Iron

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Preface

No other trace element has received more attention in relation to influences on microalgal physiology than iron (Fe). At the time of writing, a Web of Knowledge search using keywords "iron" and "phytoplankton" listed approximately 6800 references since the last edition of this textbook in 1974. To summarize all of these studies would be next to impossible. Rather, our goal in this chapter is to provide a comprehensive overview of key findings related to the acquisition and function of Fe within microalgal cells, emphasizing what we now know about how Fe is obtained, the important physiological roles of Fe and the ways in which microalgae have evolved to cope with widespread Fe limitation in aquatic environments. In addition, we have attempted to integrate a new wealth of information obtained through genomic approaches that are now common practice in microalgal physiological research. Where possible, we provide reference to extensive literature reviews that will ultimately offer a more in-depth discussion on specific Fe-related topics. Although we have made efforts to include information on all phytoplankton functional groups, including cyanobacteria, our expertise lie in diatom physiology, so there is undoubtedly a preferential focus on this group of microalgae. That being said, the vast majority of microalgal physiology studies within the scientific literature relating to transport and physiological adaptations to Fe limitation are focused on diatoms (and to some extent, freshwater green algae), as this group appears to be most affected by low Fe concentrations in the ocean and often exhibits the largest physiological response to both natural and anthropogenic Fe enrichments. Throughout this chapter, the terms microalgae and phytoplankton are used interchangeably.

1. Iron sources and distributions in aquatic environments

A review on Fe physiology of microalgae would not be complete without first describing the sources of Fe and how Fe is distributed throughout aquatic environments. Varying Fe concentrations in aquatic systems through space and time have largely shaped the evolutionary trajectories of many phytoplankton groups (Falkowski et al., 2004). Given that microalgal communities in an estimated 30-40% of the oceans are chronically deprived of Fe (Moore et al., 2002), extensive efforts have been made to obtain high-resolution measurements of Fe concentrations in the sea. As such, we will be primarily
focus on Fe distributions in seawater, although there is growing evidence that Fe may also be an important regulator of phytoplankton growth in freshwater environments (Twiss et al., 2000; Sterner et al., 2004; Havens et al., 2012; Shaked & Lis, 2012).

Iron concentrations are primarily dependent on the oxidation state. Since the first appearance of photosynthetic organisms, alternating episodes of anoxic and oxic conditions in the oceans are evident throughout Earth’s geological record with oxic conditions prevailing since the start of the Phanerozoic era (~540 mya). Increased oxygen concentrations in seawater resulted in a decline in soluble Fe content through the formation of insoluble Fe oxides that are rapidly precipitated and removed from the water column (Anbar & Knoll, 2002). The end result is that Fe requirements in microalgae remain quite high due to evolutionary constraints relative to the supply, both of which being constrained by the chemistry of Fe. Dissolved Fe concentrations in contemporary oceans are typically present in a picomolar ($10^{-12}$) to nanomolar ($10^{-9}$) concentration range. In large areas of the ocean, complexation and speciation of this Fe results in bioavailable concentrations to be far below those needed to support maximum growth rates of many microalgal species (Wells et al., 1995).

Restricted availability of Fe to microalgae is largely a consequence of its complex chemistry. Iron is present in the ocean as inorganic soluble and insoluble chemical species, dissolved organic complexes, colloidal material, mineral particles and, of course, as components of living cells. The most stable form of Fe within seawater is the oxidized ferric (Fe(III)) state. Dissolved forms of Fe(III) primarily exist as hydrolysis species bound to organic complexes with a very minimal amount existing as the free hydrated Fe(III) ion (Rue & Bruland, 1995). It is believed that these dissolved forms supply a significant proportion of the Fe flux to the cell. Ingestion of bacteria or colloidal (i.e. insoluble) forms may serve as other pathways for obtaining Fe for certain groups of phytoplankton (Maranger et al., 1998; Nishioka & Takeda, 2000), but have been considered minor as only a small percentage of phytoplankton are known to be mixotrophic. Although mixotrophy may actually be more common than previously thought (Mitra et al., 2014). Most oceanic Fe colloids are believed to consist of Fe in an
organic matrix. Inorganic Fe oxyhydroxide mineral colloids are rare in the open sea (Wells & Goldberg, 1994). Indirectly, colloidal Fe may be accessible to phytoplankton by thermal, photochemical and possibly biological reduction (Hutchins, 1995).

Iron inputs vary among regions and their proximity to land. Sources of Fe to marine euphotic zone waters include terrestrial run-off, atmospheric dust (dry deposition) and precipitation (wet deposition), upwelling of deep waters, and anthropogenic input. Such sources are crucial in determining Fe availability due to its high reactivity and low solubility (Johnson et al., 1997). Throughout the water column, Fe often exhibits a nutrient-like profile as it is significantly involved with the internal cycling of biologically derived particulate material, although in regions with high aeolian input Fe concentrations may be elevated at the surface (Bruland et al., 1994) (Fig. 1). More often, concentrations tend to be lowest in the euphotic zone due to rapid assimilation by phytoplankton and /or adsorption onto biogenic particles and then increase in the subsurface waters as sinking particles succumb to decomposition and dissolution. The residence time of Fe in seawater is relatively short (200-500 years) compared to other elements, eventually being removed from the water column through uptake and scavenging onto sinking particles (Bruland et al., 1994; Johnson et al., 1997).

Iron is the fourth most abundant element within the Earth's crust. Thus, one of the main inputs of new Fe to the ocean is continental weathering. Therefore, regions in close proximity to land or those that experience a high degree of upwelling of deep, nutrient rich waters tend to have higher Fe concentrations compared to open ocean regions that are far away from land (Fig. 1). Sources of Fe to surface waters vary depending on geographic location. Dominant inputs of Fe to coastal regions are river runoff and benthic inputs. Rivers are a primary source for most of the major ions in seawater, as well as trace metals such as Fe. Although Fe concentrations in rivers may be quite high, most of this Fe is likely removed by flocculation of strongly associated Fe-humic substances and may not make its way out of the estuary. However, there are some measurements that suggest riverine Fe inputs to the ocean can be quite high, constituting a significant
portion of new Fe inputs to the adjacent open ocean (Wetz et al., 2006; Klunder et al., 2012).

Benthic inputs of Fe to the overlying water column are primarily from two different sources, the input of continental derived material that is released into the dissolved phase and biogenic material that is exported from the surface layer. For example, off the Oregon coast, the dominant form of Fe to surface waters is a combination of reduced Fe(II) from shelf sediments and sediment that is resuspended through upwelling (Johnson et al., 1999; Chase et al., 2007; Bruland et al., 2008; Lohan & Bruland, 2008). The addition of dissolved Fe(II) to oxic seawater will quickly result in its oxidation to the less-soluble Fe(III) with the subsequent formation of colloidal and particular oxyhydroxide forms that are not readily biologically available. However the complexation of dissolved Fe(III) by strong Fe-binding ligands of microbial origin can maintain Fe(III) in the dissolved phase (see further details on siderophores below). Moore and Braucher (2008) modeled the global input of Fe from sediments and have shown the sedimentary sources along continental margins have a strong impact on open-ocean Fe concentrations, particularly in the Arctic and North Pacific and are equivalent to global estimates of Fe input from atmospheric dust (Fig. 1).

The primary source of Fe to surface waters in remote open ocean regions is through atmospheric deposition of soil dust and anthropogenic emissions from the continents. Atmospheric inputs into the ocean are often spatially and temporally patchy with regions of particularly high atmospheric Fe input within the Tropical and North Atlantic, Bay of Bengal, the Arabian Sea, and waters surrounding Australia and the southeastern continental margins of South America. Mineral aerosols consist of soil particles that are lifted into the atmosphere when high winds occur over erodible surfaces. These particles may be transported long distances before they are deposited into the ocean via settling, turbulent deposition and precipitation processes (Mahowald et al., 2005). The solubility of Fe in these various atmospheric forms depends on the source with soluble Fe fractions ranging from 0.01 to 80% (Mahowald et al., 2005).
Hydrothermal vents are considered another significant source of Fe to the ocean. In addition to emitting sulfur, high levels of trace metals, including Fe, are emitted by vents. Previously, vent-derived Fe was thought to be of limited importance due to rapid oxidation and precipitation around the vents. Yet it is now believed that hydrothermal vent inputs of Fe may in fact be long-lasting, contributing to the dissolved pool of Fe and eventually making their way to sunlit surface waters where they fuel primary productivity (Saito et al., 2013) (Conway & John, 2014). Stabilization of dissolved and particulate Fe in hydrothermal plumes is likely achieved through organic matter complexation. Modeling and observation-based studies have recently suggested hydrothermal vents may contribute substantially to overall Fe inputs into the ocean, likely being important in buffering the ocean dissolved Fe inventory against shorter-term fluctuations in dust deposition. Recent estimates suggest hydrothermal input could provide 9-22% of the Fe budget in the global deep ocean and upwards of 45-50% in the tropical Pacific (Tagliabue et al., 2010).

In polar regions, glaciers may also be an appreciable source of Fe to the ocean. Glaciers can be considered as frozen rivers, and when they reach the ocean, they discharge in the form of icebergs and water. Icebergs often will transport significant amounts of continental debris that will be released into the ocean as they melt. In addition, sea ice can act as a cap on the ocean, which will accumulate atmospheric sources of Fe and release the Fe as it melts. Sea ice is believed to be an important mechanism for Fe delivery in high-latitude regions of the Southern and Arctic Oceans. Estimates on the amount of bioavailable Fe supplied to the Southern Ocean by both aeolian dust and icebergs have been found to be similar (Raiswell et al., 2008). Free-drifting icebergs around Antarctica are often "hotspots" for chemical and biological enrichment, serving as areas of enhanced production and sequestration of organic carbon to the deep sea (Smith et al., 2007). This is likely a consequence of melting ice being a source of Fe that stimulates phytoplankton growth, although estimates on the contributions of Fe by icebergs to such regions are variable (Lin et al., 2011). As a consequence of global climate change, the importance of melting ice as a source of Fe will likely increase as temperatures continue to rise.
As in seawater, the Fe speciation in freshwater systems is highly affected by chemical composition, Fe inputs and removal processes, as well as internal recycling. In natural conditions, Fe is supplied from the products of weathered rocks and soil around the watersheds and is controlled by a number of factors including geological processes, soil composition, environmental temperature, precipitation and hydrology (Harris, 1992). In addition to these sources, anthropogenic influences such as wastewater and storm-discharge are a major supply of Fe to freshwater lakes. Iron in freshwater environments is commonly orders of magnitude higher than that of seawater, although dissolved Fe concentrations have been suggested to influence rates of NO$_3^-$ drawdown and Chl $a$ concentrations within lakes (Havens et al., 2012). Iron may also play a regulatory role in the microalgal community where cyanobacteria tend be favored over green algae under high Fe conditions (Morton & Lee, 1974; Pollingher et al., 1995; Hyenstrand et al., 2000). Similarly, Fe has been suggested to influence diatom composition in streams across the continental US. Passy (2010) identified a relationship between stream diatom richness and wetland cover, where wetlands were suggested to provide an essential source of bioavailable, dissolved organic carbon-bound Fe to stream ecosystems. More commonly, high Fe concentrations in freshwater systems, particularly rivers and streams, are a problem due to increased loads of Fe resulting from human activities such as mining of Fe enriched ores, intensified forestry, peat production and agricultural drainage.

2. **Cellular iron requirements and associated physiological processes**

Belonging to a group known as the transition metals, all of which can form cations, Fe has the ability to exist in a number of oxidation states. Biochemically, Fe has an exceptional ability to lose or gain electrons, thus effectively undergoing redox reactions. This property has made Fe essential for the growth of almost all aerobic organisms, but it is particularly important for photoautrophs due to its catalytic role in photosynthesis and respiratory electron transport. Free ions of Fe complexed to haeme or in the form of Fe-sulfur clusters are found in a variety of metalloproteins that are involved in essential components of almost all cellular pathways (Table 1).
Iron is an integral component of photosystems I and II and the cytochrome b₆f complex, which together make up the major constituents of the photosynthetic units (PSUs), comprised of light-harvesting accessory pigments and its associated photosynthetic reaction centers. The vast majority of the cellular Fe within phytoplankton may be contained within the photosynthetic apparatus, with the proportion increasing substantially when cells are Fe-limited (Raven, 1988; Greene et al., 1991). This demand is divided between PSI (12 Fe per monomer), PSII (6 Fe per monomer) and cyt b₆/f (5 Fe per monomer), with the cyt b₆/f complex alone accounting for 30% of cellular Fe.

Phytoplankton experiencing Fe stress have reduced photosynthetic pigments and pigment binding proteins when compared to Fe-replete conditions (Greene et al., 1992; Strzepek & Price, 2000) (Geider & LaRoche, 1994). Known as chlorosis, reduced chlorophyll content along with a decline in photosynthetic efficiency and reduced growth rates are the most noticeable symptom of Fe-deficiency in algae, (reviewed by Behrenfeld & Milligan, 2013). Protein (Glover, 1977; Rueter & Ades, 1987; Doucette & Harrison, 1991), lipid and carbohydrate (Milligan & Harrison, 2000; van Oijen et al., 2004) content are also observed to vary with Fe nutritional state. A reduction in chlorophyll synthesis is likely the result of both direct (reduced abundance of Fe-containing enzymes involved in chlorophyll synthesis) and indirect (a general reduction in cellular energy) effects of Fe limitation (Nouet et al. 2011). Due to iron's scarce supply to the oceans, microalgae have evolved unique strategies to reduce their photosynthetic Fe demand, a number of which are described below and have also recently been reviewed by Behrenfeld and Milligan (2013).

Iron also plays a large role in the reduction of oxidized nitrogen species, another essential element for cell growth. Phytoplankton require Fe for the assimilation of inorganic N species such as nitrate (NO₃), nitrite (NO₂) and nitrogen gas (N₂) into ammonium (NH₄). The assimilatory enzymes nitrate (NR) and nitrite reductases (NiR) contain Fe (1 Fe: 1NR and 5-6 Fe: 1NiR). Under Fe limitation in T. weissflogii, the activity of NiR decreased by 50-fold and NO₂ was excreted because NO₂ assimilation was the rate limiting step in the NO₃ assimilation pathway (Milligan & Harrison, 2000). Based on
biochemical calculations, cells growing on NO$_3$ should require 1.6 times more Fe than cells supplied with NH$_4$ due to the Fe demand associated with NO$_3$ reduction (Raven, 1988; Morel et al., 1991). Experimental evidence supports this increase in Fe demand since growth on NO$_3$ enhanced the Fe:C ratio of diatoms by 1.8 times compared with NH$_4$ (Maldonado & Price, 1996). Indirect support linking NH$_4$ use to faster growth under Fe-limiting conditions was also provided for the cyanobacterium, *Synechococcus*, where half saturation constants of Fe for growth ($K_{\mu}$; a lower $K_{\mu}$ indicates a stronger affinity for the substrate) were lower for NH$_4$ then NO$_3$- grown cells (Kudo & Harrison, 1997).

However, neither field nor laboratory experiments have observed significantly faster growth rates of Fe-limited eukaryotic phytoplankton when grown on NH$_4$ instead of NO$_3$ (Price et al., 1991; Price et al., 1994; Maldonado & Price, 1996). The lack of a N-dependent effect may, in part, be due to the profound influence of Fe limitation on photosynthesis. Conversely, Fe-stressed, low-light, NO$_3$-grown *Emiliania huxleyi*, a coccolithophore, grew significantly faster than Fe-stressed NH$_4$-grown cells (Muggli & Harrison, 1996b). The difference in growth rates was attributed to the reduction in cell volume by NO$_3$-grown cells (see discussion below about Fe effects on cell size).

Similarly, studies have shown that Fe-limited, NO$_3$-grown diatoms grew about 25% faster than Fe-limited, NH$_4$-grown cells (Price, 2005). An explanation for this discrepancy is an increase in the biologically available pool of Fe for NO$_3$-grown cells by reduction of organically complexed Fe through inducible transplasmalemma NO$_3$ reductases (Maldonado & Price, 2000).

The ability to fix nitrogen also imparts a high Fe demand on diazotrophs, many of which are cyanobacteria (e.g. *Trichodesmium*) (Kustka et al., 2003; Berman-Frank et al., 2007). This is because the enzyme that catalyzes the conversion of N$_2$ gas to biologically available ammonium requires a large amount of Fe (38 Fe per enzyme), as well as molybdenum. In many regions of the ocean, because of this increased Fe demand, the rate of N fixation in diazotrophs has been shown to be Fe-limited even when the growth rate of non-diazotrophs may not be (Moore et al., 2009).
Other examples of cellular pathways known to rely on Fe-containing enzymes and proteins include cellular respiration, vitamin synthesis, dehydrogenases and oxygenases involved in fatty acid metabolism and the dismutases of superoxides (Table 1). Superoxide dismutases (SODs) catalyze the conversion of superoxide radicals to molecular oxygen and hydrogen peroxide. Four major groups of SODs are known and distinguished by their metal co-factors: Fe, Mn, Cu/Zn, and Ni. The Fe/Mn family includes both single metal-binding and the rare cambialistic SODs which can bind either Mn or Fe. The Fe and Mn single metal-binding SODs are very similar but may be distinguished by two critical residues involved in the binding of the metal ion (Wolfe-Simon et al., 2005). Although many microalgae contain the Fe-SOD variants, interestingly, others completely lack Fe-SODs or down-regulate them when grown under low Fe conditions, which may thus contribute to lowering their Fe demands (Allen et al., 2008; Marchetti et al., 2012).

The cellular Fe requirement is often expressed by the intracellular Fe content (or quota; Q) of a microalgal cell relative to its cellular C (or P) content. The cellular quota may be given as a ratio (Fe:C/P or C/P:Fe), which allows for a measure of Fe demand and provides a means of comparison among different phytoplankton species of varying sizes and shapes. Recently, Quigg et al. (2003; 2011) suggested microalgal groups that inherited their plastids by endosymbiotic events from the green lineage tended to have higher trace metal requirements than those derived through the red lineage. This may be the result of the lineages originating in contrasting environmental conditions; the green lineage dominating under reduced ocean conditions and the red lineage dominating under oxic conditions that are more similar to present day marine environments. To examine if this is also true specifically for Fe, we compiled published Fe:C (and Fe:P) ratios measured in various phytoplankton isolates and grouped them according to taxa. Comparing Fe requirements in phytoplankton grown under Fe-replete conditions is confounded by luxury consumption of Fe in certain groups of microalgae, particularly when Fe concentrations are in excess, resulting in increased storage that contributes to the maximum Fe intracellular contents ($Q_{Fe}^{max}$) in addition to their basal requirements needed to support maximum growth rates. This is especially true for diatoms where $Q_{Fe}^{max}$
varied considerably among the isolates examined, likely as a consequence of intracellular Fe storage (see further discussion below). Therefore, we limited our data set to only those Fe:C ratios obtained when cells were grown under conditions of inorganic Fe concentrations that supported maximum or near maximum ($\mu/\mu_{\text{max}}>75\%$) growth rates, but where the Fe concentrations in the medium were below levels that would result in excessive Fe storage.

Cyanobacteria, especially $N_2$-fixing species, and microalgae from the red primary plastid lineage (red algae) have relatively higher Fe requirements than the green algae and secondary red plastid lineage groups (e.g. diatoms, dinoflagellates and haptophytes); the latter groups appear to have somewhat similar Fe requirements (Fig. 2). Although our analysis does not necessarily support a clear distinction between red and green plastid lineages, it does suggest that certain phyla have reduced their Fe requirements more effectively than others. Members of two taxa in particular, the diatoms and haptophytes, possess the lowest Fe:C ratios, which may explain their persistence (along with cyanobacteria that primarily benefit from their small size) in a number of low-Fe environments.

*Acclimation and adaptation to iron limitation*

There is considerable pressure on microalgae to evolve mechanisms to reduce their Fe requirements in order to exploit Fe-limited regions. Phytoplankton isolated from Fe-limited regions typically have lower Fe contents and higher Fe use efficiencies (the amount of C fixed per mole of Fe per day) compared to their coastal congeners. There have been numerous investigations into the mechanisms by which oceanic phytoplankton, particularly diatoms, acclimate and adapt to low Fe availability. One of the most important evolutionary responses to low Fe in the open ocean is a substantial reduction in the cellular Fe requirement for growth. Given iron's involvement in many metabolic pathways, there are numerous strategies invoked by both eukaryotic (Fig. 3) and prokaryotic (Fig. 4) microalgae to reduce their Fe demand. Possibilities include reducing cell size, using intracellular Fe pools more efficiently, minimizing the use of metabolic pathways that require large amounts of Fe and replacing Fe-containing proteins
with non-Fe containing ones that are more or less functionally equivalent.

