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Full Length Research Paper

Bioavailability of iron speciations and EDTA-iron complexes for *Thallassiosira weissflogii* (Bacillariophyta)

Selin Sayin[1]*, Oya Isik[2], Murat Ozturk[3], Leyla Hizarci Uslu[2] and Funda Turan[1]

[1]Faculty of Aquaculture and Fisheries, Mustafa Kemal University, 31200 Iskenderun, Hatay, Turkey.
[2]Faculty of Aquaculture and Fisheries, Cukurova University, Adana, Turkey.
[3]NTNU Department of Chemistry Scientific Advisor SINTEF Marine Environmental Technology, No -7491 Trondheim, Norway.

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This study aimed to determine the effects of daily and/or initial additions of various amounts of Fe (II) and Fe (III), in combination with or without EDTA, on the growth of *Thalassiosira weissflogii* cultures. The results reveal that growth parameters were higher in the cultures containing 5000 nM Fe(III) ± EDTA than those of 5 nM Fe(III) and Fe(II). *T. weissflogii* cells seemed not to have used EDTA-bound iron, but their own released ligand to bind iron when Fe(III) concentration was high, and to have preferred inorganic Fe(II). Cell abundance and chl-a level were higher in daily supplemented cultures, with regard to those supported with iron only initially, evidencing the importance of continuous supply of iron in chl-a synthesis.

Key Words: *Thallassiosira weissflogii*, iron speciation, EDTA, growth.

INTRODUCTION

Iron is perhaps the most important of all the bioactive trace metals. However, its oceanic chemistry and inorganic speciation is the most complex and have not been adequately understood yet. Iron availability controls the productivity, species composition and trophic structure of planktonic communities in several oceanic regions (for example, subarctic Pacific, equatorial Pacific and Southern Ocean) (Martin et al., 1989, 1990; Buma et al., 1991; Behrenfeld et al.,1996; Coale et al., 1996; Boyd et al., 2000; Blain et al., 2004). Bioavailability of iron depends upon every aspect of Fe chemistry (solubility, complexation, thermodynamics, and kinetics of ligand exchange) in addition to phytoplankton uptake mechanisms and kinetics. There is still no conclusive agreement describing and quantifying “bioavailable iron” (Wells et al., 1995). Some of the operationally defined iron forms may have strong correlations with bioavailability of iron to phytoplankton. It is essential to determine the biological uptake mechanism of iron, and its utilization to understand the regulation of the biological activity by iron in the oceans (Wells et al., 1995)

Despite the recognized significance of iron in phytoplankton growth and the overall biogenic carbon budget of the oceans (Martin et al., 1991), there is surprisingly little information available on the quantitative and qualitative impact of blooms on iron speciation, transformation and bioavailability of iron species. The biological uptake of iron is highly influenced by its chemical speciation, which is extremely complex and dynamic. There are two major iron transport systems: (1) siderophores that excrete strong Fe(III)-binding chelators to the external environment of cell and import the resulting Fe(III) chelates into the cells by specialized membrane proteins, and (2) ferrous or ferric ion membrane transporters that bind external Fe(II) or Fe(III) through ligand exchange with labile iron species, primarily free ions and dissolved inorganic complexes of Fe(II) or Fe(III) (Turner and Hunter, 2001). There is growing evidence that most of the dissolved iron

*Corresponding author. E-mail: helenselin@yahoo.com or ssayin@mku.edu.tr. Tel: + 90 326 614 18 66. Fax: 90 326 614 18 77.

Table 1. Experimental design of Fe availability for *Thallosiosira weisflofgii*

Group	A					B			
	0.33 nM Fe $^{\Phi}$					5 nM Fe $^{¥}$		5000 nM Fe §	
	*1	2	3	4	5	6	7	8	9
Iron	Fe^{3+}	Fe^{3+}	Fe^{3+}	Fe^{2+}	Fe^{2+}	Fe^{3+}	Fe^{3+}	Fe^{3+}	Fe^{3+}
Fe amounts	5	5	5	5	5	5	5	5000	5000
EDTA amounts	10	-	100	-	100	-	100	-	100

$^{\Phi}$ 0.33nM Fe/daily additions, $^{¥}$5nM Fe /only the first day, §5000nM Fe /only the first day, *1: Control.

