fe uptake rate by *T. erythraeum* IMS101 in cultures containing Fe(III)-citrate compared to inorganic $FeCl_3$ and Fe(II)-citrate. Rubin et al. (2011) showed that *Trichodesmium* colonies can actively increase the dissolution and acquisition of Fe from particulate sources such as dust. The release of superoxide ($O_2^{\bullet-}$) (Godrant et al., 2009) and exopolysaccharides (EPS) into the extracellular surroundings by IMS101 under Fe stress conditions have also been reported (Berman-Frank et al., 2007). However, while numerous studies focusing on $O_2^{\bullet-}$ as potential Fe reducing agent in the extracellular milieu and its role in Fe uptake rates in cyanobacteria (Fujii et al., 2010a;Godrant et al., 2009;Kranzler et al., 2011;Roe et al., 2012;Rose, 2005), very few studies have examined effect of organic exudates from cyanobacteria on Fe redox chemistry.

FeL complexation reactions, as well as the reaction of inorganic and organically bound Fe(II) with oxygen, are crucial regulators of the bioavailability of Fe in ambient seawater (Rose and Waite, 2002). While the exact role of organic complexation is not yet clear, retardation and acceleration of Fe(II) oxidation rates have both been observed depending upon the type of organic compound (Rose and Waite, 2003;Santana-Casiano et al., 2000) and/or physicochemical conditions (Gonzalez et al., 2014;Jobin and Ghosh, 1972;Liang et al., 1993). Saccharides, amino acids and phenolic compounds are the major phytoplankton exudates which have so far been characterised to form weak complexes with inorganic Fe species (Benner, 2011;Hassler et al., 2011;Santana-Casiano et al., 2014), most likely with Fe(III) (Elhabiri et al., 2007;Santana-Casiano et al., 2010). In contrast to $O_2^{\bullet-}$, which can influence Fe uptake rates by reducing Fe(III) species (Rose, 2012;Rose et al., 2005), the formation of complexes with weak Fe-binding ligands might be also beneficial for Fe uptake by *Trichodesmium* via a non-reductive ligand exchange mechanism (Roe and Barbeau, 2014). Given these gaps about the role of organic exudates from cyanobacteria in Fe nutrition, this study aimed to address the following questions:

(i) How do organic exudates released in a batch culture of the marine cyanobacterium *T. erythraeum* influence Fe(II) oxidation rates?

(ii) Does this influence depend on the Fe nutritional status and growth phase of the organism?

In an attempt to answer the questions above, two cultures of *T. erythraeum* were established under conditions corresponding to varying Fe bioavailability using different ethylenediaminetetraacetic acid (EDTA) concentrations. Subsequently, a FeLume system (Emmenegger et al., 1998;King et al., 1995) was employed to investigate the oxidation kinetics of nanomolar concentrations of Fe(II) by O_2 in the presence of organic exudates released during the various growth phases.

2 Materials and methods

2.1 Reagents and solutions

A 2 mmol L^{-1} EDTA ($Na_2EDTA.2H_2O$, Ajax Finechem Pty Ltd) stock solution was prepared by dissolving 0.0745 g in 100 mL of high purity Milli-Q water (18.2 MΩ.cm resistivity from a Milli-Q Academic Water Purification System, installed in a clean room equipped with a HEPA

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filter, hereafter denoted as HPMQ). A 500 µmol L^{-1} stock solution of ´(III) was prepared by dissolving 13.5 mg of $FeCl_3.6H_2O$ (Sigma-Aldrich) in 100 mL of HPMQ that had been acidified with 100 µL of 1 mol L^{-1} HCl (prepared from 34-37 % w/w HCl, Instrument Quality, Seastar Chemicals Inc, hereafter denoted as trace metal grade acid).

A 4 mmol L^{-1} stock solution of Fe(II) was prepared by dissolving 157 mg of ammonium iron(II) sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, Ajax Finechem Pty Ltd, reagent grade) in 100 mL of HPMQ. The stock solution was stabilised by adding 20 µL of 6 mol L^{-1} trace metal grade acid (Rose and Waite, 2003). The stock solution was then stored in an acid-cleaned polypropylene bottle and kept in the dark (wrapped in aluminium foil) at 4° C when not in use (Rose and Waite, 2003). To facilitate preparation of standards at nanomolar Fe(II) concentrations, a fresh 1 µmol L^{-1} Fe(II) solution was prepared daily by adding 25 µL Fe(II) stock solution (4 mmol L^{-1}) to 100 mL of HPMQ. The pH in the secondary stock solution was low enough to prevent oxidation of Fe(II) for one day but sufficiently high that it did not cause notable change in pH after addition to the samples. A portable meter (Hach HQ11D) was used to monitor pH in the solutions and was calibrated using standard pH buffers on the NBS scale (pH 4.00, 7.00 and 10.00 at 20° C).

