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Iron Speciation and Physiological Analysis Indicate that *Synechococcus* sp. PCC 7002 Reduces Amorphous and Crystalline Iron Forms in Synthetic Seawater Medium

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Abstract: Cyanobacteria have high iron requirements due to iron-rich photosynthetic machineries. Despite the high concentrations of iron in the Earth's crust, iron is limiting in many marine environments due to iron's low solubility. Oxic conditions leave a large portion of the ocean's iron pool unavailable for biotic uptake, and so the physiochemical properties of iron are hugely important for iron's bioavailability. Our study is the first to investigate the effect of iron source on iron internalization and extracellular reduction by *Synechococcus* sp. PCC 7002. The results indicated that the amorphous iron hydrolysis species produced by $FeCl_3$ better support growth in *Synechococcus* through more efficient iron internalization and a larger degree of extracellular reduction of iron than the crystalline FeO(OH). An analysis of dissolved iron (II) indicated that biogenic reduction took place in cultures of *Synechococcus* grown on both $FeCl_3$ and FeO(OH).

Keywords: cyanobacteria; *Synechococcus*; iron biogeochemistry; iron acquisition; iron limitation; iron speciation; photosynthesis

1. Introduction

Iron is one of the most abundant elements in the Earth's crust, but limits primary production in 25–50% of marine environments due to its solubility and consequent bioavailability [1,2]. Though the input of iron to the surface ocean fluctuates and can thus be locally and seasonally high [3–5], its low dispersion and solubility provides challenges for marine microorganisms [6]. Iron is an essential element for life: it acts as a co-factor in a number of enzymes and protein complexes involved in essential processes such as photosynthesis, nitrogen assimilation, and respiration [7]. Cyanobacteria have as much as ten times higher iron requirements than non-photosynthetic bacteria of a similar cell-size [8]. This high iron requirement is mostly due to the photosynthetic complex: a total of 12 iron atoms are needed per Photosystem I (PSI) protein complex [9], and two-to-three iron atoms are required per Photosystem II (PSII) [10]. Iron-rich proteins such as ferredoxin and cytochromes, as well as several enzymes involved in photosynthetic electron transport, account for approximately 80% of the organism's iron demand [11].

Coastal *Synechococcus* species have higher iron requirements than open ocean species, most likely due to adaption to the generally higher iron concentrations in coastal areas compared to the open

ocean [12]. Because of its relatively high iron requirements, *Synechococcus* may experience pronounced stress caused by iron limitation. Iron limitation leads to biochemical and physiological changes. The specific iron limitation responses in *Synechococcus* include the downregulation of genes involved in the assembly of PSI and PSII, increasing the PSI:PSII ratio from 1:3 to 1:1 or even higher [13,14]. Simultaneously, the iron-stress inducible protein A (IsiA) accumulates around PSI. This protein complex allows PSI to retain functionality under iron limitation by increasing the effective surface area for light absorption [15,16]. Additionally, the concentrations of pigments involved in photosynthesis, such as chlorophyll and phycobilisomes, may also decrease during iron limitation [14].

While Fe(II) is more soluble and is considered to be more bioavailable than Fe(III), the presence of O_2 and H_2O_2 results in the rapid oxidation of Fe(II) to Fe(III) with subsequent complexation, in most of the euphotic zones within the oceans [17,18]. Additionally, as much as 99% of the dissolved iron may be complexed by organic ligands (FeL complexes) and may not be readily accessible [19–21]. However, the complexation of iron with certain organic ligands may also facilitate the reduction of iron [22]. Given the limited bioavailability of iron, cyanobacteria have developed diverse iron acquisition mechanisms. Strategies include the utilization of siderophore-mediated uptake [23], and there are also indications for the extracellular reduction of Fe(III) to Fe(II) and the subsequent uptake of Fe(II) [24].

Similar to many other cyanobacterial species, *Synechococcus* possesses the necessary genes for siderophore biosynthesis [23]. This organism also produces type IV pili (T4P) [25], which may play a role in the iron acquisition of cyanobacteria [26]. T4P are extracellular protein structures consisting of type IV pilin protein PilA. Previous studies in the non-photosynthetic bacterium *Geobacter sulfurreducens* have shown that these structures are involved in extracellular electron transport to iron oxides, where Fe(III) is reduced to Fe(II) and then subsequently taken up by the cell [27]. However, which sources of iron are used by cyanobacteria and whether these organisms use intracellular or extracellular reduction to make iron bioavailable remain open questions.

This study aimed to further study the effect of iron source on iron acquisition and potential reduction in *Synechococcus* sp. PCC 7002, as well as to investigate the internalization of iron dependending on the provided iron source. It is necessary to first understand the basic responses of *Synechococcus* to iron forms of different properties. $FeCl_3$ rapidly dissolves in synthetic seawater and forms amorphous colloids, FeL and FeEDTA (Ethylenediaminetetraacetic acid) complexes. While $FeCl_3$ is not a commonly found iron salt in the open ocean, its properties mimic those of already existing dissolved iron found in the water column. Goethite, FeO(OH), is a crystalline mineral compound with poor solubility that is less likely to form complexes with EDTA and thus less likely to sustain growth in *Synechococcus*. FeO(OH) is a naturally occurring mineral found, for example, in Saharan dust [28], which is a substantial iron source for open oceans [6]. Secondly, changes in dissolved iron (II) concentrations, as well as changes in particulate and intracellular iron concentrations, can give information about how well *Synechococcus* acquires and potentially reduces the different forms of iron. The effect of iron source on iron internalization has not been previously studied in cyanobacteria, and the effect of iron source on extracellular biogenic iron reduction in cyanobacteria is understudied. This study could provide a step towards elucidating these processes. We hypothesized that $FeCl_3$ would support growth in *Synechococcus* better than FeO(OH) because $FeCl_3$-derived iron species would be reduced and acquired more readily than FeO(OH)-derived iron species by *Synechococcus*.

2. Materials and Methods

2.1. Trace Metal Clean Work and Preparation

In order to avoid contamination from iron, all plastic consumables were cleaned following the method of Achterberg et al. [29]. The experiment was carried out in a lab space covered in plastic sheeting, thus creating a temporary clean lab. A clean sluice for personnel entering and leaving the lab space was used. All personnel wore microporous laminated clean suits (Tyvek®), hair nets, and face masks when inside the clean space. Clean lab shoes were worn with plastic shoe covers.

All sample handling, filtration, and acidification were carried out inside a Class 100 Laminar flow hood (AirClean 4000).

2.2. Organisms, Culture Conditions and Abiotic Controls

Aquil was prepared according to the work of Sunda et al. (2005) [30], based on the original recipe by Morel et al. (1979) [31], by mixing solutions of hydrous and anhydrous salts. The medium was supplied with a trace metal stock solution containing 10 µM EDTA and increased concentrations of NO_3^- (10^{-2} M) and PO_4^{2-} (10^{-3} M) to reflect the nutrient concentrations necessary to avoid other nutrient limitations in *Synechococcus* sp. PCC 7002. Salt and nutrient solutions were microwave-sterilized using a 700 W microwave oven according to the work of Sunda et al. (2005) [30] and as previously outlined by Keller et al. (1988) [32] by dispensing the medium into trace-metal-clean polycarbonate bottles and subsequently microwaving it in intervals of 3, 2, 3, and 2 minutes for a total of 10 minutes of microwave treatment. The medium was mixed between heating cycles, and boiling was avoided by the careful observation of the medium in order to avoid any changes in pH. The medium was treated with Chelex-100 according to the work of Sunda et al. [30] in order to remove any trace metal contamination. Trace metal EDTA stock was filter-sterilized in order to avoid the formation and heat-aging of metal precipitates, and it was subsequently added to the sterile medium.

50 nM iron, exclusively in the form of either $FeCl_3 \times 6H_2O$ or FeO(OH) (Sigma Aldrich) was filter-sterilized and supplied, respectively, to separate 20 L containers of the prepared Aquil medium.

An axenic wildtype *Synechococcus* sp. PCC 7002 (henceforth *Synechococcus*) preculture was provided by Anne Ilse Maria Vogel (Norwegian University of Science and Technology). The preculture was washed in Chelex-treated Aquil before inoculation to avoid the transfer of extracellular iron from the previous growth medium. Axenic cultures of the cyanobacterial strain *Synechococcus* sp. PCC 7002 at 1:20,000 (cell to mL medium ratio) were grown for 24 (FeO(OH)) and 26 ($FeCl_3$) days at 25 °C.

A temperature of 25 °C was chosen in order to allow for the detection of Fe(II), which oxidizes faster at higher temperatures while avoiding the increased levels of stress exhibited by *Synechococcus* at lower temperatures [33]. The experiment was run in large batch cultures with an integrated sampling system to reduce contamination (biological or trace metal) to the system.

The pH was monitored daily in sub samples by a pH meter (WTW pH/ION 340i). Aeration was supplied by bubbling high-efficiency particulate air (HEPA)-filtered air through H_2O. Constant illumination (90 µE m^{-2} s^{-1}) was provided by LED lights.

Twenty liter abiotic controls were made up of Aquil that was treated identically to cultures in terms of nutrient and iron additions, as well as sterilization and Chelex-treatment. The experimental conditions were the same as for cultures in terms of light, aeration, and sampling scheme.

2.3. Growth

Growth was assessed by measuring optical density (OD) at 730 nm using a SPECTRONIC 200 E spectrophotometer (Thermo Scientific).

The numbers of cells were estimated based on Equation (1) [34], where y is the number of cells per mL and x is the intensity measured for OD at 730 nm. This equation was based on previous analyses by Lund et al. [35], with our calibration run for the specific organism and spectrophotometer. The calculated cell number was used only as an approximate measure to be able to calculate iron concentration per 10^9 cells.

$$y = \left(5 \times 10^8 x\right) + \left(1 \times 10^8\right) \quad (1)$$

The growth curves and simple linear regression used to evaluate growth were created using Prism version 8 (GraphPad).