in seawater is complexed to organic ligands in the forms of Fe(II) (Van den Berg, 1995; Rue and Bruland, 1995, 1997; Wu and Luther, 1995). The chemical nature of iron-binding ligands in both surface and deep seawater, however, is not obvious. There may be specialized ligands that are released by phytoplankton and binding to iron, to facilitate its uptake (Hutchins et al., 1999)

The phytoplankton growth is enhanced by EDTA, which can be explained by detoxification of toxic metal ions (such as cupric ions) upon its binding with these metals or by increasing the availability of essential metals (such as iron) to form water-soluble complexes (Sunda et al., 1991). Different EDTA concentrations have been used by several studies to chelate iron (Boye and Van den Berg, 2000; Sunda and Huntsman, 1995). In culture experiments with iron manipulation, the addition of EDTA basically has two functions: (1) to prevent iron precipitation by keeping it as a soluble organic complex, and (2) to keep soluble inorganic iron concentrations within a specific range. In most of the phytoplankton cultures, the concentration of 10 to 100 µM was used (Vuorio et al., 2005; Geringa et al., 2000). EDTA concentrations of 100 µM or more may have toxic effects on some sensitive species (Huskin et al., 2000). In addition, EDTA interferes with iron speciation and hence with bioavailability. EDTA-iron complex is readily reduced to Fe(II) when exposed to light (Vuorio et al., 2005).

Iron is known to limit the growth of phytoplankton, especially diatoms, in large regions of the oceans. Diatoms are particularly important in the export of organic matter to the deep ocean because of their large size and silica shells (Shaked et al., 2005). This study presents iron enrichment experiments conducted on *T. weissflogii*, having a costal centric diatom in the size range of 5 to 20 µm. The aim of this study was to determine the bioavailability and uptake strategies of iron species, Fe(II) and Fe(III), and to find out the effects of EDTA on cellular iron uptake mechanisms. In addition, different timing in the addition of iron to the medium [daily and initially with different (5 and 5000 nM)] concentrations of Fe (III) in laboratory cultures of *T. weissflogii* was also tested.

MATERIALS AND METHODS

The sampling site was chosen in a relatively unpolluted area off Mersin city, Turkey, north eastern Mediterranean Sea. Seawater samples were collected at 50 m depth with Niskin bottles and stored into a 50 L polyethylene bottles. The collected seawater was firstly filtered on 0.45 µm GF/6 glass fibre filter papers (1 µm pore size; Schleicher and Schuel) to take out particulate metals and then on 0.2 µm pores – sized sterile membrane filters (Millipore). The Chelex-100 (Bio-Rad, 200-400, mesh size: 74-37 µm) was used to hold and separate the total dissolved metals from the seawater (Price and Morel, 1998; Özturk et al., 2002; Isik et al., 2007).

The pH of the filtered seawater was reduced to 2 with 6 M HCl (Merck), and kept for 48 h to x facilitate dissolution of all the trace elements in order to prepare iron-free sea water. Having run the seawater through the Chelex-100 column to remove all dissolved iron, the pH was increased to 8 ± 0.1 with the addition of 3 M NaOH. During this process, the water was steadily shaken to prevent precipitation of the trace metals. Eventually, the seawater was supplemented with the main nutrients N and P, and then passed through the Chelex-100 column to purify the medium from the trace metals. The flow rate of seawater through the column was adjusted to 5 ml min^{-1}. The initial volume, 500 ml, of seawater passing through the column was discarded (observed by its decreasing Ca level). The seawater taken from the Chelex-100 column was free from trace metals, and used with the modified F/2 medium (Guillard, 1973) for *T.weissflogii* cultures. All the F/2 medium elements, except iron, were supplemented to the seawater as trace metal stock solution. EDTA stock solution was passed through the Chelex-100 column to eliminate the iron. The Chelex was not used for the vitamin stock solution to avoid the removal of some vitamins such as the Co containing vitamin B12.

In the cultures, the concentration of EDTA was arranged to 100 µM. Fe(III) and Fe(II) stock solutions were made with $FeCl_3.6H_2O$ and $FeSO4.7H_2O$, respectively. Iron treatment of cultures was in two different ways; iron was added to the cultures either once to have an initial concentrations of 5 and 5000 nM or supplemented daily to have a concentration of 0.33 nM each day and 5 nM in sum at the end of 15 days (irrespective of iron consumption by cells and iron supplied by transfer of stock culture) (Table 1). Two different forms of iron (Fe^{3+} and Fe^{2+}) were used in the daily iron supplemented experimental sets (A in Table 1), while only one form of iron (Fe^{3+}) was used in the experimental sets that was initially iron treated (B in Table 1). The effects of EDTA was also tested by supplementing one set of each of the different iron treated cultures with 100 nM EDTA, while the other were not treated with EDTA (Table 1).