A reduction in cell size often occurs when microalgae are acclimated to low light or various nutrient limitations, including Fe limitation (Sunda & Huntsman, 1997; Marchetti & Cassar, 2009). Nutrient requirements for growth decrease as a function of the cube of the cell radius ($r^3$), whereas nutrient uptake decreases as a function of the available membrane area ($r^2$), and the diffusion-limited rate as a function of the radius ($r$) (Morel et al., 1991). Decreasing cell size would increase the surface area-to-volume ratio and maximize membrane transporters and subsequently uptake rates relative to Fe requirements. A decrease in cell size also decreases the diffusion boundary layer thickness, improving nutrient uptake kinetics. Such a physiological acclimation is observed in microalgae in both field and laboratory conditions. Many Fe-limited regions are populated primarily by small pico- and nanophytoplankton (<5 µm in diameter), suggesting that this small size-fraction has an advantage in coping with low [Fe] as compared to larger phytoplankton species (e.g. diatoms). This is due to the inherent overall lower Fe requirements for small phytoplankton (on a per cell basis) as well as their more effective uptake capabilities on a cell surface area basis. As a response to Fe limitation, coastal and oceanic diatoms show a 20-50% decrease in mean cell volume per cell (Sunda & Huntsman, 1995; Muggli et al., 1996; Strzepek & Price, 2000; Marchetti & Harrison, 2007). This reduction would represent an increase in surface area-to-volume ratio of 8 to 26%. Similarly, even cyanobacteria that benefit from being relatively small, have been shown to decrease their size in response to Fe limitation (Sherman & Sherman, 1983; Wilhelm, 1995). In addition to a reduction in total cell volume, a change in shape from spherical to elongated results in an increase in the SA:V ratio (Marchetti & Cassar, 2009). However, there are likely to be constraints on the morphological changes that particular groups of microalgae may undergo, particularly hard-shelled groups such as diatoms. Similarly, although usually rare, the persistence of large cells in Fe-limited regions suggests there are benefits to large cell size, possibly in relation to nutrient (and Fe) storage capacities, periodic vertical migration and resistance to grazing (Smetacek et al., 2004).
Microalgae may also adjust their photosystems in response to Fe limitation. There is a preferential down-regulation of PSI relative to PSII (and Cyt b₆f) across prokaryotic and eukaryotic microalgae under Fe starvation and steady-state Fe stress (Greene et al., 1991; Moseley et al., 2002; Strzepek & Harrison, 2004; Behrenfeld & Milligan, 2013). Under Fe deficiency, cyanobacteria may decrease their PSI:PSII ratio from 4:1 to 1:1 (Straus, 2004), while the low-Fe adapted oceanic diatom, *Thalassiosira oceanica* has been shown to have a constitutive PSI:PSII ratio of 1:10 (Strzepek & Harrison, 2004). The decreased abundance of PSI (as well as cytochrome b₆f) may have resulted in a diminished ability by *T. oceanica* to acclimate to fluctuations in photosynthetically active radiation (see section of Fe-light interactions). Although a recent analysis of the *T. oceanica* genome and associated transcriptome under Fe-limitation demonstrated that this diatom possesses the potential to adjust many of its bioenergetic pathways (including PS1:PSII ratios) in response to Fe availability (Lommer et al., 2012). Major adjustments in the PSI:PSII ratio may also increase dependency on alternative electron pathways such as the plastoquinol oxidase (PTOX) pathway, which is present in both prokaryotic and eukaryotic microalgae (reviewed by Zehr & Kudela, 2009; Behrenfeld & Milligan, 2013). The PTOX pathway provides an electron shunt after PSII, enabling cells to bypass PSI, thus changing the stoichiometry of net O₂ production and carbon fixation. Because the PTOX pathway has a low Fe requirement (2 Fe molecules per monomer), activation of this shunt under low Fe conditions would substantially reduce a cell's Fe demand while still being able to generate ATP, however, an alternative source of reductant (e.g. NADPH) must be present for carbon fixation to occur.

Cyanobacteria and red algae also express an induced protein (isiA) under Fe stress, which can become the most abundant chl-binding protein in the cell (Ryan-Keogh et al., 2012). The precise roles of isiA remain elusive. Despite strong sequence similarity between IsiA and the gene encoding the PSII core CP43 protein, IsiA is not thought to be a CP43 replacement nor antenna complex for PSII. Rather, during Fe stress, IsiA forms superstructures around PSI trimers and monomers, resulting in a significant increase in cross-sectional absorption area of PSI ($\sigma_{\text{PSI}}$) (Bibby et al., 2001; Boekema et al., 2001). A continued up-regulation in isiA without any further increase in the $\sigma_{\text{PSI}}$ led Ryan-
Koegh et al (2012) to speculate that this protein may also be involved in other secondary roles for coping with Fe limitation.

Many of the Fe-requiring proteins in microalgae may be reduced or substituted for non-Fe containing equivalents under low Fe conditions. Some of the protein substitutions represent acclimation strategies where the Fe-containing protein is preferred when cells are growing in Fe-replete conditions. Other protein substitutions represent evolutionary adaptations where species have adapted to chronically low [Fe] by replacing Fe-containing molecules with metalloproteins that function using other trace metals more readily available in the environment. The best case of Fe metalloprotein substitution is flavodoxin for ferredoxin (La Roche et al., 1993). Ferredoxins are Fe-sulfur cluster containing proteins which mediate electron transfer in a range of metabolic reactions. Flavodoxin, on the other hand, does not contain Fe but instead utilizes a single molecule of riboflavin 5'-phosphate as a cofactor. Although flavodoxin does not have as low a redox potential as ferredoxin, the expression of flavodoxin at suboptimal [Fe] would partially alleviate Fe stress. Flavodoxins are commonly found in cyanobacteria, although not all species are considered to contain them. For example, *Anabaena* ATCC 29211 lacks the potential to synthesize flavodoxin, and simply decreases the content of ferredoxin at very low Fe concentrations (Sandmann et al., 1990). The diatom *Phaeodactylum tricornutum* increases the expression of flavodoxin 25 to 50-fold when comparing Fe limited relative to Fe-replete cells (Allen et al., 2008). The flavodoxin to ferredoxin ratio has been considered to be an *in situ* marker for Fe stress in phytoplankton (Doucette et al., 1996; La Roche et al., 1996). However, it should be used with caution given that some microalgae have several copies of flavodoxin, with gene variants not regulated by Fe availability (Whitney et al., 2011) whereas other phytoplankton groups may express flavodoxin constitutively instead of ferredoxin (Pankowski & McMinn, 2009). In *T. oceanica* there has been a permanent transfer of the ferredoxin gene from the chloroplast genome to the nuclear genome (Lommer et al., 2010). This relocation is thought to allow *T. oceanica* to better coordinate gene expression for its low-Fe response.
Another well characterized substitution is that of the copper-containing electron transport protein plastocyanin for the Fe-containing cytochrome c₆. Where plastocyanin is more commonly used in green algae (Hill & Merchant, 1995), in haptophytes, primarily cytochrome c₆ is found whereas in diatoms and cyanobacteria either transport protein may be present, and is largely dependent on growth conditions and biogeography (Sandmann et al., 1983). For example, while some oceanic diatoms have replaced cytochrome c₆ with plastocyanin, many coastal diatoms continue to use cytochrome c₆. In *T. oceanica*, the constitutive use of plastocyanin is believed to reduce the cellular Fe demand by as much as 10% (Peers & Price, 2006).

3. Iron acquisition mechanisms

Cellular transport of an essential element refers to the ability of a cell to transfer that element across the plasma membrane, from the outside of the cell to the inside. In general, the word *transport* and *uptake* are used interchangeably. Fe acquisition refers to the ability of microalgae to access Fe within a variety of complexes (both inorganic and organic) before it is transported across the plasma membrane. In contrast, assimilation of an element refers to the intracellular reactions that facilitate the incorporation of that element into organic cellular components.

Ions of essential elements enter the cell by moving across the cell boundary layer, onto the cell surface, then passing through the cell wall (if present) and plasmalemma into the cytoplasm. The thickness of the boundary layer is inversely related to the uptake rate which is determined also primarily by cell size, and as a result, uptake may be limited by the rate of diffusion across this layer (see below for further discussion). The cell wall is not a barrier to ion entry. In contrast, the plasma membrane—which consists of polar lipid bilayers interspersed with proteins and does not allow the free diffusion of charged ions or large neutral molecules. Thus, charged ions—and neutral large molecules—must be transported across the plasma membrane via facilitated diffusion (also called passive transport) or active transport, both of which require an electrochemical gradient as the driving force. Some of the common characteristics of facilitated diffusion and active transport include: a) a transmembrane transport protein (either a carrier protein or a
channel protein), b) unidirectional and ion-specific ion transport, c) Michaelis-Menten saturation kinetics, and d) competitive and non-competitive inhibition of ion transport. The greatest difference between facilitated diffusion and active transport is that in the latter, the ion is transported against an electrochemical gradient, and thus energy is required. Carrier proteins (also called carriers, permeases or transporters) undergo a series of conformational changes to transfer the bound solute across the membrane, and can be involved in facilitated diffusion or active transport. In contrast, channel proteins are only involved in facilitated diffusion. Channel proteins form an aqueous pore in the membrane through which a specific solute passes, and thus interact only weakly with the solute.

In microalgae, as in all other organisms, Fe uptake is an active transport process. The mechanisms of Fe transport in microalgae are extremely diverse and complex, and point to unique Fe uptake mechanisms acquired via horizontal gene transfer (Morrissey & Bowler, 2012). We will describe general mechanisms involved in Fe transport by discussing prokaryotic and eukaryotic microalgae separately. So far, the vast majority of work on microalgae has focused on Fe acquisition and transport, and not on Fe assimilation. The following sections thus focus on these two topics.

There are physiological and molecular aspects of Fe acquisition and transport. From a physiological perspective, the following questions can be addressed: 1) What are the Fe species (oxidation state and chemical complexes) that are bioavailable for phytoplankton for uptake? 2) What are the Fe species that react with the transporters? 3) What is the Fe species that is transported into the cell? 4) If Fe transport utilizes various proteins, what are their activities and functions? 5) What is the limiting step in Fe acquisition? and, 6) Does Fe uptake follow Michaelis-Menten kinetics?

**Physiological aspects of iron transport**

The vast majority of physiological Fe transport studies in microalgae have used marine diatoms as model organisms. However, we expect that most of the findings apply to many other microalgal taxa, and freshwater algae. There is evidence for two Fe uptake
systems, a low-affinity and a high-affinity transport system. The low affinity Fe transport mechanism is utilized when inorganic Fe species are available for uptake, and cells are either Fe sufficient or mildly Fe-limited. The high affinity Fe transport system is operational when the cells are severely Fe-limited and where the concentrations of inorganic Fe are extremely low, but there is a pool of organically bound Fe (in the nM range). In the low affinity system, Fe uptake is a function of the labile dissolved inorganic Fe(III) concentration (Fe’) in the bulk medium (Hudson & Morel, 1990), while in the high affinity system, Fe uptake is best predicted by the concentration of organically bound Fe (Maldonado & Price, 2001; Strzepek et al., 2011). These two Fe transport systems have been reconciled in a general kinetic model for Fe acquisition by marine phytoplankton (Shaked et al., 2005), where Fe(II) is an obligate intermediate in both systems. The presence of a low- and a high-affinity transport system is often termed biphasic uptake kinetics for short-term uptake rates as a function of nutrient concentration (best fit using a double rectangular hyperbola equation). Biphasic uptake kinetics have been shown for Cu in diatoms (Guo et al., 2010). For Fe, such data are still rare, though hints of biphasic uptake kinetics have been shown in the Southern Ocean prymnesiophyte, *Phaeocystis antarctica* (Strzepek et al., 2011). These two Fe acquisition mechanisms are believed to share some components/proteins, which are likely to be up-regulated under Fe limiting conditions (see below).

**Low-affinity acquisition mechanisms for inorganic iron**

Before describing Fe transport mechanisms in depth, it is essential to understand how Fe uptake is controlled by the Fe conditions in the growth medium (or the environment), as well as by cellular Fe requirements. Most studies investigating Fe physiology use a chemically well-defined medium such as Aquil (Price et al., 1988/89; Sunda et al., 2005). In this medium, a large excess of aminocarboxylate chelating agents are added to buffer a (nearly) constant concentration of dissolved free Fe(III) (pFe = -log [Fe(III)]). This provides a constant [Fe’] (inorganic Fe(III) species) in the growth medium, and thus controls the exponential growth rates by controlling Fe availability. A typical medium might have 100 μM ethylenediaminetetraacetic acid (EDTA) and nM to μM concentrations of Fe, depending on what degree of Fe limitation or sufficiency needs to
be imparted to the phytoplankton (see further discussion below). The Fe’ concentration is maintained by dissociation and formation reactions of the organically bound Fe, and thus is dependent on the [Fe], the chelator concentration and the affinity of the ligand for Fe(III). Photochemistry might also induce an increase in the Fe’ pool, due to photodegradation of the organically complexed Fe (Sunda & Huntsman, 2003).

When the concentrations of all the nutrients are in excess, except for Fe, the concentration of labile inorganic Fe [Fe’] determines microalgal growth rates. Following the Monod equation (Monod, 1942), the specific growth rate is determined by the labile dissolved inorganic Fe concentrations ([Fe’]), as well as the half-saturation constant for Fe for growth (Kμ), and the maximum specific growth rate (μmax) so that:

\[ \mu = \mu_{max} \frac{[Fe']}{(0.5 [Fe'] + K\mu)} \]  

\text{equation 1}

The specific growth rate can also be related to intracellular Fe content (Harrison & Morel, 1986), using the Droop equation (Droop, 1970). In this case, the maximum growth rate is only achieved when the microalgae are able to fulfill their cellular Fe demand:

\[ \mu = \mu'_{max} \left[ 1 - \frac{Q_{Fe_{min}}}{Q_{Fe}} \right] \]  

\text{equation 2}

where Q_{Fe_{min}} is the minimal intracellular Fe content needed to allow any growth, and Q_{Fe} is the intracellular Fe level. Here μ’_{max} refers to the ‘impossible’ growth rate at infinite quota, but in general \( \mu'_{max} / \mu_{max} \sim 1 \) and μ_{max} is achieved when the optimal cellular Fe content (Q_{Fe_{max}}) is reached (Harrison & Morel, 1986).

We can also write this equation in terms of Q_{Fe_{max}}, which is the optimal cellular Fe content when μ_{max} is achieved, thus

\[ \mu = \mu'_{max} \frac{(Q_{Fe} - Q_{Fe_{min}})}{Q_{Fe_{max}}} \]  

\text{equation 3}
When phytoplankton are growing under steady-state conditions (or exponential growth), and the rate of growth is limited by the Fe supply, the growth rate is directly proportional to the steady-state Fe uptake and inversely proportional to the cellular Fe content, so that

\[ \mu = \frac{\rho_{ss}}{Q_{Fe}} \]  

\text{equation 4}

The steady-state Fe uptake rates also follow Michaelis-Menten kinetics and can be described as:

\[ \rho_{ss} = \frac{\rho_{ss, max} \times [Fe^\prime]}{(K_{\mu Q} + [Fe^\prime])} \]  

\text{equation 5}

where \( K_{\mu Q} \) stands for half-saturation constant for steady-state Fe uptake. Equation 1, 2 and 4 are related to each other quantitatively according to \( \mu = \frac{\rho_{ss}}{Q_{Fe}} \) (equation 3). We can solve for \( \rho_{ss} \) and take the logarithm of both sides of the equation so that \( \log \rho_{ss} = \log \mu + \log Q_{Fe} \). This implies that the log of the steady-state Fe uptake rates is the sum of logarithm of \( \mu \) and the logarithm of \( Q_{Fe} \) (Morel, 1987). A graphic representation of this is given in Fig. 5.

The net result is that the half-saturation constant for steady-state Fe uptake rates is higher than that for growth, and that the quantitative relationship between \( K_{\mu Q} \) and \( K_\mu \) is related to the ability of the cells to modify their cellular Fe quotas (Harrison & Morel, 1986; Morel, 1987), such that:

\[ \frac{K_{\mu Q}}{K_\mu} = \frac{Q_{Fe \text{ max}}}{Q_{Fe \text{ min}}}, \text{ and thus, the } K_{\mu Q} \gg \gg K_\mu \]

For a phytoplankton cell growing under low Fe, a practical implication of equation 3, is that fast growth rates can be maintained by decreasing the \( Q_{Fe} \) (Harrison & Morel, 1986; Morel, 1987), even though at low \([Fe^\prime]\) \( \rho_{ss} \) will decrease according to equation 4.

Short-term Fe uptake rates reflect the rate of Fe uptake under non steady-state conditions. For example, a culture can be grown under a specific Fe limiting concentration, and can
be examined for its ability to take up Fe at various [Fe]. Normally, these short-term Fe uptake experiments should not last more than the time it take a cell to synthesize new proteins. Typically, approximately < 6 h is the recommended time for laboratory Fe uptake experiments, but this depends on the microalgal growth rate (i.e. should be increased for slower growing algae). For exponentially growing cells, when the [Fe] in the short-term Fe uptake experiments is equal to the [Fe] in the growth medium, the short-term Fe uptake rates coincide with the steady-state Fe uptake rates since they are determined by the concentration of Fe (see equation 10 below). However, in the presence of higher [Fe] (but not saturating) in the uptake medium, the short-term Fe uptake rates will be faster than the steady-state Fe uptake rates due to the higher Fe in the uptake medium compared to the growth medium.

In general, inorganic Fe acquisition (low-affinity Fe uptake system) is observed when concentrations of Fe are in the nanomolar range, and a typical oceanic species is either Fe sufficient or slightly Fe limited. Under this condition, short-term Fe transport in phytoplankton follows typical Michaelis-Menten uptake kinetics, where the rate of Fe uptake is determined by the maximum rate of uptake ($\rho_{\text{max}}$), the concentration of labile dissolved inorganic Fe species ([Fe’]), as well as the half-saturation constant for Fe uptake ($K_p$) (Hudson & Morel, 1990).

$$\rho = \rho_{\text{max}} * \frac{[\text{Fe’}]}{(K_p + [\text{Fe’}])}$$  \hspace{1cm} \text{equation 6}

The half-saturation constant for short-term Fe uptake ($K_p$) is species specific and its inverse ($1/K_p$) provides a measure of the affinity of transporter for Fe. The $K_p$ for short-term Fe uptake is less plastic than $\rho_{\text{max}}$, which may vary significantly, depending on the number of Fe transporters at the cell surface (Harrison & Morel, 1986; Hudson & Morel, 1990). Fe-limited phytoplankton have been observed to increase the number of Fe transporters by up to 20-fold, thus allowing a significant increase in their $\rho_{\text{max}}$ (Harrison & Morel, 1986). This can be graphically represented by two kinetic curves ($\rho_{\text{low}}$ and $\rho_{\text{high}}$) that have a similar $K_p$ but different $\rho_{\text{max}}$, so that $\rho_{\text{max}}$ and $\rho_{\text{max}}$ represent the $\rho_{\text{max}}$ for the Fe-sufficient and Fe-limited phytoplankton, respectively (Morel, 1987).
When the [Fe] in the uptake experiment is equal to that in the growth medium, the short-term Fe uptake rates are equal to the steady-state Fe uptake rates. Therefore, for a Fe-sufficient culture, at high [Fe] the short-term and steady-state Fe uptake rate should be the same. Similarly, for an Fe-limited cell, at low [Fe] the short-term and steady-state Fe uptake rate should be identical. As a result, the half-saturation constant for short-term Fe uptake is higher than for steady-state uptake by a factor of $\rho_{\text{max}}^{\text{high}} / \rho_{\text{max}}^{\text{low}}$, so that

$$K_{p} / K_{\mu Q} = \rho_{\text{max}}^{\text{high}} / \rho_{\text{max}}^{\text{low}},$$

and thus, the $K_{\mu Q} << < < K_{p}$

We can thus compare half-saturation constants for growth, short-term Fe uptake and steady-state Fe uptake (Fig. 5). As predicted by Morel (1987), Fig. 5 shows how the half-saturation constants for growth, short-term Fe uptake and steady-state Fe uptake vary in magnitude for *E. huxleyi* ($K_{p} = 0.1$, $K_{\mu Q} = 0.029$, and $K_{\mu} = 0.0014$ nM Fe$^+$; using data from Sunda and Huntsman, 1995), so that

$$K_{\mu} << < K_{\mu Q} << < K_{p}$$

Thus, the ratio of the half-saturation constants for growth and short-term Fe uptake rates is directly determined by the lower and upper limits of the Fe intracellular levels and of the maximum uptake rates, according to equation 7 (Morel 1987):

$$K_{\mu} / K_{p} = (Q_{Fe}^{\text{min}} / Q_{Fe}^{\text{max}}) * (\rho_{\text{max}}^{\text{low}} / \rho_{\text{max}}^{\text{high}}) \quad \text{equation 7}$$

We compiled data for a wide variety of phytoplankton to compare half-saturation constants for growth, short-term Fe uptake and steady-state Fe uptake in microalgae, as previously done by Morel (1987). As predicted by Morel (1987), Table 2 shows how the half-saturation constants for growth, short-term Fe uptake and steady-state Fe uptake vary by orders of magnitude (for cultures $K_{p} = 3.7$, $K_{\mu Q} = 0.37$, and $K_{\mu} = 0.041$ nM Fe$^+$; for field studies $K_{p} = 2.96$ vs. $K_{\mu} = 0.00032$ nM Fe$^+$). The order-of-magnitude difference among these half-saturation constants ($K_{p}$ vs. $K_{\mu Q}$ vs. $K_{\mu}$) is related to the plasticity of the cells to regulate intracellular Fe levels (lower under Fe limitation), as well as the number
of Fe transporters at the cell surface (higher under Fe limitation) under various Fe conditions.

