The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater

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The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater

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A thesis submitted in fulfilment of the requirements of the degree of

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I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

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The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater
Abstract

Iron (Fe) is an essential micronutrient for all microorganisms, and particularly nitrogen-fixing (diazotrophic) cyanobacteria. Iron in marine waters is found in two redox states of ferrous iron, Fe(II), and ferric iron, Fe(III), either chelated by organic matter or in unchelated form. Unlike Fe(III) species, Fe(II) species are more soluble and typically form weaker complexes with organic matter, and therefore can be more easily taken up by the cell. Under alkaline and oxygenated conditions, which are typical of many marine environments, the poorly soluble but thermodynamically stable Fe(III) dominates over Fe(II).

*Trichodesmium erythraeum*, a globally significant marine cyanobacterium, is able to acquire Fe from different sources including minerals, inorganic and organically complexed forms. However, little work has been performed on how the organism may influence Fe speciation and therefore its bioavailability during cell growth. The aims of this thesis are therefore to:

(i) Establish a relatively quick and reliable ferrozine (FZ) based approach for measuring total dissolved Fe in *Trichodesmium* cultures containing different types of ligands (natural and synthetic) and high concentrations of organic matter (Chapter 2); and

(ii) Investigate the effects of $O_2$ and organic exudates released by *Trichodesmium* cultures under various Fe limiting conditions and at different growth phases on the redox speciation of Fe in seawater (Chapters 3 and 4).

In relation to the first aim, acidification of samples at pH ~1 for 15 d was sufficient for full recovery of Fe in the presence desferrioxamine B (DFB) using the FZ based approach. Rapid reduction of the recovered Fe with sulfite followed by addition of FZ with subsequent incubation for 24 h gave a stable Fe(FZ)$_3$ complex, necessary for quantitative Fe determination.

In relation to the second aim, Fe(II) oxidation was accelerated in the presence of organic exudates released by *Trichodesmium*, to a greater extent when the cells were growing
exponentially. Moreover, while different pseudo-first order rate constants were observed during different growth phases, they were relatively similar for cultures grown with different iron bioavailability. Despite measuring a relatively high superoxide production rate in *Trichodesmium* cultures, a surprisingly low Fe(III) reduction rate was observed, implying the existence of other potential sink pathways for superoxide in extracellular surroundings.

Overall, this thesis provides a better understanding of biologically mediated influences on Fe speciation in seawater with fundamental findings that will assist in future development of models for Fe acquisition by marine microorganisms.
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Contribution of authors

I contributed intellectually to the design of all experiments, conducted the experiments, analyzed samples, interpreted data and wrote all manuscripts.

Andrew L. Rose and Kai G. Schulz contributed intellectually to the experimental design, interpretation of data, and editing process for Chapters 2, 3 and 4.

Andrew L. Rose also contributed to the modeling process for Chapter 3.
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Chapter 1
Introduction

Iron (Fe) is an essential micronutrient for life and can potentially limit algal biomass and primary production in the ocean with subsequent impacts on global carbon (C) and nitrogen (N) budgets. Diazotrophs (N$_2$ fixing organisms), and in particular the cyanobacterium *Trichodesmium erythraeum*, play a key role in biogeochemical cycling of N and thus ultimately C in seawater, but require additional Fe for N$_2$ fixation in comparison to other phytoplankton species.

This thesis is targeted to improve our understanding of the mechanisms employed by diazotrophic cyanobacteria to influence Fe speciation and therefore its bioavailability in the marine environment. The findings contribute fundamental knowledge with regards to modeling Fe redox cycles, particularly in low nutrient low chlorophyll (LNLC) regions including the north and east Australian shelf, where *Trichodesmium* spp. is an important contributor to marine primary production.

**Fe in Seawater: Sources and Speciation**

Fe presents an unrivaled paradox between its abundance in terrestrial and aquatic environments (Taylor and Konhauser 2011). While it is the fourth most abundant element on Earth, phytoplankton primary production in major oceanic regions is restricted by low Fe availability (Boyd et al. 2007; Boyd et al. 2000; Martin et al. 1991; Sunda and Huntsman 1995). Fe is mainly delivered to the ocean via aeolian dust deposition, shelf sediment water column interactions, riverine inputs, and hydrothermal activities (Duce and Tindale 1991; Elrod et al. 2004). However, its remineralization from sinking particulate organic matter produced at the surface is also important, particularly in the lower water column (Hutchins and Bruland 1994).

In surface seawater, the chemical speciation of Fe can be influenced by a number of factors including the abundance of oxidants (including O$_2$ and reactive oxygen species), organic
compounds and light. Generally, Fe species in aqueous systems can be grouped into three classes (Barbeau et al. 2001; Rose 2012):

(i) Unchelated Fe(II) and Fe(III), including inorganic species that can form weak complexes with Cl\(^-\), SO\(_4^{2-}\), Br\(^-\), F\(^-\), CO\(_3^{2-}\) and PO\(_4^{3-}\);

(ii) Chelated Fe(II) and Fe(III) species which can form complexes with naturally occurring organic ligands (L) including terrestrial and marine substances of biological origin (e.g. humic/fulvic materials); and

(iii) Fe oxides (FeOx), corresponding to amorphous or crystalline solids, minerals and aggregates containing Fe and potentially other organic or inorganic particles.

In addition to photochemical processes, transformations between these species can be also mediated biologically though enzymatic pathways or release of compounds with an electron donating and accepting group including labile redox active organic materials and/or free oxygen radicals.

**Cyanobacteria: High Fe Requiring Microorganisms**

Cyanobacteria or “blue green algae” are oxygenic phototrophic bacteria that can be found in almost every terrestrial and aquatic ecosystem and are characterized by the ability to synthesize both chlorophyll \(\text{a}\) and phycobilin pigments (Potts and Whitton 2000). Diazotrophic marine cyanobacteria such as *Trichodesmium* spp. contribute a large fraction of new nitrogen to the oligotrophic oceans, but little is known about how they respond to global change-driven shifts in Fe supply.

Fe is an essential micronutrient for of all organisms and is thought to limit primary production in 40% of the oceans, while occasionally co-limiting nutrient in parts of the remaining 60% (Breitbarth et al. 2008; Crichton 2001; Sunda 2002; Turner and Hunter 2001). Cyanobacteria require Fe as a co-factor in a number of redox reactions involving major cellular metabolic processes (Carpenter and McCarthy 1975; Goering et al. 1966), in particular:
For fixing N\(_2\): the enzyme responsible for this process requires 30-34 Fe atoms/molecule of nitrogenase;

- As an electron acceptor in the photosynthetic and respiratory electron transport chains;
- To scavenge or detoxify the excess reactive oxygen species (ROS including O\(_2\)\(^{-}\), H\(_2\)O\(_2\) and OH\(^{-}\)) via the Fe containing form of the enzyme superoxide dismutase; and

- During the process of assimilatory sulfate reduction.

**Bioavailability of Fe: Biological Mediators and Mechanisms**

Ferrous iron (Fe(II)) is believed to be the most readily assimilated form of Fe by microorganisms, potentially due to its higher solubility and tendency to form weaker complexes with organic matter than ferric iron (Fe(III)) (Morel et al., 2008; Shaked et al., 2005). In oxygenated surface waters, the concentration of Fe(II) in biologically available forms is typically low due to its rapid oxidation to less soluble Fe(III) (Rose and Waite 2003a; Rose and Waite 2003c). However, organic complexation, mediated by ligands which are known to actively being produced by microorganisms, can enhance the solubility of Fe(III) and therefore the concentration of bioavailable species (Santana-Casiano et al. 2014; Morel et al., 2008; Shaked et al., 2005).

In most of the conceptual models for Fe acquisition by algae, unchelated iron (Fe\(^{-}\)) species seem to be the forms which are preferentially absorbed by the cells (Dang et al. 2012; Fujii et al. 2010a; Morel et al. 2008; Shaked et al. 2005). This is based on kinetic or thermodynamic considerations and the observation that under Fe limiting conditions, the growth rate of microorganisms is usually a function of [Fe\(^{-}\)] (Fe(II)\(^{-}\), Fe(III)\(^{-}\)) and not total Fe at steady-state conditions (Dang et al. 2012; Morel et al. 2008).

Oxidative and reductive processes mediated by organic exudates, O\(_2\) and ROS species, as well as specific enzymes at the cell surface, are known to influence Fe speciation and therefore availability in the extracellular medium (Fujii et al. 2010a; Gonzalez et al. 2012; Gonzalez et
al. 2014; Rose et al. 2005; Rose and Waite 2003a; Santana-Casiano et al. 2010). Previously, the production and release of relatively strong and Fe(III)-specific binding compounds, termed siderophores, was assumed to be the major uptake mechanism employed by microorganisms under Fe-deplete conditions (Cooper et al. 1978; Granger and Price 1999; Wilhelm and Trick 1994). However, a growing number of studies suggest exudation of weaker Fe binding ligands (including saccharides, amino acids and phenolic compounds) as equally important (Gonzalez et al. 2012; Gonzalez et al. 2014; Rijkenberg et al. 2008; Santana-Casiano et al. 2014; Steigenberger et al. 2012). This is related to the finding that, unlike siderophores which typically occur at the picomolar levels, weaker Fe-binding ligands have been reported at much higher concentrations (nmol/L to µmol/L), sufficient to affect the speciation and bioavailability of Fe in the extracellular environment (Hassler et al. 2011).

In addition to organic exudates, the release of reactive oxygen species and in particular $O_2^{-}$ has been also documented under Fe stress conditions (Garg et al. 2007a; Godrant 2009; Rose et al. 2010). In contrast to the energy burden imposed on the cell by siderophore production, weak Fe binding ligands and superoxide anions may be naturally produced as by-products of metabolic processes, thereby minimizing the diffusional loss and waste of energy from the cell (Cowart 2002). Although there are numerous studies available demonstrating the influence of organic matter on Fe redox speciation, the pathways by which it modifies Fe bioavailability are not yet clear. It is assumed that organic complexation may influence the redox potential of the Fe(II)/Fe(III) species due to:

a) Changing the reactivity of Fe towards oxidants ($O_2$ and ROS) through formation of complexes and also limiting rates of Fe release by those complexes (Rose and Waite 2003a; Santana-Casiano et al. 2000);

b) The presence of redox active quinone-like moieties within organic compounds, increasing the possibility of electron transfer between organic substances and metal ions.
Fe Uptake Pathways in Cyanobacteria

Several models exist for Fe acquisition in cyanobacteria (Figure 1.1). These include:

a) The Fe(II)s model, which considers all Fe(II) (including chelated and unchelated forms) to be available to the microorganism. In this model, both Fe(II)\(^{'}\) and Fe(II)L forms can be captured by Fe(II) membrane transporters. This is consistent with the FeL model (described below), but unlike the FeL model Fe(III)\(^{'}\) is also considered to be an important precursor pool for bioavailable Fe(II) in the medium (Morel et al. 2008).

b) The FeL model, which assumes (re)oxidation of Fe(II)L complexes as a critical process in controlling the bioavailable Fe pool, itself generated by non-dissociative reduction of Fe(III)L complexes. Fe reduction is mediated either by enzymatic processes at the cell surface or by superoxide released into the surrounding environment (Salmon et al. 2006).

c) The Fe\(^{'}\) model, which assumes that both Fe(II)\(^{'}\) and Fe(III)\(^{'}\) (unchelated Fe species) can be assimilated. In this model, photochemical or biological processes, in particular active release of O\(_2^{\cdot-}\) by cells, play a key role in mediating transformations between chelated and unchelated Fe species in the extracellular medium (Fujii et al. 2010a). Dang et al. (2012) modified the model for the freshwater cyanobacterium Microcystis aeruginosa by considering Fe(II)\(^{'}\) as the only form absorbed by the microorganism.

d) The siderophore-based model, which assumes that organisms possess the ability to release siderophores into the extracellular medium and uptake the ferrisiderophore complex (FeY) as a whole into the cell (Braun and Hantke 2011). This approach has been widely studied in Gram-negative bacteria including cyanobacteria (Barbeau et al. 2003; Mirus et al. 2009; Morrissey and Bowler 2012; Wilhelm and Trick 1994). The model was later extended by Kranzler et al. (2011, 2014) and Molot et al. (2014),
considering the necessity of reducing both chelated (FeL, FeY) and unchelated (Fe(II)/Fe(III)') species before transport across the inner membrane. However, many cyanobacteria, particularly open ocean species, show neither siderophore production nor siderophore transport capabilities, implying that an alternative Fe uptake pathway is presumably involved (Hopkinson and Morel 2009; Hopkinson and Barbeau 2012).

Figure 1.1. Potential modes of Fe acquisition in cyanobacteria. (A) Fe(II)s model, (B) FeL model, (C) Fe' model and (D) siderophore-based model. The solid black arrows show irreversible reactions.

**Trichodesmium, a missing piece in the oceanic Fe puzzle: knowledge gaps**

Since *Trichodesmium* spp. forms extensive blooms in the ocean and can be easily detected by the naked eye, it is among the most studied marine diazotrophic cyanobacterium. Despite numerous experiments on its metabolic response to Fe deficiency (Rueter et al. 1990; Shi et al. 2007; Trick et al. 1995; Wilhelm 1995), only a few recent studies have examined the potential influence of *T. erythraeum* on Fe speciation or vice versa. Berman-Frank et al. (2007) and Godrant (2009) suggested the exudation of polysaccharides and superoxide anions into the
extracellular environment as a possible mechanisms to increase Fe bioavailability under Fe limitation. Chappell et al. (2010) showed expression of some specific genes under Fe limiting conditions, potentially coding for membrane proteins enhancing both Fe(II) and Fe(III) uptake capabilities. Rubin et al. (2011) showed that *T. erythraeum* colonies can actively increase the dissolution and hence acquisition of Fe from particulate sources such as dust. Roe and Barbeau (2014) reported a higher Fe acquisition rate in cultures treated with inorganic Fe and/or weak organically complexed species than Fe(III)-siderophore complexes. Finally, a recent field study highlighted the role of *T. erythraeum* colonies in introducing high concentrations of ROS to oligotrophic surface waters (Hansel et al. 2016).

Given the information above, there is still a lack of knowledge concerning:

(i) How organic exudates released by *T. erythraeum* might influence the redox state of Fe in the extracellular environment?

(ii) Whether such an influence could be modulated by the availability of Fe´ (and hence degree of Fe limitation) depending on the metabolic state of the cell, such as during different cellular growth phases in culture (lag, exponential growth, stationary and decay phase)?

(iii) Whether cellular superoxide production is an important influence on Fe speciation under various Fe limiting conditions?