To stop the medium from metal contamination, polyethylene vessels were used. 6 and 3 M HCl and TMF-DW (trace metal free distilled water was prepared by passing distilled water through the Chelex-100) was used for cleaning all materials from metals. For collection of the microalgal cells, polycarbonate (PC) membrane filters were used. The filters were kept in a 3 M HCl-bath for two days to remove iron, and then kept in 1 M trace metal free- HCl, prepared by isothermal distillation over a week with replacement of the HCl solution every day. At the end of the week, the filters were rinsed with TMF-DW several times.

1.5 L polycarbonate culture carboys containing iron-removed F/2 medium was used in triplicate for experimental sets. Initial concentrations of *T. weissflogii* (Grunow) were 70 × 10^4 cells ml^{-1}. An extra 1.6 nM Fe was introduced into each 1.5 L culture carboys from the stock culture of *T.weissflogii* which contained 500 nM Fe. The vessels were cultivated in a culture laboratory covered with PE-sheets to create a dust-free environment under continuous illumination (80 $\mu Em^{-2}s^{-1}$) with fluorescent (Philips) lights at 18 ± 2°C temperature. Light intensity was measured by a radiation sensor LI-COR (LI-250). The pH of the culture medium was measured by WTW-330 pH meter, and in order to regulate the pH of culture media trace metal, free - NH_4 (Merck) was prepared by isothermal distillation (Özturk et al., 2002). The salinity of seawater was arranged to 32% by diluting natural seawater in this experiment. During the experimental period, cell concentration (cell ml^{-1}), chl-a (µg L^{-1}), cellular chl-a content (pg $cell^{-1}$), growth rate (µ) and pH were determined every two days, and cellular Fe content (amol Fe $cell^{-1}$) was measured at the end of this study.

T. weissflogii cells were fixed with formaldehyde to have a 4% final concentration and counted with a Thoma lamella three times for each sample. The growth rates (µ) were determined according to Guillard (1973). The chl-a samples (5 ml) were filtered through 0.45 µm-GF/6 (Schleicher and Schuel). The concentrations were measured on a UV-VIS Spectrometer, SHIMADZU- 1240 after extraction in 90% acetone and held at 4°C, 24 h in the dark Parsons and Strickland (1963). The amounts of cellular chl-a were calculated using the highest cell abundance and chl-a concentrations at the stationary phase. Data were analysed statistically using one way analysis of variance (ANOVA). When treatment effects were detected as significant, Duncan's multiple range test was used to identify specific differences among treat-ment means at a probability level of 5%.

In order to determine the amount of iron, 100 ml culture samples were taken from the carboys at the end of the experiment, at 15th day; (at stationary phase) the cells were concentrated on acid washed PC filters. Then, filters were inserted into 15 ml PE falcon tubes with PE forceps and 10 ml acid mixture (3:1), HCl-HNO_3 was added for Fe extraction. After the extraction process, the tubes were settled in heated sand and the final volumes were reduced to 2 ml by evaporation. The total Fe content of cells was determined by ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrophotometry, Varian Model Liberty Series II) Azemard and Coquery (1998). The cellular iron content was calculated by dividing the total amount of Fe by the cell counts.

RESULTS

The maximum growth rates of *T. weissflogii*, 2.53 and 2.29, were obtained with 5000 nM Fe ± EDTA treatments. The growth rates were higher than those mentioned. 1.91, 1.70, 1.58, 1.46, 1.32 and 1.22 were observed in cultures of 5 nMFe only on the first day; Fe(III) + EDTA, 0.33 nM Fe/daily addition; Fe(II) + EDTA, Fe(III) + EDTA, 5 nMFe/ only on the first day; Fe(III), 0.33 nM Fe/daily addition; Fe(III) and 0.33 nM Fe/daily addition; Fe(II) groups, respectively ($p < 0.05$) (Table 2).