These half-saturation constants shown in Table 2 can also be used to define the Fe-limited condition of a phytoplankton culture or an in situ population (Morel et al., 1991). Under Fe sufficiency, when \([\text{Fe}'] > K_{\mu Q}\), near maximum growth rates are observed, and neither the number of Fe transporters are maximized, nor the \(Q_{\text{Fe}}\) is minimized. Under Fe limitation, when \(K_{\mu} < [\text{Fe}'] < K_{\mu Q}\), near maximum growth rates are observed, but the number of Fe transporters is maximized and the \(Q_{\text{Fe}}\) is minimized. Under Fe limitation when \([\text{Fe}'] < K_{\mu}\), the Fe uptake rate is too slow to fulfill their Fe requirement despite maximizing the number of Fe transporters and minimizing \(Q_{\text{Fe}}\), thus cellular growth rate decreases.

**Physical, chemical and biological factors controlling Fe transport**

Physical factors that may influence rates of Fe uptake include light, temperature and diffusion constraints. Light availability has been observed to affect Fe uptake rates by either generating changes in aquatic Fe chemistry (i.e. changes in \([\text{Fe}']\)) (Hudson & Morel, 1990; Barbeau et al., 2003; Sunda & Huntsman, 2003; Maldonado et al., 2005; Fujii et al., 2011; Sunda & Huntsman, 2011), and/or influencing energy supply to cells (ATP and/or NADPH) for Fe acquisition (Strzepek et al., 2011). In addition, a physiological interaction between light and Fe availability during growth has been shown to affect steady-state Fe transport rates (see biological factors below). The temperature effects on Fe uptake are associated with the typical microalgal temperature coefficient \((Q_{10} \text{ value is } \sim 2)\), which means that, in general, the rate of Fe uptake will be 2 times faster for every 10°C increase in temperature. Low temperature may also enhance Fe uptake by increasing the residence time of photo-chemically produced \([\text{Fe}']\) in the presence of photolabile ferric chelates (Sunda & Huntsman, 2011).

The effects of diffusion limitation of Fe uptake have been discussed in detail in Morel et al. (1991). In essence, theoretical calculations suggest that even with a very low Fe demand for growth, a phytoplankton cell with a radius \(\geq 10 \mu\text{m}\), and dividing once a day,
would be diffusion limited for Fe' uptake in the open ocean. This is due to the relationship between cellular Fe demand and Fe uptake rate. The former is proportional to the cellular volume and growth rate, while the latter is a function of the number of Fe transporters at the cell surface, and the rate at which Fe diffuses to the cell surface. At a given, low Fe transporter density (mol μm^-2), a smaller cell (relative to a bigger cell) will achieved faster volumetric Fe uptake rates (mol Fe L\(^{-1}\) cell volume h\(^{-1}\)), and thus would be better able to fulfill its Fe demand. To enhance Fe transport, a larger cell (or a Fe limited cell) may increase the number of Fe transporters at the cell surface. However, eventually, the maximum rate of Fe uptake will be limited, not by the number of Fe transporters, but instead by the rate at which Fe' diffuses to the cell surface, and this effect will be more pronounced for a bigger cell. Indeed, a large cell (r ≥ 10 μm) requires such a large number of Fe transporters at the cell surface to fulfill its Fe demand, that even if it only divides once a day, its rate of Fe uptake is diffusion limited.

The chemical factors that affect Fe uptake include the total Fe concentration in the environment, the oxidation state of Fe (Fe(II) vs. Fe(III)), its speciation (free Fe, and inorganic versus organic Fe complexes) and the concentration of ligands in the environment. These Fe binding ligands directly compete with Fe uptake ligands for dissolved inorganic Fe. The biological factors that influence Fe uptake rates are cellular Fe demand (i.e. the minimum intracellular Fe needed for maximum growth rate), the Fe limited condition of the cells (i.e. the extent to which the cellular Fe content falls short of the minimum intracellular Fe needed for maximum growth rate), their growth phase (exponential vs. stationary), and the cell population density. In addition, Fe transport has been shown to be affected by the physiological condition of the cells especially with regards to Cu (Peers et al., 2005; Maldonado et al., 2006) and pH (Schenck et al., 1988; Sunda & Huntsman, 2003; Shi et al., 2010). Extensive discussion of these chemical and biological factors follows below.

In the 1980’s and 1990’s a series of studies investigating the kinetics of Fe transport in marine phytoplankton provided the foundation of what we know about Fe transport in these organisms (e.g. Anderson & Morel, 1982; Harrison & Morel, 1986; Hudson &
Morel, 1990), and established the Fe’ model (Hudson & Morel, 1990; Sunda & Huntsman, 1995; Sunda & Huntsman, 1997). In essence, the Fe’ model predicts a dependency of Fe uptake rates on the concentrations of labile dissolved inorganic Fe. These studies also established that Fe uptake occurs via specialized active transport proteins on the plasmalemma (Hudson & Morel, 1990; 1993). Indeed, binding of Fe to the transport proteins must first occur, followed by internalization. Thus, these Fe transporters behave as surface ligands with a very high affinity for Fe ($k_L = 2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$).

\[
\begin{align*}
\text{Fe'} + L_{\text{surface}} & \leftrightarrow \text{FeL}_{\text{surface}} \rightarrow \text{Fe}_{\text{cell}} \\
k_L & \quad k_{\text{in}} \\
k_{-L} & \quad \text{equation 8}
\end{align*}
\]

In this reaction, the hydrated ferric Fe ion first needs to form an outer-sphere complex with the ligand. The binding of Fe to this surface receptor ligand is limited by the rate at which a molecule of $\text{H}_2\text{O}$ is lost from the inner coordination sphere of the Fe ($k_{-w}$)

\[
\begin{align*}
\text{Fe(O}_2\text{H})_6^Z+ + L^{n-} & \leftrightarrow \text{Fe(O}_2\text{H})_6^{Z+} \ldots L^{n-} \rightarrow \text{Fe(O}_2\text{H})_5^{Z+} \ldots L^{n-} + \text{H}_2\text{O} \rightarrow \text{Fe(O}_2\text{H})_5^{L(n-2)-} \\
k_{\text{on}} & \quad k_{-w} \\
k_{-w} & \quad \text{equation 9}
\end{align*}
\]

However, complexation of Fe by inorganic ligands, such as hydroxide or chloride, accelerates the rate of water loss by more than a 1000-fold over that of the $\text{Fe}^{3+}$ ion (Grant & Jordan, 1981). The water loss constant for Fe’ is $2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ (Hudson et al., 1992), and closely matches the rate constant for Fe’ complexation by surface ligands ($L$) in marine phytoplankton ($0.9 \times 10^6$ and $1.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$; Hudson & Morel (1990)). This strongly suggests that labile dissolved inorganic Fe species react with the cell surface transporters, and not $\text{Fe}^{3+}$ that has too low a concentration and too slow kinetics to be the species reacting with surface $L$.

As typical of active transporters, Fe uptake in marine phytoplankton saturates at relatively low Fe concentrations ($< \text{pM Fe'}$ levels) and follows typical Michaelis-Menten uptake kinetics, where the rate of Fe uptake is determined by the maximum rate of uptake.
\( \rho_{\text{max}} \), the concentration of labile dissolved inorganic Fe species ([Fe’]), as well as the half-saturation constant for Fe uptake (K_\rho).

\[ \rho = \rho_{\text{max}} \times \frac{\text{[Fe’]}}{(K_\rho + \text{[Fe’]})} \]  

Equation 10

Indeed [Fe’] is the total concentration of labile dissolved inorganic Fe species whose ‘effective’ reaction rate with the uptake L determines uptake. As a result, K_\rho is determined by the rates of metal-ligand binding (k_L) and dissociation (k_-L), as well as the rate of Fe internalization (k_{in}), such that K_\rho = (k_-L + k_{in})/k_L. Hudson and Morel (1990) demonstrated that Fe transport was under kinetic control, meaning that the rate of internalization of the Fe bound to the cell surface ligands is much faster than its rate of dissociation from the surface ligands, thus the rate of dissociation can be ignored and K_\rho = k_{in}/k_L. As discussed above, the maximum rate of Fe uptake (\( \rho_{\text{max}} \)) is highly affected by the number of transport ligands (L_T) at the cell surface, which can increase by more than 20-fold when cells are experiencing Fe limitation (Harrison and Morel 1986). The expected increase in Fe transport due to higher density of Fe transporters (up to 20-fold) is far greater than that expected from a decrease in cell size (~ 2 fold, see section above).

When phytoplankton are Fe-limited, their maximum short-term Fe uptake rates (in the presence of non-limiting [Fe’]) are determined by the number of Fe transporters at the cell surface and the rate of internalization, so that

\[ \rho_{\text{max}} = k_{\text{in}} \times L_T^{\text{max}} \]  

Equation 11

In addition, K_\rho = k_{in}/k_L, and under Fe-limiting conditions [Fe’] \ll K_\rho, so the upper limit on their steady-state Fe uptake rates can be simplified from the Michaelis-Menten equation above (equation 10) to

\[ \rho^{\text{ss}} = k_L \times L_T^{\text{max}} \times \text{[Fe’]} \]  

Equation 12

Thus, under steady-state Fe limiting conditions, the growth rate is defined as:

\[ \mu = k_L \times L_T^{\text{max}} \times \text{[Fe’]} / Q_{\text{Fe}} \]  

Equation 13
In summary, the Fe’ model predicts that Fe uptake rates are dependent on dissolved inorganic Fe(III) species (Fe’), and that these Fe’ species (be it Fe(II)’ or Fe(III)’) bind the Fe surface transporters before the Fe is internalized. Thus, the rate of Fe transport is determined by the rate of ligand exchange between these Fe species and the surface Fe transporters. This Fe’ model has been extremely useful in the last 25 years for laboratory trace metal algal physiology and toxicity studies. In these experiments, the concentration of Fe’ is buffered using synthetic organic ligands (Y) such as EDTA or DTPA, which are present in concentrations (5-100 μM) in great excess of that of Fe (1 nM-10 μM). In these typical media, Fe’ is determined by the concentration of Fe and that of Y, as well as the affinity of Y for Fe (the conditional stability constant). In some cases, Y is photochemically labile, and thus the Fe’ also depends on the light intensity, wavelength and the rate of photochemically mediated dissociation of Fe from FeY. The steady-state Fe’ concentration in the bulk solution, after accounting for the dissociation and formation of FeY, as well as the photochemical degradation of FeY, predicts well the Fe transport rates and provides robust support for the Fe’ model (Fig. 5).

High-affinity acquisition mechanisms for organically bound iron
The development of extremely sensitive analytical techniques in the 1990s allowed measurements of organic complexation of Fe in the sea. The finding that the vast majority of dissolved Fe (>99.9%) is bound to very strong organic complexes (Gledhill & van den Berg, 1994; Rue & Bruland, 1995; for a review see Gledhill & Buck, 2012) led to the reexamination of the Fe’ model because the calculated Fe’ in the open ocean was shown to be too low to support sufficient Fe' to meet cellular requirements of open ocean phytoplankton (Rue & Bruland, 1995). At the same time that Fe speciation in the ocean was being unraveled, laboratory experiments were also revealing new insights into Fe transport in phytoplankton (e.g. Allnutt & Bonner Jr., 1987; Allnutt & Bonner, 1987; Jones et al., 1987; Soria-Dengg & Horstmann, 1995; Hutchins et al., 1999b; Maldonado & Price, 2000; 2001).
Physiological studies of Fe nutrition in phytoplankton were conducted using model siderophores, such as desferrioxamine B (DFB), to induce severe Fe limiting conditions in the growth medium. Siderophores (from the Greek “iron carriers”) are the most tenacious ferric chelators known in nature (Neilands, 1995). These molecules are low molecular weight, and are secreted mainly by Fe-limited bacteria, fungi and grasses to scavenge Fe from the environment. Depending on the Fe(III) binding group, siderophores can be classified into hydroxamates, catecholates or mixed-types (containing another Fe binding group such as α-hydroxy-carboxylate next to the hydroxamate or catecholate group). Once the siderophore complexes an Fe(III) ion, the ferrisiderophore is transported into the cell via specific siderophore transporters. In the early 1990’s, eukaryotic phytoplankton had not been shown to be able to internalize ferrisiderophores. Therefore, siderophores were added to the medium to mimic extreme in situ Fe-limiting conditions, where Fe⁺ is practically undetectable.

Surprisingly, these physiological studies showed that Fe-limited phytoplankton could access Fe from these strong organic complexes, using an enzymatic reductive mechanism at the cell surface (Allnutt & Bonner Jr., 1987; Allnutt & Bonner, 1987; Soria-Dengg & Horstmann, 1995; Maldonado & Price, 2000; 2001; Weger et al., 2002; Matz et al., 2006). The ability of the phytoplankton to access these strong organic ligands was induced under Fe limitation (Allnutt & Bonner Jr., 1987; Maldonado & Price, 1999; 2001; Weger et al., 2002; Strzepek et al., 2011), suggesting that this was a high-affinity Fe transport system similar to that of other well studied eukaryotes (i.e. the yeast Saccharomyces cerevisiae) (for a review, see Van Ho et al., 2002). It is now evident that that the high-affinity Fe transport system in phytoplankton involves the activity of Fe permeases at the cell surface, as well as ferric reductases and multi-Cu containing ferroxidases (Fig. 3). Indeed, physiological evidence for the existence of a Fe reductive pathway in microalgae is widespread (see Table A1 in Shaked & Lis, 2012).

The ferric reductases are plasmalemma bound and transfer an electron from cytosolic NAD(P)H to the strong, organically bound Fe complex on the outside of the cell. Since most organic Fe chelators have a much higher affinity for Fe(III) than Fe(II), Fe
reduction results in a dissociation of the Fe(II) from the ligand, and thus an increase of inorganic Fe at the cell surface. Indeed, the rates of Fe reduction from these strong organic ligands are inversely related to the stability of the Fe(III) coordination complex (Maldonado & Price, 2001). However, once the Fe is reduced, the fraction of this Fe taken up into the cell depends critically on the relative concentration (and affinities) of the free organic ligands in solution and the free Fe transporters at the cell surface (see the Fe(II)s model below).

Once the Fe(III) is reduced to Fe(II) and dissociates from the organic complex, the free Fe(II) is then oxidized by multi-Cu oxidases, followed by internalization of the Fe(III). These reduction and oxidation steps impart specificity and selectivity to the Fe transport complex, which is crucial in the case of essential trace elements. Physiological studies with Fe-limited diatoms have measured comparable rates of Fe(III) reduction of organically bound Fe and Fe(II) oxidation (Herbik et al., 2002a; Herbik et al., 2002b; Maldonado et al., 2006). The coupling between these oxidation and reduction rates may ensure rapid oxidation of Fe(II) by the putative multi-Cu oxidase before the Fe(II) diffuses away from the cell membrane. The proximity of these putative reductases, oxidases and permeases at the cell surface of microalgae may contribute to an efficient cascade of redox reactions. Close proximity of the reductases and oxidases may also allow the formation of a ternary complex, ferric reductase–Fe(III)siderophore–putative Fe(II) oxidase, which may facilitate the reductive dissociation of Fe from very strong organic complexes (Boukhalfa & Crumbliss, 2002).

The occurrence of the reductive Fe uptake pathway to acquire Fe from strong organic Fe complexes is widespread in freshwater and marine microalgae (see Table A1 in Shaked & Lis, 2012). In many instances, physiological data have been complemented with genomic and proteomic data. One field study also measured Fe reduction rates of organically bound Fe by in situ phytoplankton (Maldonado & Price, 1999). Most recently, data from the Global Ocean Survey (GOS) metagenomes have shown that ferric reductases are characteristic of marine eukaryotic phytoplankton Fe uptake systems (Desai et al., 2012). Physiological evidence for the multi-Cu containing oxidases is still
limited to a few species, including *C. reinhartii*, *T. pseudonana* and *T. oceanica* (Herbik et al., 2002a; Herbik et al., 2002b; La Fontaine et al., 2002; Peers et al., 2005; Maldonado et al., 2006). Molecular and genomic evidence for the occurrence of multi-Cu containing oxidases is starting to emerge (Maldonado et al., 2006; Kustka et al., 2007; Paz et al., 2007a; Guo et al., 2015).

Recently, a limited set of studies has demonstrated that Fe reduction is also a necessary step for the acquisition of inorganic Fe in the presence of weak organic ligands (such as EDTA), where the dissolved inorganic Fe(III) pool is significant (Shaked et al., 2005). In this case the ferric reductase reduces Fe(III)' to Fe(II)'. As discussed above in the “inorganic Fe acquisition section”, Fe uptake in this case is still a function of the concentrations of labile dissolved inorganic Fe(III), because the production of Fe(II)' depends on concentration of Fe(III)', and can be predicted with the Fe’ model (or Fe(III)' model). However, these studies have concluded that Fe(II) is an obligate intermediate in phytoplankton Fe transport, regardless of whether the cells are acquiring Fe(III) from inorganic or organic Fe(III) complexes (Shaked et al., 2005). Yet, it is important to emphasize that the high-affinity Fe permeases ferry Fe(III) across the plasma membrane, and not Fe(II). Thus, if Fe(II) is an obligate intermediate for Fe acquisition of inorganic Fe (at non-saturating Fe’ concentrations), this Fe(II) is either oxidized by a multi-Cu containing oxidase-Fe permease complex, like that of the high-affinity Fe transport system for organically bound Fe, or alternatively, is directly internalized as Fe(II) via lower affinity divalent transporters such as ZIP and NRAMPS (see below). Further research is needed to elucidate the mechanism of uptake of Fe(II) after the enzymatic reduction of inorganic Fe complexes.

Based on the new findings that organically complexed Fe is bioavailable via a reductive mechanism at the cell surface, and that inorganic Fe acquisition also requires a reductive step, Shaked et al. (2005) proposed a new conceptual model for Fe transport by phytoplankton, “the Fe(II)s model” (Fig. 6). The name of this model was chosen to emphasize that reduction of Fe at the cell surface is an obligate intermediate in Fe
acquisition. Unfortunately, to the non-specialist, this name gives the false impression that Fe(II) is the Fe species that is internalized by the phytoplankton.

The Fe(II)s model builds on the previous kinetic model of Fe uptake (Hudson and Morel, 1990), “the Fe’ model”, which predicted Fe uptake rates based on the concentration of labile dissolved inorganic Fe(III) in the bulk medium. The Fe’ model is valid for well-buffered Fe media, in the presence of excess concentrations of aminocarboxylate chelating agents. However, the Fe’ model is incomplete because it is unable to predict rates of Fe uptake when [Fe(III)'] is practically non-existent; as observed in the presence of very strong organic Fe complexes, such as DFB. The Fe(II)s model is meant for calculating rates of Fe uptake under non-saturating [Fe(III)’]. This model reconciles the Fe’ model with the new data on the extracellular, biologically mediated Fe reduction of, not only, strong organic Fe complexes, but also inorganic Fe complexes (Shaked et al., 2005). The Fe(II)s model is not mechanistic, but is based on the premise that the Fe acquisition mechanism in marine eukaryotic phytoplankton is similar to that of yeast and involves Fe reductases at the cell surface, followed by the activity of an Fe transport complex, which contains a multi-Cu containing Fe oxidase and a permease. It aims to predict Fe uptake rates based only on reduction rates of Fe(III), either bound to organic or inorganic complexes. Specifically, the Fe(II)s model predicts Fe uptake based on the concentration of Fe(II) at the cell surface, which is determined by a) reduction of organically bound Fe (FeY) at the cell surface, b) reduction of Fe(III)’ at the cell surface, and c) the competition for free or inorganic Fe (assigned a nomenclature of Fe(II)s, but includes inorganic and free Fe(II) or Fe(III) species) between the cell surface Fe transport complexes and the free organic ligand in the medium (Z). [Note: in cases where only one ligand is present in the medium, Y = Z]. Thus, only 3 parameters need to be specified: a) cellular FeY reduction constant (k_{FeY red}; L cell^{-1} h^{-1}), b) cellular Fe(III)’ reduction constant (k_{Fe(III)’ red}; L cell^{-1} h^{-1}), and c) the ratio (k_d/k_{up}; M^{-1}) of effective formation constant of Fe(II)s complexation by excess ligand Z (k_z; M^{-1} h^{-1}) and the rate constant of Fe(II)s complexation by the membrane Fe transport complex under non-saturating [Fe(III)’](k_{up}; h^{-1}).
In the presence of weak organic Fe complexes, such as EDTA (ie. \([\text{EDTA}] = 5-100 \, \mu\text{M} \& \text{ Fe at } \sim 100 \, \text{nM}\)), Fe’ is relatively high and reduction of Fe(III)’ dominates. Since EDTA cannot compete effectively with the cell Fe transport complex for the Fe(II)s, Fe uptake rate is simply a function of \(k_{\text{Fe(III)'} \text{red}}\) and the \([\text{Fe(III)'}]\), so that Fe uptake (\(\rho_{\text{Fe}}; \text{ mol Fe cell}^{-1} \text{ h}^{-1}\)) is defined as

\[
\rho = k_{\text{Fe(III)'} \text{red}} \ast [\text{Fe(III)'}] \quad \text{equation 14}
\]

In the presence of very strong organic ligands, such as FeDFB, \([\text{Fe(III)'}]\) is practically non-existent, thus reduction of FeY dominates, and competition for Fe(II)s between the cell surface Fe transport complexes and the free organic ligand in the medium (Z) has to be incorporated into the equation (note that Z can be DFB or some other organic ligand), so that

\[
\rho = k_{\text{FeY red}} \ast [\text{FeY}] / ((k_{\text{Z}}[Z]/k_{\text{up}}) + 1) \quad \text{equation 15}
\]

The Fe(II)s model is able to reconcile the Fe(III)’ model (because in the presence of weak organic Fe complexes, the inorganic Fe(III) is the substrate for cellular surface Fe reduction and uptake) with the laboratory and field data showing Fe uptake from strong Fe organic complexes, such as siderophores. This is a kinetic model for Fe acquisition under non-saturating Fe concentrations, in which the activities of the Fe reductases and the Fe-transport complexes (multi-Cu oxidases and Fe permeases) increase proportionally to the concentrations of Fe(III) and Fe(II), respectively. These non-saturating conditions are prominent in the open ocean, as well as in laboratory experiments under Fe limiting conditions. Processes able to increase \([\text{Fe’}]\) in surface oceanic waters, such as photoreduction of organic Fe complexes (Barbeau et al., 2001), photodissolution of Fe oxides (Waite & Morel, 1984) or superoxides (reviewed by Rose, 2012) would potentially increase Fe(III)’ for cellular reduction at the cell surface. Shaked et al. (2005) estimated that in the open ocean, the Fe needed for phytoplankton growth is supplied by cellular reduction of mainly FeY (2/3rds) and some Fe(III)’ (1/3rd).
**Molecular aspects of Fe transport**

From a molecular perspective, the following questions can be addressed: 1) Is Fe uptake achieved by a single Fe transporter or an Fe transport complex? 2) What are the expression patterns of the proteins involved in Fe acquisition? 3) How are these proteins arranged in the plasmalemma? and 4) How many transmembrane domains do these transporters have?

