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AN ABSTRACT OF THE THESIS OF Dennis Raymond Ades for the
Master of Science in Biology presented September 18, 1986.

Title: The Role of Iron Nutrition in Regulating Patterns of
Photosynthesis and Nitrogen Metabolism in the Green Alga
Scenedesmus quadricauda.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

[REDACTED]
John Rueter, Chairman

[REDACTED]
Richard Petersen

[REDACTED]
Richard Tocher

The influence of iron nutrition on patterns of
photosynthetic behavior, nitrogen metabolism, and fixed-
carbon allocation is reported for a common freshwater green
alga. Cultures of Scenedesmus quadricauda were grown in
Fraquil medium in which iron concentrations ranged from 1.0
 μM to 0.01 μM (log 10^{-6} to 10^{-8} M, respectively). Carbon 14
and nitrogen uptake experiments were conducted at

photosynthetically saturating and subsaturating photon flux densities.

S. quadricauda responded to iron deficiency with reductions in chlorophyll a concentrations, cellular fluorescence characteristics, and ¹⁴C uptake. Photon flux density strongly affected the rate of carbon fixation and photosynthetically saturating light intensity compensated for moderate iron deficiencies. Cellular fluorescence capacity, a measure of light-saturated photosynthetic capacity relative to the photochemical capacity of an algal culture, was linked to photosynthetic rates and efficiencies.

Reductions in iron concentrations and irradiance were found to decrease photosynthesis sufficiently to energetically hinder the utilization of nitrate in the culture medium. However, depletion of ammonium from the culture medium was largely independent of iron nutrition and irradiance. Ammonium uptake rates exceeded nitrate uptake rates in all iron and light regimes.

Chlorophyll a concentrations and carbon-fixation rates in nitrogen-sufficient and nitrogen-deficient cultures were strongly influenced by the iron concentration of the medium. Nitrogen nutrition appeared to affect chlorophyll a concentrations only in iron-replete cultures. Cultures enriched with nitrate supported less photoassimilation of

^{14}C than ammonium-nourished cultures. This is likely due to the greater amount of photochemical energy required to fully metabolize nitrate.

Patterns of carbon incorporation into cellular components were also controlled by iron nutrition and irradiance. Much of the carbon fixed in iron-deficient cultures was allocated to protein whereas in iron-sufficient cultures, most of the ^{14}C label was recovered in the polysaccharide component. This suggests that iron-stressed algal cultures maintain the synthesis of metabolic machinery such as proteins and enzymes at the expense of other cellular components. Iron-replete cultures fixed more inorganic carbon and possibly stored the additional fixed carbon as starch. This partitioning behavior is examined briefly with a conceptual model.

THE ROLE OF IRON NUTRITION IN REGULATING PATTERNS OF
PHOTOSYNTHESIS AND NITROGEN METABOLISM
IN THE GREEN ALGA SCENEDESMUS QUADRICAUDA

by

DENNIS RAYMOND ADES

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
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Portland State University

1987

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of
Dennis Raymond Ades presented September 18, 1986.

[REDACTED]
John G. Rueter, Chairman

[REDACTED]
Richard Petersen

[REDACTED]
Richard Tocher

APPROVED:

[REDACTED]
Richard Petersen, Head, Biology Department

[REDACTED]
Bernard Ross, Dean of Graduate Studies and Research

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CHAPTER I

INTRODUCTION

Photosynthesis and algal growth rates in natural waters are determined by a myriad of biological, chemical, and physical factors. Typically, sunlight and macro-nutrients such as nitrogen and phosphorus are thought to be the primary growth-limiting parameters in aquatic ecosystems. However with advances in trace metal culturing techniques, the important role iron and other trace metals play in controlling primary productivity is becoming more apparent.

As cofactors in metalloenzymes and electron transfer proteins, trace metals are essential for the most basic metabolic processes. Iron, for example, occurs in such fundamental metabolic pathways as photosynthesis, respiration, and nitrogen metabolism. The large difference in redox potential of ferrous (Fe^{+2}) and ferric (Fe^{+3}) forms makes iron an ideal electron carrier. However, this basic biological requirement for iron and other trace metals is frequently complicated by their relatively low concentrations in natural waters. With this in mind, it is not suprising to find that iron-deficiencies do develop in

freshwater and marine ecosystems which can limit primary productivity (Goldman, 1972; Miller et al., 1974; Glover, 1977 and 1978; and Brand et al., 1983).