**Thesis Objectives**

The aim of this thesis is to increase our understanding of the controls on Fe redox speciation in seawater, with particular attention given to the potential influence of *T. erythraeum* when grown under conditions with varying Fe limitation. Chapter 2 improves the performance of the ferrozine method for detecting total dissolved Fe in samples containing high concentrations of organic matter, as well as natural and synthetic Fe binding ligands, such as in filtrates from exponentially growing phytoplankton cultures (and potentially also coastal waters). Chapter 3
investigates the effects of organic exudates released by *T. erythraeum* at various stages of growth in batch cultures on Fe(II) oxidation rates. **Chapter 4** investigates the potential of organic exudates to impact Fe(III) reduction kinetics. It also presents measurements of superoxide production rates in Fe-limited *T. erythraeum* cultures during different phases of growth. **Chapter 5** summarizes the findings related to Fe oxidation and reduction from Chapters 3 and 4, and recommendations for future studies provided.
Chapter 1

**Literature Cited**


Godrant, A. 2009. The role of superoxide in iron acquisition by marine phytoplankton. New South Wales, Australia.
Chapter 1


Chapter 1

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Roe, K. L., and K. A. Barbeau. 2014. Uptake mechanisms for


Chapter 2
Measuring total Fe concentrations in plankton cultures in the presence of synthetic and organic ligands using a modified ferrozine method

Abstract

Iron concentrations in natural waters are often in the low pico- to nanomolar range. Furthermore iron has a strong tendency to form colloids and/or complexes with organic matter. These two factors can present major challenges for measuring iron easily and accurately. This study describes an optimized ferrozine method for measurements of total iron at nanomolar concentrations in seawater samples containing relatively high concentrations of ethylenediaminetetraacetic acid (EDTA) and desferrioxamine B (DFB), commercially available synthetic and natural iron ligands which are widely used in phytoplankton cultures, and dissolved organic matter. The method involves sample acidification to liberate iron from strong complexes and/or colloids, followed by reduction of Fe(III) to Fe(II) using Na$_2$SO$_3$, and finally the formation of a complex between Fe(II) and ferrozine. The performance of each step was improved in such a way as to achieve complete dissociation of iron from the given ligands regardless their type or concentration. Storage of samples at pH 1 for 15 d was able to bring about full recovery of iron in the presence of 50 µmol/L DFB, while the duration required for full iron recovery in the presence of the same concentration of EDTA was only about 1 h. Also, the addition of ferrozine after a brief (~15 min) reduction step with subsequent incubation for 24 h resulted in more stable colour development in the samples over time. The approach was successfully applied to determine total dissolved iron in samples from coastal waters containing high concentrations of dissolved and particulate organic matter, and also in samples with an additional synthetic iron binding ligand, EDTA, from laboratory cultures of the marine cyanobacterium, Trichodesmium erythraeum.
1. **Introduction**

Iron (Fe) is an essential micronutrient for the growth and metabolism of all marine organisms and is thought to limit primary productivity in up to 40% of the ocean (Martin et al. 1990), while occasionally co-limiting phytoplankton biomass in the remaining 60% (e.g. Breitbarth et al. 2008; Hoffmann et al. 2008; Mills et al. 2004; Timmermans et al. 2001). Microorganisms require Fe as a co-factor in many redox reactions involving major cellular metabolic processes such as photosynthetic and respiratory electron transport and molecular dinitrogen (N$_2$) fixation (e.g. in cyanobacteria) (Berman-Frank et al. 2001; Chappell and Webb 2010). Thus, over the last three decades, there has been particular interest in the biogeochemical cycling of Fe in natural waters, and the associated Fe acquisition mechanisms of marine microorganisms.

Although ferrous iron (Fe(II)) is believed to be the most readily assimilated form of Fe by microbial cells, conceptual models for Fe uptake by microorganisms have primarily considered dissolved inorganic ferric iron (denoted by Fe(III)') and organically complexed Fe(III) (denoted by Fe(III) L) as the main sources of Fe for biological use (Fujii et al. 2010a; Garg et al. 2007b; Morel et al. 2008; Rose et al. 2005; Shaked et al. 2005). This arises from the fact that under alkaline and oxygenated conditions, which are typical of most marine environments inhabited by photosynthetic organisms, Fe(III) is generally regarded as the dominant and most thermodynamically stable redox state (Rose and Waite 2002). In surface waters, however, atmospheric Fe deposition, Fe(II) fluxes from anoxic sediments, Fe(III) photoreduction, as well as biologically mediated Fe(III) reduction can result in Fe(II) constituting as much as 40% of total dissolved Fe (Gonzalez et al. 2014; Lohan et al. 2005).

The analysis of Fe in natural waters and in particular seawater can be difficult as it is often present at relatively low concentrations (from <1 nmol/L in open ocean to >10 nmol/L in coastal waters), and has a propensity to form complexes with organic matter (Bruland et al. 2001; Lohan et al. 2005; Luther et al. 1996). The difficulties of measuring Fe in the presence
Chapter 2

The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater

of particulate and dissolved natural organic matter (NOM) mainly result from its ability to complex Fe and potentially modify its redox state.

Several techniques have been established for Fe measurement in natural samples, which vary depending on the targeted dissolved species of Fe(II) or Fe(III) (Lannuzel et al. 2006; Lohan et al. 2006; Lohan et al. 2005; Páscoa et al. 2009). These techniques are typically comprised of three essential steps: (1) converting organic complexes and colloids into labile dissolved inorganic forms of iron (i.e. free Fe$^{2+}$ and Fe$^{3+}$); (2) reducing or oxidizing this dissolved inorganic Fe to suit the requirements of the particular detection method; and (3) quantifying this final form of iron.

To release Fe from complexes by dissociation and transform it into a labile form, storage of samples at a low pH (often pH 1.7) for a few months is widely used in both shipboard and in situ applications (Lohan et al. 2005). This step introduces a plentiful supply of protons to replace Fe with H$^+$ in complexes and colloids, thereby releasing Fe into solution. However, due to large variability in the concentrations and types of organic matter found in natural waters, the actual time required for full dissociation of Fe from ligands remains uncertain.

Although the thermodynamically stable redox state of total dissolved iron (dFe$_T$) at low pH is Fe(II) (Johnson et al. 2003), addition of an oxidizing (Lohan et al. 2005) or a reducing reagent (Hopwood et al. 2014; Lannuzel et al. 2006; Viollier et al. 2000) into the samples is necessary to ensure consistent redox Fe speciation before measurements. However, since the performance of Fe binding compounds including chelating resins and/or complexing agents such as ferrozine (C$_{20}$H$_{13}$N$_4$NaO$_6$S$_2$·xH$_2$O, hereafter abbreviated as FZ) depends on pH, application of an efficient buffering system is another critical step for Fe quantification.

Spectrophotometric quantification of the complex between Fe(II) and ferrozine is one of the most effective procedures for measuring Fe in variety of fresh and marine waters down to low nanomolar and even picomolar concentrations (with pre-concentration techniques) (Kundra et al. 1974; Stookey 1970; Thompsen and Mottola 1984; Viollier et al. 2000). The technique is
based on the selective complexation of Fe(II) by FZ to produce a stable, purple-coloured complex with a maximum absorption at 562 nm, and a relatively high molar absorptivity of ~30,000 L/mol/cm at this wavelength, between a pH of 4 and 9 (Giokas et al. 2002; Stookey 1970; Viollier et al. 2000). An approximate incubation time of 1-30 min is typically used to allow complex formation between Fe and ferrozine (Akob et al. 2012; Dubinsky et al. 2010; González-Davila et al. 2005; Lindgren et al. 2011; Santana-Casiano et al. 2005; Stookey 1970; Tor et al. 2000).

Despite its simplicity, rapidity and high selectivity and sensitivity for measuring Fe, there are some uncertainties associated with the ferrozine method. For example, Stookey (1970) identified several alkali metals and alkaline earths other than Fe which form coloured complexes with ferrozine, as well as some anions (e.g. NO$_2^-$, CN$^-$ and C$_2$O$_4^{2-}$) that could interfere with Fe(II) measurements when present at concentrations over 500 mg/L. Dawson et al. (1990) and Luther et al. (1996) reported an incomplete reduction step, depending on the speciation of Fe(III) in the sample. Some studies have reported reduction of incubated Fe(III) with FZ over time (Im et al. 2013) where Fe(III)-FZ complexes were photosensitive (Anastácio et al. 2008). The presence of NOM in samples can also retard the Fe(II)-FZ complexation reactions, resulting in development of an asymptotic absorbance over time (Box 1984; Rose and Waite 2003c). Some workers have described a possible underestimation of Fe(II) concentrations as a result of inadequate mixing and equilibration time for FZ in the presence of NOM (Croot and Hunter 2000; Hopwood et al. 2014). Jeitner (2014) also reported the effects of some physicochemical factors (e.g. pH and temperature) on colour stability of Fe(FZ)$_3$ complexes. Furthermore multiple studies have shown poor recoveries of Fe bound to commercially available organic ligands including ethylenediaminetetraacetic acid (EDTA) and desferrioxamine B (DFB) during pre-concentration steps (Lohan et al. 2005). While DFB and EDTA are known primarily as Fe(III) complexing agents, some studies have also reported formation of Fe(II)-DFB and Fe(II)-EDTA complexes under certain conditions (Fujii et al.}
2010a; Kiss and Farkas 1998), which may result in modification of Fe redox state (Farkas et al. 2003; Harris and Aisen 1973). This issue can interfere with measurements when FZ has to outcompete ligands that are able to either form strong Fe(II) complexes or oxidize existing Fe(II) species (Rose and Waite 2003c; Shaked et al. 2005). Additional uncertainty can also result from contamination of samples during handling or reagent preparation, in the latter case primarily due to the presence of Fe impurities in consumables including acids and buffers.

Given all the possible uncertainties, the aim of this study was to optimize the FZ method for determination of nanomolar concentrations of Fe in samples with high levels of particulate and dissolved natural organic matter and, in particular, phytoplankton cultures containing both natural and synthetic ligands. This was achieved by:

(i) Assessment of the optimum duration required for complete reduction of Fe(III) to Fe(II) (Lannuzel et al. 2006; Viollier et al. 2000) and formation of a stable Fe(FZ)3 complex, using previous studies as a starting point (Hopwood et al. 2014; Im et al. 2013).

(ii) Evaluation of the minimum time required for the acidification step in the presence of high concentrations of the commonly used ligands, EDTA and DFB, in terms of full recovery of added Fe.

(iii) Application of the method to samples collected from coastal waters and cultures of the marine cyanobacterium Trichodesmium erythraeum containing particulate and dissolved organic matter.

While microwave heating and UV digestion could be also considered as other options to speed up the dissociation process, they could potentially introduce reactive oxygen species into the samples, thereby accelerating the Fe redox reactions. Therefore, in this study we only focused on use of acidification as an approach that is cheap and easily accessible in all laboratories.
2. Experimental

2.1. Reagents and solutions

To minimise the potential for Fe contamination, all the plasticware and tubes including the Liquid Waveguide Capillary Cell (LWCC) were cleaned based on the protocols described in Appendix 1. Sample acidification was performed using high purity ~12 mol/L HCl (34-37% w/w, Instrument Quality, SEASTAR CHEMICALS Inc, hereafter denoted as trace metal grade acid). Chelex-100 resin purification for removal of trace metals from artificial seawater (ASW) and the reagents (sodium hydroxide and sodium sulfite) was conducted according to Sunda et al. (2005). Artificial seawater (ASW) was prepared based on the recipe for modified YBC-II medium as described in Section 2.2 (Chen et al. 1996).

A 5 mol/L ammonium acetate (NH₄Ac) buffer was prepared with a pH of 4.5 in a fume hood by dissolving 134 g of NH₄Ac salt (C₂H₃O₂NH₄, Ajax Finechem Pty Ltd) in 187 mL of glacial acetic acid (Ajax Finechem Pty Ltd), and making up to 1 L with high purity Milli-Q water (HPMQ) in a volumetric flask. Although previous studies have used NH₄Ac buffer at pH 5.5 (e.g. Gonzalez et al. 2012; Gonzalez et al. 2014; Gonzalez-Davila et al. 2005; Santana-Casiano et al. 2005), given a pK value of 4.76 for NH₄Ac (Morel and Hering 1993), the buffering capacity of the solution is higher at pH 4.5 (Jeitner 2014). This pH is still well within the optimum pH range reported for Fe(FZ)₃ complex formation (Stookey et al. 1970; Viollier et al. 2000). The solution was stored in the dark (wrapped in aluminium foil) in an acid cleaned polypropylene bottle containing a few mL of purified Chelex-100 resin for at least 24 h before use. A solution of ~45 mmol/L sodium sulfite (Na₂SO₃, Ajax Finechem Pty Ltd) as reducing agent was prepared daily by dissolving 108 mg of Na₂SO₃ salt in 20 mL of 0.1 M NH₄Ac (itself prepared by dilution of the 5 mol/L NH₄Ac buffer with HPMQ) (Lannuzel et al. 2006) and stored with a few mL of purified Chelex-100 resin for at least 2 h before use.
A solution of ~1.65 mmol/L ferrozine for use with samples was prepared in an acid-cleaned polycarbonate bottle by dissolving 52 mg of ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate, Sigma-Aldrich) in 50 mL of trace metal clean 5 mol/L NH₄Ac and 14.4 mL of 5 mol/L sodium hydroxide (NaOH), itself prepared by dissolving 4 g of sodium hydroxide pellets into 20 mL of HPMQ. Addition of NaOH to the NH₄Ac solution ensures the final solution pH is in the optimal range (~ 4.5-5.5) for Fe(FZ)₃ complex formation after addition to the acidified samples at pH 1. A 2 mmol/L ferrozine solution for use during calibration was also prepared by dissolving 20 mg of FZ salt in 20 mL of trace metal clean 5 mol/L NH₄Ac. To avoid transferring chelating Chelex-100 resin beads into the samples, the purified buffers were filtered through acid cleaned 0.45 µm pore size syringe filters before addition to reagents or samples.

For the calibration procedure, a 5 mmol/L primary Fe(II) solution was prepared by dissolving 196 mg of FeSO₄·2H₂O (Ajax Finechem Pty Ltd) in 100 mL of HPMQ that had been acidified with 83 µL of trace metal grade acid. The pH in the secondary standard solutions was < 5 after dilution of the primary stock solution with HPMQ, which is low enough to keep the Fe(II) in reduced form for several days (Millero et al. 1987), but also high enough to avoid significant influence on the pH of the seawater samples after addition. Fe(II) standard solutions were prepared daily by diluting the 5 mmol/L Fe(II) stock solution with HPMQ. 2 mmol/L FZ reagent was added in a ratio of 100 µL per mL of standard or blank solution immediately before measurement.

To prepare experimental solutions, a 500 µmol/L stock solution of Fe(III) was prepared by dissolving 13.5 mg of FeCl₃·6H₂O (Sigma–Aldrich) in 100 mL of HPMQ that had been acidified with 100 µL of 1 mol/L trace metal grade acid. Several 500 nmol/L experimental Fe(III) solutions were made by adding 100 µL of 500 µmol/L Fe(III) stock solution per 100 mL of trace metal clean ASW and varying amounts of EDTA and DFB. All Fe(II) and Fe(III) stock solutions except the 500 µmol/L Fe(III) solution were prepared daily.
Stock solutions of 5 mmol/L EDTA (Na$_2$EDTA·2H$_2$O, Ajax Finechem Pty Ltd) and 5 mmol/L desferrioxamine B mesylate salt (DFB ~95%, Sigma-Aldrich) were prepared by dissolving 14.6 and 12.2 mg of each salt, respectively, in 10 mL of HPMQ. Experimental concentrations of 2 µmol/L and 50 µmol/L for each ligand were achieved by adding 400 µL and 10 mL, respectively, per litre of trace metal cleaned ASW.