A 0.5 mmol L^{-1} luminol reagent was prepared in 1 mol L^{-1} ammonium hydroxide solution (NH_4OH) by dissolving 89 mg of luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, Sigma-Aldrich) in 69 mL NH_4OH solution (28-30 % w/w, Sigma-Aldrich) and adding HPMQ to adjust the final volume to 1 L using a volumetric flask (Rose and Waite, 2002). The regent then was adjusted to pH 10.3 by adding 26 mL of 6 mol L^{-1} trace metal grade acid and stored in the dark at room temperature for at least 24 h before use (Rose and Waite, 2003).

2.2 Culture conditions

A non-axenic unialgal strain of *T. erythraeum* IMS101 was obtained from the National Centre for Marine Algae and Microbiota (NMCA, USA) and to minimize the occurrence of undesired organisms in significant numbers, the stock cultures were always kept in exponential phase using a semi-continuous approach. No obvious microscopic changes (e.g. bacterial colony formation) were observed in the cultures during experiments, however there was no definitive evidence for the absolute absence of heterotrophic bacteria, whose potential presence must therefore be considered when interpreting the results. Stock cultures were grown in 2.5 L polycarbonate bottles (acid-washed and sterilised) containing YBC-II medium (hereafter denoted as artificial seawater, ASW) prepared according to the recipe given in Andersen (2005) but modified to contain 10 nmol L^{-1} $FeCl_3$, 2 µmol L^{-1} KH_2PO_4, 2.1 mmol L^{-1} of $NaHCO_3$ and different EDTA concentrations as described below. To minimise the possibility of metal and biological contamination, the ASW was stored with a few mL of purified Chelex-100 resin (Sunda et al., 2005) for at least 24 h and filtered through a sterile 0.2 µm polycap TC filter capsule (polyethersulfone membrane (PES), Whatman) before the addition of nutrient solutions (including trace metals, phosphorus and vitamins).

Different levels of Fe bioavailability were established by adding either 50 nmol L^{-1} or 20 µmol L^{-1} EDTA at a constant Fe(III) concentration of 10 nmol L^{-1}. The latter was achieved by adding 20 µL of the 500 µmol L^{-1} Fe(III) stock solution per litre of ASW and allowing to equilibrate for 1 h. The concentration of unchelated iron ([Fe']) in the seawater was calculated from total Fe(III) and EDTA concentrations using an equilibrium complexation model described in

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158 Schulz et al. (2004) at a pH range from 8.1 to 8.6 corresponding to the range of pH values
159 measured in cultures (Table 1). All pH measurements were conducted on the NBS scale and
160 assumed to equal the pH on the free hydrogen ion scale to within an uncertainty of 0.005 pH
161 units (Lewis et al., 1998). This error is negligible for the purposes of this study. The Fe
162 complexation model accounts for complexes of Fe(III) with Cl^-, F^-, SO_4^{2-} and the Fe(III)OH
163 species, as well as protonated or complexed forms of EDTA with Fe^{3+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+},
164 Ca^{2+} and Mg^{2+}.
165
166 **Table 1.** Calculated concentrations of total Fe ([FeT]), unchelated Fe ([Fe']), organically
167 complexed Fe ([FeL]) and precipitated Fe ([Fe(s)]) in the presence of different EDTA
168 concentrations and a total Fe(III) addition of 10 nM.

EDTA (μmol L^{-1})	[FeT] (nmol L^{-1})	[Fe'] (nmol L^{-1})[a]	FeL (nmol L^{-1})	[Fe(s)] (nmol L^{-1})
0.05	10	1.44-2.03	0.01-0.05	8.51-7.96
20	10	0.05-0.29	9.7-9.95	0

169 [a] Fe' concentrations are reported as a range corresponding to pH varying from 8.1-8.6,
170 representing the change in pH in the culture during the entire growth cycle.
171
172 Culture media were inoculated with exponentially growing cells at a starting concentration of
173 about 500 cell/mL and incubated at 27° C, with a photon flux density of 110 µmol quanta m^{-2}
174 s^{-1} (measured with a LI-193 Spherical Quantum Sensor, LiCor) under a 14:10 dark/light cycle.
175 Exponentially growing cells were inoculated three times into fresh media over 24 d and
176 acclimated to the experimental conditions for about 12 generations. To monitor growth in the
177 cultures, 5 mL aliquots were taken every 2 days fixed in Lugol iodine solution (1%) and then
178 analysed for cell numbers using an automated particle imaging system (Morphologi G3,
179 Malvern Instruments, UK) (see Sect. S1.1 in Supplementary Material for more details).

180 **2.3 Experimental Fe(II) oxidation and analytical procedures**

181 To examine Fe(II) oxidation rates throughout the growth cycle in the batch cultures, about 250
182 mL of each culture was harvested every 2 d and adjusted to pH 8.0 by the dropwise addition of
183 1 mol L^{-1} trace metal grade acid while the sample was maintained at 20±1° C in a water bath
184 (Grant OLS 200). Subsequently, samples were gently filtered by gravity through an acid
185 cleaned 5 µm filter (47 mm, PC, Whatman) immediately before measurements. Given an
186 average width of ≥20 µm for each filaments, the filter pore size was considered small enough
187 to prevent trichomes passing into the solution, however since the cultures were not axenic it is
188 possible that some bacteria were present in the filtrate.
189 Fe(II) oxidation experiments were conducted by additions of appropriate volumes of a 1 µmol
190 L^{-1} Fe(II) stock solution to give final concentrations of 5, 10 and 20 nmol L^{-1} Fe(II) in 15 mL
191 aliquots of filtered samples. At a salinity of 35 and at 20° C the O_2 concentration in the samples
192 was calculated to be 0.225 mmol L^{-1} (Garcia and Gordon, 1992), assuming 100 % saturation at
193 continuous stirring at 120 rpm using an acid cleaned Teflon-coated magnetic stirrer during the
194 entire experiment (Rose and Waite, 2002). The changes in $[Fe(II)_T]$ (the sum of both inorganic