Iron concentrations in oxygenated waters are usually very low because the relatively soluble ferrous iron undergoes rapid oxidation to ferric iron. Ferric iron rapidly precipitates from the water column or binds with hydroxides and orthophosphate rendering it unavailable for algal use. In organic-rich waters, ferric iron forms stable colloids and organic complexes which increases the total iron concentration in the water column (Wetzel, 1975). Photodegradation of these complexes may produce transient ferrous iron concentrations which can be rapidly assimilated by algae (Colliene, 1983).

The rate of iron oxidation and colloid formation is strongly dependent on pH. In reducing environments such as the anoxic hypolimnion of a eutrophic lake, ferric iron in the sediments is reduced to ferrous iron and diffuses upward into the water column. This process, however, is generally restricted to anoxic environments and is not an important factor in controlling iron availability in the less productive surface waters of an oligotrophic lake or ocean.

Iron uptake processes in eukaryotic algae are poorly understood because of the complicated aquatic chemistry of iron. Anderson and Morel (1982) identified a membrane-bound

transfer molecule which mediated iron uptake in the marine diatom Thalassiosira weissflogii. They referred to this transport molecule as "phytotransferrin" because of its behavioral similarity to the transferrin molecule in vertebrates. Iron uptake in T. weissflogii was controlled by the free ion activity hypothesized to relate to the saturation of the phytotransferrin molecule and was not directly linked to photosynthesis or respiration. The biological availability of iron was therefore determined by the free ion activity of ferrous and ferric iron within the system, or the transient ions produced by the photo-degradation of complexed ferric iron.

Some algae have evolved specialized strategies for growing in low-iron environments. The most successful of these are the blue-green algae capable of producing siderophores, low molecular weight molecules which have high affinities for iron. Iron-stressed axenic cultures of a marine dinoflagellate have also been reported to produce a siderophore-like substance, although in much lower concentrations than cyanophytes (Trick et al., 1983). Siderophores facilitate iron uptake by dissolving iron oxides and making ferric iron available to the algal population producing the siderophores. The entire siderophore-iron complex is either transported across the cell membrane or the iron is reduced and taken up separately (Lewin, 1984).

Siderophores are species specific and sequester iron from the remainder of the algal community. This confers a competitive advantage to siderophore producing algae during periods of low iron availability. In Scenedesmus for example, growth is reduced by 80% and coenobia are disrupted and become chlorotic when grown in the presence of a siderophore extracted from cultures of Anabaena (Murphy et al., 1976). However, sensitivity to siderophores differs among the green algae; Chlorella is capable of competing for siderophore-chelated iron much more successfully than Chlamydomonas (Bailey and Taub, 1980). It appears that low iron concentrations may not only influence the level of productivity in an aquatic ecosystem, but because of the ability of some algae to produce siderophores, iron availability may strongly determine the species composition and diversity of the algal community within that ecosystem.

Two types of physiological responses to iron-deficiency may be seen in algae. Iron starvation may interfere with the biosynthesis of polymers and cellular components in a manner similar to other nutrient deficiencies, or decrease rates of inorganic carbon assimilation in a behavioral response usually seen in light-limited cultures.

Concentrations of chlorophyll a and other photosynthetic pigments and proteins are lower in iron-stressed

algal populations than they are in iron-sufficient algae (Oquist, 1971; Glover, 1977; Verstrete et al., 1980; and Sandmann and Malkin, 1983; Mueller, 1985). Iron, in the form of the photosynthetic iron-sulfur protein ferredoxin, plays an important role in activating the synthesis of heme group precursors to chlorophyll and cytochromes. The reduction in photosynthetic pigments and cytochromes results in decreased light-harvesting capacity and photosynthetic electron flow during photosynthesis (Sandmann and Malkin, 1983).

Iron enrichment has been shown to stimulate inorganic carbon-fixation in freshwater algae, although photosynthesis may be inhibited with higher concentrations (3.6 to 17.9 μM) of chelated and unchelated iron (Storch and Dunham, 1986). Photosynthetic rates and carbon assimilation numbers are also lower in iron-deficient cultures of marine algae (Glover, 1977). Simultaneous reductions in light harvesting capacity and photosynthetic rates suggest that growth rates in low-iron cultures may be at least partly controlled by the conversion of light energy into chemical energy.