2.2. Sample collection and preparation

Samples of natural coastal seawater were collected in February 2015 from Angels Beach, Ballina, Australia (153.60°E 28.85°S) in acid cleaned 1 L polycarbonate bottles from about 50 cm below the water surface. To avoid dust contamination, the bottles were uncapped and then re-capped underwater and transferred to the laboratory in double zipper plastic bags. Upon return to the laboratory, two separate unfiltered and filtered (through 0.2 µm acid-cleaned 47 mm polycarbonate filters) batches of samples (300 mL) were acidified to pH 1 as described in the procedure given later. Acidification either before or after filtration enables comparison of the particulate and dissolved Fe fractions in the samples. An additional 30 mL aliquot of each sample was vacuum filtered through precombusted glass-fiber GF/F filters (Whatman, 25mm). These filters were then dried at 60°C for 12 h and total particulate carbon (TPC) subsequently measured using an elemental analyser (Thermo Flash EA 1112).

Non-axenic, unialgal laboratory cultures of the marine cyanobacterium *T. erythraeum* (IMS101) were grown in continuous mode with constant aeration in YBCII medium prepared according to the recipe given in Andersen (2005) but modified to contain 500 nmol/L Fe, 2 µmol/L EDTA and 2 µmol/L phosphate at a dilution rate (i.e. growth rate) of 0.1 d$^{-1}$ under 110 µmol quanta m$^{-1}$s$^{-1}$ of light (LI-COR, USA) with a 14/10 h light/dark cycle at 27°C. Contamination with bacterial cells was monitored using the Morphologi (Appendix 1), however the potential presence of small amounts of bacteria would not be expected to significantly influence the experimental results. Samples for total Fe (dissolved and particulate forms) and TPC were prepared by gentle filtration in the same manner as for natural water samples.
2.3. Experimental procedures and method validation

The commonly used ferrozine method for determination of total dissolved Fe in natural water samples typically consists of (a) acidification of the sample to pH 1.7 for at least a month (Lohan et al. 2006; Lohan et al. 2005), (b) application of a reducing agent (usually ascorbic acid or hydroxylamine hydrochloride) followed by overnight storage (Hopwood et al. 2014), and finally (c) addition of FZ reagent 1-30 min prior to measurement of absorbance at 562 nm (Giokas et al. 2002; Im et al. 2013; Stookey 1970).

Compared to other commonly used reducing agents which have acidic properties, sodium sulfite was preferred due to its ability to reduce Fe(III) over a wide range of pH (Lannuzel et al. 2006; Millero et al. 1995) from very acidic (≥ 2) to mildly acidic (~ 4.5-5) conditions that were compatible with both acidification and the Fe(FZ)₃ complexation processes. This also allows the opportunity to purify the reducing agent by removal of potential Fe contamination since the pH is in the appropriate range for application of cation exchange resin columns (Lannuzel et al. 2006; Ussher et al. 2009). To ensure complete reduction of Fe(III) to Fe(II), sodium sulfite was added at 12-fold higher concentration than previously suggested by Lannuzel et al. (2006). Furthermore, a constant FZ concentration of 200 µmol/L was used since Shaked et al. (2004) described the possibility of artifacts when using concentrations higher than 400 µmol/L.

To adapt the method to the sample conditions in this work (i.e. the presence of relatively high concentrations of NOM in addition to synthetic ligands), the required incubation time for acidified samples with FZ was examined first, followed by optimization of the acidification intensity and duration. For this purpose two separate batches of ASW containing 500 nmol/L Fe(III) and 0, 2, and 50 µmol/L EDTA were acidified to pH ~1.7 and ~1.0 for durations of 24 h and 1 h, respectively (Figures 2.1 and 2.2). EDTA and Fe in the seawater batches was allowed to equilibrate for 2 d prior to acidification. Accordingly, the optimum time for sample
incubation with FZ (15-30 min, 24 and 96 h) was initially investigated in the acidified samples at pH ~1.7, and subsequently the obtained optimum time was further assessed at lower pH.

Finally, the modified procedure was applied to samples containing high organic matter concentrations, including coastal water samples and samples from a cyanobacterium culture containing an additional synthetic Fe binding ligand (EDTA) (Figure 2.3).

The minimum duration required for complete dissociation of Fe from ligands was quantified in the presence of varying concentrations of the strong siderophore-type Fe chelator desferrioxamine B (DFB). ASW batches containing 0, 2 and 50 µmol/L of DFB and 500 nmol/L Fe(III) were allowed to equilibrate for 1 h and 24 h prior to the acidification step. Triplicate aliquots from each batch were taken every 2 d and analyzed for total dissolved Fe. Subsequently, Fe(FZ)$_3$ absorbance at 562 nm was quantified with a Varian Cary 50 UV-visible spectrophotometer coupled to a 100 cm optical path Liquid Waveguide Capillary Cell (LWCC; World Precision Instruments, series 2100) via optical fibres (Waterbury et al. 1997). Approximately 2 mL of sample or standard were loaded into the LWCC at a constant flow rate of 1 mL/min using a peristaltic pump. The pump was subsequently stopped before measurements, and the absorbance determined at 1 nm resolution in a scan between 700 and 550 nm at a rate of 120 nm/min.

The system was calibrated daily with Fe(II) standards in HPMQ of known concentrations (20-700 nmol/L). Linear regression was used to relate the Fe concentration in the standards to absorbance. The difference in average absorbance from 695 to 700 nm (at which the absorbance of the Fe(FZ)$_3$ complex is negligible) between the standard blank (i.e. samples containing MQ and FZ with no added Fe) and samples, was subtracted from sample scans, thereby removing potential baseline shifts due to changes in light intensity or small air bubbles. Although some measurements were outside the calibration range, given the linearity of the calibration curves and robustness of the ferrozine method, this was not expected to contribute any significant
additional error to the measured values. Data were also corrected for potential baseline shifts or scatter by microbubbles.
3. **Results and discussion**

3.1. *Investigation of optimum incubation time for ferrozine in the presence of ligands*

The efficiency of Fe(II) complexation by FZ in the presence of different EDTA concentrations was examined at varying incubation times. Figure 2.1 clearly shows that a FZ incubation time of 15-30 min was not sufficient for full recovery of a known Fe concentration (500 nmol/L) in the presence of EDTA. However, Fe was completely recovered when the incubation period was extended to 24 h. This duration was also found to sufficient to enable formation of a stable Fe(FZ)$_3$ complex, with $< 5\%$ change in the measured Fe concentration over a further 96 h of incubation. Consistent with previous results reported by Jeitner (2014), our results suggest 24 h as an optimum incubation time for FZ in both the presence and absence of organic ligands.

After establishing the optimum duration for incubating samples with FZ, the intensity and duration of acidification optimised. As shown in the Figure 2.2, full recovery of Fe (500 nmol/L) was obtained after only 1 h acidification at pH $\sim$1, with little variation in Fe recovery after longer acidification times.
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**Figure 2.1.** Recovery of Fe by ferrozine in acidified ASW (pH 1.7) with 500 nmol/L Fe(III) and varying EDTA concentrations at 15-30 min, 24 h and 96 h of FZ incubation. Symbols represent no EDTA (filled squares), 2 µmol/L EDTA (filled triangles), and 50 µmol/L EDTA (open circles). The dashed line represents theoretical 100% recovery of Fe.

**Figure 2.2.** Recovery of Fe in the samples of ASW with 500 nmol/L Fe(III) and varying EDTA concentrations acidified to pH ~1 and FZ incubation time of 24 h. Symbols represent no EDTA (filled squares), 2 µmol/L EDTA (filled triangles), and 50 µmol/L EDTA (open circles). The dashed line represents theoretical 100% recovery of Fe.
3.2. **Total Fe recovery in the presence of the strong Fe-binding ligand DFB**

The effectiveness of the method for complete Fe recovery was also examined in the presence of varying amounts of DFB which had been allowed to equilibrate with 500 nmol/L of Fe for 1 h and 2 d before the acidification step. As shown in Figure 2.3, full recovery of Fe from DFB was achieved after ~15 d of acidification, regardless of the equilibration duration. The latter result implies relatively rapid complexation, as expected based on the complex formation rate constant \( k_f \) of \( 1.96 \times 10^6 M^{-1}s^{-1} \) reported at pH 8.1 by Witter et al. (2000).

![Figure 2.3](image)

**Figure 2.3.** Recovery of Fe bound to varying amounts of DFB (0 squares, 2 µmol/L triangles and 50 µmol/L circles) as a function of acidification time at FeDFB equilibration times of 1 h and 2 d (open and filled symbols, respectively). The dashed line indicates the added Fe concentration. Shown are means of three replicate samples with standard error. Negative values are most likely related to contamination during handling.

The increasing, and finally full Fe recovery with time in the presence of DFB suggests that (a) only the liberated iron (Fe\(^-\)) is detected by the assay, and (b) (re)complexation of dissociated Fe is negligible at pH 1. Accordingly, the dissociation rate constant \( k_d \) for the FeDFB complex was determined assuming a first order kinetic reactions with a single type of complex present as (Rose and Waite 2003b),
\[ \frac{d[FeL]}{dt} = -k_d[FeL] \] (2.1)

First order kinetics with a single complex will be valid for the samples employing known ligands such as EDTA and DFB experiments, since only one type of complex (1:1) with Fe exists (Stadtman 1993), while it is more likely that samples from coastal waters or the culture contain multiple Fe complexes with potentially different dissociation rate constants.

Under conditions where the Fe concentration was known, the rate constant for the dissociation of FeDFB was calculated from linear regression of \( \ln \left( \frac{[Fe]_{T \text{ added}} - [Fe]_{T \text{ measured}}}{[Fe]_{T \text{ added}}} \right) \) versus time with the intercept fixed at zero, where \([Fe]_{T \text{ added}}\) is the concentration of Fe corresponding to 100% recovery (i.e. 500 nmol/L total Fe) and \([Fe]_{T \text{ measured}}\) is the actual concentration of Fe measured on different days. The slope of the line thus represents the rate constant. The dissociation rate constant was therefore determined as \((2.2 \pm 0.11) \times 10^{-6} \text{ s}^{-1}\), corresponding to a half-life of \(\sim 4 \text{ d}\) for the FeDFB complex under these conditions.

Relatively few dissociation rate constants for Fe(III) complexes have been established under very acidic (pH \(\sim 1\)) conditions. Monzyk et al. (1982) reported a dissociation rate constant of \(1.9 \times 10^{-3} \text{ s}^{-1}\) for FeDFB under strongly acid conditions (at pH 0.1-1.3). This value, however, was measured at a different ionic strength and temperature than this study. The dissociation rate constant measured in this study was much lower than the one reported by Monzyk, but relatively similar to the measured value at pH 8 by Witter et al. (2000) of \((1.5 \pm 1.8) \times 10^{-6} \text{ s}^{-1}\). Even so, acidification to pH 1 is expected to greatly increase the rate at which Fe' is liberated from FeDFB because (re)complexation is also inhibited.

In contrast, dissociation proceeded so fast for the weaker ligands such as EDTA that the kinetics could not be observed on the daily time scales of our measurements (Figure 2.2). At an EDTA concentration of 50 \(\mu\text{mol/L}\), complete FeEDTA dissociation occurred within 1 h of acidification. Based on full recovery already after 1 h of acidification, the half-life must be less
than 1 h, which is significantly lower than previously reported values (1-8 d) at lower EDTA concentrations but higher pH reported by Hudson et al. (1992) and Fujii et al. (2010).

3.3. Total Fe recovery in natural samples

The modified FZ method was also applied to seawater collected from coastal waters (containing ~10.5 µmol/L POC and unknown Fe concentrations), with filtered and unfiltered acid incubations. Filtered and unfiltered acid incubations were also performed with samples from laboratory cultures of *T. erythraeum* (containing ~800 µmol/L POC and 500 nmol/L Fe initially). POC concentrations in phytoplankton cultures can be considered a proxy for dissolved organic matter (DOC) concentrations in exponentially growing cells, as 5-15% of total primary production (i.e. POC + DOC) is typically in the form of DOC (Rochelle-Newall et al. 2014). Similar to the results with EDTA present as an added ligand, the FeL dissociation rate in these samples was relatively fast with stable Fe recovery over time (Figure 2.4). Observation of a relatively constant measured total Fe concentration over time for both filtered and unfiltered samples, including cultures containing a known Fe concentration (500 nmol/L) and coastal waters with unknown Fe concentration, supports the reliability of method. However, although FZ is a strong Fe(II) complexing agent, it is possible that some Fe might not be fully captured due to limitations of the method outline in the introduction. Therefore, comparison using other methods would be also be helpful to support the validity of the method.

The recoveries of Fe in the unfiltered natural seawater and culture samples were 50-95% higher than in the filtered samples (Figure 2.4). This phenomenon was also reported by Schoemann et al. (1998) and Bruland et al. (1994), in which particulate/dissolved Fe ratios were significantly higher in coastal waters compared to open ocean samples.
Figure 2.4. Recovery of Fe in coastal waters (triangles) and samples from cultures of *T. erythraeum* (squares) for filtered and unfiltered acid incubations (open and filled symbols, respectively).

3.4. Final protocol and detection limits

In summary, the optimized procedure (Figure 2.5) involves (a) initial sample acidification at pH 1 for a minimum duration of 15 d, (b) a subsequent reduction step with 30 µL of 45 mmol/L Na$_2$SO$_3$ per mL sample (final [Na$_2$SO$_3$] = ~1.25 mmol/L) for 15 min, (c) addition of 1.65 mmol/L ferrozine reagent in a ratio of 128 µL per mL of sample (final [FZ] = 200 µmol/L) followed by 24 h of equilibration before measurement, and (d) samples were filtered (if not previously filtered when the sample was taken) and loaded into the LWCC.