**Eukaryotic microalgae:** Though we are starting to learn more about molecular aspects of Fe transport in marine eukaryotic microalgae (reviewed by Blaby-Haas & Merchant, 2012) so far, the best characterized Fe transporters in microalgae are those of the freshwater green alga *Chlamydomonas reinhardtii* (reviewed by Merchant et al., 2006)). Fe(III) transport occurs through FTR (Fe TRansporter) (Table 3). These FTRs are localized to the plasma membrane and form a complex with multi-Cu oxidases (referred to as FOXs, FET3Ps, or MUCOXs depending on the organisms). In addition, these Fe transport complexes are associated with ferric reductases (or FREs).

Three types of ferric reductases have been involved with Fe(III) reduction in eukaryotes: NAD(P)H oxidases (NOX; cytochrome b\textsubscript{558}), cytochrome b\textsubscript{5} reductases, and cytochrome b\textsubscript{561}. In *C. reinhartii*, FRE1 (a NOX enzyme) is localized in the plasma membrane and is highly induced under Fe deficiency, thus this Fe reductase is hypothesized to be involved in Fe acquisition (Allen et al., 2007). In diatoms, the following Fe reductases have been identified: *T. pseudonana* (TpFRE1 and TpFRE2, Kustka et al., 2007)), *Phaeodactylum tricornutum* (PtFRE1-4, Allen et al., 2008), and *T. oceanica* (ToFRE1, Lommer et al., 2012). TpFRE1 is similar to cytochrome b\textsubscript{5} reductase, while PtFRE3 and PtFRE4 are similar to cytochrome b\textsubscript{561} (Allen et al., 2008), and TpFRE2, PtFRE1 and PtFRE2 to NOX proteins (Blaby-Haas & Merchant, 2012). The transcript abundance of many of these diatoms’ FREs increases under Fe limitation (Kustka et al., 2007; Allen et al., 2008), thus Fe reduction is believe to be an integral component of the high-affinity Fe transport system. In addition, Fe reduction is also a required step for Fe uptake of inorganic Fe into the cell (Shaked et al., 2005), as well as mobilization through...
intracellular membranes, such as into the chloroplast or out of the vacuole (reviewed by Nouet et al., 2011).

Putative multi-Cu oxidases have been identified in many genomes, including FOX in *C. reinhardtii*, *Chlorella variabilis*, *Coccomyxa* sp. C-169, and *Volvox carteri*, FET3P in *Fragilariopsis cylindrus* & *Thalassiosira pseudonana*, and MUOX2 in *T. oceanica* (Blaby-Haas & Merchant, 2012). As with the ferric reductases, the transcript abundance of these genes is enhanced in Fe-limited cultures of *C. reinhardtii* (La Fontaine et al., 2002; Allen et al., 2007) and *T. oceanica* (Maldonado et al., 2006). Surprisingly, gene homologs to these multi-Cu containing ferroxidases have not been found in *P. tricornutum* (Allen et al., 2008), nor is its expression always enhanced in Fe-limited *T. pseudonana* (see Maldonado et al., 2006; Kustka et al., 2007). Multi-Cu oxidases contain 4 Cu atoms, and are divided in 2 types: FET3p-type and ceruloplasmin. While green microalgae multi-Cu oxidases are similar to the second type, the ones from diatoms seem more comparable to FET3p. Interestingly, the MCO of *Aureococcus anophagefferens* is different from these 2 types, and *Cyanidioschyzon merolae* is lacking a MCO (Blaby-Haas & Merchant, 2012). A physiological interaction between Fe and Cu has been shown in eukaryotic algae (Herbik et al., 2002a; Herbik et al., 2002b; La Fontaine et al., 2002; Peers et al., 2005; Maldonado et al., 2006; Annett et al., 2008; Guo et al., 2010; Guo et al., 2012), as well as cyanobacteria (Nicolaisen et al., 2010). For example, when diatoms are co-limited by Cu and Fe, the oxidation rates of Fe are slower, resulting in slower Fe uptake (Maldonado et al., 2006).

In yeast, internalization of Fe by the multi-Cu oxidase-Fe permease complex has been proposed to occur by an Fe-channeling mechanism which is activated when the multi-Cu oxidase oxidizes the Fe(II) to Fe(III) and transfers it to the Fe permease (Stearman et al., 1996). In addition, the maturation of the FET3 and FTR proteins is coupled in the secretory pathway, so that only the complex containing both proteins is present in the plasma membrane (Stearman et al., 1996). FTR1 homologs are common in microalgal genomes, except in prasinophytes and some chromalveolates (Blaby-Haas & Merchant, 2012). Fe deficiency induces FTR1 genes in *T. pseudonana* (Kustka et al., 2007) and *C.
*reinhardtii* (La Fontaine et al., 2002; Allen et al., 2007). When neither FOX1 nor FTR1 are present, as in green prasinophytes or *P. tricornutum*, direct uptake of organic Fe complexes might occur (Blaby-Haas & Merchant, 2012). Indeed, a gene encoding a putative siderophore binding protein was recently identified in *P. tricornutum* (Allen et al., 2008).

In general, algal high-affinity Fe transporters are structurally divergent from those well characterized in *Saccharomyces cerevisiae*. Novel proteins have been hypothesized to be involved in Fe transport, such as FEA1- and FEA2-like protein in *C. reinhardtii*, (Allen et al., 2007), FEA1-like protein in *M. pusilla* and *O. lucimarinus* and "Iron-starved-Induced Proteins" (ISIPs) in diatoms (Lommer et al., 2012). Since *M. pusilla* and *O. lucimarinus* lack typical, high-affinity Fe transport FTR1 homologs, these FEA1 proteins may facilitate Fe-specific transport in these organisms (Blaby-Haas & Merchant, 2012).

In addition to Fe(III) transporters, Fe(II) transporters such as those belonging to the ZIP (reviewed by Gaither & Eide, 2001) and NRAMP (reviewed by Nevo & Nelson, 2006) families are found in microalgae (Table 3). An important difference between the Fe(III) transporters discussed above and the ZIP and NRAMP Fe(II) transporters is their specificity. In essence, ZIP and NRAMP protein may transport Fe(II) in addition to other divalent metals. Thus, these Fe(II) transporters have been hypothesized to be: a) low(er)-affinity plasma membrane Fe permeases, or b) proteins involved in the trafficking of intracellular Fe to various cellular compartments. The ZIP family was named after the first discovery of a Zn transporter (Zrt1p and Zrt2p; (Zhao & Eide, 1996a; b), respectively) in *S. cerevisiae* and the Fe transporter IRT1 in *Arabidopsis* roots (Eide et al., 1996). ZIP transporters may be localized to plasma and vacuolar membranes or the secretory system. ZIP metal transporters are believed to be passive, mediating metal transport via concentration gradients (Lin et al., 2010). Within the ZIP family, there are 4 groups of transporters, depending on their phylogenetic relationships: Subfamilies I (with mainly fungal and plant proteins) and II (with plant and animal proteins), GufA (with prokaryotic and eukaryotic proteins) and LZT (with human LIV-1 Zn transporter as the founder) (Gaither & Eide, 2001). ZIP transporters are not metal specific but are able to
transport a variety of divalent cations, such as those Fe, Cu, Zn, and Mn. In *C. reinhardtii*, there are 13 ZIP family members, including IRT1 and IRT2 (from the GufA family), both of which are upregulated under Fe deficiency (Allen et al., 2007). In addition, IRT1 and IRT2 are induced under Zn and Cu limitation (Castruita et al., 2011), respectively. Since IRT2 is also a potential target for the Cu responsive transcription factor CRR1, IRT2 has been hypothesized to be an intracellular Fe(II) transporter delivering Fe to cytochrome c₆, or an alternative plasma membrane Fe transporter when Cu is limiting and the Fe transporter-complex is not functional (Blaby-Haas & Merchant, 2012). The diatoms *P. tricornutum* (Allen et al., 2008) and *T. pseudonana* (Kustka et al., 2007) also have ZIP homologs. As in *C. reinhardtii*, the gene transcript of a ZRT, IRT-like protein, belonging to the ZIP family, was enriched under Fe deficiency in *P. tricornutum* (Allen et al., 2008). In *T. pseudonana*, 5 genes encoding ZIP-like transporters have been identified, and the expression of some of these genes are enhanced under Fe deficiency or repressed after a Cu addition to a low Cu culture (Guo et al., 2015).

Another family of metal permeases able to transport Fe(II) is the NRAMP family (reviewed by Cellier et al., 2001). This family is named after its first identified member, a Natural-Resistance-Associated Macro-Phage protein 1. Most NRAMP permeases are able to transport divalent cations, mainly Fe and Mn, into the cytoplasm. As with ZIP proteins, NRAMPs may be localized to the secretory system, plasma and vacuolar membranes. NRAMPs proteins rely on a proton gradient for metal transport. Phylogenetically, the NRAMP family is divided into 4 subfamilies: prokaryotic MntH group A, B, and C, as well as a eukaryotic group (Cellier et al., 2001). Within the eukaryotic group, three clusters are found: subgroup I (with NRAMPs from major eukaryotic lineages), II (NRAMPs from plants, prasinophytes and *C. merolae*), and III (fungal proteins). Most microalgal genomes to date contain one NRAMP homolog (Blaby-Haas & Merchant, 2012), except for *P. tricornutum*, which lacks an NRAMP homolog (Kustka et al., 2007). The *Chlamydomonas* genome encodes four NRAMPs: NRAMP1, NRAMP2, RET1 and NRAMP4, although some of these proteins do not have metal transport activity (Blaby-Haas & Merchant, 2012). An ortholog of NRAMP4 is highly upregulated in Fe-limited
T. pseudonana, however whether this permease is located to the plasma or vacuolar membrane remains to be established (Kustka et al., 2007). In a more recent study, the expression of TpNRAMP was up-regulated not only by Fe limitation, but also by low Cu (Guo et al., 2015). Once Fe enters the cell, it must be taken to its sites of incorporation or storage (for a review, see Nouet et al., 2011).

Prokaryotic microalgae: As siderophore production is more prominent in prokaryotic organisms and fungi than in eukaryotes, since the 1970s many cyanobacteria have been tested for their ability to produce siderophores. Cyanobacteria, both marine and freshwater, are able to produce hydroxamate- (i.e. synechobactin A-C, (Ito & Butler, 2005); schizokinen, (Simpson & Neilands, 1976)) and catecholate-type siderophores (ie. anachelin-H, anachelin-1, and -2; (Beiderbeck et al., 2000)), though the former is more commonly synthesized (reviewed by Gademann & Portmann, 2008)). Interestingly, some of the marine cyanobacterial siderophores are photolabile, and have a fatty tail, which is believed to enhance the affinity of the siderophore for the bacterial membrane (Ito & Butler, 2005), as previously shown for marine bacteria (Barbeau et al., 2003; Martinez et al., 2003). These two characteristics might aid in Fe acquisition in the dilute oceanic environment.

Siderophores can be synthesized by the non-ribosomal peptide synthase (NRPSs) pathway or by the NRPS-independent siderophore (NISs) biosynthesis pathway. NRPSs are normally associated with polyketide synthases (PKSs) (Table 3). Several genes coding for NRPSs and PKSs have been found in cyanobacteria genomes (Silva-Stenico et al., 2011), but in some cases the specific siderophore that is produced by these synthases is unknown. Both NRPSs and PKSs genes are more prevalent in filamentous and heterocystous strains, but were absent in many Synechocystis, Prochlorococcus, and Synechoccocus (Ehrenreich et al., 2005). In general, NRPSs genes seem to be more prominent in cyanobacteria genomes than NISs (Hopkinson & Morel, 2009). However, many NRPSs pathways synthesize secondary metabolites, instead of siderophores. Thus, the presence of NRPSs genes only suggests the potential for siderophore synthesis. In contrast, finding genes encoding NIS pathways are indicative of siderophore biosynthesis
(mainly hydroxamates and/or mixed-hydroxamates). In a study on marine prokaryotic genomes, genes for the NIS siderophore biosynthesis pathway were only found in 15% of the marine prokaryotic genomes, and were completely absent from the oceanic environmental samples (Hopkinson & Barbeau, 2012). The low abundance of siderophore biosynthesis genes in the ocean suggests that many marine cyanobacteria are involved in siderophore Fe piracy, and may be able to strip the Fe off the ferrisiderophore at the cell surface (via a reductive mechanism or a ternary complex formation). Recent studies on the diversity of iron uptake systems in the ocean examined sequenced genomes of marine microbes (eukaryotes and prokaryotes), as well as metagenomes from GOS (Desai et al., 2012). They demonstrated the presence of some of the components of rhizobactin siderophore synthesis in cyanobacteria and eukaryotic phytoplankton genomes, as well as in the metagenomes (though at much lower abundance). One of the few eukaryotic microalgae previously shown to have a siderophore biosynthesis pathway is the prasinophyte *O. lucimarinus* (Palenik et al., 2007).

Little is known about how cyanobacteria secrete siderophores to the environment after intracellular synthesis. The only siderophore secretion pathway studied in detail is that of *Anabaena* sp. PCC 7120. Export through the inner membrane is suggested to be mediated by a transporter from the Resistance, Nodulation and cell Division (RND), the Major Facilitator (MFS) or the ATP-binding cassette (ABC) superfamilies, while transport through the outer membrane is suggested to occur by a TonIC-type protein, as in other bacteria (Bleuel et al., 2005; and reviewed by Miethke & Marahiel, 2007). In *Anabaena* sp. PCC 7120, siderophore schizokinen secretion is mediated by the MFS-type protein SchE in the inner membrane and by the TonIC-type protein HgdD in the outer membrane (Nicolaisen et al., 2010).

Siderophore acquisition has been studied in detail in *E. coli* and *Pseudomonas aeruginosa*. Siderophore-specific transporters are found in the outer membrane of Gram-negative bacteria, and are part of the TonB-dependent transporters (TBDTs). Although that TBDTs are known to transport a variety of substrates, their structure consists of a membrane bound 22-stranded β-barrel (poorly conserved), and a N-terminal plug domain
(highly conserved) located within the barrel. Molecular dynamic simulations indicated that binding of siderophores, such as ferrichrome, initiates a signaling mechanism that ultimately leads to the TonB-mediated partial or total removal of the core domain from the β-barrel, thus opening up a permeable pore (Faraldo-Gomez & Sansom, 2003; Faraldo-Gomez et al., 2003). In many cases, the substrate-binding site in the extracellular pocket of the transporter lacks sequence conservation (Chimento et al., 2005), thus allowing TBDTs to have specificity for a variety of substrates, besides siderophores, such as haeme, vitamin B\textsubscript{12} and Fe-citrate (reviewed by Schauer et al., 2008).

Even though many TonB-dependent receptor (TBDT) have been identified in cyanobacteria (Mirus et al., 2009), their function in Fe transport has only been shown in a few species. For example, a TBDT belonging to \textit{Anabaena} sp. PCC 7120 has been identified (SchT) for the transport of a specific siderophore, schizokinen (Fig. 4) (Nicolaisen et al., 2008). SchT is encoded by \textit{alr}0379, spans across the outer membrane, and it is fueled by a “Ton” system anchored in the periplasmic membrane. Bacterial TonB-dependent transporters consists of an energy transducing unit, TonB, and 2 stabilizing units, ExbB and ExbD (reviewed by Noinaj et al., 2010). The interaction between TBDT and the “Ton” system is mediated by TonB. The TBDT contains a TonB-box in front of its N-terminal plug domain, which is recognized by TonB. In the absence of a ferrisiderophore, the plug domain is blocked. The interaction between a ferrisiderophore-loaded TBDT and TonB results in a conformational change in the plug domain, allowing passage of the siderophore through the TBDT pore (Fig. 4).

Other components of siderophore-mediated iron transport were recently identified in \textit{Anabaena} sp. PCC 7120, namely TonB3, the ExbB3/ExbD3 and the Fhu systems (Stevanovic et al., 2012) (Table 3). The transcript abundance of these genes is enhanced under under iron-limiting conditions, as it is typical of a high-affinity Fe transport systems (Stevanovic et al., 2012). In general, transport of ferrisiderophore across the periplasmic and inner membrane is then accomplished by a three-component system: a periplasmic Fe binding protein (e.g. FhuD/FutA/FecA), a membrane-embedded permease (e.g. FhuB/FutB/FecCD) and a ATP-binding protein (e.g. FhuC/FutC/FecB). The
FhuD/B/C-system that allows transport of ferrichrome in *E. coli* is well established (reviewed by Krewulak & Vogel, 2011). So far, in *Anabaena* sp. PCC7120, 5 gene clusters similar to Fut-, Fec- and Fhu-system have been annotated, but only the Fhu-cluster has been linked to periplasmic ferrisiderophore transport (Stevanovic et al., 2012). The expression of some of these genes in *Anabaena* sp. is enhanced by not only low Fe, but also by high Cu (Stevanovic et al., 2012).

Genes encoding putative TonB-dependent receptors are found in many freshwater cyanobacterial genomes, but are much less common in marine cyanobacteria, and practically absent in picocyanobacteria (Hopkinson & Morel, 2009; Hopkinson & Barbeau, 2012). Interestingly, the only TBDT-like gene found in picocyanobacteria in this latter study, is a TBDT-type heme transporter (as well as its associated TonB energy translocation system, ExbB/Exbd) in the genome of *Prochlorococcus* sp. MIT9202. These results suggest that direct internalization of ferrisiderophores might not be a widespread physiological strategy to acquire Fe in the ocean. However, the most recent genomic study by Desai et al. (2012) found that TBD hydroxymate uptake components were relatively abundant in the metagenomes, in agreement with the abundance of hydroxymate siderophores in the open ocean.

Axenic cultures, as well as natural populations of marine *Synechococcus* have been shown to use a reductive mechanism at the cell surface, similar to that of eukaryotic microalgae, to access the Fe within siderophore complexes (Lis & Shaked, 2009; Kranzler et al., 2011). This strategy is advantageous in the ocean because it allows access to Fe from a wide variety of naturally occurring strong organic Fe complexes. One of the pending questions in this research is where does the reduction of Fe bound to strong organic complexes occur in cyanobacteria? Iron reduction is most likely to occur either on the surface of the outer membrane or in the periplasmic space. Studies investigating reduction of Fe in strong organic Fe complexes use the Fe(II) trapping agent ferrozine (FZ). These studies measure Fe(II)FZ in solution, suggesting that Fe(II)FZ₃ is produced outside the cell. Since Fe(II)FZ₃ is not available for uptake, it is possible that FZ crosses...
the outer membrane, traps the Fe(II) produced in the periplasmic space and is then secreted by the cell as Fe(II)FZ$_3$.