Maximum cell concentrations of *T. weissflogii*, 96.77 × 10^4 cells ml^{-1} and 55.00 × 10^4 cells ml^{-1}, were detected in cultures supplemented with 5000 nM Fe(III) ± EDTA. The lowest cell densities were observed in the cultures daily supplemented with 0.33 nM Fe(III) ± EDTA (7.67 × 10^4 cells ml^{-1} and 7.92 x 10^4 cells ml^{-1}) and 0.33 nM Fe(II) + EDTA (8.67 × 10^4cells ml^{-1}). The cultures initially supplemented with 5 nM Fe(III) ± EDTA reached cell abundances of (9.58 × 10^4 cells ml^{-1} and 10.25 × 10^4 cells ml^{-1}), and the abundance of daily 0.33 nM Fe(II) added cultures was 17.42 × 10^4 cells ml^{-1}.

The maximum chl-a concentration of 225 µg L^{-1} was determined in 5000 nM Fe(III) treatment while in the 5000 nM Fe(III) + EDTA treatment, chl-a reached a concentration of 139.98 µg L^{-1}. During the stationary phase, chl-a concentrations were lower in the carboys having low iron concentrations. Following chl-a, values were obtained in the different experimental sets; 43.22 µg chl-a L-1 in 0.33 nM Fe(III) added carboys, 51.14 µg chl-a L^{-1} in 0.33 nM daily Fe (II) + EDTA added carboys, 51.54 µg chl-a L^{-1} in 0.33 nM daily Fe(III) + EDTA added cultures, 56.28 µg chl-a L^{-1} in 5 nM initial Fe(III) supplemented carboys, 6.18 µg chl-a L^{-1} in 5 nM initial Fe(III) + EDTA amended carboys and 94.23 µg chl-a L^{-1} in 0.33 nM daily Fe(II) added cultures ($p < 0.05$) (Table 2).

The highest cellular chl-a content (0.67 pg $cell^{-1}$) was found in cultures daily enriched with Fe(III) + EDTA to have 0.33 nM concentration. The lowest cellular chl-a contents were obtained in cultures of 5000 nM Fe (III) + EDTA and Fe (III) addition. The highest cellular Fe contents (125.12 amol $cell^{-1}$, 70.05 amol $cell^{-1}$) were detected in the experimental sets of 5000 nM initial Fe(III) ± EDTA addition. The lowest cellular Fe content (9.51 amol $cell^{-1}$, 15.22 amol $cell^{-1}$) was found in the carboys to which 5 nM Fe (III) ± EDTA was added initially. The maximum pH value (9.46) was observed in the culture initially enriched with 5000 nM Fe(III) + EDTA. The second and third-highest pH values were determined in cultures of 0.33 nM daily Fe(II) added (9.35) and 5000 nM initial Fe(III) (9.31), respectively ($p < 0.05$) (Table 2).

In this present study, cellular carbon biomass of *T. weissflogii* was considered as 13.3 pmoll $cell^{-1}$ based on previously reported carbon measurement of this species with CHN analyzer (Huskin et al., 2000). The highest C:chl-a ratios (64.58, 58.61) were found in the cultures initially enriched with 5000 nM Fe ± EDTA , while the lowest ones (22.31) were obtained in those with daily Fe(III) + EDTA additions ($p < 0.05$) (Table 2). Moreover, the lowest Fe:C ratio (0.71) was determined in the cultures initially enriched with 5 nM Fe(III). The second and third- lowest Fe:C ratios were found in the cultures initially enriched with 5 nM Fe(III) + EDTA (1.14) and 0.33 nM daily Fe(III) addition (1.76). Cultures enriched with 5000 nM Fe(III) ± EDTA had the highest Fe:C ratios (9.40, 5.26). The highest Fe:chl-a ratio (538.67) was found in the cultures initially treated with 5000 nM Fe(III), whereas the lowest Fe:chl-a ratio was found in those initially enriched with 5 nM Fe(III) (15.91) ($p < 0.05$) (Table 2).

DISCUSSION

In this study, the uptake strategies and bioavailability of iron species, Fe(II) and Fe(III), by *T. weissflogii* were studied in the presence and absence of artificial organic

Table 2. Gowth rates, cell densities, chl-a concentrations at the stationary phase, Chl-a content of the cells, Chl-a:C ratios, Fe contents of cells, Fe:C ratios and Fe:Chl-a ratios of *T. weissflogii* cultures, and the pH values of culture media in all iron and EDTA treatments applied in this present study.