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and organically complexed Fe(II) concentrations) over time were monitored by an automated continuous chemiluminescence flow system (Rose and Waite, 2002). The system is based on the reaction of the added Fe(II) with O_2 and luminol reagent at high pH (Rose and Waite, 2001, 2002). Sample and the reagent are mixed in a spiral-shaped flow cell positioned beneath a photomultiplier tube (PMT) using a peristaltic pump at a flow of 1 mL/min (Rose and Waite, 2002). The reaction results in the production of chemiluminescence at 426 nm. The emitted photons were recorded over a fixed time period of 600 s at one second integrals.

To account for potential effects of EDTA or other components of the culture media on Fe(II) oxidation rates, two different blank treatments (i.e. without Fe(II) additions) were analysed for baseline correction in the samples: (i) ASW containing the same nutrient (i.e. vitamins, trace metals, $FeCl_3$ and phosphorus) and EDTA concentrations as in the culture samples, which provides the background signal in the absence of organic exudates; and (ii) culture samples which were filtered at least about 5 h prior to analysis and kept in the dark (wrapped in aluminium foil) to ensure that all pre-existing Fe(II)/Fe(II)L species were fully oxidised. The first set of blank values were used as an indicator of Fe(II) oxidation rate in the absence of organic exudates, while the second set of blank values were subtracted from filtrate samples after Fe(II) additions.

The system was calibrated directly with the experimental data based on the additions of known amounts of Fe(II) (yielding 5, 10 and 20 nmol L^{-1}) to the samples in each experiment. All measurements were performed in duplicate and then the highest and lowest calculated rate constants were removed from each data set, leaving four replicates. Mean values were then considered for data analysis. The FeLume signals were related to Fe(II) concentrations by a power law calibration relationship, which was developed as follows. In the continuous flow system, the first reliable measurements of Fe(II) concentration cannot be made until about 70 s after adding Fe(II) to the sample due to the delay in mixing the sample with the reagent. During this time, a substantial proportion of the added Fe(II) may oxidise. Thus, to calculate initial Fe(II) concentrations at time zero, the data points obtained under steady flow conditions (70-600 s) were extrapolated back to the time at which Fe(II) was added to the samples via linear regression of the signal logarithm versus time (see Sect. S1.3 for details) (Rose and Waite, 2003). As the signal to noise ratio decreased towards the end of measurements, only data collected in the interval of 110-530 s after adding Fe(II) was used for kinetic analysis.

Calibration curves were prepared by plotting the added log [Fe(II)] versus the extrapolated log (signal), and a linear trend ($R^2 \geq 0.9$) on the log-log plot (i.e. a power law function) was observed in all cases. The detection limit was about 0.5 nmol L^{-1} based on three times the calculated Fe(II) concentrations in the blanks.

2.4 Analysis of Fe(II) oxidation kinetics

For analysis of Fe(II) oxidation kinetics, it was assumed that Fe(II) is mainly oxidised by O_2 due to its presence at a much higher concentration than other potential oxidants such as reactive oxygen species. This is a reasonable assumption given that with the relatively low (at most 20 nmol L^{-1}) Fe(II) concentrations used, at most 10 nmol L^{-1} $O_2^{\bullet-}$ and at most 5 nmol L^{-1} H_2O_2 could potentially accumulate due to reaction of Fe(II) with O_2, and that previous studies have shown that the contribution of these species to Fe(II) oxidation under similar conditions is negligible (Rose and Waite, 2002). Moreover, the rate constants for reaction between H_2O_2 and

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Fe(II)EDTA, as well as between H_2O_2 and other organic Fe(II) complexes, are orders of magnitude less than the rate constant for reaction between H_2O_2 and inorganic Fe(II) (Miller et al. 2009; Miller et al. 2016). This means that micromolar concentrations of H_2O_2 are needed to outcompete O_2 and affect the oxidation rate; therefore only the reaction between Fe(II) and O_2 was assumed to be important here. The kinetics of the reaction of Fe(II) with O_2 are given by:

$$\frac{\mathrm{d[Fe(II)]}}{\mathrm{d}t} = -k[\mathrm{Fe(II)}][\mathrm{O_2}] \qquad \text{Eq. (1)}$$

where k is a second order rate constant for the reaction involving the two species Fe(II) and O_2. However, since O_2 was present at much higher concentration than Fe(II) under our experimental conditions, its concentration can be considered constant. Thus letting

$$k' = k[\mathrm{O_2}] \qquad \text{Eq. (2)}$$

Then Eq. (1) can be written in the form of a first order reaction, where k' represents a pseudo-first order rate constant:

$$\frac{\mathrm{d[Fe(II)]}}{\mathrm{d}t} = -k'[\mathrm{Fe(II)}] \qquad \text{Eq. (3)}$$

Moreover, since the rate constants were all calculated based on observed overall reaction, hereafter k' is referred to "apparent" pseudo-first order rate constant.