![Figure 2.5. Simplified scheme of the experimental procedure.](image)

With care, a detection limit of 0.7-1 nmol/L total Fe can be achieved based on three times the standard deviation of the blank (Lohan et al. 2006). This is close to the theoretically calculated
value (~0.5 nmol/L) for the 100 cm pathlength LWCC system. Given that Fe is typically found in the pico- to low nanomolar range (0.05-2 nmol/L) in open ocean waters (Achterberg et al. 2001), the approach presented here is most suitable for coastal and groundwater samples, and in particular plankton cultures containing low to high nanomolar concentrations of Fe, as well as organic and synthetic ligands (Sanders et al. 2015; Wells and Mayer 1991; Windom et al. 2006; Wong et al. 2010). The molar absorption coefficient for the Fe(FZ)₃ complex in this study was calculated at 22,970 L⁻¹mol⁻¹cm⁻¹ (theoretical detection limit, 0.4 nmol/L) which is at the lower end of the range of previously reported coefficient values (26,500-39,500 L⁻¹mol⁻¹cm⁻¹) (Carter 1971; Giokas et al. 2002; Hanson 2000; Stookey 1970).
4. Conclusions

This chapter presented a modified ferrozine method for measuring total dissolved Fe in the presence of dissolved organic matter and organic and synthetic ligands, optimized specifically for measurements in phytoplankton cultures. The dissociation kinetics of Fe complexes in samples stored at pH 1 were found to depend on ligand strength and concentration, with 15 d being sufficient time to fully recover Fe in the presence of 50 µmol/L of DFB and 1 d being sufficient for full recovery of Fe in the presence of EDTA. A detection limit as low as 0.7 nmol/L total Fe can be achieved using the method with a 100 cm Liquid Waveguide Capillary Cell. The method described was also successfully applied to determine total Fe concentrations in coastal marine waters, and should additionally be suitable for total Fe determination in groundwater.
Chapter 2

**Literature cited**


The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater


The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater


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Chapter 3
Growth phase and organic exudates dependent modification of Fe(II) oxidation rates in Fe limited cultures of *Trichodesmium erythraeum*

**Abstract**

The 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 Fe(II) oxidation rate constant was studied during different phases of growth for batch cultures grown with Fe⁺ concentrations of 0.1 and 1.59 nmol/L. Fe(II) oxidation was accelerated in the presence of organic exudates, intensifying when the cells were growing exponentially and then plateauing towards the stationary phase. The best fit of the kinetic model to the data also demonstrated clear differences in oxidation rate constants during varying growth phases. However, no significant difference was observed in oxidation rate constants between the two Fe⁺ treatments. These findings suggest that organic exudates released by *Trichodesmium* promote Fe(II) oxidation, suggesting organic complexation as possible strategy for increasing the bioavailable pool of dissolved Fe in the extracellular matrix (seawater) by *Trichodesmium*. 
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 constituents 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 DOM exudation rate with decreasing nutrient availability over time is an important cellular metabolic strategy to dissipate excess light energy during nutrient starvation (Myklestad 2000). Release of DOM has been also documented as a strategy to regulate the speciation, bioavailability and toxicity of trace metals in the external milieu (Jones 1998; Moffett et al. 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 world’s 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 N₂ 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 my knowledge, no systematic study has yet been performed on how cyanobacterial exudates may influence Fe chemistry during the various growth phases that occur in batch cultures.

In marine surface waters, Fe exists in two oxidation states of Fe(II) and Fe(III), 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 (i.e. Fe(II)´ and Fe(III)´) are assumed to be the most bioavailable pools in the milieu, dissociation of organic Fe complexes (FeL) is usually a precursor step in their 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 dissociative conditions (Morel et al. 2008; Shaked et al. 2005). However, under alkaline (pH 8) and oxygenated conditions, Fe(II) is rapidly oxidized to the more dominant and thermodynamically stable Fe(III). Thus, while a pre-reduction step seems to be necessary 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 some eukaryotic microorganisms prior to uptake (Garg et al. 2007a; Maldonado et al. 2006). This conflict poses new challenges for Fe(II) studies 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. 2011b). For instance, Roe et al. (2011) 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 homologous genes within the genome, feoB and futABC, coding transporters for inorganic Fe 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 FeCl₃ and Fe(II)-citrate forms. Rubin et al. (2011) showed that *Trichodesmium* colonies can actively increase the dissolution and acquisition of Fe from particulate sources such as dust. Release of superoxide (O₂⁻⁻) (Godrant et al. 2009) and exopolysaccharides (EPS) (Berman-Frank et al. 2007) into the extracellular surroundings have also been reported in IMS101 under Fe stress conditions. Despite the numerous studies on the influence of superoxide on Fe uptake rates in cyanobacteria, and in particular *Trichodesmium* (Fujii et al. 2010a; Godrant et al. 2009;...
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Kranzler et al. 2011; Roe et al. 2012; Rose et al. 2005), very few studies have examined the effect of their organic exudates 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 2003a; 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 been characterized so far to form weak complexes with inorganic Fe species (Benner 2011; Hassler et al. 2011b; Santana-Casiano et al. 2014), most likely with Fe(III) (Elhabiri et al. 2007; Santana-Casiano et al. 2010). In contrast to superoxide, which can influence Fe uptake rates by reducing Fe(III) species (Rose 2012; Rose and Waite 2005), 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). This study aimed to address the following questions:

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

2. Are the oxidation rate constants for organically complexed Fe(II) species dependent on 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 different Fe nutrition conditions using different 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 O2 in the presence of organic exudates released during the various growth phases.
2. Experimental

2.1. Reagents and solutions

A 2 mmol/L EDTA (Na₂EDTA.2H₂O, 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 filter, hereafter denoted as HPMQ). A 500 µmol/L stock solution of Fe(III) was prepared by dissolving 13.5 mg of FeCl₃.6H₂O (Sigma-Aldrich) in 100 mL of HPMQ that had been acidified with 100 µL of 1 mol/L HCl (prepared from 34-37% w/w HCl, Instrument Quality, SEASTAR CHEMICALS Inc, hereafter denoted as trace metal grade acid).

A 4 mmol/L stock solution of Fe(II) was prepared by dissolving 157 mg of ammonium iron(II) sulfate hexahydrate (Fe(NH₄)₂(SO₄)₂·6H₂O, Ajax Finechem Pty Ltd, reagent grade) in 100 mL of HPMQ. The stock solution was stabilized by adding 20 µL of 6 mol/L trace metal grade acid (Rose and Waite 2003a). 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 2003a). To facilitate preparation of standards at nanomolar Fe(II) concentrations, a fresh 1 µmol/L Fe(II) solution was prepared daily by adding 25 µL Fe(II) stock solution (4 mmol/L) 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 significant 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 NBS standard pH buffers (4.00, 7.00 and 10.00 at 20°C).

A 0.5 mmol/L luminol reagent was prepared in 1 mol/L ammonium hydroxide solution (NH₄OH) by dissolving 89 mg of luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, Sigma-Aldrich) in 69 mL NH₄OH (28-30% w/w, ≥ 99.99% trace metals basis, Sigma-Aldrich, final concentration 1 mol/L) 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 trace metal grade acid and stored in the dark at room temperature for at least 24 h before use (Rose and Waite 2003a).

### 2.2. Culture conditions

A non-axenic unialgal strain of *T. erythraeum* IMS101 was obtained from the National Center for Marine Algae and Microbiota (NMCA), USA. 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, see Appendix 1), using a batch culture approach in 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 FeCl₃, 2 µmol/L KH₂PO₄, 2.1 mmol/L of NaHCO₃ and different EDTA concentrations as described below. To minimize 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 polyethersulfone membrane (polycap TC filter capsule, PES, Whatman) before addition of nutrient solutions (including trace metals, phosphorus and vitamins).

Different levels of Fe bioavailability were established by adding two concentrations of ethylenediaminetetraacetic acid (EDTA) into the YBC-II medium. To do so, 50 nmol/L and 20 µmol/L EDTA solutions were prepared by adding 25 µL and 10 mL of 2 mmol/L EDTA stock solution per litre of ASW, respectively. Subsequently, a final 10 nmol/L Fe(III) concentration was achieved by adding 20 µL of the 500 µmol/L Fe(III) stock solution per litre of ASW and allowed to sit for 1 h to equilibrate. The concentration of unchelated iron ([Fe']) in the seawater was calculated from total Fe(III) and EDTA concentrations using the equilibrium complexation model at pH 8.2 given by Schulz et al (2004) (Table 3.1). All the measurements were conducted on the NBS scale and assumed to equal the free scale, which results in an error of about 0.005
pH units (Lewis et al., 1998). This error is negligible for the purposes of this study. This model accounts for all complexed forms of Fe(III) with Cl\(^-\), F\(^-\), SO\(_4^{2-}\) and the Fe(III)OH species, as well as protonated or complexed forms of EDTA with Fe, Cu, Co, Mn, Zn, Ca and Mg.

**Table 3.1.** Calculated concentrations of total Fe ([Fe\(_T\)], unchelated Fe ([Fe\(^-'\)], organically complexed Fe ([FeL]) and precipitated Fe ([Fe(s)]) in the presence of different EDTA concentrations and a Fe(III) addition of 10 nmol/L at pH 8.2 (free scale).

<table>
<thead>
<tr>
<th>EDTA (µmol/L)</th>
<th>[Fe(_T)] (nmol/L)</th>
<th>[Fe(^-')] (nmol/L)</th>
<th>FeL (nmol/L)</th>
<th>[Fe(s)] (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>10</td>
<td>1.59</td>
<td>0.03</td>
<td>8.37</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>0.10</td>
<td>9.90</td>
<td>0</td>
</tr>
</tbody>
</table>

Culture media were inoculated with exponentially growing cells at a concentration of ~500 cell/mL and incubated at 27°C, with a photon flux density of 110 µmol quanta m\(^{-2}\)s\(^{-1}\) (measured with a LI-193 Spherical Quantum Sensor, LiCor) and a 14:10 dark/light cycle. Exponentially growing cells were inoculated three times into fresh media over 24 d and acclimatized for 12 generations. To monitor growth of the cultures, 5 mL of each culture was taken every 2 d, fixed in Lugol iodine solution (1%) and then the cells counted using an automated particle imaging system (Morphologi G3, Malvern Instruments, UK). The procedures for counting cells using the Morphologi G3 and calculating the growth rates are described in Appendix 1.

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

To examine Fe(II) oxidation rates throughout the growth cycle in the batch cultures, about 250 mL of each culture was harvested every 2 d and adjusted to pH 8.0 by the dropwise addition of 1 mol/L trace metal grade acid while the sample was maintained at 20±1°C in a shaking water bath (Grant OLS 200). Subsequently samples were gently filtered by gravity through an acid cleaned 5 µm filter (47 mm, PC, Whatman) immediately before measurements. Given an average width of ≥20 µm for each filaments, the filter pore size was considered small enough to prevent trichomes passing into the solution, however since the cultures were not axenic it is
possible that some bacteria were present in the filtrate. Fe(II) oxidation experiments were conducted by standard addition of appropriate volumes of 1 µmol/L Fe(II) stock solution to give final concentrations of 5, 10 and 20 nmol/L Fe(II) in 15 mL aliquots of filtered samples. At a salinity of 35 and at 20°C the O$_2$ concentration in the samples was calculated to be 0.225 mmol L$^{-1}$ (Garcia and Gordon 1992), assuming 100% saturation at continuous stirring at 120 rpm using an acid cleaned Teflon-coated magnetic stirrer during the entire experiment (Rose and Waite 2002). The changes in [Fe(II)$_T$] (the sum of both inorganic 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 (10.3). Accordingly, sample and the reagent are mixed in a spiral-shaped flow cell positioned beneath a photomultiplier tube (PMT) using a peristaltic pump with a flux of 1 mL/min (Rose and Waite 2002). This reaction results in the production of chemiluminescence at 426 nm. The emitted photons were collected over a fixed time period of 600 s and recorded in 1 s intervals.

To account for potential effects of EDTA or other components of the culture media on Fe(II) oxidation rates, two different blank treatments (without Fe(II) additions) were analysed for baseline correction in the samples: (1) ASW containing the same nutrient (i.e. vitamins, trace metals, FeCl$_3$ and phosphorus) and EDTA concentrations as in the samples, which provides the background signal in the absence of organic exudates; and (2) culture samples which were filtered at least ~5 h prior to analysis and kept in the dark (wrapped in aluminium foil) to ensure that all pre-existing Fe(II)/Fe(II)species were fully oxidized.

The system was calibrated directly from the experimental data based on the standard addition of known Fe(II) concentrations (5, 10 and 20 nmol/L) to the samples in each experiment. All the measurements were performed in duplicate and then the highest and lowest rate constant values were removed from each data set as potential outliers which gave a minimum of four replicates. Mean values were then considered for data analysis. The FeLume signals were
proportional to Fe(II) concentrations at a particular time. In the continuous flow system, the first reliable measurements of Fe(II) concentration cannot be made until ~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 oxidize. 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 log (signal) versus time (see Appendix 2) (Rose and Waite 2003a). Calibration curves were prepared by plotting log [Fe(II)] versus log (signal), and a linear trend ($R^2 > 0.9$) on the log-log plot (i.e. a power law function) was observed in all cases. The detection limit was ~0.5 nmol/L based on three times the calculated Fe(II) concentrations in the blanks.

2.4. Analysis of Fe(II) oxidation kinetics

In this study, we assumed that Fe(II) is mainly oxidized by $O_2$ due to its presence at a much higher concentration than reactive oxygen species. This is reasonable given that with the relatively low (at most 20 nmol/L) Fe(II) concentrations used, at most 10 nmol/L $O_2^-$ and at most 5 nmol/L $H_2O_2$ could potentially accumulate due to reaction of Fe(II) with oxygen, 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 Fe(II)EDTA, as well as $H_2O_2$ and other organically complexed Fe(II) 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 hydrogen peroxide are needed to outcompete $O_2$ and affect the oxidation rate, therefore here only the reaction between Fe(II) and $O_2$ was assumed to be important. The kinetics of this reaction are represented by the equation:

$$\frac{d[Fe(II)]}{dt} = -k[Fe(II)][O_2]$$

(3.1)
where $k$ is a second order rate constant for the reaction involving the two species Fe(II) and O$_2$. However, since oxygen was present at much higher concentration than Fe(II) under these experimental conditions, its concentration can be considered to be constant. Thus letting

$$k' = k[O_2]$$  \hspace{1cm} (3.2)

then equation (3.1) can be written in the form of a first order reaction, where $k'$ represents a pseudo first order rate constant.

$$\frac{d[Fe(II)]}{dt} = -k'[Fe(II)]$$  \hspace{1cm} (3.3)

The solution to equation (3.3) is:

$$[Fe(II)] = [Fe(II)]_0 e^{-k't}$$  \hspace{1cm} (3.4)

which can also be written as:

$$\ln([Fe(II)]) = \ln([Fe(II)]_0) - k't$$  \hspace{1cm} (3.5)

where $[Fe(II)]_0$ is the initial Fe(II) concentration. The pseudo-first order rate constant can therefore be determined from linear regression of $\ln[Fe(II)]$ against time. In the first instance, the kinetics of Fe(II) oxidation were therefore analysed by this approach using Microsoft Excel.

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). In the present study, however, since the oxidation of organically complexed Fe(II) was affected by both EDTA and organic exudates (L), a three-step kinetic modeling 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 oxygen in ASW matrix.

(ii) Considering two more reactions, namely the complexation of Fe(II) by EDTA and subsequent oxidation of the Fe(II)EDTA by oxygen, which allowed determination of the oxidation rate constant for Fe(II)EDTA complex in ASW; and
(iii) Similarly, addition of two more reactions accounting for Fe(II)L complexation and its subsequent oxidation in the presence of organic ligand(s) (of unknown type and concentration) in the exudates.