Parameter	Treatment							
	0.33 nM Fe/daily additions				5 nM Fe /only the first day		5000 nM Fe/only the first day	
	Fe(III)	Fe(III) + EDTA	Fe(II)	Fe(II) + EDTA	Fe(III)	Fe(III) + EDTA	Fe(III)	Fe(III) + EDTA
Growth rate (μ)	1.70 ± 0.058^{e}	1.46 ± 0.006^{c}	1.91 ± 0.025^{f}	1.32 ± 0.025^{b}	1.58 ± 0.057^{d}	1.22 ± 0.021^{a}	2.29 ± 0.015^{g}	2.53 ± 0.012^{h}
Cell density (x10^{4} cells ml^{-1})	7.92±0.025^{a}	7.67 ± 0.044^{a}	17.420 ± 0.176^{a}	8.67 ± 0.157^{a}	9.58 ± 0.020^{a}	10.25 ± 0.021^{a}	96.77 ± 30.395^{b}	55.00 ± 1.527^{b}
Chl a conc. at sp$^{\alpha}$ (μg ml^{-1})	43.22 ± 0.168^{a}	51.54 ± 0.395^{b}	94.23 ± 0.232^{e}	51.14 ± 0.488^{b}	56.28 ± 0.026^{c}	61.18 ± 0.050^{d}	225.00 ± 3.605^{g}	139.98 ± 1.548^{f}
Chl a content (pg cell^{-1})	0.54 ± 0.003^{c}	0.67 ± 0.007^{e}	0.54 ± 0.003^{c}	0.59 ±1.667^{d}	0.60 ± 0.010^{d}	0.60 ± 0.000^{d}	0.23 ± 0.003^{a}	0.25 ± 0.003^{b}
Chl a:C (weight)	27.47 ± 0.058^{c}	22.31 ± 0.138^{a}	27.71 ± 0.199^{c}	25.43 ± 0.646^{b}	25.09 ± 0.393^{b}	25.12 ± 0.043^{b}	64.58 ± 0.780^{e}	58.61 ± 0.693^{d}
Fe content (amol cell^{-1})	23.42^{c} ± 0.296	31.09^{d} ± 0.543	39.55^{e} ± 0.142	31.09^{d} ± 0.826	9.51^{a} ± 0.081	15.22^{b} ± 0.078	125.12^{g} ± 0.931	70.05^{f} ± 0.546
Fe:C	1.76 ± 0.024^{c}	2.34 ± 0.062^{d}	2.97 ± 0.010^{e}	2.33 ± 0.039^{d}	0.71 ± 0.007^{a}	1.14 ± 0.007^{b}	9.40 ± 0.070^{g}	5.26 ± 0.043^{f}
Fe: Chl *a* (pmol:ng)	42.89 ± 0.494^{ab}	46.26 ± 1.508^{ab}	73.07 ± 0.785^{b}	52.69 ± 0.815^{ab}	15.91 ± 0.295^{a}	25.49 ± 0.148^{a}	538.67 ± 9.844^{d}	236.66 ± 35.890^{c}
pH	9.15 ± 0.012^{d}	9.01 ± 0.023^{c}	9.35 ± 0.006^{f}	8.92 ± 0.007^{b}	8.55 ± 0.006^{a}	8.99 ± 0.003^{c}	9.31 ± 0.009^{e}	9.46 ± 0.010^{g}

P < 0.05, there is a difference among the means, single-factor ANOVA.Values are means and different letters in the same row denote significant differences (P < 0.05). Mean C measurement of Huskin et al. (2000): 13.3 (pmol C cell^{-1}) for *Thalossiosira weissflogii* cells was used. sp$^{\alpha}$ = stationary phase.

chealator EDTA. We have tested daily and initial additions of iron to investigate how *T. weissflogii* responds to continuous or episodic iron supply to seawater (for example, either by the atmosphere or any vertical/horizontal transport). Furthermore, with EDTA supplements, we aimed to simulate an environment organic ligands already existing in seawater, and investigated if this iron-type was preferred by cells. Although, our results showed that growth rate, abundance of cells and chl-a concentration were affected with the addition of EDTA into 5000 nM iron enriched groups, the highest growth rate, cell abundance and chl-a concentrations were obtained from these groups. The results showed that growth rate, abundance of cells and chl-a concentrations decreased in the Fe(II) added groups with EDTA, as in previous studies by Muggli and Harrison (1996) and Isik et al. (2007). Our study detected that abundance of cells, chl-a concentration, chl-a content per cells, as well as Fe quota were generally higher in cultures grown without EDTA. Except for initially 5000 nM iron without EDTA added cultures, growth rate was found to be higher in cultures with EDTA addition. Probably, *T.weissflogii* cells could better take up iron by releasing their own iron-binding ligand rather than using Fe-EDTA complex. This pattern of release is observed in many marine microorganisms which secrete specific strong-iron ligands (siderophores) to take up iron under iron-limited conditions (Isik et al., 2007; Barbeau et al., 2001; Soria-Dengg et al., 2001).