2.4. Fluorescence Emission Analysis at 77 K

An analysis by fluorescence emission analysis (FEA) was done at days 24 (FeO(OH)) and 26 ($FeCl_3$) to evaluate the state of the photosynthetic machinery after iron limitation. Subsamples of 2 mL were collected in triplicate from cultures and concentrated via centrifugation (13,000 rpm for 10 min) before being diluted to a maximum of 2 µg/mL chlorophyll *a* in the Aquil medium. Samples of 1 mL of the prepared cells were transferred to sample glass tubes (inner diameter of 2 mm and outer diameter of 5 mm) and frozen in liquid nitrogen at −196 °C (77 K) until analysis, which was also performed at 77 K. The low temperature used in the 77 K FEA sharpens spectral characteristics because it inhibits most photosynthetic electron transport reactions, with the exception of those involved in primary charge separation and charge stabilization within photosystems [36]. Compared to fluorescence analyses performed at room temperature, the fluorescence yield of a PSII complex increases approximately twofold, while the fluorescence yield of PSI increases by a factor of about 20 [37,38], thus enabling the separate analysis of fluorescence from each of the two photosystems. Emission spectra in triplicate were recorded upon excitation with a 440 nm diode using a setup described by Lamb et al. [39]. These spectra were baseline-corrected and normalized to 725 nm in MATLAB R2017a (TheMathWorks, Inc.). Graphs were made in Prism version 8 (GraphPad). A two-way ANOVA with uncorrected Fisher's least significant difference (LSD) were performed in Prism version 8 (GraphPad) in order to assess significant differences between peak ratios.

2.5. Iron Measurements

All samples for total iron were analyzed using high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS; Element 2, Thermo Scientific), and graphs were made using Prism Version 8 (GraphPad). A two-way ANOVA with uncorrected Fisher's LSD were performed in Prism version 8 (GraphPad) in order to assess significant differences between each treatment on different sampling days, as well as between separate treatments on each sampling day. The separate sampling procedures are described below.

2.5.1. Total Dissolvable Fe

Subsamples for total dissolvable iron (TFe) were collected in triplicate from both cultures and abiotic controls and acidified to pH < 2 using ultra-pure (UP) nitric acid (HNO_3, 65%) at the start, middle, and end of the experiment. Samples were analyzed by HR-ICP-MS.

2.5.2. Total Particulate and Intracellular Iron

Subsamples for particulate (PFe) and intracellular iron (InFe) were collected in triplicate and filtered through an acid-washed 0.2 µm polycarbonate track-etch membrane filter (Sartorius Stedim), using a Nalgene filtration system (Thermo Scientific). Sampling was done on selected sampling days (Supplementary Table S1). Filters for particulate iron were frozen at −20 °C for at least a week, and they were subsequently digested by Ultra Clave before analysis by HR-ICP-MS. Blank filters for pFe were frozen, digested by Ultra Clave, and analyzed by HR-ICP-MS.

Filters for intracellular iron were washed after filtration using an oxalate wash and NaCl rinse according to the work of Tang and Morel [40]. The filters were then frozen at −20 °C for at least a week and subsequently digested by Ultra Clave before analysis by HR-ICP-MS. Blank filters for InFe were treated with oxalate wash and NaCl rinse before being frozen, digested by Ultra Clave, and analyzed by HR-ICP-MS.

2.5.3. Total Dissolved Iron

Subsamples for total dissolved iron (dFe) were collected in triplicate from both cultures and abiotic controls and filtered through a 0.2 µm polycarbonate track-etch membrane filter (Sartorius Stedim) on

selected sampling days (Supplementary Table S1). The filtrate was acidified to pH < 2 using UP HNO_3 (65%). Samples were analyzed by HR-ICP-MS.

2.6. Iron (II) Measurements

Dissolved iron (II) (dFe(II)) was measured on selected sampling days (Supplementary Table S1) using flow injection analysis (FIA) with a chemiluminescence (CL) detector. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma-Aldrich) was used as a reagent, prepared according to the work of Hopwood et al. (2016) [41] using 0.53 g/L of potassium carbonate (K_2CO_3), 0.13 g/L of luminol, 0.25 M ammonia (NH_4OH, trace metal grade), and 11 g/L of ultra high purity (UHP) hydrochloric acid (HCl). The final luminol pH was between 10.0–10.2.

All standards for iron (II) measurements were prepared gravimetrically. A primary standard (500 µM ammonium iron (III) sulfate ($NH_4Fe(SO_4)_2$) in 0.1 M HCl, kept for a maximum of one month) was used to prepare a secondary standard (50 µM $NH_4Fe(SO_4)_2$ in 0.01M HCl, kept for a maximum of 3 days) that was used to prepare a tertiary standard (1 µM $NH_4Fe(SO_4)_2$ in MilliQ water, prepared daily). The tertiary standard was used to prepare daily calibration standards of 0.5, 1, 2, 3, and 4 nM $NH_4Fe(SO_4)_2$ in an Aquil salt solution. Standards were prepared seconds before analysis by adding tertiary stock solution to a gravimetrically prepared amount of water in order to avoid the oxidation of Fe(II). Each standard was run in quintuplicate or sextuplicate for a five-point calibration curve prior to sample analysis. Sample concentrations were calculated based on the daily calibration curve. The FIA system (Waterville Analytical) and software (Labview run FIA v. 2.03) were run according to method of Croot and Laan [42] without pre-concentration.

Subsamples for dissolved iron (II) were collected from both cultures and abiotic controls, and then they were filtered through a 0.2 µm acid-washed polycarbonate track-etch membrane filter (Sartorius Stedim) before being introduced to the FIA system. The filtration was done to avoid contamination of the system, as well as to avoid any potential autofluorescence from the cells.

2.7. Chemical Equilibrium Calculations

Metal speciation and solubility equilibria were calculated using Visual MINTEQ 3.1 (available for free download at https://vminteq.lwr.kth.se/download/). The software was run with concentration of Aquil as reported in methods Section 2.2, with a pH of 8.

3. Results and Discussion

In order to assess influence of iron source on iron limitation, *Synechococcus* sp. PCC 7002 was grown on either $FeCl_3$ or FeO(OH) as exclusive iron sources. These iron sources were chosen to investigate the difference in response from *Synechococcus* to an amorphous, more bioavailable form and a crystalline, less bioavailable form of iron, respectively. Hydrolysis species of iron, such as those produced when $FeCl_3$ is dissolved in water, are considered more available for complexation by EDTA than poorly soluble oxyhydroxides such as FeO(OH) [30,43]. As the growth medium contains EDTA, these hydrolysis species are kept in solution and thus more available for uptake than in the absence of EDTA or other chelator. FeO(OH) precipitates more readily into a bio-unavailable form, making it difficult for the organism to access and utilize for growth.

3.1. Growth

Growth measurements were recorded every 24 hours (Figure 1) for the entirety of the experiment. With $FeCl_3$ as sole iron source, *Synechococcus* achieved a higher final cell count than on FeO(OH). These results were in line with expectations because FeO(OH) is considered less bioavailable than $FeCl_3$ due to its low solubility [44] and reported growth curves. The more rapid growth could potentially be attributed to the higher initial total iron concentration (Supplementary Figure S1) in the $FeCl_3$ culture. However, despite this higher initial total iron concentration in the $FeCl_3$ culture, the culture and abiotic control on $FeCl_3$ showed no significant differences in initial dissolved iron concentrations

(14.65 ± 9.5 nM in the culture and 8.99 ± 2.7 nM in the control), as evidenced by a two-way ANOVA with uncorrected Fisher's LSD (p-value: 0.4137), thus indicating that the difference in initial total iron concentrations did not determine growth because the dissolved iron fraction is considered more relevant for supporting growth. Both cultures were considered iron-limited, as also evidenced by results from the 77 K FEA.

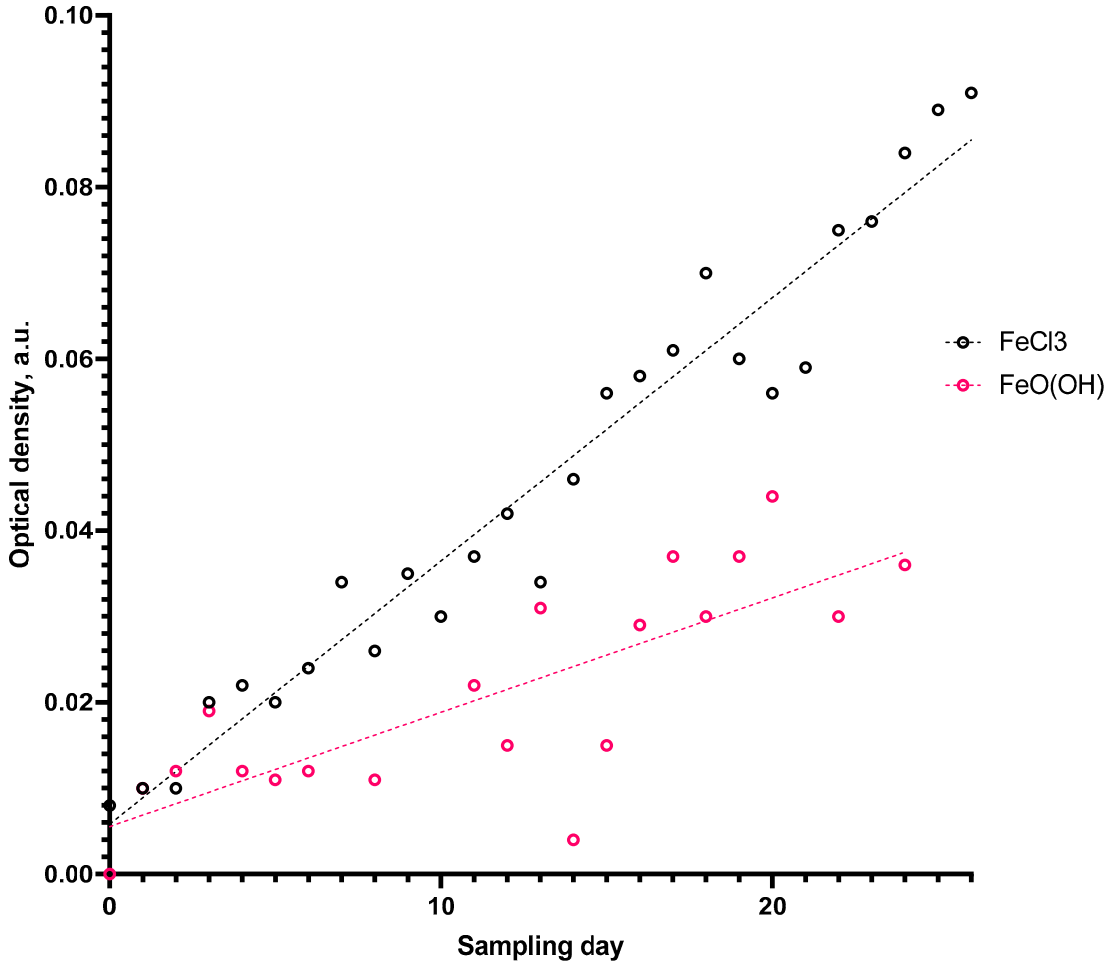

Figure 1. Optical density at 730 nm as a measure of growth of *Synechococcus* grown on an Aquil medium supplied with $FeCl_3$ (black) and FeO(OH) (red), with simple regression curves shown.