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.
3. **Results and discussion**

3.1. *Growth rates in the cultures*

Growth rates are shown in Figure 3.1, illustrating that the culture containing a lower Fe’ concentration (squares) exhibited a lower growth rate than the culture with a higher Fe’ concentration. However, the biomass carrying capacity for the cultures containing higher EDTA concentrations was remarkably higher due to the absence of Fe precipitation, which was not the case for the other culture. The slope of a linear fit to log(cell density) versus time during exponential phase was considered as the growth rate. The observation of a significant difference between biomass under conditions in which the Fe concentration was the only variable in the cultures implies two well-established different Fe-limited conditions. The average observed growth rate of 0.35 d⁻¹ in this study is in the mid-range of values reported in other studies under Fe-limited conditions (Berman-Frank et al. 2001; Chappell and Webb 2010; Kustka et al. 2003; Shi et al. 2012).

![Figure 3.1. Comparison between growth rates of cells grown in the presence of different concentrations of unchelated Fe. The filled circles and open squares correspond to Fe’ concentrations of 1.59 and 0.1 nmol/L, respectively. Growth rates (µ, in units of d⁻¹) have been calculated as described in Appendix 2 and are annotated on the plot for each curve.](image-url)
3.2. Fe(II) oxidation kinetics in the absence of organic exudates

Formation of Fe(II)-EDTA complexes has previously been observed to enhance the Fe(II) oxidation rate (Santana-Casiano et al. 2000). This may result from a higher propensity for oxygen to react with Fe(II)L complexes rather than inorganic Fe(II) (Voelker and Sulzberger 1996). Similarly, we observed a significant difference \( (p < 0.01) \) between the slope of \( \log([\text{Fe(II)}]) \) vs time (over the period 110-530 s) in the presence of varying EDTA treatments (0, 0.05 and 20 µmol/L) using a general one-way analysis of variance (ANOVA) (data not shown). However, due to using much lower EDTA concentrations in this study, as well as differences between the ionic strength of the media used, a lesser influence of EDTA on Fe(II) oxidation rate was observed compared to Santana-Casiano et al. (2000). Figure 3.2 shows a comparison between pseudo first order Fe(II) oxidation rate constants obtained from linear regression of \( \log([\text{Fe(II)}]) \) vs time based on the first (110-170 s) and last (480-530 s) sixty seconds of data for each EDTA treatment. The ANOVA revealed no significant difference between rate constants for the first and last 60 s of data for each treatment, indicating the assumption of pseudo first order kinetics in the media was valid in the absence of organic exudates.

![Figure 3.2. Comparison between pseudo first order rate constants obtained from the first and the last 60 s of measurement for ASW containing different EDTA concentrations. The control is ASW without EDTA addition. Error bars represent the standard deviation of four replicates.](image-url)
To more accurately determine rate constants, the oxidation of nanomolar concentrations of added Fe(II) in ASW was modeled in both the presence and the absence of EDTA (Figure 3.3). 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$_2^*$ was ignored. Based on kinetic modeling, the second order oxidation rate constant of inorganic Fe(II) over a range of initial Fe(II) concentrations (5, 10 and 20 nmol/L) was determined to be 5.94 ±0.03 M$^{-1}$s$^{-1}$ at pH 8.0 and 20±1°C which was in agreement with values reported in previous studies ranging from 2.2 to 8.5 M$^{-1}$s$^{-1}$ (Millero et al. 1987; Murray and Gill 1978; Waite and Morel 1984). This value was used for all subsequent modeling of Fe(II) oxidation in the presence of EDTA and organic exudates.
Figure 3.3. Oxidation of Fe(II) in ASW containing (a) no EDTA, (b) 50 nmol/L EDTA and (c) 20 µmol/L 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.
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 (Figure 3.2) 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 2003a). 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 rate constant for oxidation of Fe(II)EDTA in seawater, 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 modeling purposes. There is little information about the kinetics of formation and dissociation of Fe(II)EDTA in seawater (Fujii et al. 2010b; Santana-Casiano et al. 2000). 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, 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. Therefore an apparent stability constant $(K = k_f/k_d)$ of 16200 M$^{-1}$ was obtained for Fe(II)EDTA in seawater at pH 8.0 and 20±1°C.

### 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 assuming pseudo-first order oxidation kinetics by linear regression of log([Fe(II)]) against time in Microsoft Excel. Pearson moment correlation coefficients ($R^2 > 0.98$) values were 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 filtrate compared to ASW containing EDTA (Figures 3.4 and 3.5). Similarly, acceleration of Fe(II) oxidation in the presence of various kinds of terrestrial originated organic matter had been previously noted in some studies (Millero et al. 1987; Rose and Waite 2002; Rose and Waite 2003a; Santana-Casiano et al. 2000).

**Figure 3.4.** Changes in Fe(II) oxidation rate at different times during growth of the culture in the presence of organic exudates produced in the cultures grown with 1.59 nmol/L Fe concentration. The shaded area shows the range determined for 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.
**Figure 3.5.** Changes in Fe(II) oxidation rate at different times during growth of the culture in the presence of organic exudates produced in the culture grown with 0.1 nmol/L Fe´ concentration. The shaded area shows the range determined for 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.

To investigate the assumption of 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 the organic ligand concentration ([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 ($k_d = 0$). The alternative possibility that Fe(II)L oxidation was very rapid was also investigated. These correspond to the two most likely possible limiting scenarios under which oxidation would be pseudo-first order (Rose and Waite 2003a). In both cases, the ligand concentration was thus arbitrarily assumed to be 1 µmol/L then either the rate constant for oxidation of the complex, $k_{ox}$, was set to an arbitrarily large
value and $k_f$ fitted, or $k_f$ 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 (see Appendix 2). 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 modeling.

An average second order rate constant of $9.60 \pm 1.97 \ M^{-1}s^{-1}$ for $k_{ox}$ was calculated from the model fit to the data, given that statistical analysis revealed no significant difference ($p > 0.97$) between the rate constants for exponentially growing cultures between days 2-8 and 2-15 at 1.59 and 0.1 nmol/L Fe’ concentrations, respectively. Comparing this value with the earlier rate constant obtained for oxidation of inorganic Fe(II) and the rate constant reported by Fujii et al. (2010b) for oxidation of Fe(II)EDTA suggests that IMS101 organic exudates oxidise Fe(II) at a rate 38% faster than inorganic Fe(II), but 25% slower than Fe(II)EDTA complexes.

In comparison, there are a few studies showing a decrease in inorganic 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, respectively, values of apparent oxidation rate constants ($k_{app}$) of 1.09 M$^{-1}$s$^{-1}$ and 6.9 M$^{-1}$s$^{-1}$ in the presence of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* (Gonzalez et al. 2014; Santana-Casiano et al. 2014). 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, temperature, dissolved oxygen concentration and measuring approach, 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 the possibility that Trichodesmium produces a different class of dissolved organic matter which enhances Fe(II) oxidation. This enhancement could be explained by Marcus theory (Marcus, 1964) which implies that as a result of the outer-sphere electron transfer from $O_2$ to Fe(II)/Fe(II)L during the oxidation process, Fe(II)L
complexes are dissociated resulting in subsequent Fe(III) formation. This theory also implies that the rate of electron transfer between O₂ and Fe(II)L is proportional to the ratio of the stability constants (i.e. formation constant) for Fe(III)L and Fe(II)L complexes. Meaning that the accelerant in Fe(II) oxidation rate results from a higher prosperity for IMS101 exudates to form more stable complexes with Fe(III) than Fe(II). However, the observation of pseudo first order reaction kinetics and similar oxidation rate constants for the cultures grown with different Fe´ concentrations suggest that a single type of Fe(II) complex forms in the presence of exudates. Moreover, measurement of a lower oxidation rate constant 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

Figures 3.6 and 3.7 show variation in 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. Consistent with the oxidation data (Figure 3.4 and 3.5), these results showed a significant (p <0.01) (difference between oxidation rate constants in the presence and in the absence of organic exudates. The figures also show a decrease in oxidation rate constants when the growth rate tends to level off towards the stationary phase (i.e. approaches µ=0).
Figure 3.6. Relationship between growth rate and Fe(II) oxidation rate constant in the culture grown with 1.59 nmol/L Fe\(^-\) concentration. The triangles, shaded area illustrate mean of 4 replicates and background, respectively. To elucidate the difference between the slopes during positive and negative growths, two separate regressions were considered (solid lines). Each symbol represents the mean ± standard deviation of four replicate determinations.

Figure 3.7. Relationship between growth rate and Fe(II) oxidation rate constant in the culture grown with 0.1 nmol/L Fe\(^-\) concentration. The triangles, solid line and shaded area illustrate mean of 4 replicates, linear regression and background, respectively. Each symbol represents the mean ± standard deviation of four replicate determinations.
These results are consistent with previous studies implying that exponentially growing cells could actively influence the redox state of Fe in their surroundings by releasing dissolved organic matter (Gonzalez et al. 2014; Santana-Casiano et al. 2014). Fe complexes formed in the presence of exudates may not necessarily be with specific Fe binding ligands such as siderophores or porphyrins, as other metabolic products such as carbohydrates and proteins are much more abundant and positively correlated with cell density (Gonzalez et al. 2014; Hassler et al. 2011a). However, despite an expected increase in DOC concentration towards the end of exponential growth phase, an average (and relatively constant) 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 Trichodesmium might produce some different form of organic matter (Achilles et al. 2003; Hutchins et al. 1999) compared with diatoms (Gonzalez et al. 2012; Rijkenberg et al. 2008; Steigenberg et al. 2009) and green algae (Gonzalez et al. 2014) which accelerates 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. The oxidation data in the presence of exudates were well described using a pseudo-first model, meaning that the concentrations of organic Fe binding ligands were higher than the maximum concentration of Fe added (20 nmol/L). This suggests that most likely the ligands 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 could be significantly different 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 characterization of the organic matter released by this microorganism.
Chapter 3

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The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater

Chapter 3


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Chapter 4
Extracellular superoxide production and its potential to influence Fe reduction in cultures of *Trichodesmium*

**Abstract**

Extracellular production of superoxide has been documented in both prokaryotic and eukaryotic microorganisms. While the role of superoxide in mediating many physiological and biochemical processes has been well-established, its contribution to Fe redox speciation in seawater and therefore potential pathways for cellular Fe acquisition is still not clear. In this study, an attempt was made initially to measure extracellular superoxide production and Fe(III) reduction rates in *Trichodesmium* cultures grown with varying Fe' concentrations at close to natural cell densities using the Methyl Cypridina Luciferin Analogue (MCLA) and ferrozine techniques. An extracellular superoxide production rate of 4.06 fmol/cell/h and Fe(III) reduction rate of ≤5 amol Fe(III)/cell/h were measured in exponentially growing cultures of *Trichodesmium*. The results are in good agreement with previously reported values for the marine diatoms *Thalassiosira weissflogii* and *Thalassiosira pseudonana*. Despite producing superoxide at relatively high rates, Fe(III) reduction rates and resulting steady-state Fe(II) concentrations were surprisingly low. This finding suggests the involvement of potential reduction pathways or mechanisms that don’t involve superoxide for Fe(III) reduction by *Trichodesmium* in order to generate bioavailable Fe(II) in the extracellular environment.
1. Introduction

Under mildly basic pH conditions, which is the case in the majority of cytoplasmic areas (Salhany et al. 1975) and in many natural surface waters, superoxide ($O_2^{•−}$) exists at concentrations several orders of magnitude higher than other reactive oxygen species (Rose 2012). In aquatic microorganisms, superoxide is naturally generated by both eukaryotes and prokaryotes (Diaz et al. 2013; Kustka et al. 2005; Marshall et al. 2005; Rose et al. 2005) during the light-dark cycle as a by-product of respiration and photosynthesis (Hansel et al. 2016). Cells exploit $O_2^{•−}$ as a co-factor in many biological processes such as: (a) for physiological purposes during signaling or cellular proliferation and differentiation (Hancock et al. 2001; Tsukagoshi et al. 2010); (b) enzymatically mediated bioreduction of metals in the cell membrane (Kim et al. 2000; Kustka et al. 2005; Lovley 1993; Saragosti et al. 2010); (c) degradation of carbonic compounds including lignin and organic pollutants (Goldstone and Voelker 2000; Hansel et al. 2016); (d) immune defence against oxidative stress and invading microorganisms (Babior et al. 1973; Fridovich 1998); and (e) extracellular biochemical cycling of trace metals (Hansard et al. 2011; Li et al. 2014; Voelker et al. 2000).

Within the last two decades, increasing attention has been given to redox active extracellular electron transport compounds, termed electron shuttles, and their role in dissolution of poorly soluble iron (Fe) minerals in various sedimentary environments. Humic quinone moieties (Klüpfel et al. 2014; Lovley et al. 1996; Newman and Kolter 2000; Scott et al. 1998) and superoxide (Du et al. 1998; Huycke et al. 2001; Rose 2012) are the most frequently cited compounds for use as electron shuttles in both oxic and anoxic conditions. In contrast to the anoxic conditions which are a dominant feature of sediments (Klüpfel et al. 2014), in the ocean surface layer the redox potentials of the $O_2/O_2^{•−}$ and Fe(III)/Fe(II) species are closely tied, and the relationship between these two redox couples has been frequently considered in conceptual models of Fe acquisition by phytoplankton. In these models, enzymatically produced $O_2^{•−}$ at the cell surface could reduce stable but less soluble Fe(III) (chelated and unchelated) to Fe(II),
which is thought to be readily taken up by the cells (Fujii et al. 2010; Kranzler et al. 2011; Morel et al. 2008; Salmon et al. 2006). Despite several O$_2$•− production rate measurements in a variety of microorganisms (Diaz et al. 2013; Garg et al. 2007; Godrant et al. 2009; Kustka et al. 2005; Rose et al. 2005), its potential to aid Fe uptake by supplying Fe(II) to Fe-stressed cells is still not clear. Rose et al. (2005) observed a clear decrease in Fe(II) uptake rates by the coastal diazotroph Lyngbya majuscula, when superoxide production was inhibited, but such links between extracellular superoxide production and Fe uptake are not always clear.