The solution to equation 3 is:

$$[\mathrm{Fe(II)}] = [\mathrm{Fe(II)}]_0 e^{-k't} \qquad \text{Eq. (4)}$$

which can also be written as:

$$\ln([\mathrm{Fe(II)}]) = \ln([\mathrm{Fe(II)}]_0) - k't \qquad \text{Eq. (5)}$$

where $[\mathrm{Fe(II)}]_0$ is the initial Fe(II) concentration. The pseudo-first order rate constant can therefore be determined from linear regression of $\ln[\mathrm{Fe(II)}]$ against time.

While this approach has shown to be suitable for analysing oxidation of inorganic Fe(II), the presence of organic ligands may result in non-pseudo first order kinetics due to formation of organic Fe(II) complexes followed by the parallel oxidation of inorganic Fe(II) and organically complexed Fe(II). Since the oxidation of Fe(II) could also be affected by EDTA, a three-step kinetic modelling approach was employed using the software Kintek Explorer. The steps were:

(i) The oxidation of inorganic Fe(II) was modelled in the absence of any organic compounds, which allowed determination of the rate constant for the reaction between Fe(II) and O_2 in ASW matrix.

(ii) Considering two more reactions, namely the complexation of Fe(II) by EDTA and subsequent oxidation of the Fe(II)EDTA complex by O_2, which allowed determination of the oxidation rate constant for the Fe(II)EDTA complex in ASW; and

(iii) The addition of two more reactions accounting for Fe(II)L complexation with organic matter and its subsequent oxidation by O_2. The assumptions for organically Fe complexation and oxidation rate constants are described further in the results and discussion.

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3 Results and discussion

3.1 Growth rates in the cultures

The culture containing a lower Fe' concentration exhibited a lower growth rate ($p \leq 0.05$) than the culture with a higher Fe' concentration (Fig. 1). However, the biomass carrying capacity for the culture containing higher EDTA concentration was considerably higher, despite the lower Fe' concentration, due to the absence of Fe precipitation in this culture. Comparing the values obtained in this study with other studies shows that the growth rates are within the range previously reported for Fe-limited conditions for this microorganism (Berman-Frank et al., 2001;Chappell and Webb, 2010;Kustka et al., 2003;Shi et al., 2012).

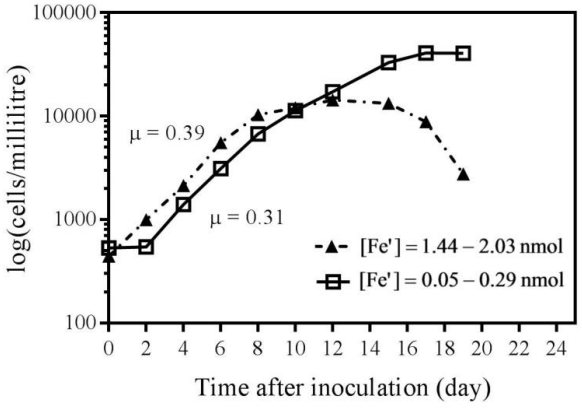

Figure 1. Growth rates of *T. erythreum* grown in batch cultures with different concentrations of unchelated Fe. Growth rates (μ, in units of d^{-1}) were calculated based on the slope from linear regression of log(cell density) against time for each culture during exponential phase (i.e. days 2-8 for the culture shown as triangles and days 2-15 for the culture shown as squares; see Sect. S1.2 for details) and are annotated on the plot for each curve.

3.2 Fe(II) oxidation kinetics in the absence of organic exudates

The formation of Fe(II)-EDTA complexes has previously been observed to accelerate Fe(II) oxidation in seawater (Santana-Casiano et al., 2000). This may result from a higher propensity for O_2 to react with Fe(II)L complexes rather than inorganic Fe(II) (Voelker and Sulzberger, 1996). Consistent with this, we observed a significant difference ($p \leq 0.01$) between the slope of log([Fe(II)]) vs time (over the period 110-530 s) in the presence of varying EDTA concentrations in artificial seawater (0, 0.05 and 20 μmol L^{-1}) using a general one-way analysis of variance (ANOVA) (Fig. 2). However, due to use of much lower EDTA concentrations in this study, and differences between the ionic strength of the media used, a lesser influence of EDTA on Fe(II) oxidation rate was observed compared to that reported by Santana-Casiano et al. (2000). ANOVA additionally revealed no significant ($p \geq 0.5$) difference in the slope of log([Fe(II)]) vs time during the first (110-170 s) and last (480-530 s) 60 seconds of data within each treatment, indicating Fe(II) oxidation obeyed pseudo-first order kinetics in the media in the absence of organic exudates.