3.2. Fluorescence Emission Analysis at 77 K

To evaluate changes in the photosynthetic machinery, cultures of *Synechococcus* were investigated using 77 K fluorescence emission spectroscopy (Figure 2).

Chlorophyll is found in both photosystems and produces fluorescence under photosynthetically active radiation (PAR) excitation. As mentioned above, PSI emits limited fluorescence at room temperature. However, at 77 K cyanobacterial PSI emits measurable fluorescence in a wavelength range of 727–760 nm, depending on the species [45] and the potential polymerization of the complex. Some cyanobacterial species form monomeric, trimeric, and tetrameric PSI with different optical characteristics, resulting in distinctive chlorophyll fluorescence emission bands at a temperature of 77 K—the monomer at 725 nm, the trimer at 730 nm, and the tetramer at 715 nm [46]. PSII, on the other hand, does exhibit measurable fluorescence at room temperature—predominantly from chlorophylls fluorescing at 695 nm. When freezing a sample at 77 K however, a distinct fluorescence at 685 nm may also be observed [39]. When evaluating iron limitation in cyanobacteria, a third protein complex may be of interest. IsiA forms an antenna around PSI when the organism is experiencing iron stress,

and this can be observed by an increase in fluorescence emission at 685 nm [47–49], as well as an increase in energy partitioning to PSI that is reflected by an increased fluorescence emission by the PSI reaction center antenna chlorophylls at around 720 nm [39]. This, in turn, leads to a decrease in the 685/725 nm (PSII/PSI) spectral ratio and an increase in the 685/695 nm (PSII/IsiA) spectral peak ratio.

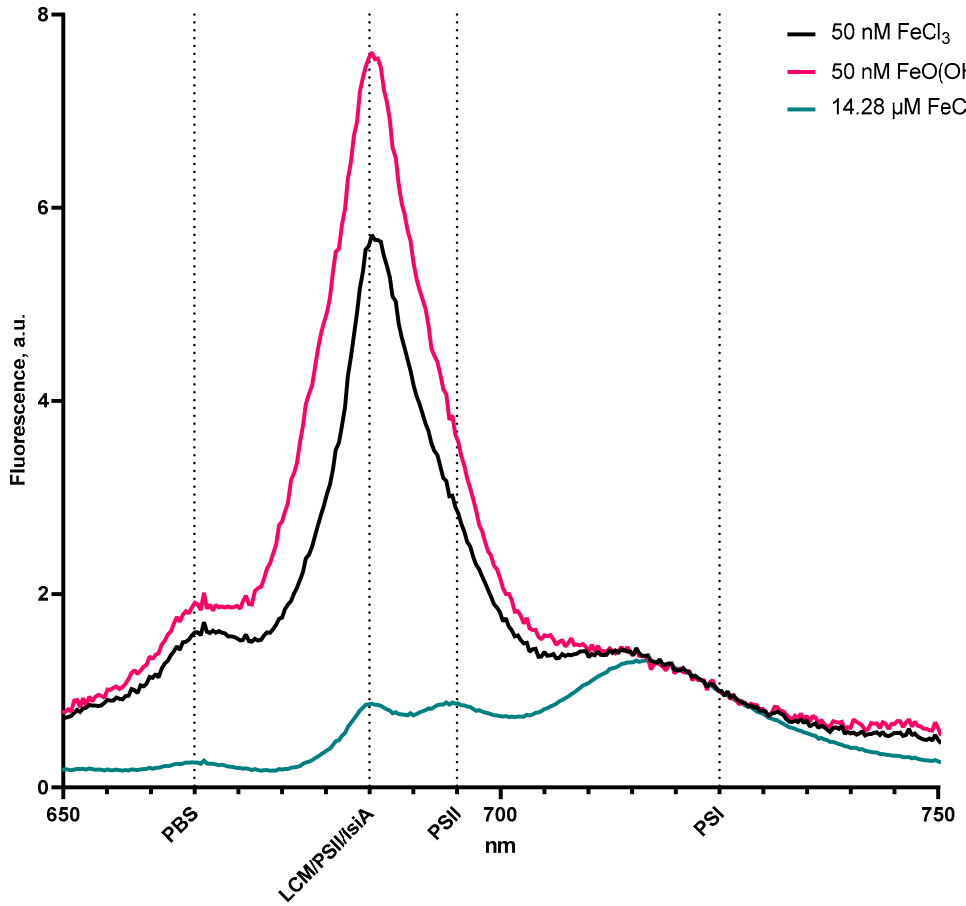

Figure 2. 77 K fluorescence emission spectra of cultures grown on $FeCl_3$ and FeO(OH) that were acquired with an excitation wavelength of 440 nm. Peaks attributed to phycobilisomes (PBS), a combined contribution from photosystem II (PSII), the protein complex iron-stress inducible protein (IsiA) and the terminal phycobilisome emitter (LCM), PSII, and photosystem I (PSI) are indicated with dotted lines. The green line shows the fluorescence emission signal from the *Synechococcus* culture grown under iron-replete conditions of 14.28 μM $FeCl_3$ [25]. The signal was normalized to the 725 peak for better visual representation.

The characteristic fluorescence emission peak of IsiA at 685 nm was observed in the cultures grown on both $FeCl_3$ and FeO(OH). IsiA is considered one of the most common indicators for iron stress [50], and a signal at 685 nm would thus be more prominent when iron limitation (or high light/oxidative stress) is present. PSI has a fluorescence emission peak ranging from 715 to 725 nm. During iron stress, IsiA is upregulated, thus increasing the 685 nm/725 nm spectral ratio with increasing iron stress. While both cultures here produced a substantial IsiA signal, the IsiA/PSI ratio clearly shows that the stress response was more pronounced in the culture grown on FeO(OH).

The 77K FEA spectra were used to obtain indicative ratios between PSII (695 nm) and PSI (725 nm) and between PSII (695 nm) and IsiA (685 nm) for both cultures (Figure 3). In iron-limiting conditions, where the IsiA peak dominates the fluorescence signal, this peak would also have an effect on the height of the 695 nm peak. This is due to the fact that the 685 and 695 nm peaks are so close together that the 695 nm peak (indicative of PSII) rides the tail of the 685 nm peak (indicative of IsiA) when IsiA emission

is strong. A *Synechococcus* culture grown under iron-replete conditions (14.28 μM, $FeCl_3$) during a separate experiment exhibited a relative PSII/PSI ratio of 0.89 ± 0.02 and a relative PSII/IsiA ratio of 1.0 ± 0.04 (data kindly provided by Anne Ilse Maria Vogel, whose manuscript is in preparation [25]). This clearly shows the difference in emittance at 685 nm between non-iron stressed and iron-stressed cultures. The iron limited culture grown on FeO(OH) exhibited a relative PSII/PSI ratio of 3.7 ± 0.03 and a relative PSII/IsiA ratio of 0.48 ± 0.008, while the iron limited culture grown on $FeCl_3$ exhibited a relative PSII/PSI ratio of 2.9 ± 0.05, and a relative PSII/IsiA ratio of 0.50 ± 0.004. Comparing these ratios to those of the culture grown under iron-replete conditions indicated the presence of iron stress. The downregulation of PSI is a sign of iron limitation [14] and increases the relative PSII/PSI ratio. While a high relative PSII/PSI ratio was seen in both cultures, the *Synechococcus* grown on FeO(OH) had a significantly higher relative PSII/PSI ratio compared to that grown on $FeCl_3$ (p-value: <0.0001), thus indicating that the stress increased when the available iron was in a more crystalline form. This confirmed the hypothesis that FeO(OH), like other crystalline iron (oxyhydr)oxides, has a lower bioavailability than amorphous iron forms such as $FeCl_3$ [43]. Cultures grown on both $FeCl_3$ and FeO(OH) exhibited markedly higher relative PSII/IsiA ratios than the culture grown under iron-replete conditions, which was a clear indication of iron stress in both cultures under low iron concentrations The iron concentration used in this experiment was comparable to naturally occurring iron concentrations in coastal areas [29]; therefore, these results indicate that *Synechococcus* may experience iron stress under naturally occurring iron concentrations. Alternatively, it may also by a possibility that the used *Synechococcus* strain was adapted to lab conditions and thus had higher iron requirements than its natural counterparts.