The marine diazotrophic cyanobacterium, Trichodesmium spp., has been shown capable of producing extracellular O$_2$•− from both free trichomes and colonies (Godrant et al. 2009; Hansel et al. 2016). In the ocean, it is assumed that Trichodesmium blooms initially start with exponentially growing single trichomes and subsequently form colonies as a response to nutrient deprivation (most likely Fe) (Bell et al. 2005; Berman-Frank et al. 2007). Rubin et al. (2011) and Hansel et al. (2016) showed that there is a correlation between the shape and size of colonies, the dissolution rate of Fe(III) minerals, and superoxide production in naturally occurring Trichodesmium. The colony organization may also be partly attributed to spatial and temporal segregation of nitrogen (N$_2$) and carbon fixation during the photoperiod as a self-regulatory strategy that alleviates inhibitory effects of O$_2$ on nitrogenase activity (Berman-Frank et al. 2007). Colonies, however, rarely occur within Trichodesmium cultures (Hansel et al. 2016), where Fe is most often provided in the form of organically complexed ferric iron such as various complexes between Fe(III) and EDTA (Sunda and Huntsman 2003), although clear evidence for a causal connection is missing. Nevertheless, in cultures, depending upon the amount of ligand-bound Fe added, growth usually occurs as a function of the concentration of non-chelated Fe ([Fe']) (Dang et al. 2012), but biomass can be limited by total dissolved Fe or typically by another macro nutrient (for diazotrophic microorganisms, usually phosphorus) (Ihssen and Egli 2004; Sañudo-Wilhelmy et al. 2001).
The aim of this study was to measure extracellular production rates of superoxide and subsequently evaluate its potential to reduce Fe(III) in *Trichodesmium* cultures grown with different concentrations of Fe'. To do so, an optimised Methyl Cypridina Luciferin Analogue (MCLA) chemiluminescence technique and the ferrozine method were employed, respectively, to measure production rates of extracellular superoxide and Fe(II) in the cultures. The findings were expected to provide a better understanding of the role of cell generated O$_2$•− in regulating Fe(II) concentrations in the extracellular environment and its potential effects on mechanisms of Fe uptake in *Trichodesmium*. 
2. Experimental

2.1. Reagents and solutions

Stock solutions of 125 µmol/L Methyl Cypridina Luciferin Analogue (MCLA, Sigma-Aldrich), 3 kU/mL superoxide dismutase from bovine erythrocytes (SOD, Sigma-Aldrich), and 1 mmol/L SOTS-1 (superoxide thermal source, Cayman Chemicals) were prepared soon after having received these chemicals, stored frozen at −80°C and then thawed at room temperature just before use. A secondary SOTS-1 stock solution was prepared by 10-fold dilution of 1 mmol/L SOTS-1 in artificial seawater (ASW) that was previously treated with a small amount of SOD (~1 nmol/L) that can’t outcompete the MCLA for superoxide produced by the organism and therefore doesn’t affect the chemiluminescence signal but also is able to remove any pre-existing $O_2^-$. The ASW treated with SOD was then aged in the dark for at least a month at room temperature. A stock solution of 3 mmol/L diethylenetriaminepentaacetic acid (DTPA, Sigma-Aldrich) was also prepared and stored at room temperature. All primary solutions mentioned above were prepared in Milli-Q water (18.2 MΩ·cm resistivity from a Milli-Q Academic Water Purification System, hereafter denoted as MQ) as the only solvent, as the organic solvent DMSO, which has previously been used to aid dissolution (Heller and Croot 2010), was found to interfere with measurements. Instead, the dissolution of SOTS-1 was facilitated using a short (30 s) ultrasonic treatment, unless otherwise stated. A stock solution of 250 µmol/L red-CLA (TCI scientific) was prepared in 50% (vol/vol) ethanol/MQ.

Two different solutions of ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p’-disulfonylic acid monosodium salt hydrate, FZ) were also prepared for calibration purposes and measurements of Fe(II) production rates in the cultures. For the solution used in calibrations, initially a 5 mol/L ammonium acetate (NH$_4$Ac) buffer was prepared by dissolving 134 g of NH$_4$Ac salt (C$_2$H$_3$O$_2$NH$_4$, Ajax Finechem Pty Ltd) in 187 mL of glacial acetic acid (Ajax Finechem Pty Ltd), and making up to 1 L with MQ in a volumetric flask. Subsequently, 2 mmol/L FZ solution was made by the addition of 20 mg ferrozine salt into 20 mL of 5 mol/L
The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater

NH₄Ac at pH 4.5. For Fe(II) production rate measurements in the cultures, another FZ solution (200 mmol/L) was prepared at pH 8 in a 1 mmol/L NaHCO₃ solution (by dissolution of 0.081 mg NaHCO₃ salt in 10 mL MQ) buffered at pH 8 by dropwise additions of 2 mol/L NaOH. For calibration standards, a 5 mmol/L Fe(II) stock solution was prepared by dissolving 196 mg of FeSO₄.2H₂O (Ajax Finechem Pty Ltd) in 50 mL of MQ that had been acidified with 83 µL of 12 M HCl acid (34-37% w/w, Instrument Quality, SEASTAR CHEMICALS Inc). The pH measurements were carried out with a Hach pH meter and the electrode was calibrated with pH 4, 7 and 10 buffers before use.

2.2. Culture conditions and cell density and growth monitoring

Unialgal batch cultures of *Trichodesmium erythraeum*, strain IMS101 (NCMA; Bigelow) were established in YBCII media, an artificial seawater matrix (Andersen 2005) modified to contain 2.1 mmol/L NaHCO₃ and 2 µmol/L KH₂PO₄ and either 0.05 or 20 µmol/L EDTA, and 10 or 155 nmol/L FeCl₃, (see Table 4.1 for details on the EDTA/Fe combinations). The stock cultures (2.5 L) were maintained under a 14:10-h light-dark cycle with 110 µmol quanta m⁻²s⁻¹ (measured using a Li-COR light meter) at 27°C in a standard incubator. Fe⁺ concentrations were calculated from total Fe(III) and EDTA concentrations, using an equilibrium complexation model at a culture pH range from 8.1 to 8.6 (i.e. the measured pH range throughout the entire growth cycle), taking into account the inorganically complexed forms of Fe³⁺ with Cl⁻, F⁻, SO₄²⁻ and OH⁻, as well as EDTA complexation with Fe³⁺, Cu²⁺, Co²⁺, Mn²⁺, Zn²⁺, Ca²⁺ and Mg²⁺ (Schulz et al. 2004). All measurements were conducted on the NBS scale and assumed to equal the free scale with an uncertainty of about 0.005 pH units (Lewis et al. 1998).

During acclimatisation of the cultures, exponentially growing cells were inoculated into fresh media for at least 12 generations prior to use in the experiments. Cell densities and growth rates in cultures grown at different Fe⁺ concentrations were monitored using a Malvern Morphologi G3 particle characterisation system (See Appendix 1). To obtain sufficient cell density for
superoxide measurements, in one set of experiments 200 mL of exponentially growing culture water was gently filtered by gravity through a 5 μm pore size 47 mm diameter polycarbonate filter until the volume was reduced to 20 mL.

**Table 4.1.** Concentrations of added EDTA and total Fe ([Fe\text{T}]) with corresponding calculated concentrations of dissolved non-chelated iron(III) ([Fe\text{−}]), FeEDTA complexes (FeL) and precipitated Fe ([Fe(s)]) in the pH range from 8.1-8.6 (on the free hydrogen ion scale).

<table>
<thead>
<tr>
<th>EDTA (µmol/L)</th>
<th>[Fe\text{T}] (nmol/L)</th>
<th>[Fe\text{−}] (nmol/L) (^a)</th>
<th>FeL (nmol/L)</th>
<th>[Fe(s)] (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>10</td>
<td>1.44-2.03</td>
<td>0.05-0.01</td>
<td>8.51-7.96</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>0.05-0.29</td>
<td>9.95-9.7</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>155</td>
<td>0.78-2.02</td>
<td>154-66.9</td>
<td>0-3.10</td>
</tr>
</tbody>
</table>

\(^a\) Fe\text{−} concentrations are reported as a range corresponding to pH varying from 8.1-8.6, representing the change in pH in the culture during the entire growth cycle.

**2.3. Procedure for superoxide measurements in the cultures**

The O\text{2−} production rate in the cultures was assessed at 27°C, the culturing temperature, using an optimized MCLA method. Although similar to that used by Godrant et al. (2009), some parts of the procedure used here were modified to improve its performance under our experimental conditions. However, the general procedure involves reacting superoxide released by the cells with the chemiluminescence (CL) probe MCLA, resulting in continuous CL emission which was detected by a Fluostar Optima microplate reader (BMG Labtech). Unlike prior calibration procedures using the xanthine/xanthine-oxidase system, an optimized calibration method using SOTS-1 was utilized in ASW, both in the presence and the absence of cells (Heller and Croot 2010). Calibrations were performed by additions of known aliquots (2, 4, 10, 20 µL) of the secondary SOTS-1 solution to the sample matrix and correlating the CL signals with the calculated O\text{2−} production rates with repeated measurements over a time period of 24 min (see
below for details). In this method, the thermal decomposition of SOTS-1 mediates the reduction of oxygen to produce superoxide. Separate calibrations were performed in ASW and culture samples including cells, resulting in a linear trend in all cases. Calibration curves accounted for the time needed for preparation or thawing of SOTS-1 (half-life of about 280 min at 27°C) before the addition to the samples and also the measurement time.

The decomposition of SOTS-1 in seawater has been shown to follow pseudo-first order decay with:

\[
[SOTS](t) = [SOTS]_i \times e^{\left(\frac{-t}{\tau}\right)} \tag{4.1}
\]

where \([SOTS](t)\) represents the concentration of SOTS-1 (nmol/L) remaining at time \(t\) (s) after the addition of an initial amount of SOTS-1 ([SOTS]_i) and \(\tau\) is the mean lifetime or exponential time constant, equal to \(1/k\) (where \(k\) is the first order decay rate constant). As decay of SOTS-1 is a thermal process, the superoxide production rate is highly temperature dependent. Heller et al. (2010) established a linear relationship between \(1/T\) and \(\ln(k/T)\), with \(T\) being the temperature in Celcius. Thus, \(k\) was calculated for 27°C, the incubation and measurement temperature, to be \(4.07 \times 10^{-5}\) s\(^{-1}\), corresponding to a values of \(\tau = 409\) min.

The \(O_2^{-}\) production rate was computed based on the equation below, assuming a 40% superoxide production efficiency by SOTS-1 (Heller and Croot 2010).

\[
O_2^{-} \text{ production rate} = [SOTS](t) \times 0.4 \tag{4.2}
\]

Accordingly, net superoxide production rates were obtained by subtracting the \(O_2^{-}\) production rate of the background (i.e. only media in the absence of cells) from measured values in the cultures (i.e. in the presence of cells). The layout of the microplate (96 wells) for each run was the same, consisting of background media or culture (without SOD or SOTS-1), the blank (background media or culture with SOD) and the standards (background or culture with SOTS-1 additions). The background media was ASW containing trace metals and different EDTA concentrations (depending on culture conditions). Inclusion of the trace metal mix in the
background was important as some metals (e.g. Cu and Mn) can act as a sink for $O_2^{−}\cdot$ (Heller and Croot 2010). Phosphorus and vitamins were excluded to prevent bacterial growth, while aging background media, as needed to remove pre-existing $O_2^{−}\cdot$ (Heller and Croot 2010).

To obtain maximum signal intensity and therefore an optimal detection limit, the microplate reader acquisition settings were adjusted to a gain setting of 3600 with no wavelength filter applied, thus assuming that the detected luminescence was from MCLA only. Additionally, certain precautions were necessary to minimize the standard deviations between replicates and contamination possibilities during sample preparation:

(i) 10 µL of DTPA stock solution was dispensed at the bottom of each well (yielding $[\text{DTPA}]_{\text{final}} = 100 \, \text{µmol/L}$) to chelate trace metals that would otherwise be capable of acting as a sink for superoxide (Hansel et al. 2016).

(ii) Measurements were performed in triplicates for the blanks (SOD added), samples (no SOTS-1 additions) and standards (SOTS-1 additions). The layout of the microplate containing the culture samples was identical to the one containing the background media. To avoid cross-contamination, two separate plates were used, and the plates were washed with detergent (Decon 90), acid (1 mol/L HCl) and rinsed with MQ and dried immediately after use. The final sample volume for each well was 300 µL and therefore the volumes of culture or background media added were adjusted to compensate for varying amounts of reagents added (SOTS-1 and SOD).

(iii) 5 µL of the SOD stock solution was added to one set of wells (culture or background media) (yielding $[\text{SOD}]_{\text{final}} = 50 \, \text{kU/L}$) to remove any superoxide and establish a baseline signal. The baseline signal, termed blanks, consists of background CL produced by auto-oxidation of MCLA (Fujimori et al. 1993; Hansard et al. 2010; Hansel et al. 2016). The blank signals were subtracted from sample signals to calculate net superoxide production rates.

(iv) For calibration, 2, 4, 10 and 20 µL of secondary SOTS-1 solution (0.1 mmol/L) was
added to samples to obtain final concentrations of 0.67, 1.33, 3.33 and 6.67 µmol/L.

(v) 6 µL of MCLA stock solution was injected automatically by the plate reader into the wells (yielding [MCLA]$_{\text{final}}$ = 2.5 µmol/L) and the emitted CL was measured for 2 s per well, over 6 cycles being 4 min apart (total measurement time of 24 min). For comparison, 12 µL of red-CLA (250 µmol/L) stock solution was also used during initial testing.

2.4. Fe(II) production rate measurements

Fe(II) production rates were determined spectrophotometrically both in the presence of cells (i.e. in culture samples) and in the absence of cells (i.e. in filtrates after passing culture samples through 0.2 µm PES syringe filters) using the ferrozine (FZ) method (for a more detailed FZ method description see Chapter 2). To avoid filter clogging and minimise the potential for rupture of cells, the sample was gently passed through a new filter using a syringe at a very slow flow rate. To do so, 1 µL of a ferrozine stock solution (200 mmol/L) was added per mL of culture or filtrate sample (yielding [FZ]$_{\text{final}}$ = 200 µmol/L), which were then kept in the incubator under the same light and temperature conditions used for culture maintenance. Subsequently, duplicate aliquots of 2 mL were taken from each batch at selected time intervals (0.5, 1.5, 3.5, 5, and 7.5 h) and the Fe(II) concentration determined by absorbance measurements of the Fe(II)FZ complex at 560-565 nm in a spectrophotometer (Ocean Optics) connected to 1 m Liquid Waveguide Capillary Cell (LWCC; World Precision Instruments) by optical fibers. An extra batch of culture media (ASW containing all nutrients and EDTA) and FZ reagent was simultaneously incubated with the other samples for background correction. All samples were filtered (0.2µm) before loading (via a peristaltic pump at a flux of 1 mL/min) into the LWCC. The system was calibrated on each measurement day by the addition of 100 µL of the ferrozine stock solution (2 mmol/L) per mL of pure ASW (no EDTA or inorganic nutrients) containing a range of six Fe(II) concentrations (5-200 nmol/L), resulting in a linear correlation fit between absorbance and Fe(II) concentration in all cases. Final Fe(II) concentrations in the culture, filtrate and ASW samples were corrected for potential interference
from other compounds (e.g. organic exudates) by subtracting the absorbance of culture, filtrate and ASW samples without the addition of FZ.