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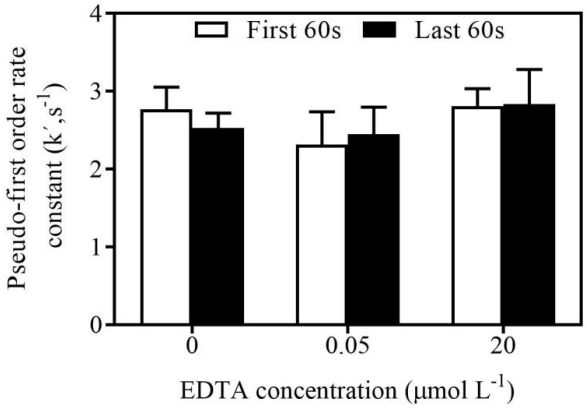

Figure 2. Comparison of apparent pseudo-first order rate constants for Fe(II) oxidation obtained from the first and the last 60 s of measurement for ASW containing different EDTA concentrations. Error bars represent the standard deviation of four replicates.

To explore the potential impact of ligands on Fe(II) oxidation kinetics, the oxidation of nanomolar concentrations of added Fe(II) in ASW was modelled in both the presence and the absence of EDTA (Fig. 3a). As previously stated, the reaction of Fe(II) with oxygen (O_2) was the only oxidation reaction considered and the potential back reaction of Fe(III)/Fe(III)L with $O_2^{\bullet-}$ was ignored. Based on kinetic modelling, the second order oxidation rate constant for oxidation of inorganic Fe(II) over a range of initial Fe(II) concentrations (5,10 and 20 nmol L^{-1}) was determined to be 5.94±0.03 $M^{-1}s^{-1}$ the physicochemical conditions described in Sect. 2.3, which is in agreement with values reported in previous studies ranging from 2.2 to 8.5 M^{-1} s^{-1} (Millero, 1987;Murray and Gill, 1978;Waite and Morel, 1984). This value of the inorganic Fe(II) oxidation rate constant in ASW was subsequently used for modelling of Fe(II) oxidation in the presence of EDTA and organic exudates.

Given that EDTA is known to form complexes with Fe(II) that accelerate Fe(II) oxidation, the observation of pseudo-first order Fe(II) oxidation kinetics in the presence of EDTA (based on the model fit to the data, Fig. 3b and Fig. 3c) implies that the reaction mechanism involves either rapid complex formation then rate limiting oxidation of the complex, or rate limiting complex formation followed by rapid oxidation of the complex (Rose and Waite, 2003). Since both formation and oxidation rate constants for the Fe(II)EDTA complex were unknown and could not be independently constrained under our experimental conditions due to use of relatively low EDTA concentrations, the oxidation rate constant value was obtained from the literature. Previously, Fujii et al. (2010b) and Santana-Casiano et al. (2000) have determined values of 12 $M^{-1}s^{-1}$ and 70.6 $M^{-1}s^{-1}$, respectively, for the oxidation rate constant of Fe(II)EDTA in seawater, implying that oxidation of the Fe(II)EDTA complex occurs on a timescale of several minutes and therefore suggesting that the oxidation step is rate limiting and complex formation is rapid. Given that the ionic strength in the latter study was much greater than that used in this study, the Fe(II)EDTA oxidation rate constant reported by Fujii et al. (2010) was used here for modelling purposes. There is little information about the kinetics of formation

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and dissociation of Fe(II)EDTA in artificial seawater (Santana-Casiano et al., 2000). However, assuming that Fe(II)EDTA complexation and dissociation occur rapidly on the timescale of the Fe(II) oxidation experiments at an EDTA concentration of 20 µmol L^{-1} (i.e. highest [EDTA] used in this study), the dissociation rate constant k_d was arbitrarily set to a value of 1000 s^{-1} and the complex formation rate constant k_f was fitted to the data. On this basis, an apparent stability constant ($K = k_f/k_d$) of 16200 M^{-1} was obtained for Fe(II)EDTA in ASW at pH 8.0 and 20±1° C.