3.3. Particulate and Intracellular Iron

HR-ICP-MS was used to analyze the particulate and intracellular iron concentrations of *Synechococcus* grown on $FeCl_3$ or FeO(OH) as exclusive iron sources to evaluate any differences in the iron uptake dependence on the iron source (Figure 4). Iron per 10^9 cells values were obtained by dividing the measured iron concentration (nM) by the number of 10^9 cells per liter (data obtained from OD_{730} using Equation (1)) and used in order to make comparisons between the cultures possible across culture densities. While inorganic iron particulates may have formed in the medium, they were only considered to contribute a small fraction of the total particulate iron concentration. (Visual MINTEQ calculations placed 99.82% of all iron in the medium as complexed to EDTA via $FeEDTA^-$ or $FeOHEDTA^{2-}$; full details in Supplementary Table S2). This was also supported by the low particulate iron concentrations measured in the cultures with a low optical density, and we therefore omit a discussion of the formation of inorganic iron particulates even though they surely existed—especially in the FeO(OH) cultures. Particulate iron concentrations are thus discussed as a reflection of the total iron concentration of the cell, both incorporated and associated with the cell surface. Intracellular concentrations reflected the iron that was incorporated into the cell and could not be easily washed away by use of an iron-chelating washing procedure. Our analysis showed that *Synechococcus* adsorbed and incorporated more iron when $FeCl_3$ was provided as an iron source compared to FeO(OH). From start to finish, both particulate (p-value: 0.0111) and intracellular (p-value: 0.0021) iron concentrations per cell increased significantly in the culture grown on $FeCl_3$ from a particulate iron concentration of 0.059 ± 0.003 nmol Fe per 10^9 cells and an intracellular iron concentration of 0.029 ± 0.012 nmol Fe per 10^9 cells on day 10 to a particulate iron concentration of 0.139 ± 0.016 nmol Fe per 10^9 cells and an intracellular iron concentration of 0.059 ± 0.014 nmol Fe per 10^9 cells on day 26. A two-way ANOVA with uncorrected Fisher's LSD showed no significant changes throughout the experiment in particulate (p-value: 0.4313) or intracellular (p-value: 0.5239) iron concentrations in the culture grown on FeO(OH). Particulate concentrations in the culture grown on FeO(OH) ranged from 0.016 ± 0.004 nmol Fe per 10^9 cells on day 6 to 0.025 ± 0.011 nmol Fe per 10^9 cells on day 24 and an intracellular iron concentration of 0 nmol Fe per 10^9 cells on day 6 to 0 nmol

Fe per 10^9 cells on day 24. The particulate and intracellular iron concentrations of the cultures grown on $FeCl_3$ remained higher than for the cultures grown on FeO(OH) throughout the experiment.

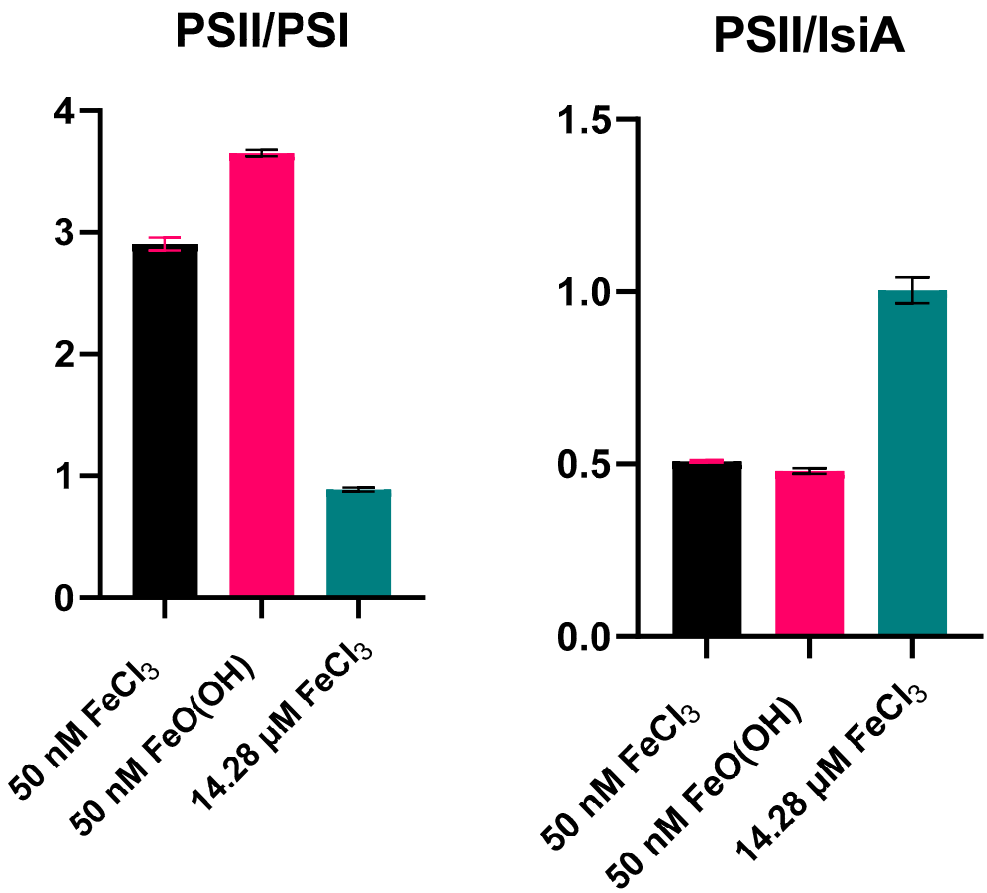

Figure 3. Pigment ratios detected with 77 K fluorescence emission. A two-way ANOVA with uncorrected Fisher's LSD showed significant differences between the PSII/PSI ratios from the two cultures (p-value: < 0.0001). The PSII/PSI ratio increased with the downregulation of PSI, and a higher ratio was evidence of a higher degree of stress response. Both ratios would be closer to 1 if no iron stress was present, as evidenced by the PSII/PSI ratio from the iron-replete culture of *Synechococcus* (green). The PSII/IsiA ratio decreased with higher degrees of iron stress, as the signal from IsiA overshadowed that from PSII. No significant differences were found between the PSII/IsiA ratios of the $FeCl_3$ and FeO(OH) cultures (p-value: 0.2985), but a *Synechococcus* culture grown on 14.28 µM $FeCl_3$ exhibited a markedly higher PSII/IsiA ratio, thus evidencing the iron stress in both of the cultures in this experiment.

Comparing particulate and intracellular fractions from the culture grown on $FeCl_3$ indicated that a large amount of the cell-associated iron was bound to or associated with the outside of the cell, and the relationship between the two fractions changed little throughout the experiment. Intracellular iron concentrations ranged from 48.72% (day 10) to 60.44% (day 13) to 42.73% (day 26) of the total particulate concentration. Seen in relation to the growth for the two different cultures, it became evident that the more soluble and bioavailable $FeCl_3$ was associated with a higher growth compared to the less bioavailable FeO(OH). With $FeCl_3$ as the available iron source, it appeared that iron adsorbed to the cell surface of *Synechococcus* without immediately being internalized. One could speculate that extracellular iron storage may have been in place to prevent intracellular iron toxicity [51]. However, the results from the 77 K FEA showed a high degree of iron-related stress, even in the $FeCl_3$ culture; therefore, toxicity was likely not the major mechanism responsible for this cell-surface "storage" of iron. The accumulation of cell-surface iron may have happened due to both biotic and abiotic

processes, and it is possible that iron uptake rates were limited under the experimental circumstances. An explanation for this may be that the extracellularly-stored iron was not bioavailable or that the transport of extracellularly produced iron (II) into the cell became the rate-limiting step for iron acquisition, as transporters in the cell membrane could not keep up with the rapid cell-surface reduction [52,53]. Any extracellular-associated iron (II) in this experiment would have contributed to the PFe concentration, as HR-ICP-MS does not distinguish between the oxidation states of iron. Additionally, cultures were transferred from an iron-rich medium and not (yet) adapted sufficiently to long term iron starvation, which may have caused less effective iron uptake rates. Further research should focus on analyzing gene expression data to investigate the underlying transcriptomic changes and adaptations.