2.5. *Fe(II) concentration estimates in steady state based on Fe(III) reduction rate and Fe(II) oxidation kinetic measurements*

At steady state, measured Fe(III) reduction rates will be equal to Fe(II) oxidation rates. Fe(II) oxidation kinetics (see Chapter 3 for details) are represented by:

$$\frac{d[Fe(II)]}{dt} = -k[Fe(II)][O_2] \quad (4.3)$$

where $k$ denotes the second order rate constant for the reaction between the two species Fe(II) and oxygen. Under constant O$_2$ conditions, this equation can be reformulated as:

$$\frac{d[Fe(II)]}{dt} = -k'[Fe(II)] \quad (4.4)$$

with $k' = k[O_2]$ being the apparent pseudo-first order rate constant of Fe(II) oxidation. Given that at steady state the rate of Fe(III) reduction is equal to the Fe(II) oxidation rate under these conditions, equation (4.4) can be rewritten as:

$$\frac{d[Fe(III)]}{dt} = -k'[Fe(II)] \quad (4.5)$$

Thus, Fe(II) concentrations at steady state can be estimated by dividing measured Fe(III) reduction rates, $\frac{d[Fe(III)]}{dt}$, (this Chapter) by measured apparent pseudo-first order rate constants for Fe(II) oxidation, $k'$ (see Chapter 3 for details).

Chemiluminescence and cell counting experiments were carried out on the same day as measurements of Fe(III) reduction rates, allowing to enable direct comparison of both rates.
3. Results and discussion

3.1. Growth rates and cell densities in the cultures

Figure 4.1 illustrates growth rates and carrying capacities at different total Fe and EDTA concentrations (Table 4.1). Growth rates were expected to be related to Fe<sup>-</sup> concentrations while the carrying capacity was expected to be related to total dissolved Fe (or phosphate).

**Figure 4.1.** (a) Cell densities and (b) log-normalized cell densities and resulting growth rates in the cultures grown at different total Fe and EDTA concentrations. Filled triangles and open circles represent the cultures grown with 0.78-2.02 and 1.44-2.03 nmol/L Fe<sup>-</sup> (155 and 10 nmol/L [Fe<sub>T</sub>] with 20 μmol/L and 50 nmol/L of EDTA, respectively) and filled squares represent the culture grown at 0.05-0.24 nmol/L Fe<sup>-</sup> (10 nmol/L [Fe<sub>T</sub>] and 20 μmol/L EDTA). Solid lines depict the linear regressions calculated for the exponential growth phase, with the slope being equal to daily growth rates, µ (annotated on the graph).
At the lowest Fe´ concentration, which ranged from 0.05-0.29 nmol/L (depending on the pH in the culture, which varied during the whole growth cycle), growth rates were lowest at 0.3 d⁻¹ (Figure 4.1b), while increasing to 0.44 d⁻¹ at with Fe´ concentrations of 0.78-2.02 nmol/L and 0.5 d⁻¹ at Fe´ concentrations of 1.44-2.03 nmol/L. This is consistent with previous observations that growth rates in marine phytoplankton are typically related to Fe´ concentrations (the sum of all hydrolysis species and complexes with weak inorganic ligands) rather than to individual iron species such as, for instance, Fe³⁺ (Dang et al. 2012). The lower carrying capacity (maximum cell density in stationary phase) in the culture with the lowest Fe´ concentration is probably connected to lower total dissolved iron concentrations due to significant precipitation (Table 4.1).

3.2. Challenges to detect superoxide production rates in cultures at environmentally-relevant cell densities

The maximum cell densities in exponentially growing cultures (Figure 4.1a) were about one order of magnitude lower than cell densities used in previous studies for superoxide measurements in cyanobacterium cultures grown under Fe sufficient conditions (e.g. 4 × 10⁵ to 4 × 10⁶ cells/mL) (Godrant et al. 2009; Rose et al. 2008). The fact the background signal (in absence of cells) is usually relatively high, which is unavoidable due to MCLA auto-oxidation, resulted in some challenges in measuring superoxide production rates in the cultures when the cell densities were relatively low, as is representative of the natural environment (see Appendix 3 for more detail). As a result, no significant superoxide production rates were observed in background corrected samples throughout exponential growth phase, as shown in Figure 4.2a. To increase the amount of superoxide generated in order to obtain a measureable signal, the cell density in the samples was therefore increased by gentle gravity filtration (see Section 2.2).
Figure 4.2. Relationship between superoxide production rates in the cultures with (a) time and growth phase and (b) cell density. Filled triangles represent the culture grown with 0.78-2.02 nmol/L Fe$^-$ (155 nmol/L Fe$_T$ and 20 μmol/L EDTA), open circles represent the culture grown with 1.44-2.03 nmol/L Fe$^-$ (10 nmol/L Fe$_T$ with 50 nmol/L EDTA), and filled squares represent the culture grown with 0.05-0.24 nmol/L Fe$^-$ (10 nmol/L Fe$_T$ and 20 μmol/L EDTA). Blue symbols depict rates measured in concentrated cultures.

A quenching effect on the MCLA signal intensity has been previously reported when cell/trichome density was increased due to association of the MCLA with cell membranes as a result of its hydrophobic nature (Marshall et al. 2005; Nakano 1998). Quenching was also partly attributed to absorption of light by chlorophyll a, which absorbs in the wavelength range similar
to that emitted by MCLA (Campbell et al. 1998; Phillip et al. 1996). Godrant et al. (2009) suggested replacing MCLA by red-CLA reagent to measure superoxide in dense cultures. Hence, MCLA and red-CLA probes were both tested under the experimental conditions of this study and, although a slightly higher signal was observed in the presence of red-CLA, the difference was not statistically significant ($p > 0.25$). Additionally, using the same instrument settings (no filter, 3600 gain adjustment), MCLA was found to give better results in terms of reproducibility and consistency between replicates (data not shown). Hence, in this study, as in previous studies of *Trichodesmium* colonies (Hansel et al. 2016; Rose et al. 2010), MCLA was used.

### 3.3. Superoxide production rates in exponentially growing cultures at environmentally-relevant cell densities concentrated by gravity filtration

Having been unable to measure significant superoxide production rates throughout the initial 15 d incubation of cultures, addition cultures grown under the same Fe´ conditions were used to prepare 10-fold concentrated samples by gentle gravity filtration. Superoxide production rates were measured at 0.54 and 0.10 and 0.01 nmol/L/s for the three Fe´ ranges (0.05-0.29, 0.78-2.02 and 1.44-2.03 nmol/L, respectively) (blue symbols in Figure 4.2a). The measured rates for the first condition, at least, were significantly greater than zero, most likely because this culture was still in exponential growth phase (at culture cell densities of 50,000 cells/mL with a carrying capacity of about 100,000 cells/mL). In contrast, the two other cultures were already in or just had reached stationary phase. Normalized to cell density, these extracellular superoxide production rates correspond to 4.06, 0.12 and 0.18 fmol/cell/h (for Fe´ ranges of 0.05-0.29, 0.78-2.02 and 1.44-2.03 nmol/L, respectively).

These results are in good agreement with those reported previously (Garg et al. 2007; Godrant 2009), with measurable $O_2^{-}$ production rates for the actively growing cultures (i.e. middle of exponential phase) in comparison to those in late exponential or stationary phase. The regulation of superoxide production during varying growth phases has been previously
observed in some bacteria (Carlioz and Touati 1986; Storz et al. 1987), in which expression of a periplasmic SOD in the outer membrane resulted in a decline in extracellular \( \text{O}_2^- \) concentrations by 80% during stationary phase (Benov and Fridovich 1994; Oda et al. 1995).

Previously, Godrant et al. (2009) and Hansel et al. (2016) reported \( \text{O}_2^- \) production rates of ~1.59 pmol/trichome/h and 11.7 pmol/colony/h for free and aggregated trichomes of *Trichodesmium*, respectively. Considering an average of 88 cells/trichome and 150 trichomes/colony (Godrant et al. 2009; Hansel et al. 2016), the superoxide production rates in these studies would amount to 18.0 and 0.89 fmol/cell/h, respectively, compared to 4.06 fmol/cell/h determined in this study. Considering the differences in culture conditions (temperature, light and micro- and macronutrients, including Fe’ levels) between studies and different procedures used for superoxide production measurements, the cellular superoxide production rate measured in this study falls well within the reported range.

Based on the measured values here and those reported from previously published studies, the extracellular \( \text{O}_2^- \) production rate by *Trichodesmium* (0.89-18.0 fmol/cell/h) can be much higher than that of diatoms (0.84-1.4 fmol/cell/h) (Kustka et al. 2005; Milne et al. 2009; Rose et al. 2008), but is significantly lower than that of the raphidophyte *Chattonella marina* (0.29 to 4 pmol/cell/h) (Garg et al. 2007; Oda et al. 1998). The latter species is, however, suspected of causing mortalities in fish via production of significant amounts of reactive oxygen species during red tide events (Lee et al. 1995; Marshall et al. 2005).

### 3.4. Fe(III) reduction rates in the extracellular milieu of *Trichodesmium* cultures

To test whether *Trichodesmium* cells could impact Fe(III) reduction rates in the extracellular milieu, Fe(II) accumulation rates were measured in both cultures and filtrates in the presence of FZ. Non-significant Fe(II) production rates were measured in ASW and also in samples in the absence of cells (i.e. filtrates) and containing cells (i.e. cultures) within the measurement period of about 8 h (Figure 4.3). Since similar results were observed on different days during the growth cycle, results from only one day are presented here as an example.
Given that the reported detection limit of the FZ method used here of 1 nmol/L (see Chapter 2) is only achievable under optimum conditions, a significant accumulation rate could have been detected with confidence when exceeding 4 times that limit during the incubation period of about eight hours, i.e. exceeding 0.5 nmol/L/h (0.139 pmol/L/s). Thus Fe(II) production rates must have been lower than this value in the cultures for the entire experiment. Using a maximum cell density in the cultures of ~100,000 cells/mL equates to a detection limit of $5 \times 10^{-18}$ mol Fe(III)/cell/h. This is in the range of reported values for marine diatoms and unicellular cyanobacteria from $7 \times 10^{-21}$ to $1 \times 10^{-18}$ mol Fe(III)/cell/h (Kranzler et al. 2011; Kustka et al. 2005; Shaked et al. 2004), although at the upper end.

Finally, the Fe(III) reduction rate detection limit can be used to estimate maximum Fe(II) concentrations at steady state using measured values for the apparent pseudo-first order rate constant for Fe(II) oxidation (see Section 2.5 for details). Based on a maximum rate of 0.139 pmol/L/s for Fe(III) reduction and pseudo-first order oxidation rate constant of $k' = 0.001$ s$^{-1}$, Fe(II) concentrations could be as high as 139 pmol/L. Considering a maximum total iron addition in the cultures of 155 nmol/L, this would constitute < 0.1% of the total dissolved iron pool. Furthermore, considering that this Fe(III) reduction rate estimate is at the higher end, steady state Fe(II) levels are probably even lower.
**Figure 4.3.** Representative Fe(II) accumulation in (a) ASW, (b) filtrate and, (c) culture media on a sample after 7 h of incubation with FZ. Here, ASW samples are considered as control treatments including all media ingredients except the cells. Filled triangles represent the culture grown with 0.78-2.02 nmol/L Fe$^-$ (155 nmol/L Fe$_T$ and 20 μmol/L EDTA), open circles represent the culture grown with 1.44-2.03 nmol/L Fe$^-$ (10 nmol/L Fe$_T$ with 50 nmol/L EDTA),
and filled squares represent the culture grown with 0.05-0.24 nmol/L Fe\(^+\) (10 nmol/L Fe\(_T\) and 20 µmol/L EDTA). The presented data have been also corrected for any possible interferences resulting from coloured compounds (e.g. organic matter) in the media and filtrates before FZ additions. The negative values imply measured Fe(II) concentrations were lower than the detection limit.

4. **Conclusions**

In this study a superoxide production rate of 4.06 fmol/cell/h was measured in exponentially growing cultures of *T. erythraeum*, which is well within the previously reported range of 0.89-18.0 fmol/cell/h (Godrant et al., 2009; Hansel et al., 2016). In parallel measurements, a Fe(III) reduction rate of \(\leq 5 \times 10^{-18}\) mol Fe(III)/cell/h was estimated, which is consistent with previously reported values of 2-8 \(\times 10^{-18}\) mol Fe(III)/cell/h by Roe and Barbeau (2014) for *Trichodesmium* cultures treated with \(^{55}\)Fe and also is well within the upper range of previously reported Fe(III) reduction rates for unicellular microorganisms (Kranzler et al. 2011; Kustka et al. 2005; Shaked et al. 2004). Using the apparent pseudo-first order rate constant for Fe(II) oxidation (k\(^-\)) obtained previously (see Chapter 3) and the Fe(III) reduction rate determined here, a Fe(II) concentration constituting < 0.1% of total Fe was calculated under steady state conditions. The large ratio of measured extracellular superoxide production rates to Fe(III) reduction rates indicates that most of the produced superoxide does not result in Fe(III) reduction, similar to observations for marine diatoms in batch culture (Kustka et al., 2005). The majority of the superoxide produced must therefore be consumed by alternative pathways, presumably by reaction with other compounds (e.g. Cu, Mn and organic matter), at least under culture conditions. Consistent with the findings in Chapter 3 that exudates from *Trichodesmium* cultures accelerate Fe(II) oxidation, the negligible rate of Fe(III) reduction suggests that *Trichodesmium* is unlikely to use a reductive strategy to increase Fe bioavailability under the culture conditions employed, even when its growth is Fe limited.
Literature cited


Godrant, A. 2009. The role of superoxide in iron acquisition by marine phytoplankton. New South Wales, Australia.


Chapter 5
1. Conclusions

The results of the studies in this thesis provide new insights into the biological mediation of Fe speciation in seawater by the cyanobacterium *Trichodesmium erythraeum*, particularly in regards to: (i) measuring total Fe concentrations in plankton cultures in the presence of natural and synthetic organic ligands using a modified ferrozine method; (ii) modification of Fe(II) oxidation rates by organic exudates in Fe limited cultures of *Trichodesmium* as a function of their growth phase; and (iii) extracellular superoxide production and its potential to influence Fe reduction in Fe limited cultures of *Trichodesmium*. The main conclusions are summarized below.

1.1. Chapter 2: Measuring total Fe concentrations in plankton cultures in the presence of synthetic and organic ligands using a modified ferrozine method

The aim of this study was to optimize the performance of the ferrozine method for measuring total dissolved Fe in the presence of high concentrations of organic matter including synthetic ligands (e.g. commercially available ligands such as EDTA) and naturally occurring organic ligands (e.g. DFB and organic exudates released by microorganisms) under typical plankton culture conditions. The major findings of this study are:

(i) Acidification of samples at a pH of about 1 for a minimum duration of 15 days brought about a full recovery of Fe from ligands regardless of their Fe binding strength or concentration.

(ii) Increasing the incubation time for contact between the sample with FZ from ~30 min to 24 h resulted in formation of more stable Fe(FZ)_3 complexes, whereas previous studies have shown that these complexes form rapidly but don’t dissociated readily.
1.2. Chapter 3: Growth phase and organic exudates dependent modification of Fe(II) oxidation rates in Fe limited cultures of *Trichodesmium*

The aim of the studies described in this chapter was to gain insight into the kinetics of Fe(II) oxidation in the presence organic exudates released by *Trichodesmium* cultures grown at different Fe´ concentrations in various growth phases. The major findings of this study are:

(i) Fe(II) oxidation was accelerated in the presence of organic exudates (L) released by *Trichodesmium*, apparently due to formation of Fe(II)L complexes that exhibited pseudo-first order oxidation kinetics with an oxidation rate constant intermediate between rate constants for oxidation of inorganic Fe(II) and organically complexed forms of Fe with EDTA presented in the media.