Recently, a reductive pathway for the transport of inorganic Fe has also been proposed for the freshwater *Synechococcus* (PCC6803) (Kranzler et al., 2013a) (Fig. 4). Inorganic Fe(III) (or Fe(III)’’) is first transported across the outer membrane, possibly through a non-specific porin (Fujii et al., 2011). Once in the periplasma, Fe(III) is complexed by the high-affinity Fe binding protein FutA2, thus establishing a chemical gradient for Fe(III) influx through the outer membrane. The reduction of Fe(III) within the Fe(III)FutA2 protein that follows is mediated by the integral plasma membrane cytochrome c oxidase (Alternate Respiratory Terminal Oxidase). The reductive step results in the release of Fe(II) from the Fe(II)FutA2 complex and the subsequent Fe(II) transport across the plasma membrane via the Fe(II)-specific transporter FeoB (Kranzler et al., 2013a). The Fe(II)-specific transporter FeoB genes have been identified in freshwater and coastal cyanobacteria, but appear to be absent in marine picocyanobacteria and eukaryotic microalgae (Desai et al., 2012). One coastal strain of *Synechococcus* has genes encoding an Fe(II)-specific transporter FeoB (Palenik et al., 2006). This might be an adaptation to the faster redox cycling of Fe in coastal waters due to photo-reduction of Fe(III) in the presence of hydroxycarboxylic acids (Kuma & al., 1992). Other divalent transporters, such as ZIPs and NRAMPs have been identified in marine picocyanobacteria, and thus may mediate their Fe(II) uptake, as previously demonstrated in other organisms. In oceanic metagenomes, Fe(II) transporters are not very abundant, but ZIPs, NRAMPs are more common than FeoB-type.

Even though Fe reduction and a plasma membrane Fe(II) transporter seem to be the dominant Fe transport mechanism in the model cyanobacterium *Synechococcus* (PCC6803), the presence of an Fe(III) transport system, such as FutABC is also expected, given that some of these genes have been identified (Katoh et al., 2001a; Katoh et al., 2001b). This transport system consists of a soluble Fe(III)-binding periplasmic protein (FutA2), a permease (FutB), a peripheral plasma membrane associated ATPase (FutC), and a intracellular FutA1 (Fig. 4) (Katoh et al., 2001a; Katoh et al., 2001b). The
intracellular subunits FutA1 and FutC of this Fe(III) transport pathway are believed to regulate the reductive Fe(II) uptake pathway (Kranzler et al., 2013a). Genomic analyses have revealed the presence of Fe(III)-specific ABC transport systems in many marine cyanobacteria, as well as picocyanobacteria (Desai et al., 2012; Hopkinson & Barbeau, 2012), suggesting that Fe(III) uptake is very common, in agreement with the thermodynamic stability of Fe(III) and the prevalence of Fe(III) organic complexing ligands in seawater. In addition, in oceanic metagenomes Fe(III)ABC transport systems are much more common than Fe(II) uptake systems (Desai et al., 2012; Hopkinson & Barbeau, 2012).

4. Iron storage and luxury uptake

Iron storage is another strategy that enables microalgae to cope with low and intermittent Fe supplies. Because many of the new Fe inputs to the ocean are sporadic, being able to rapidly take up and store Fe from ephemeral inputs would be particularly advantageous. The capacity to take up more Fe beyond than required to satisfy biochemical functions for maximum growth is often termed luxury uptake and likely an important Fe-acquisition strategy of microalgae, in particular diatoms. The Fe storage capacity can be approximated by the ratio between the intracellular Fe requirements when microalgae are grown in excessively high [Fe] and the concentrations in which growth starts to decrease due to Fe-limiting conditions. The resulting ratio reflects a phytoplankter’s potential to store intracellular Fe. Among the phytoplankton examined, the general trend is that oceanic centric microalgae can achieve a lower minimum $Q_{Fe}$ whereas coastal microalgae have a higher maximum $Q_{Fe}$ (Fig. 7) (Sunda & Huntsman, 1995; Maldonado & Price, 1996; Marchetti et al., 2006). Microalgae, particularly bloom-forming species, may benefit from being able to take up and store large quantities of Fe during periods of surplus Fe availability because these reserves can then be drawn upon as Fe concentrations become exhausted. The relatively high storage capacities observed in diatom isolates from the Fe-limited regions of the Equatorial Pacific relative to other coastal and oceanic diatoms isolates were suggested to be an evolutionary adaptation for living in low Fe environments with sporadic Fe inputs (Maldonado & Price, 1996).
The Fe storage capacities of pennate diatoms belonging to the genus *Pseudo-nitzschia*, a cosmopolitan diatom genus found in both coastal and oceanic environments have also been examined (Marchetti et al., 2006). Members of this genus are near-universal responders to artificial Fe enrichment in Fe-limited regions of the ocean (Marchetti et al., 2008; Trick et al., 2010). In Fe-limited oceanic waters, *Pseudo-nitzschia* abundance is usually low compared to other phytoplankton although species richness may be high. Upon Fe-enrichment, *Pseudo-nitzschia* numerically dominates the Fe-induced diatom community and can make up a significant proportion of the phytoplankton biomass. The Fe storage capacities for the oceanic *Pseudo-nitzschia* spp. examined were markedly higher than those calculated for oceanic *Thalassiosira* spp. Differences in Fe:C ratios of the oceanic *Pseudo-nitzschia* isolates compared to oceanic *Thalassiosira* spp. were a consequence of a higher maximum $Q_{Fe}$ rather than a lower minimum $Q_{Fe}$ observed (Fig. 7), suggesting that *Pseudo-nitzschia* spp. have exceptionally high Fe storage capabilities.

A molecular basis for the enhanced Fe storage in *Pseudo-nitzschia* was provided with the identification of ferritin (FTN) genes within several pennate diatoms (Marchetti et al., 2009). Due to the high redox potential of Fe, extensive intracellular concentrations of free Fe could be detrimental to cells. Ferritin is an Fe-storage protein used by plants, animals, cyanobacteria and other microorganisms to safely concentrate and store Fe, thereby minimizing potential cell damage from reactive oxygen species and oxidative stress. In cyanobacteria, there are three different types of ferritin family proteins that have been identified including bacterioferritins, ferritins and DNA-binding proteins from starved cells (DPS) ferritins (Kranzler et al., 2013b). Ferritins and bacterioferritins oxidize Fe(II) to Fe(III), thereby functioning as ferroxidases while generating hydrogen peroxide. Bacterioferritins differ from ferritins in the presence of a haeme molecule, anchored at the interface between two adjacent subunits. In eukaryotic ferritins (24 subunits, 480 kDa), up to 4500 Fe(III) atoms can be stored as an Fe oxide mineral and releases reduced Fe on cellular demand (Liu & Theil, 2005). Such storage compounds allow Fe to be readily released and utilized when needed, thus acting as a buffer against Fe deficiency. Although ferritins appear to be ubiquitously present in green algae and cyanobacteria, the discovery of ferritin in pennate diatoms was the first indication of the
Fe storage protein in any member of the Stramenopiles with whole genomes sequenced, including *T. pseudonana*. Thus, the acquisition of a ferritin gene in diatoms, likely resulted from a horizontal gene transfer event. The diatom ferritin genes contain signal and target peptides that predicted the protein to be targeted to the chloroplast to control the distribution and storage of Fe for proper functioning of the photosynthetic machinery (Marchetti et al., 2009).

The absence of a ferritin gene in *T. pseudonana* in combination with the significantly reduced capacity of *T. oceanica* to store Fe compared to *Pseudo-nitzschia* led to the speculation that centric diatoms do not contain ferritin. Although centric diatoms lacking ferritin can certainly store Fe, the mechanism as to how this is achieved is not well understood. For example, in the centric diatoms *T. pseudonana* and *T. weissflogii*, Nuester et al. (2012) observed the elemental stoichiometry of cellular Fe to be consistent with a potential vacuolar Fe storage pool mechanism. Recent transcriptomic sequencing of a number of centric and pennate coastal diatoms has now identified the sporadic presence of ferritin-like genes within some centric diatoms. In addition, multiple homologs of FTN genes have been identified in *C. reinhardtii* and several diatom genomes, including *Pseudo-nitzschia multiseries*. Whether these homologs perform different functions is unknown. This new and rather unanticipated patchy diversity of ferritins throughout the diatom lineage necessitates a more systematic examination into the differences in the Fe storage capabilities of diatoms that either contain or lack ferritin.

While the results from bulk studies with natural phytoplankton assemblages suggest that some microalgae can accumulate Fe following pulsed inputs, these experiments do not clarify which species are accumulating the Fe, or whether the Fe is simply adsorbed onto the outside of the cells or actually accumulated internally. Synchrotron x-ray fluorescence microscopy (SXRF) has provided this missing information, as it enables quantitative elemental analysis of individual cells, as well as spatial information about where the elements are located within the cells (Twining et al., 2003; Twining & Baines, 2013). During the SOFeX Fe fertilization experiment in the Southern Ocean, diatoms increased their Fe:C ratios nearly 5-fold after two Fe additions, while autotrophic
flagellated cells increased their Fe quotas by only 3-fold (Twining et al., 2004). Additionally, two-dimensional element maps showed that the extra Fe was accumulated largely in the regions of the chloroplasts, indicating that uptake was internal and not the result of abiotic precipitation and adsorption. SXRF has also been used to measure Fe accumulation by microalgae in the Equatorial Pacific. Baines et al. (2011) found Fe quotas of *Pseudo-nitzschia*-like diatoms to increase 24-fold in 48-h following addition of 2 nM Fe to deckboard incubations. Further, this Fe was largely localized in Fe storage bodies adjacent to the chloroplasts, as predicted for ferritin (Marchetti et al. 2009). Over the final two days of the 96-h incubation, cellular Fe contained within the storage bodies dropped exponentially at a rate which approximated the growth of the diatom population, while overall cellular Fe quotas decreased more slowly (Twining et al., 2011). These data demonstrate the potential for stored Fe to support subsequent population growth, as well as the unique ability of SXRF to quantify luxury uptake of Fe by phytoplankton in natural systems.

5. Iron and light co-limitation

Light and Fe availability interact in a complex and bi-directional manner, and have a profound effect on the ability of phytoplankton to photosynthesize. Indeed, Fe limitation impairs (especially at low light) the capacity of the cells to absorb light energy, as well as convert light energy into chemical energy. Some phytoplankton acclimate to limiting Fe levels by lowering the abundance of the Fe-rich PSI relative to PSII, as previously discussed. High light may enhance Fe availability under low Fe concentrations by promoting photochemical reactions that increase the pool of inorganic Fe ([Fe']) in the presence of photolabile organic Fe complexes (Anderson & Morel, 1982; Sunda & Huntsman, 2011). In addition, under low Fe, high light may enhance Fe uptake by providing more photosynthetically derived NAD(P)H, which is needed for the functioning of the membrane reductases involved in Fe uptake (Askwith et al. 1996).

Phytoplankton acclimate to low photon flux by changing the ratio of accessory pigments to chl *a*, as well as the abundance and stoichiometry of Fe-rich photosynthetic electron carriers (Falkowski et al., 1981). Under low light conditions, phytoplankton may modify
their PSUs to enhance light harvesting by increasing their numbers and/or their size. These two physiological strategies alter distinctively their Fe requirements for photosynthesis. A higher number of PSUs will increase their Fe demand, as the photosynthetic electron transport protein complexes associated with PSU are Fe-rich. In contrast, an increase in the size of PSU involves higher concentrations of light-harvesting accessory pigments, and does increase Fe demand (Geider et al., 1993).

Support for the first strategy has been predicted theoretically (Raven, 1990) and documented empirically by showing higher intracellular Fe quotas under low light, both in laboratory cultures (Sunda & Huntsman, 1997; Strzepek & Price, 2000; Strzepek & Harrison, 2004; Finkel et al., 2006; Weng et al., 2007; Sunda & Huntsman, 2011), and field populations (Maldonado et al., 1999; Hopkinson & Barbeau, 2008). These higher Fe quotas are allocated towards the synthesis of additional Fe-rich photosynthetic electron transport protein complexes to maintain high rates of C fixation at low light (Sunda and Huntsman 1997). Indeed, in the case of T. weissflogii, the relative increase in Fe quotas under low light matches the predicted relative increase in abundance of PSI relative to PSII during photoacclimation (Strzepek and Price, 2000). However, the higher Fe quotas observed under low light are partly a consequence of slower growth rates ($p_{Fe}^{ss} = \text{Fe quota} \times \text{growth rate}$, Sunda and Huntsman 2011)). In addition, significantly higher half-saturation constants for Fe ($K_{\mu}$) have been shown for cyanobacteria and diatoms grown under low light than under high light (Kudo & Harrison, 1997; Bucciarelli et al., 2010). The higher $K_{\mu}$ under low light are partly explained by lower [Fe$^+$] in a medium with photolabile organic Fe complexes ($[\text{Fe}^+]_{HL} = [\text{Fe}^+]_{LL} \times 1.14$, Bucciarelli et al. (2010)). The remaining difference between these $K_{\mu}$s (e.g. for T. oceanica $K_{\mu HL}$ and $K_{\mu LL} = 1.29$ and 3.01 pM, respectively; Bucciarelli et al. (2010)) is likely due to higher Fe demand by low-light-adapted phytoplankton.

The second strategy, increasing the size of PSUs, has been shown in cyanobacteria, the green alga Dunaliella salina and Southern Ocean diatoms and haptophytes. Southern Ocean phytoplankton are extremely well adapted to limiting Fe levels and cope with low light by an unprecedented change in the size (6-fold larger) of the effective absorption
cross section ($\sigma_{PSII}$) of their PSII reaction centers, without an increase in cellular Fe (Strzepek et al. 2012). In a similar manner, Fe-limited cyanobacteria have fewer Fe-rich PSIs, but promote the expression of IsiA antenna proteins associated with PSI. This increases the PSI absorption cross-section and maintains rapid electron flow through PSI (Chauhan et al., 2011). Likewise, under low Fe, the green alga *Dunaliella salina* induces the expression of chlorophyll a/b-binding proteins associated with PSI (Varsano et al., 2006). However, this physiological photo-acclimation strategy has its limitations, as the size of the PSU has an optimal maximum, beyond which the energy transfer efficiency between the light harvesting pigments and PSUs decreases sharply (Raven 1990).

6. Iron limitation effects on elemental composition

Phytoplankton are responsible for approximately half of the carbon fixation on Earth. Thus minor alterations in the Redfield composition (i.e. 106C:16N:1P) and overall elemental composition due to Fe limitation could have large influences on global biogeochemical cycles. Diatoms, in particular account for approximately one-half of marine primary productivity (Nelson et al., 1995). One important effect of Fe limitation on the elemental composition of diatoms is a relative increase in their Si content. A study in the California coastal upwelling region by Hutchins and Bruland (1998) noted a 2-3-fold increase in the silicic acid-to-nitrate ($\text{Si}([\text{OH}]_4:\text{NO}_3$) and silicic acid-to-dissolved inorganic carbon ($\text{Si}([\text{OH}]_4:\text{DIC}$) drawdown ratios of diatom-dominated communities in Fe-limited controls relative to Fe-enriched seawater samples. The observed decrease in the dissolved nutrient ratios upon the alleviation of Fe limitation was primarily due to the rapid increase in $\text{NO}_3$ utilization with little or no change in $\text{Si}([\text{OH}]_4$ utilization. This lead to the speculation that Fe-limited diatoms utilize more Si per cell or unit biomass relative to Fe-replete diatoms and thus were more heavily silicified (Boyle, 1998). At the same time, in addition to studying the response to Fe enrichments in natural assemblages, Takeda (1998) found that Fe-limited Antarctic diatoms had higher Si:N ratios relative to when they were Fe-replete. In a *Nitzschia* sp., the increase in the ratio was due to more Si per cell, whereas in *Chaetoceros dichaeta* less N per cell drove the increase in the ratio. Since then, additional studies have documented changes in the Si:N and $\text{Si}([\text{OH}]_4:\text{NO}_3$ uptake/consumption ratios in Fe-limited diatoms or diatom-dominated
natural assemblages and have attributed these changes to enhancements in silicification and/or reductions in N and C, or have suggested that no changes in the ratios occur when both new (NO$_3$-based) and regenerated (NH$_4$-based) forms of N are considered (reviewed by Marchetti & Cassar, 2009). In addition, coordinated gene expression responses to Si and Fe limitation within *T. pseudonana* suggest both elements have a similar influence on diatom cell wall processes (Mock et al., 2008).

Discrepancies in the causal element influencing the Si:N ratios among these laboratory and field studies may be due to varying levels of Fe deficiency, differences in growth conditions, implemented methodology, shifts in microalgal species composition and/or the intrinsic variability among different diatoms (reviewed by Marchetti and Cassar 2009). Despite the variety of possible causes behind changes in the element stoichiometries, the observed outcome of Fe-limited diatoms is generally consistent – an increase in the cellular Si:N ratios, although there are exceptions with certain diatoms under severe Fe limitation (Bucciarelli et al., 2010). This result supports the assertion that Fe-limitation is an important factor in contributing to the preferential export of Si relative to N (Dugdale & Wilkerson, 1998; Hutchins & Bruland, 1998). However, the specific physiological mechanism driving the changes in elemental stoichiometry of diatoms (i.e. an increase in cellular Si content and/or a decrease in both N and C contents) are important for predicting diatom biogeochemical fluxes to the ocean floor. Cell size and the degree of silicification may affect how effectively a certain diatom species can sequester carbon due to the possibility of relatively larger and thicker-shelled diatoms sinking faster (Smayda, 1970; Smetacek, 1985; 1999; Raven & Waite, 2004; Assmy et al., 2013), being less susceptible to grazing (Smetacek, 1999; Hamm et al., 2003; Pondaven et al., 2007) and more effectively preserved in ocean sediments.

Although the C:N ratios of phytoplankton are relatively unaffected by nutrient limitation, including Fe, there are reports of an decrease in N:P (and by inference C:P) ratios in Fe-limited phytoplankton. In *T. weissflogii*, this decrease was primarily a result of accumulation of P under low-Fe conditions due to a more rapid decline in growth rate than in steady-state uptake rate of phosphorus (Price, 2005). Under the most severe Fe-
limiting conditions, *T. weissflogii* contained 1.5 time more P per liter cell volume than when Fe-replete. The trend observed in the laboratory isolate is supported by field observations from Fe-limited natural assemblages dominated by diatoms where lower N:P and C:P ratios have been observed. Thus, as Fe inputs to Fe-limited regions around the globe vary in space and over geological timescales, considerable changes in the elemental composition of sinking phytoplankton debris would be anticipated.

Iron status may also influence the stoichiometry of other trace elements. For example, as previously discussed, diatoms that contain a multi-copper oxidase as part of their high affinity Fe uptake pathway have an increased demand for copper when this pathway is induced under low Fe-limiting conditions (Peers et al., 2005; Maldonado et al., 2006). Even under Fe-replete conditions, the copper requirements of many oceanic diatoms are higher due to their dependence on the copper-containing electron transport protein plastocyanin, whereas many coastal diatoms use cytochrome c₆, which is an Fe-containing protein (Peers & Price, 2006).

7. Microalgal response to iron fertilization

In vast expanses of our world’s oceans, the biomass of phytoplankton is low even though macronutrient concentrations are quite high. These areas are termed “high nutrient (nitrate), low chlorophyll” or HNLC regions. Until recently, the factors most responsible for minimizing new production in HNLC regions remained unknown, forcing oceanographers to speculate about possible causes. Competing hypotheses that emerged in the late 1980s attempted to explain these paradoxical HNLC regions. As reviewed by Cullen (1991), factors believed to control primary productivity from a “bottom-up” approach included the effects of low temperature (Tilzer et al., 1986), light limitation and/or water column instability and strong turbulence (Chavez et al., 1991; Miller et al., 1991; Mitchell et al., 1991), “shift-up” in NO₃ assimilation (Dugdale & Wilkerson, 1991) and micronutrient (Fe) limitation (Martin & Fitzwater, 1988). “Top-down” or “grazer” control was also suggested as the primary factor regulating phytoplankton biomass in these oceanic regions (McAllister et al., 1960; Banse, 1991; Miller et al., 1991). The possibility that the regulation of primary productivity in HNLC regions may be a
combination of all these factors rather than a single one was also considered (Harrison et al., 2004).

Martin and Fitzwater (1988) proposed that low Fe concentrations may limit growth of phytoplankton in many regions of the ocean. Although early investigators had proposed the possibility of Fe limitation (Hart, 1934), supportive experimental data was difficult to obtain due to the lack of trace metal clean sampling methods that inevitably resulted in Fe contamination. Martin et al. (1989) showed that surface concentrations of dissolved Fe in these HNLC regions were very low (<<1 nM) and dissolved Fe exhibited a nutrient-like profile. Furthermore, bottle experiments consisting of the addition of Fe to the HNLC Subarctic Pacific resulted in an increase in chl $\alpha$ and a subsequent drawdown of NO$_3$ (Martin and Fitzwater 1988). In particular, the growth rate of large phytoplankton increases, resulting in a species composition shift from cyanobacteria and small flagellates to large diatoms. These experiments provided compelling evidence that Fe may indeed limit phytoplankton growth and validated the “Iron Hypothesis” (Martin, 1990). There are three main regions of the world’s oceans where the primary producers may be consistently or periodically limited by the supply of Fe. These include parts of the Equatorial Pacific, the Subarctic Pacific and most of the Southern Ocean (de Baar et al., 2005; Boyd et al., 2007). There is also compelling evidence for Fe limitation of phytoplankton in coastal upwelling regions (Hutchins et al., 1998; Firme et al., 2003), as well as seasonal Fe limitation (primarily of N$_2$ fixation rates) within the North Atlantic (Moore et al., 2009).