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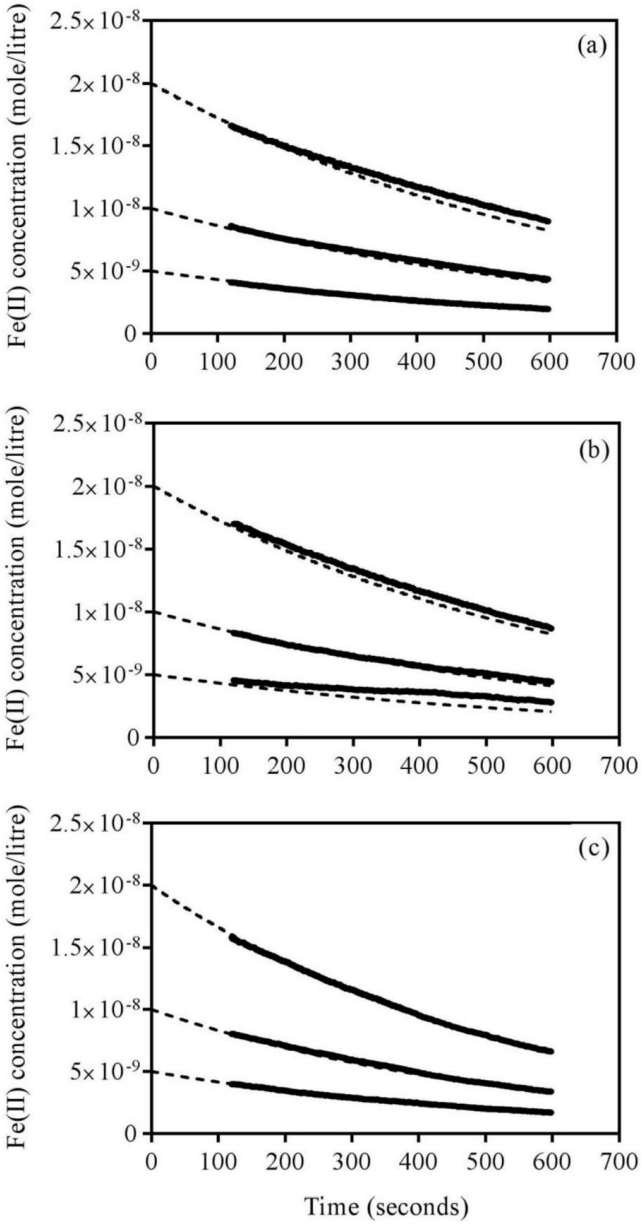

Figure 3. Oxidation of Fe(II) in ASW containing (a) no EDTA, (b) 50 nmol L^{-1} EDTA and (c) 20 µmol L^{-1} of EDTA in the absence of organic exudates. Data points represent the mean from two measurements and dashed lines indicate the fit of the kinetic model to the data. Initial Fe(II) concentrations were 5, 10 and 20 nmol L^{-1}. Further details on rate constants are provided in Table S1.

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3.3 Fe(II) oxidation kinetics in the presence of organic exudates

Fe(II) oxidation data in the presence of organic exudates from IMS101 cultures were initially fitted by linear regression of log([Fe(II)]) against time, assuming pseudo-first order oxidation kinetics. Pearson correlation coefficient values of $r \geq 0.98$ were obtained from all linear regression fits to Fe(II) oxidation data throughout the entire growth phase of the cultures, suggesting that this approach based on the assumption of pseudo-first kinetics was reasonable. Fe(II) oxidation was accelerated in the presence of IMS101 exudates from exponentially growing cells under Fe limited conditions, as demonstrated by increasing values of the overall pseudo first order rate constant for Fe(II) oxidation in culture filtrates compared to the media (Fig. 4). Acceleration of Fe(II) oxidation in the presence of specific types of organic matter (mostly of terrestrial origin) has been previously noted in several other studies (Millero et al., 1987;Rose and Waite, 2002, 2003;Santana-Casiano et al., 2000).

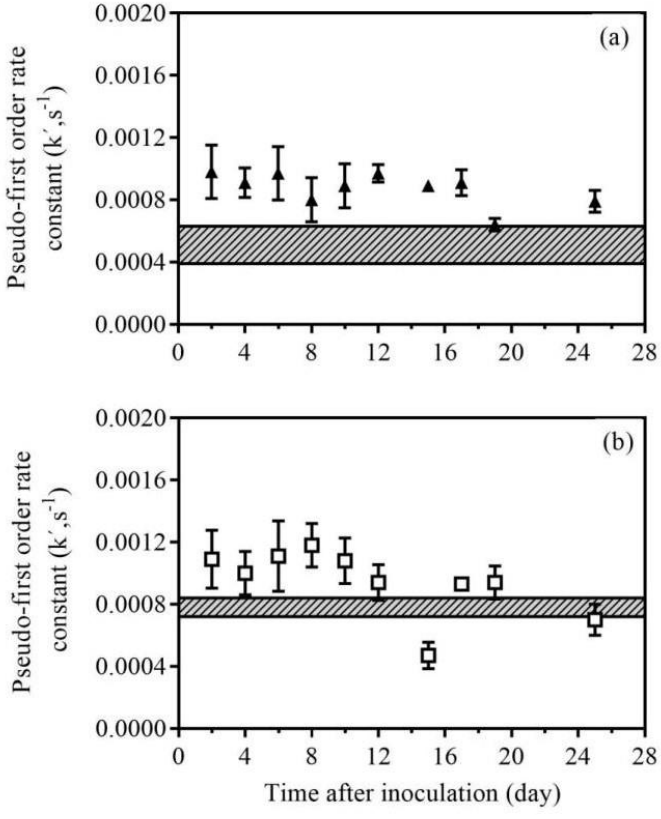

Figure 4. Changes in the apparent pseudo-first order rate constant for Fe(II) oxidation in filtrate from batch cultures of *T. erythraeum* grown with (a) 1.44-2.03 nmol L^{-1} and (b) 0.05-0.29 nmol L^{-1} Fe' concentrations at different times during the growth cycle of the culture. The shaded area shows the range of measured values of the Fe(II) oxidation rate constant in ASW containing the same nutrient and EDTA concentrations as filtrates in the absence of cells. Symbols represent the mean and error bars represent the standard deviation of the mean from four replicates. Values of oxidation rate constants are reported in Table S1.