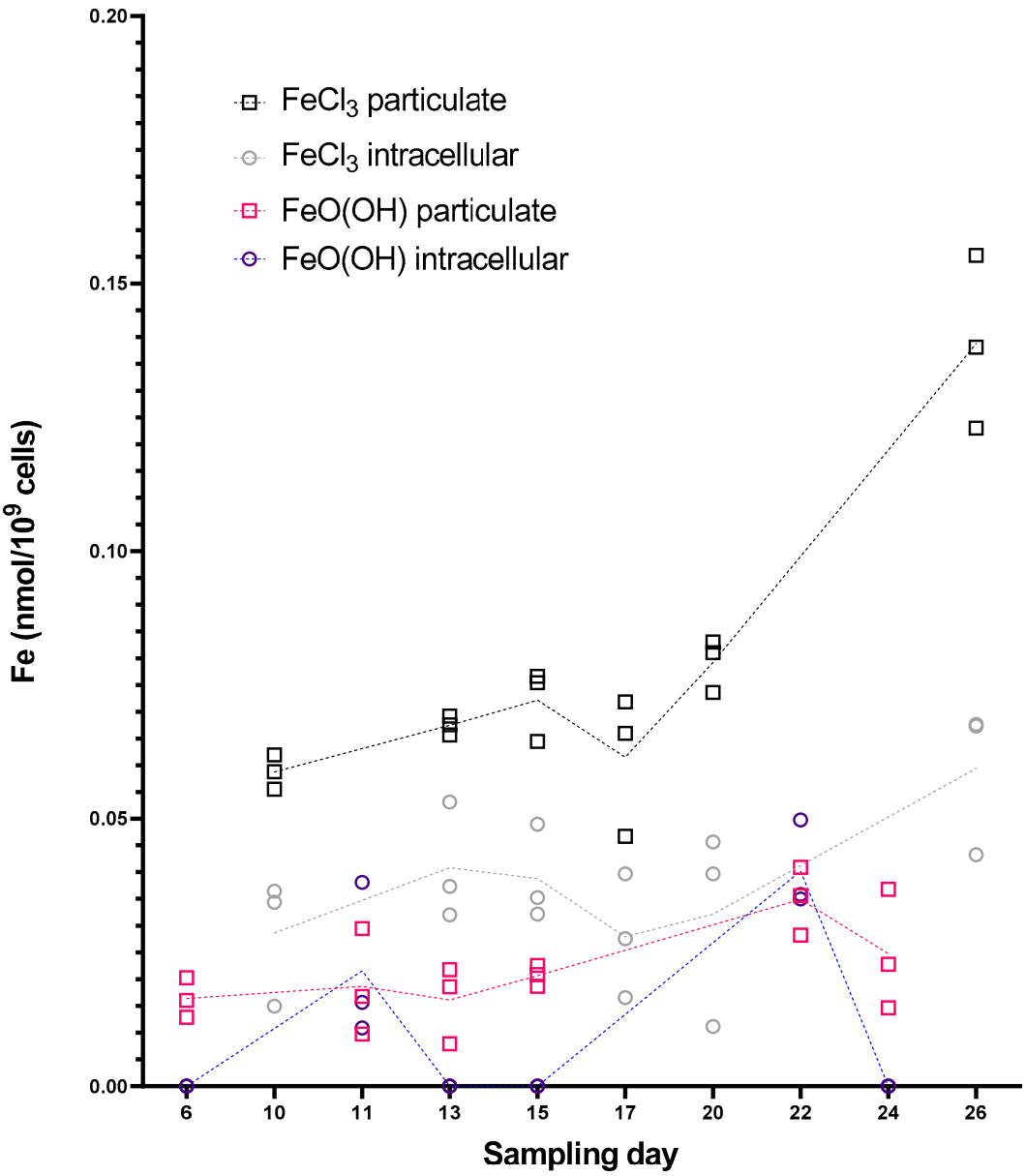

Figure 4. Particulate (PFe) and intracellular iron (InFe) concentrations in nmol Fe per 10^9 cells for cultures of *Synechococcus* grown on $FeCl_3$ and FeO(OH) in triplicate with added trend lines. Intracellular iron concentrations were obtained using an oxalate wash. Concentration per 10^9 cells values were obtained by dividing the total measured iron concentration by measured number of 10^9 cells per L sample (data obtained from optical density at 730 nm (OD_{730})).

As seen in Figure 4, InFe concentrations were either below the limit of detection (concentration set to 0) or higher than PFe in the culture grown on FeO(OH). However, a two-way ANOVA with uncorrected Fisher's LSD revealed that there were only significant differences between PFe and InFe on days 6 (p-value: 0.0026), 13 (p-value: 0.0092), and 15 (p-value: 0.0195) of the experiment, where intracellular iron concentrations were measured to 0 nmol Fe per 10^9 cells. The lack of differences between PFe and InFe concentrations throughout the rest of the experiment could have been due to the low initial iron concentration and/or the fact that cell iron concentrations were so low that there would have been a larger potential for contamination during sample processing. This lack of concentration difference between particulate and intracellular iron could also indicate a highly efficient internalization of iron under a low bioavailable iron concentration once the crystalline iron source was in close proximity to the cell. It is also possible that FeO(OH) was forming colloidal particles with a size of <0.2 µm, which would have passed through the filter and thus avoided detection. This clearly contrasted with the *Synechococcus* grown on $FeCl_3$ as the sole iron source: a larger difference between particulate and intracellular iron in the culture grown on $FeCl_3$ showed that iron was not immediately internalized. Previous studies have shown the increased internalization of iron and the upregulation of iron transporter genes under iron limitation in other species [54,55], but the effect of specific iron sources on the internalization of iron is understudied in cyanobacteria. Previous studies on *Synechocystis* sp. PCC 6803 have shown that identical concentrations of different iron sources provide different degrees of stress in organisms, with FeO(OH) resulting in the highest stress levels [26]. The kinetic and thermodynamic properties of the two iron forms used in this study are well-known [56], and their interactions with EDTA and organic chelators of biological origin [57] suggest that $FeCl_3$ should indeed be more bioavailable than FeO(OH), and this difference in bioavailability should be enough to induce different degrees of iron-related stress mechanisms in *Synechococcus*.

3.4. Dissolved Total Iron and Iron (II)

dFe concentrations were measured using HR-ICP-MS after filtering the samples through an acid-cleaned 0.2 µm polycarbonate track-etch membrane filter. Dissolved iron (II) concentrations were measured using FIA-CL after filtering samples through a 0.2 µm acid-washed polycarbonate track-etch membrane filter in order to establish whether there were any marked differences between the dissolved iron (II) concentrations in *Synechococcus* cultures compared to abiotic controls using $FeCl_3$ and FeO(OH) as exclusive iron sources, as well as whether the dissolved iron (II) concentrations would vary depending on iron source. Abiotic controls were used to assess whether dissolved iron (II) may be of biogenic origin or whether its presence was due solely to abiotic processes, such as the photochemical reduction of iron (III) to iron (II). All dissolved iron concentrations are showed in Figure 5.

The results for the total dissolved iron showed a clear difference between iron sources, as cultures and abiotic controls followed similar general trends in relation to iron source. In the culture and control provided with $FeCl_3$, the total dissolved iron increased in the first week of the experiment from 14.65 to 40.53 nM in the culture and 8.99 to 16.07 nM in the control before it decreases sharply towards the end of the experiment, reaching final concentrations of 3.26 nM in the culture and 5.10 nM in the control. In the culture provided with FeO(OH), the increase happened slower from the start of the experiment, from 7.69 to 12.00 nM in the first ten days, with a large increase towards the end of the experiment, reaching a final concentration of 35.49 nM. The abiotic control supplied with FeO(OH) showed no significant differences (p-value: 0.6765) in the total dissolved iron between initial sampling (3.37 ± 0.2 nM) and the end of the experiment (3.027 ± 1.4 nM), thus pointing to a lack of abiotic solubilization of FeO(OH). Despite the difference in total iron concentrations between the culture and control provided with $FeCl_3$ at the start of the experiment, the measured total dissolved iron indicated that the contamination of the $FeCl_3$ culture was negligible, as there were no significant differences (p-value: 0.4137) between the initial total dissolved iron concentrations in the culture (14.65 ± 9.5 nM) and abiotic control (8.99 ± 2.7 nM) on $FeCl_3$. The dFe fraction is considered to be the iron fraction that

is most available for biotic uptake and is thus a better measure for initial available iron concentration than TFe. The amorphous $FeCl_3$ solubilizes better and forms complexes with EDTA more easily than FeO(OH) [30,43], thus explaining the higher initial concentrations of the total dissolved iron in the $FeCl_3$ control (8.99 ± 2.6 nM)) compared to the FeO(OH) control (3.37 ± 0.2 nM). The fact that there were no significant changes in the total dissolved iron concentration in the abiotic FeO(OH) control between the start and end of the experiment could point to the sampled fraction mostly consisting of FeEDTA, or iron complexed to EDTA, most likely the only truly soluble iron species in the solution. Higher concentrations of FeEDTA, as well as true Fe′, was expected in the abiotic $FeCl_3$ control, and the larger degree of fluctuation in the total dissolved iron concentration could be attributed to photochemical transformations of FeEDTA complexes, as well as transformations of amorphous iron (hydr)oxides [58].

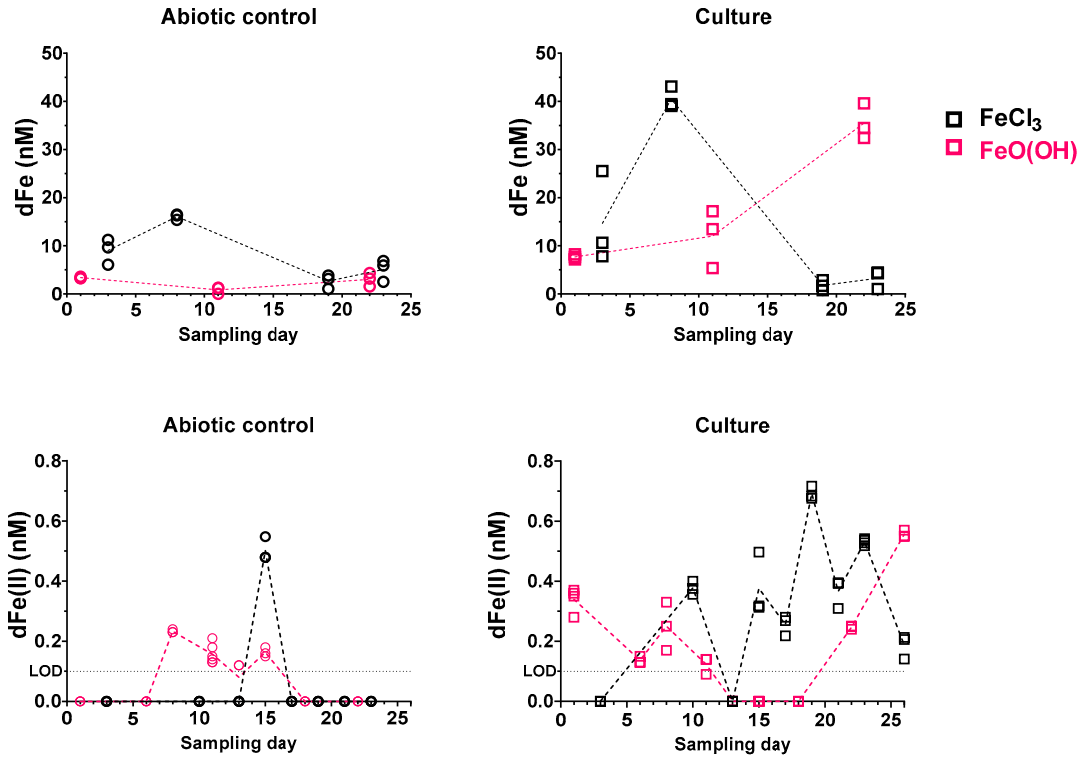

Figure 5. Changes in total dissolved iron concentration (top) and dissolved iron (II) (bottom) for abiotic controls and cultures of *Synechococcus* grown on $FeCl_3$ (black) and FeO(OH) (red) in triplicate with trend lines. Limit of detection (LOD) is indicated for total dissolved iron II (dFe(II)).