The confirmation that phytoplankton may be limited by a trace metal that is very abundant on land has lead to the concept of performing mesoscale open ocean Fe enrichment experiments to mitigate increases in atmospheric CO$_2$ caused by the burning of fossil fuels. In addition, since the molar stoichiometry of most phytoplankton is $\sim 10^5$ C:1Fe, compared to $\sim 7$C:1N, it is considerably very economical to add Fe to large areas of the ocean rather than N. Though met with considerable debate and skepticism by many scientists (Strong et al., 2009), human-induced Fe enrichment is viewed by some as a possible solution to dealing with climate change and reductions in commercial fish
stocks. The unprecedented rate of increase in atmospheric levels of CO$_2$ is considered to be the major contributor to global warming, although methane, nitrous oxides and chlorofluorocarbons also play a role. A number of such Fe enrichment experiments have been performed in the equatorial Pacific (IronEx I and IronEx II), in the Southern Ocean (SOIREE, EisenEx, SOFex, Eifex and Lohafex) and North Pacific (SEEDS I and II and SERIES) (Fig. 8) (Boyd et al., 2007; Smetacek et al., 2012). Each of these experiments demonstrated varying degrees of increased phytoplankton growth in response to Fe additions to surface waters that were dependent on the initial environmental conditions and the initial phytoplankton community composition. The phytoplankton response to Fe seems to be dependent on the initial environmental conditions, as well as the phytoplankton seed populations. With the exception of a few experiments, Fe enrichments resulted in increased diatom growth that subsequently partially or fully depleted macronutrient concentrations leading to bloom termination. The resulting diatom blooms are often large enough to be visible from space (Fig. 8).

Following the initial Fe enrichment studies performed in each of the well-characterized HNLC regions, subsequent rounds of large-scale Fe addition experiments have focused on the Southern Ocean, as it is the largest of the HNLC regions containing a vast pool of underutilized macronutrients. The SOFeX experiments added Fe to two separate north and south patches, with the north patch containing low Si(OH)$_4$ concentrations and the south patch containing high Si(OH)$_4$ concentrations (Coale et al., 2004). Although an increase in phytoplankton biomass was observed in both patches, the phytoplankton assemblage differed, as the north patch contained a significantly lower proportion of diatoms due to Si(OH)$_4$ limitation. During Eifex, Fe was added to a vertically coherent, mesoscale eddy of the Antarctic Circumpolar Current (Smetacek et al., 2012). An ensuing large diatom bloom was monitored for five weeks verifying that at least half of the phytoplankton bloom biomass sank to a depth of 1000 meters and a substantial portion likely reached the sea floor. Similar to natural Fe fertilization observations in the Southern Ocean (Blain et al., 2007; Pollard et al., 2009), Eifex verified a substantial deep carbon export associated with the ensuing Fe-induced diatom bloom.
A molecular basis for the phytoplankton response to Fe enrichment and their mechanism of recovery was recently achieved through a comparative metatranscriptome analysis of an Fe-limited plankton community in HNLC waters of the NE Pacific Ocean (Marchetti et al., 2012). Consistent with the large-scale Fe enrichment experiments, a large diatom bloom ensued after Fe addition to mesocosms. The immediate diatom response to Fe enrichment was to continue to express genes encoding non-Fe containing proteins rather than carrying out a widespread replacement of these proteins with their Fe-containing counterparts. For example, transcripts associated with the Fe-free electron transfer protein flavodoxin were abundant in all libraries and over-represented after Fe enrichment, suggesting that some or all diatoms continue relying on this protein for photosynthetic electron transfer rather than switching entirely to use the Fe-containing version, ferredoxin. Additionally, diatom transcripts for copper-containing plastocyanin were highly abundant under all conditions whereas very few transcripts for cytochrome c₆, the Fe-requiring counterpart, were readily identified. Continued dependence on Fe-free proteins may also provide diatoms with a more rapid acclimation to a return to low-Fe conditions.

The addition of Fe to diatoms stimulated what appears to be a system-wide pattern of gene expression in alleviation of chlorosis and preparation for rapid cell division. Specifically, significant increases in transcripts for genes encoding proteins that catalyze the biosynthesis of nucleic and amino acids, sugars, chlorophyll (and its precursors, i.e. porphyrin) as well as the prerequisites for long-chain polyamines, which may play an important role in silica precipitation for diatom cell wall formation, were observed. Components of the diatom urea cycle were suggested to likely play a significant role in facilitating the Fe response leading to bloom formation following the alleviation of Fe limitation and/or low-Fe-induced NO₃ limitation. The ability of diatoms to divert their newly acquired Fe towards nitrate assimilation may underlie why diatoms consistently dominate Fe enrichments in HNLC regions. The distinctiveness of the diatom response was even more apparent when compared to the haptophytes, the other dominant member of the phytoplankton community after Fe enrichment, which displayed a more typical response to Fe enrichment (i.e. increased transcripts of ferredoxin, Fe-SODs and other
Fe-requiring protein-encoding genes). In addition, rhodopsin transcripts assigned to diatoms, haptophytes and dinoflagellates were highly abundant in the Fe-limited libraries, decreasing in abundance following Fe enrichment. Rhodopsins are light-driven proton pumps that may be associated with ATP generation (Beja et al., 2000; Fuhrman et al., 2008), which suggested a role for rhodopsins in eukaryotic phytoplankton to deal with low-Fe conditions (Marchetti et al. submitted).

8. Climate change effects on iron-related microalgal physiology

As the amount of CO$_2$ in the atmosphere continues to increase, equilibrium of atmospheric CO$_2$ with the surface ocean will decrease the pH of seawater, the consequences of which have often been referred to as ocean acidification. An extensive amount of research has been conducted examining ocean acidification effects on the calcification processes of marine organisms, including microalgae (e.g. coccolithophores) (for a review, see Doney et al., 2009). As seawater pH is reduced, the most prominent effect is a decrease in the carbonate ion concentration that could impede calcification rates, although the responses of marine organisms appear to be varied. Less is known about other potential effects of ocean acidification and warming on marine ecosystems, including how the speciation and distributions of trace metals such as Fe will be affected (Hoffmann et al., 2012). In addition to changes in carbon chemistry (i.e. a reduction in carbonate ion), a decrease in pH will result in the reduction of hydroxide in most natural surface waters. Because Fe forms strong complexes with hydroxides, there will likely be significant changes in Fe speciation, where Fe(III) in seawater will increase in solubility and there will be increases in ionic Fe(II) as opposed to it forming carbonate complexes (Millero et al., 2009).

To some degree, an increase in Fe solubility along with proposed changes in kinetics could lead to increases in primary productivity in Fe-limited regions where Fe is present in its free form, and constitutes a measurable fraction of the Fe pool. However as the vast majority of Fe$_{\text{diss}}$ is bound to strong organic ligands (Gledhill & van den Berg, 1994; Rue & Bruland, 1995), the changes in complexation of Fe with these organic ligands at a reduced pH are what will largely drive how ocean acidification affects Fe availability.
The effects of reduced pH on Fe availability to marine phytoplankton when Fe is bound to a variety of organic ligands were recently examined (Shi et al., 2010). In all examined species, which included both coastal and oceanic diatoms as well as the coccolithophore (*E. huxleyi*), Fe uptake rates were proportional to Fe', indicating that the effect of pH is largely due to a change in the chemical speciation of Fe and not a physiological response of the organisms. When Fe was bound to chelators with acidic binding groups that are not protonated in seawater (e.g. carboxylic acid group chelators such as EDTA), the extent of decrease in Fe' with decreasing pH is dependent on the number of protons released upon dissociation of Fe. Likewise, if the uptake of Fe is dependent on enzymatic reduction of the Fe-chelator complex, a decrease in pH could affect rates of the enzymatic reaction if protons are liberated. This was proposed to be the case for a decrease in Fe uptake rates with decreasing pH when Fe is bound to the trihydroxomate siderophore, DFB (Shi et al., 2010). In contrast, there was a negligible effect of pH on Fe uptake when Fe was bound to chelators that are protonated in seawater (e.g. catechols). Thus, in general, it was determined that the availability of Fe to microalgae decreases at lower pH and are largely dictated by the acid-base chemistry of the chelating ligands.

Field experiments have shown mixed responses to alteration in CO₂ and Fe concentrations within natural phytoplankton assemblages from HNLC regions. In Fe-limited waters within the Gulf of Alaska, a marginal increase in biomass and production was observed at high CO₂ and was attributed to the energy savings associated with a down-regulation of the carbon-concentrating mechanisms (CCM) and, in some cases, a reduction of Fe rich photosynthetic proteins (Hopkinson et al., 2010). Alternatively, under Fe-enriched conditions, increases in growth and biomass at high CO₂ compared to low CO₂ treatments were suggested to be the result of lower rates of respiration, leading to reductions in carbon loss. Similarly, in coastal waters off Norway, at high CO₂ levels, increases in Fe₅₃ and Fe(II) concentrations were observed within mesocosm experiments, suggesting increased Fe availability as a result of CO₂ enrichment (Breitbarth et al., 2010). In contrast, experiments conducted with natural seawater samples in coastal and oceanic waters in the North Atlantic (Shi et al. 2010) and Fe-limited waters in the Bering Sea (Sugie et al., 2013) inferred decreases in Fe uptake rates,
Fe availability and growth of phytoplankton with increasing CO₂ levels. In the Bering Sea study, these CO₂-mediated influences disappeared under Fe-replete conditions. Such discrepancies could be due to a number of factors, including differences in the Fe concentrations, Fe speciation and the phytoplankton community composition.

Apart from affecting Fe availability, interactive effects of ocean acidification and Fe have been shown to influence other aspects of microalgal physiology and community dynamics. For example, in the coastal diatom *Pseudo-nitzschia pseudodelicatissima*, Si:C and Si:N elemental ratios decreased, whereas N:P ratios increased with increasing pCO₂ (Sugie & Yoshimura, 2013). Thus, in a high CO₂ world, these patterns could buffer the changes in elemental ratios that occur when diatoms are Fe-limited, which tend to be in the opposite direction. Within Fe-enriched natural assemblages present in the Weddell Sea, Antarctica, CO₂-dependent changes in productivity were accompanied by a taxonomic transition from weakly to heavily silicified diatoms (Hoppe et al., 2013). These transitions did not occur under Fe-limiting conditions, suggesting differences in carbon acquisition strategies and/or pH-mediated cellular physiologies among diatoms that were only evident after the alleviation of Fe stress.

Although our knowledge of ocean acidification effects on microalgae has advanced tremendously over the last decade, ocean acidification is only one of multiple factors that will be altered within marine systems as a consequence of climate change. Along with increases in atmospheric CO₂, significant warming of the land and oceans will occur which will result in changes to water column stratification, salinity and ocean circulation (Doney et al., 2012). In addition, changes in nutrient content and oxygen input to the ocean surface waters are predicted. All of these factors will undoubtedly effect Fe distributions and speciation, perhaps more so than ocean acidification, which will then have downstream biological consequences.

9. Techniques for studying iron-related microalgal physiology

*Achieving iron limitation in the laboratory*
One of the primary means by which physiologists investigate ways in which microalgae acclimate to varying Fe availabilities is by attaining steady-state Fe-limitation of growth rates in laboratory isolates. Achieving steady-state Fe limitation is now common practice provided that there is access to trace metal clean culturing facilities and implementation of certain culturing protocols (Price et al., 1988/89; Sunda et al., 2005). These protocols include the use of chemical-resistant plastics that contain lower concentrations of trace elements for culturing (such as polycarbonate, low-density polyethylene and teflon), acid-cleaning of all culture tubes and bottles, chelexing of the seawater and performing all preparation of medium and culture transfers within a trace metal clean laminar flowhood. Either synthetic ocean water (i.e. Aquil or ESAW; Harrison & Berges, 2005) or low-Fe natural seawater can be used as growth medium. In most seawater medium recipes, synthetic aminocarboxylate chelating agents, such as EDTA are commonly used to regulate trace metal speciation. Concentrations of EDTA vary among the different types of medium, but typically range between 5-100 μM. When EDTA is added to the medium, the concentrations of low level Fe and natural Fe chelators within seawater become almost negligible and they are unlikely to have a significant influence on metal speciation. [Fe]_{diss} exceeding ca. 0.07 nM is expected to precipitate and is likely no longer bioavailable to phytoplankton (Johnson et al., 1997), whereas in the presence of EDTA, Fe' concentrations can reach up to 0.7 nM before exceeding the empirically observed threshold for precipitation of Fe hydroxides (Sunda & Huntsman, 1995). By adjusting the concentrations of Fe and EDTA in the culture medium, the amount of Fe’ can be lowered to the point where the availability of Fe regulates the growth rate of the phytoplankton. As described previously, EDTA will continuously buffer the concentration of Fe, maintaining a low, but constant pool of Fe’ until another nutrient (typically a macronutrient such as silicic acid when growing diatoms) is depleted and the culture enters stationary phase. Fig. 9 provides total Fe, Fe' and Fe(III) concentrations reportedly used in laboratory culture experiments to achieve Fe replete (e.g. pFe 18 and 19) and Fe-limited (pFe > 20.5) growth of microalgae.

Although useful for laboratory studies, the relevance of using synthetic chelators such as EDTA to buffer trace metals within culture medium to determine the Fe requirements and
Fe-related physiology of microalgae has limitations. As previously discussed, the use of large amounts of EDTA results in the biological availability of Fe to be primarily controlled by concentrations of Fe'. Yet our current understanding is that the dissolved Fe pool is predominantly bound by natural organic ligands (Gledhill & van den Berg, 1994; Rue & Bruland, 1995). Therefore, the corresponding calculated inorganic Fe in natural seawater is likely far too low for sustaining growth of even the smallest phytoplankton species, suggesting some of these natural organic Fe complexes are bioavailable (e.g. Strzepek et al. 2011). The question can then be posed whether EDTA provides for an accurate representation of these natural organic ligands (Muggli & Harrison, 1996a)? Although it is unlikely, it is difficult (but certainly not impossible) to perform Fe physiological investigations without the use of such chelators when growing phytoplankton in natural low-Fe seawater unamended with additional Fe complexes. Therefore, there are only a few studies examining Fe physiology without the use of synthetic chelators such as EDTA (e.g. Timmermans et al., 2001; Timmermans et al., 2004). Alternatively, natural organic ligands such as DFB can be used (see section below). If the objective is to grow microalgae without the use of such chelators, practicing strict trace metal clean procedures, in addition to quantifying and characterizing the naturally present organic and Fe ligand pools are essential.

Probing the iron nutritional status of natural phytoplankton

The physiological state of phytoplankton with regards to Fe nutrition can be assessed by measuring rates of Fe uptake in the presence of DFB or EDTA using Fe radionuclides (i.e. \(^{55}\)Fe or \(^{59}\)Fe). The transport of Fe in the presence of excess DFB at sub-saturating Fe concentrations is indicative of Fe limitation. In contrast, the transport of Fe at saturating concentrations, achieved by nM additions of Fe bound to EDTA (to prevent Fe precipitation), are a measure of the number of Fe transporters at the cell surface. The reason is that at saturating Fe levels, maximum rates of Fe transport are observed and are determined by the number of Fe transporters at the cell surface and the rate of internalization, so that \(\rho_{max} = k_{in} \cdot L_{T}^{max}\).

Determining sub-saturating rates of iron uptake in the presence of DFB
The hydroxamate siderophore desferrioxamine B (DFB) has a conditional stability constant for Fe similar to those of in situ strong organic ligands (Rue and Bruland, 1995). Most recently, hydroxamate siderophores have been identified and quantified in the Atlantic, and seem to be widely distributed in the ocean (Mawji et al., 2008). Therefore, DFB is a good model for the strong naturally occurring organic ligands for Fe in seawater. Many studies (e.g. Hutchins et al., 1999a; Eldridge et al., 2004; Taylor et al., 2013) have used DFB to induce Fe limitation in bottle incubations, by adding high concentrations of DFB that significantly decrease the concentration of inorganic Fe and result in an excess of free DFB. Whether additions of DFB induce Fe limitation depends on the molar ratio of [Fe] to [DFB]. If the concentration of DFB is greatly in excess of that of Fe, the free DFB competes directly with the cell surface Fe transport complexes for the biologically generated free Fe, and thus the ability of the cells to take up Fe is hampered, and results in Fe-limited growth. For example, many oceanic and Southern Ocean species are able to grow at maximum rates when Fe and DFB are at equimolar concentrations (i.e. 4 nM), while coastal species are Fe-limited in these growth conditions. As the concentration of DFB is increased, oceanic and Southern Ocean species start to experience Fe limitation at various degrees. For example, *T. oceanica* grew at 50% of $\mu_{\text{max}}$ in the presence of 4nM Fe:40nM DFB, while to achieve similar reductions in growth rates in Southern Ocean isolates (*Phaeocystis antarctica* and *Proboscia inermis*) additions of 400nM DFB (bound to 4nM Fe) were needed (Strzepek et al., 2011). In another study, *T. oceanica* did not achieve Fe limitation at 12.9 nM Fe: 1.29 μM DFB, nor 40 nM Fe: 4 μM DFB. However at 120 nM Fe:12 μM DFB, a 50% reduction in growth rates was observed (Maldonado & Price, 2001). In the Arctic, at in situ [Fe]$_{\text{diss}} = 0.15$ nM, Fe limitation was induced with additions of 1 nM DFB (Taylor et al., 2013). In the Weddell Sea, at in situ [Fe]$_{\text{diss}} = 1.12$ nM, Fe limitation was induced with a 10 nM DFB addition (Hoppe et al., 2013). Therefore, it is essential to know the initial concentration of dissolved Fe. As a rule of thumb, a DFB addition at a concentration 10- to 20-fold in excess of that of Fe should be sufficient to induce Fe limitation.

However, Fe uptake in the presence of DFB can also be used to test the Fe-limited conditions of phytoplankton growth. This is due to the fact that phytoplankton are only
able to acquire significant amounts of Fe bound within FeDFB when they are Fe-limited, and thus, have up-regulated their high-affinity Fe transport system (Maldonado & Price, 1999; Strzepek et al., 2011). Thus, although the rates of Fe uptake in the presence of FeDFB are orders of magnitude slower than those in the presence of only inorganic Fe or FeEDTA, these FeDFB uptake rates can be used as a proxy for the Fe-limited condition of the phytoplankton (Maldonado & Price, 1999). Thus, the rates of $^{55}$Fe uptake from $^{55}$FeDFB ($\rho$FeDFB) can be determined in order to establish whether plankton in the various treatments or locations are Fe-limited. The addition of Fe and DFB can vary depending on the study. For example, we have used 2 nM Fe bound to 20 nM DFB (100% of the Fe as $^{55}$Fe, Maldonado and Price 1999; Maldonado et al. 2005), or 100 nM Fe bound to 105 nM DFB (5% of the Fe as $^{55}$Fe, Taylor et al. 2013). Another advantage of using DFB for Fe uptake experiments in situ is that because the FeDFB complex is a strong Fe ligand ($K_{Fe' L_{cond}} = 10^{16.5} \text{M}^{-1}$) (Rue & Bruland, 1995) the addition of FeDFB to seawater does not significantly increase the in situ inorganic Fe concentration over the course of the 24 h uptake assay. For example, if the natural seawater has an [Fe’] of $1.5 * 10^{-12}$ M, in an Fe uptake experiment with additions of 100 nM Fe and 105 nM DFB, the calculated [Fe’] ($\sim 6.3 * 10^{-16}$ M [Fe’]) in the presence of DFB, calculated using $[\text{Fe’}] = \frac{\text{[FeDFB]}}{([\text{DFB’}] * K_{Fe' L_{cond}})}$, where $[\text{DFB’}] = [\text{DFB}]_{\text{free}}$, and $K_{Fe' L_{cond}} = 10^{16.5} \text{M}^{-1}$, Rue and Bruland, 1995) does not exceed the in situ [Fe’].

To perform these Fe uptake experiments, before FeDFB is added it to the 1 L incubation bottles, the cold Fe and the $^{55}$Fe (specific activity 740-1480 MBq mg$^{-1}$) must be complexed to DFB in small aliquots of Milli-Q water. All the manipulations must be performed inside a laminar flow hood. The pH of the $^{55}$Fe stock solution ($\sim 0.11$ mM $^{55}$Fe in 0.005 M HCl) used to prepare the Fe complexes should be below 3, so that all of the Fe is truly dissolved. The DFB stock solution is prepared in sterile, Milli-Q water, and is kept refrigerated to prevent fungal growth. Complete complexation of the Fe to DFB is ensured by allowing the DFB to react with the Fe for 5-8 h at pH 3.3 (in 18.2 MΩ, MilliQ-H$_2$O with 0.0005 M HCl). At pH > 3, FeDFB is completely coordinated and very stable with respect to dissociation (Monzyk & Crumbliss, 1982). Indeed, at pH 3.3 the concentration of DFB is $10^{13}$ times higher than that of DFBH$^+$, so most of the DFB is free.
and available to bind to Fe(III) (Maldonado and Price 2001). After FeDFB complexation has been achieved, the FeDFB can be added to triplicate 1L polycarbonate bottles with seawater. The bottles are then capped and sealed with Parafilm™, and then placed on-deck incubators at in situ light intensity and temperature. Every 4 h, samples are drawn from the incubation bottles, collected on polycarbonate filters of varying porosity and rinsed with Ti(III) citrate EDTA reagent (Hudson and Morel, 1989) or oxalate wash (Tang & Morel, 2006). Control samples should be poisoned with glutaraldehyde (0.5 ml of 25% glutaraldehyde L⁻¹) and then processed in an identical manner to that of the samples. These controls are used to correct for abiotic Fe uptake and ⁵⁵Fe adsorption by the filters, and normally represent <5% of the total Fe uptake by the living cells.