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To investigate the assumption pseudo-first order kinetics in more detail, a kinetic model was developed in Kintek Explorer that accounted for the parallel oxidation of inorganic Fe(II), Fe(II)EDTA complexes, and a single organic complex between Fe(II) and ligands in the culture exudate. Since concentration of organic ligands in the exudate ([L]) was unknown, the kinetic model was developed based on the assumptions that the ligand is present at a much higher concentration than Fe(II) (which would be necessary for pseudo-first order kinetics over the entire range of Fe(II) concentrations examined), that Fe(II)L complexation occurred rapidly compared to Fe(II) oxidation, and that Fe(II)L dissociation was negligible on the timescale of the Fe(II) oxidation measurements (k_d = 0). The alternative possibility that Fe(II)L oxidation was very rapid was also investigated. These alternatives correspond to the two most likely possible limiting scenarios under which oxidation would be pseudo-first order (Rose and Waite, 2003). In both cases, the ligand concentration was thus arbitrarily assumed to be 1 µmol L^{-1} then either the rate constant for oxidation of the complex, k_{ox}, was set to an arbitrarily large value and k_f fitted, or k_f was set to an arbitrarily large value and k_{ox} fitted. In both cases, the model fit to the data was comparable and did not suggest any reason to reject the assumption of pseudo-first order kinetics. Given that it was not possible to distinguish which of the scenarios was most reasonable in reality, and that the actual ligand concentration was also unable to be determined from this approach, no further analysis was undertaken using detailed kinetic modelling.

If we assume that oxidation is rate limiting, however, then k_{ox} is approximately equal to the overall apparent second-order rate constant for Fe(II) oxidation, k_{app}, enabling comparison with previously reported values of k_{app}. Under this assumption, an average second order rate constant of 9.60±1.97 $M^{-1}s^{-1}$ for k_{ox} was calculated from the model fits to the data during exponential phase, given that statistical analysis revealed no significant difference (p >0.97) between the rate constants for actively growing cultures between days 2-8 and 2-15 at ranges 1.44-2.03 and 0.05-0.29 nmol L^{-1} Fe' concentrations, respectively. In comparison, several studies have reported a decrease in Fe(II) oxidation rate in the presence of organic exudates released by some eukaryotic algae. For instance, Santana-Casiano et al. (2014) and Gonzalez et al. (2014) reported apparent second-order Fe(II) oxidation rate constants (k_{app}) of 1.09 $M^{-1}s^{-1}$ and 6.9 $M^{-1}s^{-1}$ in filtrates from cultures of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, respectively. In these studies, however, the value of k_{app} in the seawater used (17.4 $M^{-1}s^{-1}$) was much higher than that reported here due to the differences in ionic strength and composition (e.g. silicate or chloride), temperature and dissolved oxygen concentration, resulting in a much greater apparent effect of exudates on Fe(II) oxidation compared to in the absence of exudates. The results presented in this study suggest that *Trichodesmium* produces a different class of dissolved organic matter which enhances Fe(II) oxidation. According to Marcus theory (Marcus, 1964), which applies in the case of outer-sphere electron transfer from O_2 to Fe(II)/Fe(II)L during the oxidation process, the rate of electron transfer between O_2 and Fe(II)L is proportional to the ratio of the stability constants (thermodynamic formation constants) for Fe(III)L and Fe(II)L complexes. Therefore, the observed increase in k_{ox} values for organically complexed Fe(II) compared to the k_{ox} value for inorganic Fe(II) implies that the organic exudates released by *T. erythraeum* IMS101 likely form more stable complexes with Fe(III) than Fe(II). The observation of pseudo-first order reaction kinetics and similar oxidation rate

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constants for the cultures grown with different Fe' concentrations furthermore suggest that the effect of exudates on Fe(II) oxidation was likely due to formation of a single type of Fe(II) complex. Moreover, the lower value of k_{ox} for Fe(II)L compared to that for Fe(II)EDTA suggests the presence of a potentially weak class of Fe(III) binding ligand in IMS101 exudates.

3.4 Effects of growth phase on Fe(II) oxidation kinetics

The apparent pseudo-first order rate constant for overall Fe(II) oxidation varied with changes in growth rate over the ~25 d duration of culture growth (Fig. 5). The results showed a significant ($p \leq 0.01$) difference between oxidation rate constants in the presence and in the absence of organic exudates. The oxidation rate constants were also observed to decrease as the growth rate approached zero when the cultures entered stationary phase.