These results suggest that microorganisms may have developed mechanisms to solve fresh inputs of iron, thereby, preventing their loss via formation of iron hydroxides or adsorption onto settling particles.

Another purpose of Fe additions to the cultures with different timing, once at the beginning and with daily intervals, was to compare possible changes with precipitation of iron in the former ones and with fresh inputs in the latter cultures.

Although, higher cell abundance and total chl-a values were determined in the cultures grown with initially 5 nM Fe(III) + EDTA than cultures only 5 nM Fe(III) added, the growth rate was decreased in this group. Presumably, the cell number declined with decreasing iron concen-tration in the medium. Similar to previous findings, iron limitation is responsible for a low growth rate in many marine phytoplankton species (Jolanta and Kosakoswska, 2004).

The highest Fe quota values were found in the cultures with 5000 nM Fe(III) ± EDTA. It is likely that 5 nM Fe was not enough to enhance the cell division because the highest growth rates, cell densities, chl-a concentrations, Fe contents, Fe:C ratios, Fe:Chl-a ratios and pH values were observed in cultures containing 5000 nM Fe(III) ± EDTA (Table 2). Blain et al. (2002) reported that the environmental and physiological history of phytoplankton in the experiments may also have an importance in their apparent iron requirements, given that oceanic algae are thought to indulge in ‘‘luxury’’ uptake and storage of iron. Thus, it is conceivable that phytoplankton may adapt their cellular iron content and growth response to iron according to the availability of iron in the water column (Hutchins et al., 1999).

These results are consistent with those of Fitzwater et al. (1996) and Jolanta and Kosakoswska, (2004) who suggested that low concentrations of dissolved Fe may limit phytoplankton production in the medium. Additionally, several experiments using well-characterized media and phytoplankton cultures have also shown that low iron levels may limit the growth rate of phytoplankton, especially diatoms (Sunda et al., 1991; Brand, 1991; Hudson and Morel, 1990; Price et al., 1991; Sunda, 2001). All iron content measurement was performed on the 15th day when stationary phase was already over in the cultures initially enriched with 5 nM Fe(III) + EDTA. Thus, these cultures were excluded when comparing the iron contents. Jolanta and Kosakoswska, (2004) reported that measurements of chl-a at the end of the serial experiments (after 10 days) showed an appa-rent response of the diatom cells, demonstrating that they were dependent on the availability of iron in the medium. It was previously reported that the iron enrichment resulted in higher chl-a:C ratios in phytoplankton. In contrast, in this present study, when iron concentrations were very high (5000 nM), chl-a:C ratios were the lowest among all other treatments. C values were not measured in this present study, but the constant value of Huskin et al. (2000) was used for the different iron concentrations. However, these low chl-a: C ratios in the cultures having high iron concentrations must not be due to high C content of cells, because several studies have reported that C contents of *T. weissflogii* cells were high under iron or any other nutrient limitation (Muggli and Harrison, 1997).

Low chl-a:C ratios in the cultures with 5000 nM iron added could be due to early termination of the log phase growth after consuming other nutrients with high cell abundance. Additionally, 5000 nM Fe(III) ± EDTA-added cultures in this study showed higher cell abundance, photosynthetic activity and pH levels than cultures containing 5 nM Fe(II) ± EDTA and 5 nM Fe(III) ± EDTA, indicating stronger limitation under low iron concentrations. Fe(II) is known to be more soluble, kinetically more labile, and form weaker organic chelates than Fe(III) (Eker-Develi et al., 2006). In this present study, the daily Fe(II) added cultures had much higher growth rate, cell abundance, total chl-a concentration and chl-a content per cell when compared to the corresponding daily Fe(III)-added cultures. This finding may show the importance of rain droplets carrying Sharan dust and having high iron content, since iron can be photochemically reduced in cloud droplets to the +2 oxidation state (Kuma et al., 1992).

Similarly, Fe quota was found to be higher in the daily Fe (II)-added culture with regard to daily Fe (III)-added cultures.

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