Seen in relation to the particulate iron concentrations (Figure 4), it is clear that the total dissolved iron concentration in the cultures decreased as cellular iron concentrations increased, and that biotic processes affected the solubilization of iron. This was seen most strongly in the $FeCl_3$ culture where increasing amounts of iron were being adsorbed and internalized by *Synechococcus* as the experiment progressed. An increase in total dissolved iron concentration on day eight in both the abiotic control and the culture on $FeCl_3$ pointed to the pure physiochemical solubilization of iron and biotic processes affecting iron concentrations simultaneously. The decrease in the total dissolved iron concentration in the culture grown on $FeCl_3$ at the end of the experiment may point to the more efficient internalization of iron by *Synechococcus*, which may have been due to an increased adaptation to iron limitation. The similar trend in the abiotic control indicated that abiotic processes diminish the solubilized iron fraction, with more iron seeming to precipitate towards the end of the experiment. Over time, more iron will have a chance to precipitate but is unlikely to resolubilize without changing environmental conditions [56]. The sharp increase in the total dissolved iron towards the end of the experiment in the culture grown on FeO(OH) pointed to biotic processes solubilizing even this insoluble iron form

over time. This delay could have been due to the need of the organism to adapt to the specific iron limitation conditions.

Dissolved iron (II) production was higher in the cultures than in the abiotic controls throughout the experiment for the $FeCl_3$ treatment, with a peak of 0.69 ± 0.02 nM on day 19. For most sampling days, no abiotic production of dissolved iron (II) was observed, and the highest recorded concentration in the controls was recorded on day 15 at 0.50 ± 0.04 nM. For the FeO(OH) treatment, sampling days 8, 11, 13, and 15 had a higher abiotic than biotic production of dissolved iron (II). The highest dissolved iron (II) concentration was recorded on day 26 for the culture (0.56 ± 0.01 nM) and on day 8 for the abiotic control (0.23 ± 0.006 nM) on FeO(OH). Higher concentrations of dissolved iron (II) in the *Synechococcus* culture with $FeCl_3$ as the iron source compared to the abiotic controls suggested that *Synechococcus* may be able to reduce iron extracellularly. Electron donation to inorganic iron complexes with a subsequent uptake of reduced iron forms has been suggested to occur in cyanobacteria [59]. A study investigating the function of T4P in *Synechocystis* sp. PCC 6803 found that the presence of the PilA1 gene enhanced its growth ability on iron oxides [26]. This was linked to a possible utilization of pili as donors of electrons to iron oxides, thus facilitating a reduction of iron, as has been shown in non-photosynthetic soil bacteria [60]. As *Synechococcus* also possess the necessary genes for expression on PilA [25], further research into the function of PilA in *Synechococcus* may elucidate any potential link between pili and extracellular iron reduction. The higher concentration of dissolved iron (II) in the cultures compared to abiotic controls could also be attributed to the interactions between dissolved organic matter (DOM) and Fe(III). DOM may provide ligands for the complexation of iron, but it may also serve as an electron source for the photoreduction of iron (III) [22,61]. However, the quantification and characterization of the produced DOM is necessary to determine whether the DOM is indeed able to facilitate such a reduction of iron in these cultures.

As photochemical reactions may have reduced iron (III) to iron (II) in the light regiment used [62,63], the detection of dissolved iron (II) in the abiotic controls was not unexpected. Aquil contains EDTA, which may have facilitated the production of iron (II) because it can function as a substrate for photoreduction [64].

Some of the iron (II) detected in the *Synechococcus* cultures may thus have been of abiotic origin, but it is important to consider that *Synechococcus* would have just as efficiently taken up abiotically produced iron (II) as it became available in the medium. There is no apparent way of distinguishing abiotically produced iron (II) from that produced on cell surfaces, and abiotic production may also be affected by biological activity. This means that a true biotically produced iron (II) concentration could not be found by simply subtracting the abiotically produced iron (II) concentration found in the controls. Where higher iron (II) concentrations were seen in the abiotic controls compared to the cultures, a rapid uptake by *Synechococcus* may have explained why similar values were not seen in the cultures.

We hypothesized that if *Synechococcus* was able to reduce iron, the more amorphous form of $FeCl_3$ would be more easily reducible than FeO(OH) because $FeCl_3$ rapidly forms amorphous iron oxides and FeEDTA complexes, both of which have a higher solubility than the crystalline forms of FeO(OH) [65]. The higher concentrations of dissolved iron (II) in these cultures supported the initial hypothesis. The results for dissolved iron (II) were also in agreement with the results showing a higher relative uptake of iron in the cells grown on $FeCl_3$ compared to those grown on FeO(OH).

The amorphous iron oxides and FeEDTA complexes formed by dissolving $FeCl_3$ are less thermodynamically stable than FeO(OH) and its EDTA complexes, and, thus, reduction rates could be expected to be more rapid for these iron species independent of whether the reduction is of biotic of abiotic origin [66]. This line of thought could explain why iron (II) could be detected on more sampling days throughout the experiment in the cultures grown on $FeCl_3$ compared to those on FeO(OH). This could also be connected to the PFe and InFe results for *Synechococcus* grown on $FeCl_3$, where iron was not immediately internalized but was stored extracellularly. While iron (II) may have been produced relatively rapidly by *Synechococcus*, the transport of iron (II) into the cell itself may have

become the rate-limiting step, as transporters in the cell membrane may not have kept up with the rapid reduction [52,53]. This allowed for the detection of iron (II), and it may be a partial explanation for the larger amount of cell surface-associated iron in the $FeCl_3$ culture. However, the interaction between iron (II) and EDTA, as well as the rapid re-oxidation from iron (II) to iron (III), may have removed portions of the iron (II) pool from the medium faster than could be detected due to time-consuming analysis procedures, explaining the low iron (II) concentrations and the variations in Fe(II) concentrations throughout the experiment. A simplified schematic of some of the interactions between iron forms and other constituents of the seawater medium is shown in Figure 6.

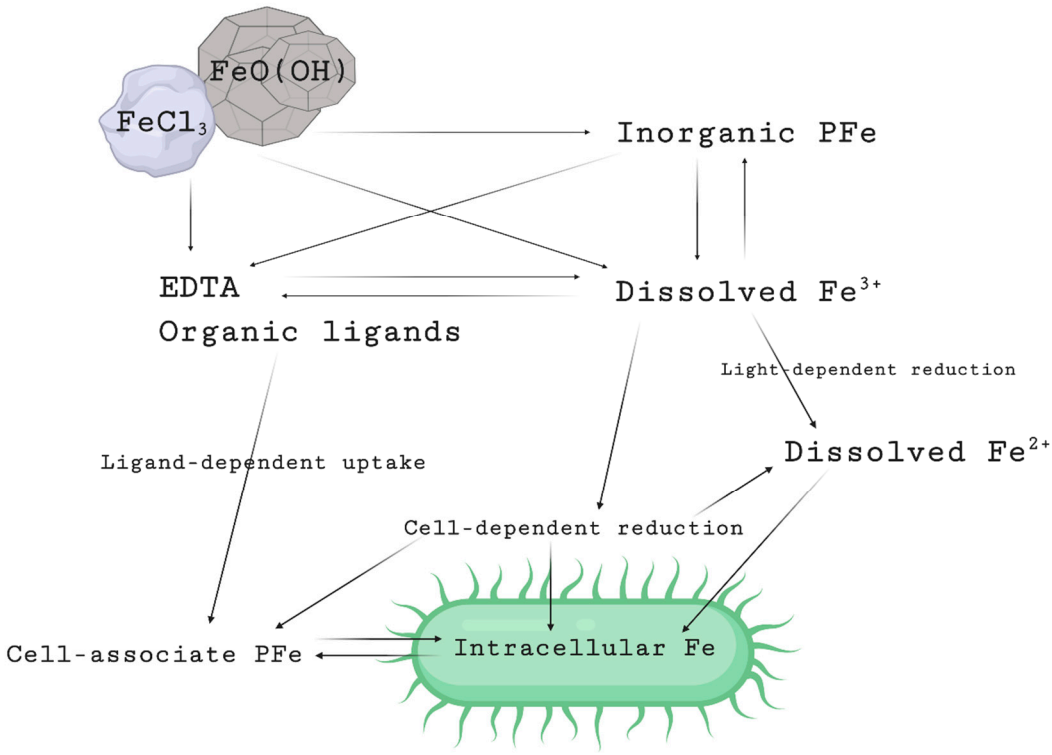

Figure 6. Interactions within an iron pool are complex and often mediated by other constituents within a seawater medium. Iron can be internalized by a cell through a range of mechanisms but usually only after specific modifications from the original iron source.

4. Conclusions

$FeCl_3$ as the exclusive iron source supported growth in *Synechococcus* better than FeO(OH) as the exclusive iron source. *Synechococcus* grown on FeO(OH) exhibited a more pronounced degree of stress than when grown on $FeCl_3$, as shown by differences in the relative PSII/PSI and IsiA/PSI ratios obtained by the 77 K FEA. These findings suggested that the physiochemical properties of the iron source affect the physiological responses of *Synechococcus*.

Higher concentrations of dissolved iron (II) in *Synechococcus* cultures compared to the controls indicate that extracellular iron reduction took place, something which has been previously understudied in cyanobacteria. This extracellular reduction could have been facilitated by PilA on the cell surface or the result of siderophore or other DOM-mediated reduction of iron due to increased siderophore/DOM production in the cultures. The concentrations were generally higher in the cultures supplied with $FeCl_3$, suggesting that a more soluble amorphous iron form better supports biotic reduction. With $FeCl_3$ as the exclusive iron source, the extracellular accumulation of iron was more prominent in *Synechococcus* than with FeO(OH) as the iron source. This extracellular iron storage coincided with the higher concentration of dissolved iron (II) that was found in the *Synechococcus* cultures grown on $FeCl_3$, with a limited iron transport into the cell as a possible reason for slower iron acquisition under these

conditions. The effect of iron source on the internalization and extracellular reduction of iron has not previously been studied in *Synechococcus*, and we showed that the iron source greatly influenced both the extracellular association of iron and internalization into the cell itself, as well as the biogenic production of Fe(II).