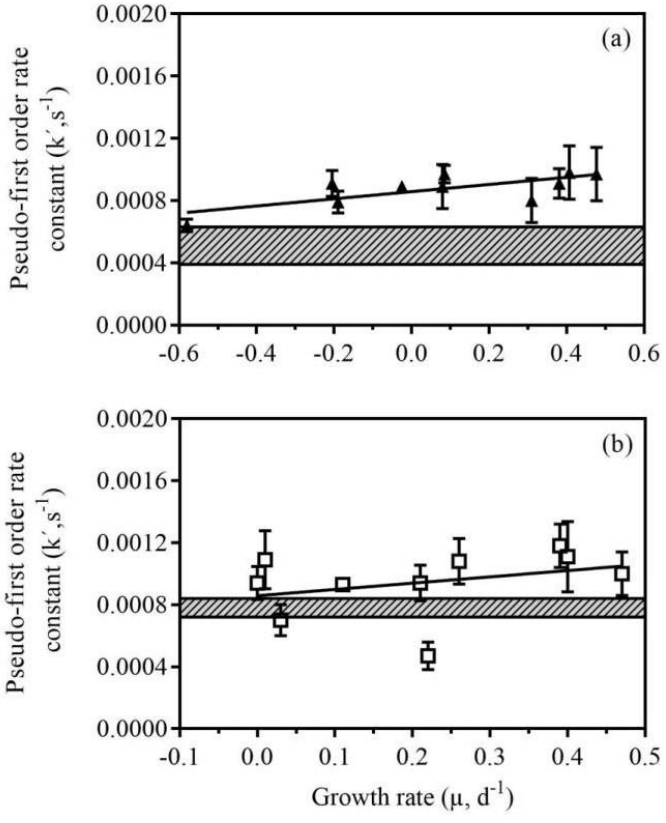

Figure 5. Relationship between growth rate and Fe(II) oxidation rate constant in the culture grown with (a) 1.44-2.03 nmol L^{-1} and (b) 0.05-0.29 nmol L^{-1} Fe' concentrations. The shaded area shows the range of measured values of the Fe(II) oxidation rate constant in ASW containing the same nutrient and EDTA concentrations as filtrates in the absence of cells. Symbols represent the mean and error bars represent the standard deviation of the mean from four replicates. Lines represent linear regression of the data.

These results are consistent with previous studies implying that exponentially growing cells can actively influence the redox dynamics of Fe in their surroundings by releasing dissolved organic matter (Gonzalez et al., 2014;Santana-Casiano et al., 2014). This influence cannot be due to the physicochemical conditions under which the experiments were performed (i.e. temperature, DO and/or pH), which were adjusted to maintain similar values in all samples. Fe complexes formed in the presence of exudates may not necessarily be due to complexation by specific Fe binding ligands such as siderophores or porphyrins, as other metabolic products such as carbohydrates and proteins are much more abundant, with abundance often strongly correlated with cell density (Santana-Casiano et al., 2014;Hassler et al., 2011). However, despite an expected increase in DOC concentration towards the end of exponential growth phase, an average (and relatively constant) apparent pseudo first order oxidation rate constant of 0.001 s^{-1} was observed in both cultures grown with different Fe' concentrations. These findings further support the notion that *T. erythraeum* might exude different organic compounds (Achilles et al. 2003; Hutchins et al. 1999) to diatoms (Gonzalez et al. 2014; Rijkenberg et al. 2008; Steigenberg et al. 2009) and green algae (Gonzalez et al. 2014), and that these exudates may accelerate Fe(II) oxidation.

4 Conclusions

In this study, it was found that Fe(II) oxidation was accelerated in the presence of organic exudates released by *T. erythraeum*. This occurred to a greater extent when the cells were growing exponentially. Fe(II) oxidation kinetics in the presence of exudates were well described using a pseudo-first order model, implying that the concentrations of organic Fe binding ligands were much higher than the maximum concentration of Fe(II) added (20 nmol L^{-1}). This suggests that the ligands most likely complexed Fe(II) relatively rapidly, with oxidation of the complex being rate limiting in terms of the overall mechanism by which Fe(II) oxidation was accelerated. Analysis of the kinetics showed that the oxidation rate constants differed significantly depending on the growth phase of the organism in batch culture, with the Fe(II) oxidation rate declining as the culture approached stationary phase. Moreover, no significant difference was observed between the oxidation rate constants for Fe(II) in the presence of exudates from the two cultures grown under different Fe' conditions. These results suggest organic complexation of Fe as a potentially important mechanism which may permit *Trichodesmium* to facilitate dissolution of solid phase Fe and therefore increase the solubility of Fe in its surroundings. In addition, observation of an increase in Fe(II) oxidation rates in the presence of organic exudates strengthens the hypothesis for existence of some (probably weak) organic ligands that may complex Fe(III), followed by reduction or ligand exchange mechanisms at the cell surface. This hypothesis, however, needs to be further tested by more detailed characterisation of the organic matter released by this microorganism.

5 Author contribution

Hanieh T. Farid contributed intellectually to the design of all experiments, conducted the experiments, analysed samples, interpreted data, modelling and wrote the manuscript. Andrew L. Rose and Kai G. Schulz contributed intellectually to the experimental design, interpretation of data, and editing process. Andrew L. Rose also contributed to the modelling process.

6 Competing interests

The authors declare that they have no conflict of interest.

7 Data availability

Fe(II) oxidation data are publically available from ePublications@SCU (http://epubs.scu.edu.au/).

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