Adding nM concentrations of Fe in the ρFeDFB experiments (or ρFeEDTA, see below) allows Fe uptake rates to be calculated without a priori knowledge of the in situ Fe concentration, at least in most open ocean surface waters where [Fe]ₐₐ₈ ≈ 0.07 nM (Johnson et al., 1997). Volumetric ⁵⁵Fe uptake rates (mol L⁻¹ h⁻¹) are then calculated from the linear regression of particulate ⁵⁵Fe concentration as a function of incubation time. These ⁵⁵Fe uptake rates are then converted to total Fe uptake rates using the ratio of dissolved concentrations of ⁵⁵Fe to cold Fe in the experimental Fe uptake bottles. In field Fe uptake studies, the volumetric Fe uptake rates can be normalized to phytoplankton biomass (e.g. chlorophyll concentrations). These chl a-normalized rates can then be normalized to C using published C to chl a ratios (Geider, 1987). In laboratory Fe uptake experiments, the rates of Fe transport (ρFe) should be normalized to cellular surface area. Reporting Fe uptake rates as mol Fe μm⁻² h⁻¹ allows easy comparison between species of different sizes, or between Fe-limited growth treatments of the same species (given that a reduced cell size is a common acclimation to Fe limitation). In addition, Shaked and Lis (2012) suggested normalizing sub-saturating Fe uptake rates to the concentration of dissolved Fe, given that sub-saturating Fe uptake rates by a cell vary with Fe concentration. The resulting parameter is the uptake rate constant (k_uₚ in Shaked and Lis 2012; or k_in in Lis et al. 2014), and has units of L cell⁻¹ h⁻¹ or L μm⁻² h⁻¹, depending on whether the ρFe are, in the first place, expressed as cellular rates or normalized to surface area, respectively. Therefore, k_uₚ (or k_in) allow easy comparisons among multiple studies.
that used various sub-saturating dissolved Fe concentrations. Indeed, Lis et al. (2014) compared the uptake rate constants \( (k_{in}) \) of many Fe-limited phytoplankton and discovered a tight correlation and proportionality between their \( k_{in} \) and their respective surface areas, suggesting a physical constraint on the number of transporters at the cell surface allocated toward Fe uptake. Interestingly the \( k_{in} \) for Fe’ was 1000-fold higher than the \( k_{in} \) for strong organic complexes like FeDFB. Furthermore, many other Fe substrates have \( k_{in} \) between the values for Fe’ and FeDFB. This finding suggests that these two substrates (Fe’ and FeDFB) provide an empirical range for the bioavailability of all Fe substrates for uptake by phytoplankton.

**Determining saturating rates of iron uptake in the presence of EDTA**

Short-term Fe uptake rates from \(^{55}\text{FeEDTA} \) \( (\rho\text{FeEDTA}) \) can be determined to investigate saturating rates of Fe uptake, which are a direct function of the density of plasmalemma-bound Fe transporters, and are faster for Fe-limited phytoplankton (Harrison and Morel, 1986). The experiments are performed in an identical manner to those of FeDFB, except that the Fe is added at saturating concentrations and is bound to EDTA. Even though the Fe could be added as \( \text{FeCl}_3 \), the addition of EDTA prevents Fe precipitation. We have used various concentrations of Fe:EDTA, from 20 nM Fe bound to 500 nM EDTA (5% of the Fe as \(^{55}\text{Fe}; \text{Maldonado and Price}, 1999\)), to 100 nM Fe bound to 1\( \mu \)M EDTA (1% of the Fe as \(^{55}\text{Fe}; \text{Taylor et al.}, 2013\)). The high concentrations of EDTA in the Taylor et al. (2013) experiment were chosen so that competitive complexation between EDTA and in situ ligands or DFB (in the induced Fe-limiting treatment bottles) was negligible. As a general rule, the lower concentrations of EDTA used in Maldonado and Price (1999) are recommended.

**10. Conclusions and future directions**

Our knowledge of the important roles that iron plays within microalgae and the vast number of acquisition strategies invoked to cope with a limited Fe supply has increased substantially since the discovery of widespread Fe limitation in aquatic environments. Over the last century, we have gained an appreciation for the incredible amount of genetic, physiological and species diversity that exists within microalgae (Andersen,
Yet despite this diversity, much of our understanding of microalgal physiology is based on only a handful of model species, with only a subset of these having their genomes sequenced. Over the next decade, we anticipate a substantial increase in the number of sequenced genomes of microalgae, covering all taxonomic groups, both prokaryotic and eukaryotic. This will certainly illustrate the complexity and variety that exists among the microalgae with respect to the distribution of Fe-requiring proteins, uptake and storage strategies, and ways in which they have evolved to cope with Fe limitation. In addition, the number of studies implementing the 'omic' approaches (i.e. genomics, transcriptomics, proteomics and metabolomics) to examine both laboratory isolates and natural assemblages will continue to increase. Their findings will provide new insights into how microalgae interact with their biotic and abiotic environments and how Fe-related processes are affected by these interactions. Clearly, we are just at the beginning of a molecular era in microalgal research.

Another growing area of research will be investigations into the degree to which microalgae experience co-limitation between Fe and other essential elements and compounds required for growth. Although it has been well acknowledged that Fe and light may be co-limiting for microalgal growth in their natural environment, other types of co-limitation exist (Saito et al., 2008) and may be widespread. For example, recent studies suggest that in certain HNLC regions, there is co-limitation of phytoplankton growth by Fe and vitamin B$_{12}$. Additions of both Fe and vitamin B$_{12}$ have been shown to either further stimulate the growth of entire phytoplankton communities (Bertrand et al., 2007) or a subset of microalgal species, resulting in shifts in community composition when vitamins are added in addition to Fe (Koch et al., 2011). There have also been suggestions of possible co-limitation effects by Fe and N (Behrenfeld et al., 2006; North et al., 2007; Taylor et al., 2013), P (Mills et al., 2004), Si (Marchetti et al., 2010; Brzezinski et al., 2011), as well as other bioessential trace elements including Cu (Guo et al., 2012), Co (Saito et al., 2005) and Zn (Crawford et al., 2003). Our current understanding of how such co-limitations will impact Fe-related microalgal physiology is still limited.
It has been over two decades since John Martin provided compelling evidence of widespread Fe limitation in the sea, suggesting Fe regulates the primary productivity of large areas of the ocean and has a profound effect on global climate. He will forever be revered for proclaiming, "Give me a tanker of Fe and I'll give you an ice age". With the growing concerns over climate change, large-scale Fe fertilization continues to be considered as a geo-engineering strategy to curtail atmospheric CO\textsubscript{2} increases. To be successful, the efficiency in which an increased supply of Fe has the ability to result in carbon sequestration (i.e. the ratio of Fe added to the amount of particulate carbon exported to a fixed depth below the ocean surface) would need to be well constrained. However, an important lesson the previous era of large-scale Fe fertilization experiments has taught us is that there is extensive biological, chemical and physical variability within the ocean and Fe-limited regions are no exception. With every Fe-stimulated phytoplankton bloom, whether it be by natural or anthropogenic causes, the phytoplankton response and associated carbon export is influenced by the entire plankton community composition, their degree of Fe limitation, the ambient nutrient concentrations as well as a myriad of other variables; all of which can change on very small spatial and short temporal scales making the outcome somewhat unpredictable.

How future climate change will affect Fe-related processes in microalgae is presently uncertain. As the ocean environment responds to the increasing effects of ocean acidification and warming, there are many unknowns as to how microalgae will acclimate or adapt. If ocean acidification affects Fe distributions and speciation as predicted, Fe availability to phytoplankton will be affected. Indirectly, ocean acidification could also influence the distributions of other bioactive metals that interact with Fe nutrition. For example, it is predicted that Cu availability will increase as Fe availability decreases (Millero et al., 2009; Hoffmann et al., 2012). Depending on the resulting dissolved Cu concentrations relative to those of Fe, this increase in Cu availability could be beneficial or detrimental. If the Fe concentration is very low and the Cu concentration is not toxic, higher Cu might enhance the activity of the high-affinity Fe transporters. However, if Fe concentration is very low but Cu concentration is at toxic levels, phytoplankton growth would be impaired. The availability of these metals may also affect the phytoplankton
production of the climate-influencing osmolyte, dimethylsulfiniopropionate (DMSP) (Sunda et al., 2002). Recent evidence has also shown ocean acidification slows down N\textsubscript{2} fixation in the diazotroph \textit{Trichodesmium} when they are Fe-limited (Shi et al., 2012). How all of these changes will alter microalgal Fe requirements and their elemental compositions as well as important cellular processes such as photosynthesis, respiration and the production of organic compounds such as DMSP is unclear. Most importantly, will all microalgae be affected in the same way and how will these changes influence microalgal community composition throughout the different aquatic environments? Resolving the answers to these questions is critical given the significant roles microalgae play in aquatic food chains and global biogeochemical cycles.

\textbf{Tables and Figures:}

\textbf{Table 1} Common Fe-related proteins in prokaryotic and eukaryotic microalgae. Also provided are the protein abbreviations, their cellular function, the form of Fe associated with each protein and the microalgal groups in which these proteins are known to be present. The form of Fe and microalgal groups identified for each protein are tentative, based on available published data and should therefore be interpreted with caution. Fe forms: H, haeme; nH, non-haeme; Fe-S, iron-sulfur cluster

\textbf{Table 2} Kinetics of Fe transport and growth in microalgae. Half-saturation constants for steady-state Fe uptake rates ($K_{\mu Q}$, [Fe']\textsubscript{nm}), short-term Fe uptake rates ($K_{\rho}$, [Fe']\textsubscript{nm}), as well as Fe-limited growth ($K_{\mu}$, [Fe']\textsubscript{nm} or [Fe\textsubscript{diss}]\textsubscript{nm}). In some instances the kinetic parameters were not determined in the original manuscript, thus the available data were used to calculate $K$, using the hyperbolic, Michaelis-Menten kinetic function [$\rho_{Fe} = \rho_{max} * [Fe']/(K_{\rho} + [Fe'])$, or the Monod equation [$\mu_{Fe} = \mu_{max} * [Fe']/(K_{\mu} + [Fe'])]. Most culture studies utilized 100 \textmu M EDTA. Otherwise, the chelator used and the concentrations are noted under “growth conditions or dominant microalgae”. For culture studies where [Fe’] was not specified, concentrations were calculated using MINEQL

\textbf{Table 3} Most common proteins involved in Fe transport separated between prokaryotic and eukaryotic microalgae. Also provided are the protein abbreviations, their localization
within the cell, the Fe oxidation states and the microalgal groups in which these proteins are known to be present. The microalgal groups identified for each protein are tentative, based on available genomic data and should therefore be interpreted with caution.

**Fig. 1** Distributions of Fe in the world ocean. a) Mean profiles of dissolved iron in the North Atlantic, North Pacific, and Southern Ocean averaged over the depth intervals: 0–100 m, 100–250 m, 250–500 m, 500–1000 m, 1000–2000 m, 2000–3000 m, 3000–4000 m, and 4000–5000 m, b) Observed Fe concentrations from depths >1000 m in the eastern subtropical Pacific Ocean (20–50°N) plotted as a function of distance from the continental land mass and c) Modeled estimates of the two most important sources of Fe to the world ocean: mineral dust (24 x 10^9 mol Fe) and shallow (<502 m) sediments (21 x 10^9 mol Fe). Figures are reproduced from Moore and Braucher (2008). Panel c is courtesy of Bruland et al. (2014).

**Fig. 2** Box and whisker plots of Fe requirements (presented in the form of Fe:C ratios) for microalgae grouped by phyla. Eukaryotic groups are separated by the green plastid lineage (green) and the red plastid lineage (red). All quotas were obtained from microalgal isolates grown in medium that enabled maximum or near maximum (μ/μ_max >75%) growth rates and the Fe concentrations were below levels in which significant amounts of luxury uptake of Fe would occur ([Fe'] < 0.25 nM). Data are compiled from Sunda and Huntsman (1995), Maldonado and Price (1996), Ho et al. (2003), Marchetti et al. (2006), Lane et al. (2009), Quigg et al. (2011) and Strzepek et al. (2011).

**Fig. 3** Major forms of Fe in seawater and depiction of a eukaryotic microalgal cell. a) Bioavailable Fe primarily exists as a dissolved ferric Fe (Fe(III)) ion or tightly bound to organic ligands (L). Ferrous Fe (Fe(II)) is supplied primarily either through wet deposition and/or photoreduction of ferric Fe. Due to the high oxygen content and short residence time, concentrations of free ferrous Fe are extremely low in seawater. Much of the Fe within oxic seawater will eventually form colloids that precipitate out of the water column. The bioavailability of Fe oxyhydroxides and colloidal Fe is not well known, but assumed to be minimal. b) Fe uptake may occur through a number of mechanisms. Only
Fe transport pathways into the cells are illustrated. In general, Fe bound to organic ligands must be reduced before taken up by the cell. Many microalgae have a high-affinity Fe uptake mechanism that couples the Fe reductase with a multicopper-oxidase and Fe permease. Ferrous Fe may also be taken up through non-Fe specific divalent transporters. There is no illustration for proteins involved in intracellular Fe tracking (which are listed in Table 3). The bulk of the Fe demand in eukaryotic microalgae is used within the photosynthetic reaction centers and for the assimilation of NO$_3$ (also shown). Iron containing proteins are indicated in red. Some non-Fe containing proteins that may substitute when Fe levels are low are indicated in purple. Low-Fe induced proteins and processes are indicated in orange, which include an increased dependence on reduced forms of nitrogen (e.g. NH$_4$ and urea) as well as an increased ratio of PSII : PSI reaction centers. In some (but not all) microalgae, the presence of rhodopsins may compensate for a decreased production of ATP from photosynthesis in Fe-limited cells. The localization of rhodopsins to the vacuolar membrane is speculative. Similarly, some microalgae contain ferritins for Fe storage that are located in the plastid. Full protein names are provided in Table 1 (Fe-related proteins) and Table 3 (Fe transport and associated proteins). Others: AMT, NH$_4$ transporter; ATP, ATP-synthase; FLD, flavodoxin; GS, glutamine synthetase; NiRT, nitrite transporter; NRT, nitrate transporter; PCY, plastocyanin; RHO, rhodopsin

**Fig. 4** Major forms of Fe in seawater and depiction of a prokaryotic microalgal cell. a) See legend in Fig. 3. b) Putative Fe transport pathways in prokaryotic microalgae, as predicted (by genomic analyses, protein determination and physiological studies) for model organisms: Pathway I: Schizokinen transport by *Anabaena* sp., pathway II: reductive transport of organic & inorganic Fe(III) complexes (*Synechococcus*, PCC6803) and pathway III: Fe(II) transport in various cyanobacteria. Predicted locations and common substrates for each transporter are shown. The bulk of the Fe demand in prokaryotic microalgae is used within the photosynthetic reaction centers and for the assimilation of N (not shown). Iron containing proteins are indicated in red. Some non-Fe containing proteins that may substitute when Fe levels are low are indicated in purple. Low-Fe induced proteins and processes are indicated in orange, which include an
increased ratio of PSII : PSI reaction centers. Most cyanobacteria contain ferritins for Fe storage most commonly in the form of bacterioferritin or DPS ferritin. Full protein names are provided in Table 1 (Fe-related proteins) and Table 3 (Fe transport and associated proteins). Others: Fld, flavodoxin; isiA, Fe-starved induced protein, PC, plastocyanin

**Fig. 5** a) Steady-state Fe uptake rates, b) Fe quotas and c) growth rates as a function of inorganic Fe concentration ([Fe’]; 10^{-12} M) for *Emiliania huxleyi* (Sunda and Huntsman 1995). These log-log graphs show how the half-saturation constant for growth, steady-state and short-term Fe uptake rates are related to each other, so that the half-saturation constant for growth is much lower than those for steady-state and short-term Fe uptake rates (Morel 1987). The short-term Fe uptake rates for Fe-limited and Fe-sufficient *E. huxleyi* are computed, based on our knowledge in other marine phytoplankton species (Harrison and Morel 1986). Our calculated K values are: for growth: K_μ = 1.46 pM (Monod equation); for Fe quota: K_QFe = 26 pM (Droop equation); for steady-state Fe uptake rates: K_ρ^{ss} = 29 pM \left[ ρ^{ss} = ρ^{ss}_{max} * [Fe’]/(K_ρ^{ss} + [Fe’]) \right]; and for short-term Fe uptake rates: K_ρ = 100 pM (Michaelis-Menten kinetics). See text for equations

**Fig. 6** Cartoon schematic of the most common Fe transport kinetic models in microalgae: Fe’ model (Hudson and Morel, 1990) and the Fe(II)s model (Shaked et al., 2005). These kinetic models are for a medium with an excess of a photolabile organic ligands, inorganic Fe(III)’, and inorganic Fe(II)’. Fe(III)’ is more chemically stable than Fe(II)’ and is the dominant species for Fe transport. The concentration of Fe(III)’ is maintained at equilibrium by dissociation (rate constant k_d) and complexation (rate constant k_f) reactions with the organic ligands, as well as photoreduction of Fe(III) bound to photolabile organic ligands (rate constant k_{hv}) to Fe(II)’, which quickly oxidizes to Fe(III)’ (rate constant k_{ox}) at seawater pH (~8.1). In the Fe’ model, Fe(III)’ are the species reacting with the Fe transporter. In the Fe(II)s model, both the Fe(III)’ and the organic Fe have to be enzymatically reduced to Fe(II) before Fe is transported into the cell through the Fe(III) transporter complex. This complex is hypothesized to contain a multi-Cu containing oxidase and an Fe permease. Fe(II)’ may also be directly
internalized, but these divalent transporters are low affinity and are not illustrated here. For details of the Fe(II)s model and the Fe’ model, see Shaked et al. (2005), and Hudson and Morel (1990), respectively. Most model studies have used eukaryotes, though some prokaryotes have proven to have similar kinetics.

**Fig. 7** A comparison of Fe quotas ranges (as measured by the Fe:C ratios) in marine microalgae grown under Fe-limited (low range) and Fe-replete (high range) conditions. Shown are the average ranges for oceanic (o) and coastal (c) isolates of the diatom genera *Pseudo-nitzschia* and *Thalassiosira*, several haptophytes (*Emiliana huxleyi*, *Phaeocystis pouchetti* and *Chrysochromulina polylepis*) and the dinoflagellate *Prorocentrum minimum*. The dashed horizontal line represents the Fe:C ratio for phytoplankton from low Fe regions (derived from Sunda et al. 1991) that is commonly used in marine biogeochemical models (e.g. see Moore et al. 2002). Data are compiled from Sunda and Huntsman (1995), Maldonado and Price (1996), Marchetti et al. (2006) and Lane et al. (2009).

**Fig. 8** a) Map of global surface NO$_3$ concentrations along with the locations of 12 Fe enrichment experiments (indicated by yellow circles). 1. IronEx-I (1993), 2. IronEx-II (1995), 3. SOIEREE (1999), 4. EisenEx (2000), 5. SEEDS-I (2001), 6. SERIES (2002), 7. SOFeX North (2002), 8. SOFeX South (2002), 9. SEEDS-II (2004), 10. EIFEX (2004), 11. SAGE (2004), 12. LOHAFEX (2009). Also indicated is an Fe addition experiment performed by the Haida Salmon Restoration Corporation in 2012 that was not government approved (red circle). NO$_3$ are World Ocean Atlas climatological spring values. b) SeaWiFs satellite image of the phytoplankton bloom resulting from the SERIES Fe enrichment experiment in the northeast Pacific Ocean (indicated by the number 6 in panel a). The coast of Alaska is shown. Warm colors (reds and yellows) indicate high concentrations of chlorophyll *a* and thus high phytoplankton biomass; cool colors (blues) indicate low chlorophyll *a* concentrations. Dark areas over the ocean result from cloud cover. The white-circled region indicates the 700 km$^2$ region of high chlorophyll *a* concentrations resulting from the addition of Fe. c) Light microscope image of the oceanic diatom *Pseudo-nitzschia granii*; members of this genus are common
responders to Fe enrichment in all HNLC regions. Scale bar is 10 μm. Adapted from figures in Behrenfeld et al. (2009), Armbrust (2009) and Morrisey and Bowler (2012). SERIES satellite image courtesy of J. Gower, Orbimage/NASA

Fig. 9 Predicted dissolved inorganic Fe (Fe’), free ferric Fe (Fe(III)) concentrations, and pFe values for growth mediums as a function of total Fe concentrations. The conditional stability constants used to predict Fe’ and Fe(III) were obtained from Sunda et al. (2005), with a 100 μM EDTA metal ion buffer system in seawater at 20 °C, pH of 8.2, and salinity of 36. pFe values are the -log[Fe(III)]. At Fe’ above ~750 pM (black bar), significant iron hydroxide precipitation is likely to occur (Sunda and Huntsman 1995). For simplicity, the Fe' and Fe(III) are also shown above this maximum.