The iron source also affected the solubilization of total iron. While the cultures of *Synechococcus* and the abiotic controls showed similar trends related to changes in the total dissolved iron concentrations, more total dissolved iron was detected in the cultures than the controls for both $FeCl_3$ and FeO(OH), suggesting that biotic mechanisms, such as cell-related reduction and siderophore complexation, along with abiotic mechanisms, such as photochemical reactions and EDTA complexation, contributed to the solubilization of iron. Solubilization happened slower for FeO(OH) than for $FeCl_3$, suggesting that *Synechococcus* requires more time to adapt to FeO(OH) as its source of iron.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-1312/8/12/996/s1, Figure S1: Total iron concentrations, Table S1: Sampling days, Table S2: Visual Minteq Output and percentage distributions

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Martin, J.H.; Gordon, R.M.; Fitzwater, S.E. The Case for Iron. *Limnol. Oceanogr.* **1991**, *36*, 1793–1802. [CrossRef]
2. Behrenfeld, M.J.; O'Malley, R.T.; Siegel, D.A.; McClain, C.R.; Sarmiento, J.L.; Feldman, G.C.; Milligan, A.J.; Falkowski, P.G.; Letelier, R.M.; Boss, E.S. Climate-driven trends in contemporary ocean productivity. *Nature* **2006**, *444*, 752. [CrossRef] [PubMed]
3. Norman, L.; Cabanes, D.J.E.; Blanco-Ameijeiras, S.; Moisset, S.A.M.; Hassler, C.S. Iron Biogeochemistry in Aquatic Systems: From Source to Bioavailability. *Chimia* **2014**, *68*, 764–771. [CrossRef] [PubMed]
4. Yucel, M.; Gartman, A.; Chan, C.S.; Luther, G.W. Hydrothermal vents as a kinetically stable source of iron-sulphide-bearing nanoparticles to the ocean. *Nat. Geosci.* **2011**, *4*, 367–371. [CrossRef]
5. Sarthou, G.; Baker, A.R.; Blain, S.; Achterberg, E.P.; Boye, M.; Bowie, A.R.; Croot, P.; Laan, P.; de Baar, H.J.W.; Jickells, T.D.; et al. Atmospheric iron deposition and sea-surface dissolved iron concentrations in the eastern Atlantic Ocean. *Deep-Sea Res. Part I* **2003**, *50*, 1339–1352. [CrossRef]
6. Mahowald, N.M.; Baker, A.R.; Bergametti, G.; Brooks, N.; Duce, R.A.; Jickells, T.D.; Kubilay, N.; Prospero, J.M.; Tegen, I. Atmospheric global dust cycle and iron inputs to the ocean. *Glob. Biogeochem. Cycle* **2005**, *19*. [CrossRef]
7. Andrews, S.C.; Robinson, A.K.; Rodriguez-Quinones, F. Bacterial iron homeostasis. *FEMS Microbiol. Rev.* **2003**, *27*, 215–237. [CrossRef]
8. Raven, J.A.; Evans, M.C.W.; Korb, R.E. The role of trace metals in photosynthetic electron transport in O-2-evolving organisms. *Photosynth. Res.* **1999**, *60*, 111–149. [CrossRef]
9. Jordan, P.; Fromme, P.; Witt, H.T.; Klukas, O.; Saenger, W.; Krauß, N. Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution. *Nature* **2001**, *411*, 909–917. [CrossRef]
10. Kamiya, N.; Shen, J.-R. Crystal structure of oxygen-evolving photosystem II from Thermosynechococcus vulcanus at 3.7-A resolution. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 98–103. [CrossRef]
11. Blanco-Ameijeiras, S.; Cosio, C.; Hassler, C.S. Long-Term Acclimation to Iron Limitation Reveals New Insights in Metabolism Regulation of *Synechococcus* sp. PCC7002. *Front. Mar. Sci.* **2017**, *4*, 247. [CrossRef]
12. Sunda, W.G.; Huntsman, S.A. High iron requirement for growth, photosynthesis, and low-light acclimation in the coastal cyanobacterium Synechococcus bacillaris. *Front. Microbiol.* **2015**, *6*, 561. [CrossRef] [PubMed]

13. Riethman, H.; Bullerjahn, G.; Reddy, K.J.; Sherman, L.A. Regulation of Cyanobacterial Pigment-Protein Composition and Organization by Environmental-Factors. *Photosynth. Res.* **1988**, *18*, 133–161. [CrossRef] [PubMed]
14. Singh, A.K.; McIntyre, L.M.; Sherman, L.A. Microarray analysis of the genome-wide response to iron deficiency and iron reconstitution in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Physiol.* **2003**, *132*, 1825–1839. [CrossRef] [PubMed]
15. Ryan-Keogh, T.J.; Macey, A.I.; Cockshutt, A.M.; Moore, C.M.; Bibby, T.S. The Cyanobacterial Chlorophyll-Binding-Protein Isia Acts to Increase the in Vivo Effective Absorption Cross-Section of Psi under Iron Limitation. *J. Phycol.* **2012**, *48*, 145–154. [CrossRef] [PubMed]
16. Melkozernov, A.N.; Bibby, T.S.; Lin, S.; Barber, J.; Blankenship, R.E. Time-resolved absorption and emission show that the CP43′ antenna ring of iron-stressed *Synechocystis* sp. PCC6803 is efficiently coupled to the photosystem I reaction center core. *Biochemistry* **2003**, *42*, 3893–3903. [CrossRef]
17. Santana-Casiano, J.M.; Gonzalez-Davila, A.; Millero, F.J. The role of Fe(II) species on the oxidation of Fe(II) in natural waters in the presence of O-2 and H2O2. *Mar. Chem.* **2006**, *99*, 70–82. [CrossRef]
18. Hopwood, M.J.; Birchill, A.J.; Gledhill, M.; Achterberg, E.P.; Klar, J.K.; Milne, A. A Comparison between Four Analytical Methods for the Measurement of Fe(II) at Nanomolar Concentrations in Coastal Seawater. *Front. Mar. Sci.* **2017**, *4*, 192. [CrossRef]
19. Gledhill, M.; Van Den Berg, C.M.G. Determination of Complexation of Iron(Iii) with Natural Organic Complexing Ligands in Seawater Using Cathodic Stripping Voltammetry. *Mar. Chem.* **1994**, *47*, 41–54. [CrossRef]
20. Rue, E.L.; Bruland, K.W. Complexation of Iron(Iii) by Natural Organic-Ligands in the Central North Pacific as Determined by a New Competitive Ligand Equilibration Adsorptive Cathodic Stripping Voltammetric Method. *Mar. Chem.* **1995**, *50*, 117–138. [CrossRef]
21. Gledhill, M.; Buck, K.N. The organic complexation of iron in the marine environment: A review. *Front. Microbiol.* **2012**, *3*, 69. [CrossRef] [PubMed]
22. Kuma, K.; Nakabayashi, S.; Matsunaga, K. Photoreduction of Fe(III) by hydroxycarboxylic acids in seawater. *Water Res.* **1995**, *29*, 1559–1569. [CrossRef]
23. Swanner, E.D.; Wu, W.; Hao, L.; Wüstner, M.L.; Obst, M.; Moran, D.M.; McIlvin, M.R.; Saito, M.A.; Kappler, A. Physiology, Fe(II) oxidation, and Fe mineral formation by a marine planktonic cyanobacterium grown under ferruginous conditions. *Front. Earth Sci.* **2015**, *3*, 60. [CrossRef]
24. Kranzler, C.; Lis, H.; Finkel, O.M.; Schmetterer, G.; Shaked, Y.; Keren, N. Coordinated transporter activity shapes high-affinity iron acquisition in cyanobacteria. *ISME J.* **2014**, *8*, 409–417. [CrossRef]
25. Vogel, A.I.M.; Winther-Larsen, H.C.; Hoofgaard, A.; Roos, N.; Busche, T.; Rückert, C.; Kalinowski, J.; Hohmann-Mariott, M.F. Identification of SYNPCC7002_A2804 as the main pilin-encoding gene in the synthetic biology chassis *Synechococcus* sp. PCC 7002. 2020; Manuscript in Preparation.
26. Lamb, J.J.; Hill, R.E.; Eaton-Rye, J.J.; Hohmann-Marriott, M.F. Functional Role of PilA in Iron Acquisition in the Cyanobacterium *Synechocystis* sp. PCC 6803. *PLoS ONE* **2014**, *9*, e105761. [CrossRef]
27. Reguera, G.; McCarthy, K.D.; Mehta, T.; Nicoll, J.S.; Tuominen, M.T.; Lovley, D.R. Extracellular electron transfer via microbial nanowires. *Nature* **2005**, *435*, 1098–1101. [CrossRef]
28. Formenti, P.; Caquineau, S.; Chevaillier, S.; Klaver, A.; Desboeufs, K.; Rajot, J.; Belin, S.; Briois, V. Dominance of goethite over hematite in iron oxides of mineral dust from Western Africa: Quantitative partitioning by X-ray Absorption Spectroscopy. *J. Geophys. Res. Atmos.* **2014**, *119*, 12–740. [CrossRef]
29. Achterberg, E.P.; Holland, T.W.; Bowie, A.R.; Fauzi, R.; Mantoura, C.; Worsfold, P.J. Determination of iron in seawater. *Anal. Chim. Acta* **2001**, *442*, 1–14. [CrossRef]
30. Sunda, W.G.; Price, N.M.; Morel, F.M.M. Trace metal ion buffers and their use in culture studies. In *Algal Culturing Techniques*; Andersen, R.A., Ed.; Elsevier Academic Press: London, UK, 2005.
31. Morel, F.M.M.; Rueter, J.G.; Anderson, D.M.; Guillard, R.R.L. Aquil—Chemically Defined Phytoplankton Culture-Medium for Trace-Metal Studies. *J. Phycol.* **1979**, *15*, 135–141. [CrossRef]
32. Keller, M.D.; Bellows, W.K.; Guillard, R.R.L. Microwave Treatment for Sterilization of Phytoplankton Culture Media. *J. Exp. Mar. Biol. Ecol.* **1988**, *117*, 279–283. [CrossRef]
33. Ludwig, M.; Bryant, D.A. *Synechococcus* sp. strain PCC 7002 transcriptome: Acclimation to temperature, salinity, oxidative stress, and mixotrophic growth conditions. *Front. Microbiol.* **2012**, *3*, 354. [CrossRef] [PubMed]