(ii) The pseudo-first order oxidation rate constants varied significantly between different growth phases of the cultures, with higher values observed when cells were growing exponentially but then decreasing towards the stationary phase.

(iii) Kinetic modeling of the data indicated relatively similar oxidation rate constants for both cultures grown at different Fe´ concentrations.

1.3. Chapter 4: Extracellular superoxide production and its potential to influence Fe reduction in Fe limited cultures of *Trichodesmium*

The aim of the studies described in this chapter was to determine extracellular superoxide production rates in batch cultures of *Trichodesmium* during different growth phases and to evaluate its potential impact on Fe(III) reduction in the extracellular environment. The major findings of this study are:

(i) Superoxide production rate measurements using the MCLA method were highly dependent on cell density and growth phase of the culture.
(ii) Extracellular superoxide production rates were within the previously reported range, and were significantly higher in exponentially growing cells compared to those which were already in or approaching stationary phase.

(iii) Fe(II) production/Fe(III) reduction rates were observed to be lower than the detection limit of the method used in all cases, suggesting a very small Fe(II) concentration (less than 0.1% of the total Fe) under steady state conditions.

(iv) The observation of a low ratio of extracellular Fe(III) reduction to superoxide production suggests some other potential sink pathways for the superoxide produced, such as the reaction with other trace metals (e.g. Cu and Mn) in the *Trichodesmium* culture medium.

2. **Implications of the findings**

This work provides compelling evidence of the connection between the metabolic state of *Trichodesmium* cells (changing with growth phases in batch cultures) and the speciation of Fe in seawater when grown at different total dissolved Fe concentrations and/or different Fe’ concentrations by using EDTA as an added ligand under laboratory conditions. The findings show that *Trichodesmium* is capable of producing a relatively high amount of superoxide and organic exudates in its extracellular environment, potentially enhancing the solubility and dissolution of Fe in the extracellular surroundings, which could potentially increase Fe uptake by the microorganism. However, the oxidation of Fe(II) was accelerated by exudates from *Trichodesmium*, in contrast to other studies reporting a retardation of Fe(II) oxidation in the presence of organic exudates released by some eukaryotic microorganisms. This suggests that cyanobacteria likely exploit different Fe uptake pathways compared to eukaryotic microorganisms. Furthermore, calculation of an almost negligible steady-state Fe(II) concentration in cultures, despite the fact that they were actively produced extracellular superoxide at substantial rates, implies that the role of cell-mediated redox reactions in the extracellular milieu of *Trichodesmium* was unlikely to have been significant in terms of
assisting Fe uptake, at least under the culture conditions used. Whether or not this is also the case in the real oceans requires further investigation, given the differences in conditions compared to those in laboratory cultures.

3. Recommendations for future work

In order to further improve our understanding of the influence of *T. erythraeum* on Fe speciation in natural waters, the following additional studies are suggested:

(i) In Chapter 2, it was shown that measured total Fe concentrations in *Trichodesmium* cultures were much less after filtration, suggesting that considerable amounts of Fe in the cultures was present in particulate forms. However, there is still a lack of knowledge about the precise nature of these particulate forms, and in particular how much of this Fe is associated with the cells themselves or present as colloids in the extracellular environment.

(ii) In Chapter 3, it was shown that Fe(II) oxidation was accelerated in the presence of organic exudates released by *Trichodesmium* cultures grown at different Fe´ concentrations, seemingly independent of cell density during exponential phase, but with decreasing importance as the cultures approached stationary phase. While our results suggest possible production of a weak class of Fe binding ligands by *Trichodesmium*, there are few studies that have characterized organic exudates in cyanobacteria cultures, and in particular those released by *Trichodesmium*.

(iii) In Chapter 4, extracellular superoxide and Fe(II) measurements were challenging at the low, yet environmentally relevant, cell densities in the cultures. It would also be worthwhile to perform a similar experiment using both concentrated culture samples and filtrates at different Fe´ concentration and growth phases, potentially using a longer (500 cm) LWCC, and/or employing more sensitive methods such as isotopic labelling of Fe.
Appendix 1
1. General protocols

1.1. Cleaning procedures

All plasticware (polycarbonate if not stated otherwise) was rigorously cleaned prior to use as follows:

1) Plastic flasks were first cleaned in 50% Decon 90, subsequently rinsed with tap water and Milli-Q water (18.2 MΩ.cm resistance from a Milli-Q Academic Water Purification System, hereafter denoted as MQ) three times each, then filled to the brim with 1 mol/L HCl (prepared from 32% w/w HCl, Analytical Grade, Ajax Finechem), capped and stored for 3 d.

2) Afterwards, flasks were emptied, transferred to a clean room supplied with HEPA-filtered air (>99.9% of 0.3 μm particles retained), rinsed with MQ three times, filled with 0.02 mol/L HCl (prepared from 34-37% w/w HCl, Instrument Quality, SEASTAR CHEMICALS Inc, hereafter denoted as trace metal grade acid) and capped.

3) After 3 d, flasks were emptied and rinsed with high purity Milli-Q water (18.2 MΩ.cm resistivity from a Milli-Q Element Water Purification System installed in a clean room supplied with HEPA-filtered, hereafter denoted as HPMQ) five times and then were filled with 0.01 mol/L trace metal grade acid and stored for an additional 3 d.

4) Finally, flasks were emptied and rinsed with HPMQ five times and dried under a laminar flow hood for 24 h, capped tightly and kept in double plastic bags until use.

5) Syringe filters (0.45 μm pore size, Minisart® High Flow with poly ethersulfone membrane) were cleaned by passing through 1 mol/L trace metal grade acid three times and then HPMQ six times immediately before use. Membrane filters (0.22 μm pore size, 47 mm diameter, polycarbonate, Whatman) were cleaned by soaking for at least 3 d in a similar manner. Pump tubing and the LWCC were cleaned before use based on
standard cleaning procedure (Floge et al. 2009), and by flushing the system with 1 mol/L HCl and HPMQ for 1 min at a flow rate of 1 mL/min after finishing measurements.

1.2. Procedure for counting cells using a Morphologi G3

The Morphologi G3 is an automated tool for characterization of particles in a variety of dry and wet mixtures including soils and/or solutions based on their shape and size (0.5µm to several millimeters) (Figure A1.1). This instrument was successfully used for the purpose of counting the cells by discriminating trichomes from other particles in the seawater matrix based on defined properties (e.g. elongation, circularity and fibers width). The Morphologi G3 instrument basically comprises three major parts: (1) wet cell (Figure A1.2); (2) scanning area including the microscope optics (2.5X, 5X, 10X, 20X and 50X) and wet-cell sample holder (Figure A1.3); and (3) software.

The entire procedure for cell counting using the Morphologi G3 can be summarized by the following steps:

- Establishment of a standard operating procedure (SOP), including the software adjustments which need to be performed to ensure that all trichomes have been identified by the instrument, as follows:
  - Measurement control – fixed number of slides/plates and a new result for each slide/plate
  - Sample carrier – wet cell plate (100×80×0.125mm) and compensate for plate tilt
  - Illumination settings – discopic (bottom light), 80 calibration intensity and 0.5 intensity tolerance
  - Optics selection (2.5X)
  - Threshold (adjusted based on the best contrast between trichomes and background)
Appendix 1

- Scanned area (mm$^2$) – is determined based on the culture density (e.g. bigger scanned area for less dense cultures).
- Analysis settings – analysis ID 3, minimum pixels 50, calculate fiber parameters
- Filters – to exclude/classify the scanned particles based on the defined properties at specific range (e.g. area (pixels) <100, fiber elongation <0.8, fiber width <8 and circularity >0.5).

Gentle injection (to prevent introduction of tiny bubbles) of about ~2.5 mL of well-mixed cell suspension (fixed with 1% Lugol’s iodine) into the wet cell (Figure A1.4), placement into cell holder and start of particle counting (Figure A1.5).

Afterwards, the instrument provides a library of images of each individual particle (in this case trichomes) by scanning the sample underneath the microscope optics, while keeping the particles in focus. It is also able to measure a range of morphological properties (e.g. fiber length) for each particle (Figure A1.6).
Appendix 1

The effect of the marine cyanobacterium *Trichodesmium erythraeum* on iron speciation in seawater

**Figure A1.1.** Morphologi G3 instrument

**Figure A1.2.** Wet cell

**Figure A1.3.** Scanning area

**Figure A1.4.** Injection of sample

**Figure A1.5.** Placing wet cell

**Figure A1.6.** Library of images
1.3. Procedure for calculation of growth rates using Morphology G3 data

To calculate number of cells per mL, initially the measured lengths for all the trichomes were summed to obtain the total length (µm) and then this value was divided by the average length of a cell (µm) to get total number of cells. The average length of cell was calculated by dividing total length of several trichomes (at least three) by their number of cells (counted annually using the 20X objective lens). The number of cells was then divided by volume in milliliters (i.e. scanned area (mm²) × 0.25 mm (gap between the cell glasses)/1000) to obtain number of cell/mL. The growth rate (µ) was then calculated from equation below,

\[ \mu = \frac{(ln(f)-ln(i))}{d} \]

where d, f and i represent, respectively, number of days, final and initial cell densities, respectively.
Literature cited

Appendix 2
1. Experimental

1.1. Data analysis and calibration

For a better understanding of the data analysis procedure, an example is illustrated for data obtained by addition of 20 nmol/L of Fe(II) into a sample. As shown in Figure A2.1, a time period of 70 s is required to load and mix sample with the reagent prior to obtaining a stable reading. This duration, which is shown in red, is excluded from further data analysis while the remaining data (i.e. 70-600 s) are extrapolated back to time zero via linear regression on a plot of log signal versus time (Figure A2.2).

![Figure A2.1](image)

**Figure A2.1.** Display of primary data points collected by the chemiluminescence system. Red and black colors illustrate, respectively, data values excluded from and included in subsequent data analysis. The curve represents the mean from two replicates.
Figure A2.2. Plot of log signal versus time illustrating extrapolation procedure. The dashed line shows the extrapolated data points at intervals of 70-600 s to time zero. The equation of the line contains the slope and the Fe(II) concentration at time zero/intercept (highlighted in red).
2. Results

2.1. Factors affecting FeLume and Fe(II) oxidation experiments

EDTA is the most effective metal chelating agent among the synthetic compounds used in complexation studies, owing to both the carboxyl and amino groups (Morel 1983). This compound is widely used in algal culture media to form complexes with Fe and keep it soluble at typical seawater pH (Andersen 2005). Santana-Casiano et al. (2000) demonstrated that EDTA is able to enhance the Fe(II) oxidation rate by forming complexes with Fe(II) (Santana-Casiano et al. 2000). However, its effect was not significant under our experimental conditions due to ionic strength differences in the media, as well as using much lower EDTA concentrations (i.e. nmol/L to µmol/L) compared to those used in their study (mM) (see Chapter 3 for more detail). Moreover, Fe(II) oxidation rates in culture media were considered to be positively correlated with pH (Gonzalez et al. 2014; King et al. 1995; Millero et al. 1987). Thus, to avoid changes in pH during phytoplankton growth, the biologically harmless buffering agent of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was tested. The oxidation rate of Fe(II) was also examined in the presence of HEPES buffer within the FeLume measurements. Accordingly, 20 nmol/L Fe(II) was added to various ASW stock solutions containing 0, 5, 20 and 100 mmol/L of HEPES, adjusted to pH 8 (by dropwise addition of 1 mol/L HCl and 2 mol/L NaOH), immediately before FeLume measurements. A decrease in chemiluminescence signal associated with an increase in oxidation rates for samples containing HEPES buffer (Figure A2.3) suggests it as a possible weak Fe binding ligand. Accordingly, since HEPES had the potential to outcompete organic exudates released by the microorganism, for the experiments presented in Chapter 3, pH was adjusted by the addition of acid at a controlled constant temperature of 20°C.
Figure A2.3. The effect of HEPES buffer on Fe(II) chemiluminescence measurement. The green, red, blue and gray solid lines show 0, 5, 20 and 100 mmol/L of HEPES, respectively.

2.2. Comparison between Fe(II) oxidation rate constants obtained from linear regression analysis and detailed kinetic modeling

Table A2.1. Comparison between Fe(II) oxidation rate constants in the presence and in the absence of organic exudates using linear regression and kinetic modelling.

<table>
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<tr>
<th>Type of ligand</th>
<th>Culture age (d)</th>
<th>Ligand concentration (µmol/L)</th>
<th>$k' \times 10^4$ (s$^{-1}$)</th>
<th>Linear regression</th>
<th>$k$ (M$^{-1}$s$^{-1}$)</th>
<th>Kinetic modeling</th>
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</thead>
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<tr>
<td>ASW (i.e. no ligand)</td>
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<td>N/A</td>
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<td>EDTA</td>
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<td>EDTA</td>
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<th>Culture age (d)</th>
<th>Ligand concentration (µmol/L)</th>
<th>$k \times 10^4$ (s$^{-1}$) Linear regression</th>
<th>$k$ (M$^{-1}$s$^{-1}$) Kinetic modeling</th>
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<tbody>
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<td>Organic exudates from culture containing 1.59 nmol/L Fe$^{2+}$</td>
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Table A2.1 (continued).

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<th>Type of ligand</th>
<th>Culture age (d)</th>
<th>Ligand concentration (µmol/L)</th>
<th>$k \times 10^4$ (s$^{-1}$)</th>
<th>$k$ (M$^{-1}$s$^{-1}$)</th>
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2.3. *Comparison of Fe oxidation rate in the presence and absence of organic exudates*

As shown in Figure A2.4, the oxidation rate is faster in the filtrate compared to the background, implying the mediating role of organic exudates in accelerating the Fe(II) oxidation rate.
Figure A2.4. Changes in Fe(II) concentration after addition of 10 nmol/L Fe(II) into media (blue line) or culture filtrate (green line). Fe(II) was added at time zero, however Fe(II) oxidation data are only shown for the time interval 71-560s.
Appendix 2

Literature cited


Appendix 3
1. Results

1.1. Examples of raw data collected by microplate reader

A comparison between signals collected using a microplate reader for background media and cultures containing different Fe⁺ concentrations is shown in Figure A3.1. Although the signals were slightly higher in the cultures compared to the media (i.e. in the absence of cells), the measured signals were not above the method detection limit after performing corrections for the background signals.

Figure A3.1. Superoxide production rate in (a) media and (b) cultures. The red, orange and green colours represent, respectively, signals obtained in media containing 50 nmol/L EDTA + 10 nmol/L Fe, 20 µmol/L EDTA + 10 nmol/L Fe, and 20 µmol/L EDTA + 155 nmol/L Fe.