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Steady-state Fe uptake rate (μmol Fe mol C\(^{-1}\) d\(^{-1}\))

- \(K_{\rho_{Fe}}^{ss}\)
- \(K_{\rho_{Fe}}\)

\(\rho_{Fe-limited}\)
\(\rho_{Fe}^{ss} = \mu^\star Q_{Fe}\)
\(\rho_{Fe-sufficient}\)

FeC (μmol Fe mol C)

- \(K_{Q_{Fe}}\)

Growth rate (d\(^{-1}\))

- \(K_{\mu}\)

[Fe\(^{'}\)] pM
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<td>Respiratory terminal oxidase</td>
<td>RTO</td>
<td>ATP formation through reduction of O2 to H2O</td>
<td>H</td>
<td>c,r</td>
</tr>
<tr>
<td>Cellular Respiration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aconitase</td>
<td>ACO</td>
<td>isomerization of citrate to isocitrate</td>
<td>Fe-S</td>
<td>all</td>
</tr>
<tr>
<td>Alternative oxidase</td>
<td>AOX</td>
<td>alternative route for electron transport</td>
<td>nH</td>
<td>all</td>
</tr>
<tr>
<td>Cytocrome c oxidase</td>
<td>COX1</td>
<td>RC IV; electron transfer for the formation of ATP</td>
<td>H</td>
<td>all</td>
</tr>
<tr>
<td>Cytocrome c</td>
<td>Cyt c</td>
<td>electron transport</td>
<td>H</td>
<td>all</td>
</tr>
<tr>
<td>Ferredoxins</td>
<td>FDX or Fd</td>
<td>electron transport</td>
<td>Fe-S</td>
<td>all</td>
</tr>
<tr>
<td>NADH: ubiquinone oxidoreductase</td>
<td>NQR</td>
<td>RC I; electron transfer from NADH to coenzyme Q10</td>
<td>Fe-S</td>
<td>all</td>
</tr>
<tr>
<td>Succinate:ubiquinone oxidoreductase</td>
<td>SQR</td>
<td>RC II; oxidation of succinate to fumarate</td>
<td>Fe-S, H</td>
<td>all</td>
</tr>
<tr>
<td>Ubiquinol-cytochrome c oxidoreductase</td>
<td>Cyt bc1</td>
<td>RC III, electron transfer from ubiquinol to ubiquinone</td>
<td>Fe-S, H</td>
<td>all</td>
</tr>
<tr>
<td>Nitrogen Assimilation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate synthase</td>
<td>GLT</td>
<td>requires FDX in the conversion of glutamine to glutamate</td>
<td>Fe-S</td>
<td>all</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>NR</td>
<td>reduction of nitrate to nitrite</td>
<td>H</td>
<td>a except some c</td>
</tr>
<tr>
<td>Nitrite reductase</td>
<td>NiR</td>
<td>reduction of nitrite to ammonium</td>
<td>H</td>
<td>all</td>
</tr>
<tr>
<td>Nitrogenase</td>
<td>NiFH</td>
<td>fixes atmospheric nitrogen gas</td>
<td>Fe-S</td>
<td>nitrogen-fixing c</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-carotene monooxygenase</td>
<td>BCMO</td>
<td>final step in synthesis of retinal</td>
<td>nH</td>
<td>all</td>
</tr>
<tr>
<td>Biotin synthase</td>
<td>BIOB</td>
<td>final step in synthesis of biotin</td>
<td>Fe-S</td>
<td>all</td>
</tr>
<tr>
<td>Coproporphyrinogen oxidase</td>
<td>CPOX</td>
<td>synthesis of chlorophyll and heme</td>
<td>nH</td>
<td>all</td>
</tr>
<tr>
<td>Fe-superoxide dismutase</td>
<td>SOD</td>
<td>dismutation of superoxide into oxygen and hydrogen peroxide</td>
<td>nH</td>
<td>some but not all</td>
</tr>
<tr>
<td>Ferritin</td>
<td>FTN</td>
<td>iron storage protein</td>
<td>nH</td>
<td>some, but not all</td>
</tr>
<tr>
<td>Ferrochelatase</td>
<td>FECH</td>
<td>final step in synthesis of heme</td>
<td>nH</td>
<td>all</td>
</tr>
<tr>
<td>Lipoxygenases</td>
<td>LOX</td>
<td>dioxygenation of polyunsaturated fatty acids to lipids</td>
<td>nH</td>
<td>all</td>
</tr>
<tr>
<td>Peroxidases</td>
<td></td>
<td>oxidation of a substrate by a peroxide</td>
<td>H</td>
<td>all</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>XO</td>
<td>oxidation of hypoxanthine to xanthine and uric acid</td>
<td>Fe-S</td>
<td>all</td>
</tr>
</tbody>
</table>

*Abbreviations: PCY, plastocyanin; RC, respiratory complex; ch, chlorophytes; d, diatoms; c, cyanobacteria; r, rhodophytes*
<table>
<thead>
<tr>
<th>Organism or Field Location</th>
<th>Growth conditions or dominant microalgae</th>
<th>Cultures</th>
<th>Field (or no chelators added to cultures)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechococcus</em> sp.</td>
<td>High light (110 µm⁻³ s⁻¹, 100 nM EDTA, NO₃⁻)</td>
<td>0.74</td>
<td></td>
<td>Kudo &amp; Harrison 1997</td>
</tr>
<tr>
<td></td>
<td>Low light (26 µm⁻³ s⁻¹, 100 nM EDTA, NO₃⁻)</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thalassiosira oceanica</em></td>
<td>High light (75 µm⁻³ s⁻¹, 14L:10D)</td>
<td>0.00129</td>
<td></td>
<td>Buccinelli et al. 2010</td>
</tr>
<tr>
<td><em>Ditylum brightwellii</em></td>
<td>High light (75 µm⁻³ s⁻¹, 14L:10D)</td>
<td>0.0254</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thalassiosira pseudonana</em></td>
<td></td>
<td>0.0365</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Emiliania huxleyi</em> (Fig. 4)</td>
<td>w/ various EDTA &amp; DFB additions</td>
<td>0.000001</td>
<td></td>
<td>Straepel et al. 2011</td>
</tr>
<tr>
<td><em>Phaeocystis antarctica</em> (Southern Ocean)</td>
<td></td>
<td>0.000163</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phaeocystis antarctica</em> (Southern Ocean)</td>
<td></td>
<td>0.118</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em> (Southern Ocean)</td>
<td></td>
<td>0.01346</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp.</td>
<td></td>
<td>0.00915</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cylindrotheca fusiformis</em></td>
<td></td>
<td>0.0006</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thalassiosira pseudonana</em></td>
<td></td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thalassiosira oceanica</em></td>
<td></td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Synechococcus</em></td>
<td></td>
<td>0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Prasinomonas</em></td>
<td></td>
<td>0.045</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phaeocystis antarctica</em> (cultured)</td>
<td></td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Eukaryotes

#### Group A: Fe transport into the cytoplasm (from outside of cell or from the vacuole)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Abbreviation</th>
<th>Localization</th>
<th>Fe oxidation state</th>
<th>Microalgal Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III) transport systems (from inorganic and organic Fe(III) complexes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(III) reductase (coupled to NAD(P)H oxidase)</td>
<td>FRE (Ferric REDuctase)</td>
<td>Plasmatic membrane</td>
<td>Fe(III)</td>
<td>all</td>
</tr>
<tr>
<td>cytochrome b_553</td>
<td>NOX (RBOK)</td>
<td>Plasma membrane</td>
<td>green, diatoms</td>
<td></td>
</tr>
<tr>
<td>cytochrome b_5 reductase</td>
<td>CBF1 (Cytochrome b_5 reductase)</td>
<td>Plasma membrane</td>
<td>diatoms</td>
<td></td>
</tr>
<tr>
<td>cytochrome b_561 (ascorbate as electron donor)</td>
<td>Riboflavin</td>
<td>Plasma membrane</td>
<td>diatoms</td>
<td></td>
</tr>
<tr>
<td>High-affinity Fe(III) Transporter Complex (associated with ferric reductases, see above)</td>
<td>MCO/FOX/MUCOX, FET3P</td>
<td>Plasma membrane</td>
<td>Fe(II)</td>
<td></td>
</tr>
<tr>
<td>Fe(III) permease</td>
<td>FTR (Fe TTransporter)</td>
<td>Plasma membrane</td>
<td>Fe(III)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>all, except prasinophytes and some chromalveolates</td>
<td></td>
</tr>
<tr>
<td>Uncommon/Novel Fe(III) transporters</td>
<td>FEA-like</td>
<td>Plasma membrane</td>
<td>Fe(III)</td>
<td>C. reinhardtii, M. pusillo, and O. lucimarianus, chromalveolates</td>
</tr>
<tr>
<td>Fe(III) binding proteins in the extracellular space, may deliver Fe to FTR</td>
<td>ISIP (Iron-Starvation Induced Protein)</td>
<td>Plasma membrane</td>
<td>Fe(III)</td>
<td>diatoms</td>
</tr>
<tr>
<td>Iron-starvation induced protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin-like Fe(II) transport protein</td>
<td>TRF (TransFerrin-like)</td>
<td>Plasma membrane</td>
<td>Fe(III)</td>
<td>Dunaliella</td>
</tr>
<tr>
<td>Ferrichrome binding protein</td>
<td>FBP (Ferrichrome Binding Protein)</td>
<td>Plasma membrane</td>
<td>Fe(III)</td>
<td>Phaeodactylum</td>
</tr>
<tr>
<td>Fe(II) transport systems</td>
<td>ZIP-IRT</td>
<td>Plasma and vacuolar membrane &amp; secretory system</td>
<td>Fe(II)</td>
<td>C. reinhardtii, P. tricornutum</td>
</tr>
<tr>
<td>Passive divalent transporters (non-Fe specific, Zn transporter)</td>
<td>ZIP, ZnC (Transporter)</td>
<td>Plasma and vacuolar membrane &amp; secretory system</td>
<td>Fe(II)</td>
<td></td>
</tr>
<tr>
<td>Iron Regulated Transporter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAMP</td>
<td>NRAMP (Natural-Resistance-Associated-Macro-Phage Protein)</td>
<td>Plasma and vacuolar membrane &amp; secretory system</td>
<td>Fe(II)</td>
<td>all, except P. tricornutum</td>
</tr>
<tr>
<td>Subgroup I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subgroup II</td>
<td></td>
<td></td>
<td></td>
<td>major lineages</td>
</tr>
</tbody>
</table>

#### Group B: Fe trafficking within the cell, to sites of Fe assimilation or storage

<table>
<thead>
<tr>
<th>Protein</th>
<th>Abbreviation</th>
<th>Localization</th>
<th>Fe oxidation state</th>
<th>Microalgal Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDF</td>
<td>CDF (Cation Diffusion Facilitator)</td>
<td>Various intracellular compartments</td>
<td>Fe(II)</td>
<td>diatoms, Ectocarpus, Aureococcus, Micromonas</td>
</tr>
<tr>
<td>Fie-like</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSC</td>
<td>MSC (Mitochondrial Solute Carrier)</td>
<td>Mainly localized to mitochondria</td>
<td>Fe(II)</td>
<td>C. reinhardtii</td>
</tr>
<tr>
<td>MFL1 (MitoFerrin-Like 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MML1 (MitoFerrin-Like 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIT1/Ccc1</td>
<td>VIT (Vaccualr Iron Transporter) and Ccc (Cation-Chloride Co-transporter)</td>
<td>Vacuolar membrane</td>
<td>Fe(II)</td>
<td>C. reinhardtii</td>
</tr>
<tr>
<td>CVL1/CVL2 (Poi1- subgroup)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

### Prokaryotes

#### Ferric reductase

<table>
<thead>
<tr>
<th>Protein</th>
<th>Abbreviation</th>
<th>Localization</th>
<th>Fe oxidation state</th>
<th>Microalgal Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytochrome c oxidase (associated w/ FeoB)</td>
<td>ARTD (Alternate Respiratory Terminal Oxidase)</td>
<td>Periplasmic membrane</td>
<td>Fe(III)</td>
<td>Synechocystis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Fe(III) transport systems (ABC transport system, from inorganic and organic Fe(III) complexes)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Abbreviation</th>
<th>Localization</th>
<th>Fe oxidation state</th>
<th>Microalgal Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III) binding protein</td>
<td>IdIA/FutA/AfuA</td>
<td>Periplasmic space</td>
<td>Fe(III)</td>
<td>Trichodesmium, Prochlorococcus, Synechococcus, Synechocystis, Crocosphaera (abundant in picocyanobacteria)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(III) permease</td>
<td>IdIB/FutB</td>
<td>Periplasmic membrane</td>
<td>Fe(III)</td>
<td></td>
</tr>
<tr>
<td>ATPase</td>
<td>IdIC/FutC</td>
<td>Fe(III)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Fe(II) transport systems

<table>
<thead>
<tr>
<th>Protein</th>
<th>Abbreviation</th>
<th>Localization</th>
<th>Fe oxidation state</th>
<th>Microalgal Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small soluble protein</td>
<td>FeoA</td>
<td>Periplasmic membrane</td>
<td>Fe(II)</td>
<td>Synechococcus, Trichodesmium., Synechocystis, rare in Prochlorococcus</td>
</tr>
<tr>
<td>Predicted Fe(II) permease</td>
<td>FeoB</td>
<td>Periplasmic membrane</td>
<td>Fe(II)</td>
<td></td>
</tr>
</tbody>
</table>

---

Recent studies have highlighted the importance of iron transport and assimilation in microalgae, particularly in the context of iron deficiency and nutritional requirements. The presence of Fe(III) transport systems in both eukaryotic and prokaryotic microalgae underscores the evolutionarily conserved mechanisms that enable efficient Fe acquisition and incorporation into cellular structures, including iron-containing proteins and enzymes. These systems are crucial for the survival and growth of microalgae in diverse environments, including iron-limited conditions found in natural waters and the open ocean. The intricate interplay between these transporters and the regulatory mechanisms that govern iron uptake and utilization underscores the complexity of iron homeostasis in microalgae, a topic of ongoing research and critical importance for understanding the ecological and physiological implications of Fe availability in microalgal communities.
<table>
<thead>
<tr>
<th>Predicted regulator</th>
<th>FeoC</th>
<th>Fe(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRAMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZIP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TonB-dependent siderophore transporters (TBDTs)**

- For schizokinen transport into the cell (SchT) periplasmic binding protein (FhuD/FutA/FesA) membrane embedded permease (FhuB/FutB/FeCDD) ATP-binding protein (FhuC/FutC/FecB) TonB-energy translocation system (ExbB/ExbD) periplasmic membrane

- For heme TonB & energy translocation system (ExbB/ExbD)

**Siderophore synthesis**

- NRPS pathway and PKS (polyketide synthases) Non-Ribosomal Peptide Synthase (NRPS) Condensation NIS synthesis pathway for hydroxamate & mixed-hydroxamate siderophores Nrps-Independent Siderophore synthesis (NIS)

**Acetyl transferase (AlcB)**

- Siderophore synthetase for Aerobactin (IucA/IucC & IucD)
- Pyridoxal 5'-phosphate dependent decarboxylase for Rhizobactin (RhhB)
- Diaminobutyrate-2-oxoglutarate aminotransferase for Rhizobactin (RhhA)

**Fe(III) chelates**

- Anabaena sp.
- Prochlorococcus

**NIS synthesis pathway for hydroxamate & mixed-hydroxamate siderophores**

- More prevalent in filamentous and heterocyst cyanobacteria, picocyanobacteria *P. marinus* (absent in Prochlorococcus, *Synechococcus* and *Synechocystis*)

- *Anabaena* sp., *Synechococcus* and *Prochlorococcus* (prasinophyte *O. lucimarinus*, an eukaryote)

- *P. marinus* cyanobacteria
<table>
<thead>
<tr>
<th>Strain/Species</th>
<th>Fe Content</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phaeocystis antarctica (colonial)</td>
<td>20 μE m⁻³ s⁻¹</td>
<td>0.45 Sedwick et al. 2007</td>
</tr>
<tr>
<td>Pseudo-nitzschia delicatissima</td>
<td>0.013</td>
<td>LeLong et al. 2013</td>
</tr>
<tr>
<td>Pseudo-nitzschia grunii</td>
<td>0.005</td>
<td>Marchetti et al. 2008</td>
</tr>
<tr>
<td>Pseudo-nitzschia multiseries</td>
<td>0.023</td>
<td></td>
</tr>
<tr>
<td>Freshwater microalgae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dunaliella salina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-replete (Fe citrate additions)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-limited (Fe citrate additions)</td>
<td>8.00</td>
<td>Paz et al. 2007</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>0.013</td>
<td>Eischhardt and Buckhout 1998</td>
</tr>
<tr>
<td>Chlorella kessleri</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microcystis aeruginosa</td>
<td>0.000018</td>
<td></td>
</tr>
<tr>
<td>Tetraselmis suecica</td>
<td>0.000036</td>
<td></td>
</tr>
<tr>
<td>Synechocystis sp. PCC 6803</td>
<td>0.27</td>
<td>Kranzler et al. 2011</td>
</tr>
<tr>
<td>Modelled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(II) model work (using T. weissflogii data)</td>
<td>0.23</td>
<td>Sunda and Huntsman, 1995</td>
</tr>
<tr>
<td>Fe(II) model work (using P. minimum data)</td>
<td>0.12</td>
<td>Suna and Huntsman, 1995 [Marine chemistry]</td>
</tr>
<tr>
<td>Field populations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERIES (NE Pacific) 12°C</td>
<td>Nanoplankton</td>
<td>0.08 Kudo et al. 2006</td>
</tr>
<tr>
<td>SERIES (NE Pacific) 16°C</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>SEEDS (NW Pacific) 9°C</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>SERIES (NE Pacific) 12°C microplankton</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>SERIES (NE Pacific) 16°C</td>
<td></td>
<td>0.19</td>
</tr>
<tr>
<td>SEEDS (NW Pacific) 5°C</td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>Humboldt Current</td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td>Peru upwelling</td>
<td></td>
<td>0.26 Hutchins et al. 2002</td>
</tr>
<tr>
<td>Polar Front Zone (PFZ, 0.33 nM Fe)</td>
<td>Small pennate diatoms</td>
<td>0.41 Blain et al. 2002</td>
</tr>
<tr>
<td>Confucence subtropical &amp; subtropical front (SAF/STF, 0.29 nM Fe)</td>
<td>Mixed assemblage</td>
<td>0.45</td>
</tr>
<tr>
<td>Southern Subtropical zone (STZ, 0.09 nM Fe)</td>
<td>Small pennate diatoms</td>
<td>0.055</td>
</tr>
<tr>
<td>Ross Sea</td>
<td></td>
<td>0.09 Cochlé et al. 2002</td>
</tr>
<tr>
<td>Equatorial Pacific Ocean</td>
<td></td>
<td>0.12 Coale et al. 1996</td>
</tr>
<tr>
<td>Equatorial Pacific Ocean</td>
<td></td>
<td>0.12 Fitzwater et al. 1996</td>
</tr>
<tr>
<td>High Si water of the ACC</td>
<td></td>
<td>0.27 Coale et al. 2003</td>
</tr>
<tr>
<td>Ross Sea (PII3 &amp; PII4) community</td>
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<td>0.004</td>
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<tr>
<td>ACC in spring, community</td>
<td></td>
<td>0.091</td>
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<tr>
<td>ACC in summer, community</td>
<td></td>
<td>0.131</td>
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<tr>
<td>Southern Ocean, diatoms dominated, Pacific sector</td>
<td>0.237</td>
<td>Cullen et al. 2003</td>
</tr>
<tr>
<td>Southern Ocean Fe enrichment</td>
<td>&gt; 20 μm size-fraction</td>
<td>3.3 Maldonado et al. 2001</td>
</tr>
<tr>
<td></td>
<td>20-2 μm size-fraction</td>
<td>2.6</td>
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<table>
<thead>
<tr>
<th>Cultures</th>
<th>Field (or no chelators added to cultures)</th>
<th>( K_p )</th>
<th>( K_m )</th>
<th>( K_d )</th>
<th>( K_d )</th>
<th>( K_m )</th>
<th>( K_d )</th>
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<tbody>
<tr>
<td>AVERAGE</td>
<td></td>
<td>42.0</td>
<td>0.37</td>
<td>0.040</td>
<td>802.37</td>
<td>0.802</td>
<td>2.36</td>
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<tr>
<td>Average excluding values in italics</td>
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<td>0.37</td>
<td>0.041</td>
<td>0.32</td>
<td>0.0032</td>
<td>2.96</td>
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</tbody>
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1 Obtained from Maldonado et al. (2002)