34. Vogel, A.I.M.; Lale, R.; Hohmann-Marriott, M.F. Streamlining recombination-mediated genetic engineering by validating three neutral integration sites in *Synechococcus* sp. PCC 7002. *J. Biol. Eng.* **2017**, *11*, 19. [CrossRef] [PubMed]
35. Lund, J.W.G.; Kipling, C.; Le Cren, E.D. The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. *Hydrobiologia* **1958**, *11*, 143–170. [CrossRef]
36. Lamb, J.; Forfang, K.; Hohmann-Marriott, M. A Practical Solution for 77 K Fluorescence Measurements Based on LED Excitation and CCD Array Detector. *PLoS ONE* **2015**, *10*, e0132258. [CrossRef]
37. Dekker, J.; Hassoldt, A.; Pettersson, A.; Van Roon, H.; Groot, M.; Van Grondelle, R. On the nature of the F695 and F685 emission of photosystem II. *Photosynth. Light Biosphere* **1995**, *1*, 53–56.
38. Mukerji, I.; Sauer, K. Temperature-dependent steady-state and picosecond kinetic fluorescence measurements of a photosystem I preparation from spinach. *Plant Biol.* **1989**, *8*, 105–122.
39. Lamb, J.J.; Rokke, G.; Hohmann-Marriott, M.F. Chlorophyll fluorescence emission spectroscopy of oxygenic organisms at 77 K. *Photosynthetica* **2018**, *56*, 105–124. [CrossRef]
40. Tang, D.G.; Morel, F.M.M. Distinguishing between cellular and Fe-oxide-associated trace elements in phytoplankton. *Mar. Chem.* **2006**, *98*, 18–30. [CrossRef]
41. Hopwood, M.J.; Connelly, D.P.; Arendt, K.E.; Juul-Pedersen, T.; Stinchcombe, M.C.; Meire, L.; Esposito, M.; Krishna, R. Seasonal Changes in Fe along a Glaciated Greenlandic Fjord. *Front. Earth Sci.* **2016**, *4*, 15. [CrossRef]
42. Croot, P.L.; Laan, P. Continuous shipboard determination of Fe(II) in polar waters using flow injection analysis with chemiluminescence detection. *Anal. Chim. Acta* **2002**, *466*, 261–273. [CrossRef]
43. Notini, L.; Byrne, J.M.; Tomaszewski, E.J.; Latta, D.E.; Zhou, Z.; Scherer, M.M.; Kappler, A. Mineral Defects Enhance Bioavailability of Goethite toward Microbial Fe(III) Reduction. *Environ. Sci. Technol.* **2019**, *53*, 8883–8891. [CrossRef] [PubMed]
44. Boyd, P.W.; Ellwood, M.J. The biogeochemical cycle of iron in the ocean. *Nat. Geosci.* **2010**, *3*, 675–682. [CrossRef]
45. Karapetyan, N.V.; Bolychevtseva, Y.V.; Yurina, N.P.; Terekhova, I.V.; Shubin, V.V.; Brecht, M. Long-wavelength chlorophylls in photosystem I of cyanobacteria: Origin, localization, and functions. *Biochemistry* **2014**, *79*, 213–220. [CrossRef]
46. Li, M.; Semchonok, D.A.; Boekema, E.J.; Bruce, B.D. Characterization and evolution of tetrameric photosystem I from the thermophilic cyanobacterium *Chroococcidiopsis* sp. TS-821. *Plant Cell* **2014**, *26*, 1230–1245. [CrossRef]
47. Burnap, R.L.; Troyan, T.; Sherman, L.A. The Highly Abundant Chlorophyll-Protein Complex of Iron-Deficient Synechococcus Sp Pcc7942 (Cp43) Is Encoded by the Isia Gene. *Plant Physiol.* **1993**, *103*, 893–902. [CrossRef]
48. Falk, S.; Samson, G.; Bruce, D.; Huner, N.P.A.; Laudenbach, D.E. Functional analysis of the iron-stress induced CP 43′ polypeptide of PS II in the cyanobacterium *Synechococcus* sp. PCC 7942. *Photosynth. Res.* **1995**, *45*, 51–60. [CrossRef]
49. Park, Y.-I.; Sandström, S.; Gustafsson, P.; Öquist, G. Expression of the isiA gene is essential for the survival of the cyanobacterium *Synechococcus* sp. PCC 7942 by protecting photosystem II from excess light under iron limitation. *Mol. Microbiol.* **1999**, *32*, 123–129. [CrossRef]
50. Singh, A.K.; Sherman, L.A. Reflections on the function of IsiA, a cyanobacterial stress-inducible, Chl-binding protein. *Photosynth. Res.* **2007**, *93*, 17–25. [CrossRef]
51. Shcolnick, S.; Keren, N. Metal homeostasis in cyanobacteria and chloroplasts. Balancing benefits and risks to the photosynthetic apparatus. *Plant Physiol.* **2006**, *141*, 805–810. [CrossRef]
52. Shaked, Y.; Kustka, A.B.; Morel, F.M.M. A general kinetic model for iron acquisition by eukaryotic phytoplankton. *Limnol. Oceanogr.* **2005**, *50*, 872–882. [CrossRef]
53. Lis, H.; Shaked, Y.; Kranzler, C.; Keren, N.; Morel, F.M.M. Iron bioavailability to phytoplankton: An empirical approach. *ISME J.* **2015**, *9*, 1003–1013. [CrossRef] [PubMed]
54. Paz, Y.; Shimoni, E.; Weiss, M.; Pick, U. Effects of iron deficiency on iron binding and internalization into acidic vacuoles in Dunaliella salina. *Plant Physiol.* **2007**, *144*, 1407–1415. [CrossRef] [PubMed]
55. Hernández-Prieto, M.A.; Schön, V.; Georg, J.; Barreira, L.; Varela, J.; Hess, W.R.; Futschik, M.E. Iron deprivation in Synechocystis: Inference of pathways, non-coding RNAs, and regulatory elements from comprehensive expression profiling. *G3 (Bethesda)* **2012**, *2*, 1475–1495. [CrossRef]
56. Cornell, R.M.; Schwertmann, U. *The Iron Oxides: Structure, Properties, Reactions, Occurrences and Uses*; 2nd Completely Rev. and Extended Edition ed.; Wiley-VCH: Weinheim, Germany, 2006; p. 703.
57. Ngwack, B.; Sigg, L. Dissolution of Fe(III) (hydr) oxides by metal-EDTA complexes. *Geochim. Cosmochim. Acta* **1997**, *61*, 951–963. [CrossRef]

58. Lueder, U.; Jorgensen, B.B.; Kappler, A.; Schmidt, C. Photochemistry of iron in aquatic environments. *Environ. Sci. Process Impacts* **2020**, *22*, 12–24. [CrossRef]
59. Kranzler, C.; Lis, H.; Shaked, Y.; Keren, N. The role of reduction in iron uptake processes in a unicellular, planktonic cyanobacterium. *Environ. Microbiol.* **2011**, *13*, 2990–2999. [CrossRef]
60. Gorby, Y.A.; Yanina, S.; McLean, J.S.; Rosso, K.M.; Moyles, D.; Dohnalkova, A.; Beveridge, T.J.; Chang, I.S.; Kim, B.H.; Kim, K.S.; et al. Electrically conductive bacterial nanowires produced by Shewanella oneidensis strain MR-1 and other microorganisms. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11358. [CrossRef]
61. Kuma, K.; Nakabayashi, S.; Suzuki, Y.; Kudo, I.; Matsunaga, K. Photo-reduction of Fe(III) by dissolved organic substances and existence of Fe(II) in seawater during spring blooms. *Mar. Chem.* **1992**, *37*, 15–27. [CrossRef]
62. Miller, W.L.; Kester, D. Photochemical Iron Reduction and Iron Bioavailability in Seawater. *J. Mar. Res.* **1994**, *52*, 325–343. [CrossRef]
63. Wells, M.L.; Mayer, L.M.; Donard, O.F.X.; Sierra, M.M.D.; Ackelson, S.G. The Photolysis of Colloidal Iron in the Oceans. *Nature* **1991**, *353*, 248–250. [CrossRef]
64. Barbeau, K.; Rue, E.L.; Trick, C.G.; Bruland, K.T.; Butler, A. Photochemical reactivity of siderophores produced by marine heterotrophic bacteria and cyanobacteria based on characteristic Fe(III) binding groups. *Limnol. Oceanogr.* **2003**, *48*, 1069–1078. [CrossRef]
65. Millero, F.J. Solubility of Fe(III) in seawater. *Earth Planet Sci. Lett.* **1998**, *154*, 323–329. [CrossRef]
66. Aeppli, M.; Voegelin, A.; Gorski, C.A.; Hofstetter, T.B.; Sander, M. Mediated Electrochemical Reduction of Iron (Oxyhydr-)Oxides under Defined Thermodynamic Boundary Conditions. *Environ. Sci. Technol.* **2018**, *52*, 560–570. [CrossRef] [PubMed]