Iron nutrition also controls patterns of inorganic nitrogen metabolism in algae, contributing to each step of nitrate reduction and assimilation. Iron occurs in the nitrate reductase and nitrite reductase enzymes and in ferredoxin, which regulates the flow of photoreductant to

the nitrogen metabolism pathway. A generalized illustration of nitrate reduction and assimilation is shown in Figure 1.

Iron nutrition has been suggested to regulate patterns of nitrogen fixation activity in blue-green algae by controlling the synthesis of glutamine synthetase (Verstreate et al., 1980). Glutamine synthetase apparently inhibits the activity of nitrogenase, the enzyme responsible for reduction of atmospheric nitrogen. When iron-nutrition is sufficient and nitrate concentrations are low, glutamine synthetase is not synthesized and nitrogen-fixation takes place. Blue-green algae may be energetically stressed if iron supply is low. In this condition glutamine synthetase is synthesized and the metabolically expensive process of nitrogen fixation is inhibited.

The role of iron in controlling nitrogen metabolism in green algae has not been well studied. In a study with Ankistrodesmus and Chlorella, enrichment of the culture medium with EDTA-chelated iron specifically increased the reduction of nitrite without affecting the reduction of nitrate to nitrite (Kessler and Czygan, 1967). It is not clear whether iron is required for the synthesis of the enzyme nitrite reductase or to insure an adequate supply of reductant. More recently, Verstreate et al. (1980) found that iron concentrations greater than $0.2 \mu\text{M Fe}^{+2}$ inhibited nitrate reductase and glutamine synthetase activity in

Figure 1. The nitrate reduction and assimilation pathway. Reductant generated in photosynthesis and respiration flows through nicotinamide adenine dinucleotide phosphate (NADPH H⁺) and ferredoxin (Fd) to enzymes in the nitrate reduction pathway. NRase-nitrate reductase; NiRase-nitrite reductase; GS/GOGAT- glutamine synthetase/glutamate synthetase. Not shown is the involvement of ATP in the active transport of nitrate and glutamine synthetase/ glutamate synthetase activity.

Scenedesmus. The cause for such inhibition was not identified or speculated although lower concentrations of these enzymes were also observed in cultures with greater iron concentrations.

The relative abundance of cellular components such as proteins, lipids, and polysaccharides may reflect the nutritional and physiological status of an algal culture. Patterns of inorganic carbon metabolism, like nitrogen, are also affected by iron nutrition. Glover (1977) reported a greater proportion of fixed ^{14}C was found in protein in iron-deficient cultures than in iron-sufficient cultures. This pattern of carbon metabolism is similar to that observed in energetically-limited cultures grown at low-light intensities (Morris et al., 1974).

Iron nutrition can control the synthesis of photosynthetic pigments and proteins, patterns of photosynthesis, nitrogen metabolism, and carbon assimilation. This effort has focused on identifying these patterns in the green alga, Scenedesmus quadricauda. Chlorophyll *a* concentrations, fluorescence response, and carbon-fixation rates will be examined as a function of the iron concentration in the culture medium and light intensity in chapter III. This will provide information on the energetic and nutrient interactions of iron-deficiency. Energetic interactions are further examined as they pertain to nitrogen metabolism in

Chapter IV. Nitrogen uptake and assimilation is an energetically costly yet basic metabolic process in algae. To further elucidate the overall physiological response to iron-nutrition chapter V will discuss short-term patterns in carbon metabolism at various levels of iron nutrition and irradiance. This information will be compiled and viewed from an environmental perspective in chapter VI.

CHAPTER II

METHODS

Semi-continuous batch cultures of the chlorophyte Scenedesmus quadricauda (Culture II, Freshwater Institute, Winnipeg, Canada) were grown in complete Fraquil medium (Morel et al., 1979; as modified by Petersen, 1982; Table 1) and used as maintenance stock for experimental cultures. Cultures were incubated at 22°C with continuous cool-white light at 100 μ Einsteins/m²/sec in 500 ml erlenmeyer flasks. Culture flasks were swirled daily to facilitate gas exchange and to keep cells in suspension.

Fraquil medium was prepared with concentrated nutrient stock solutions which were added to Nanopure reagent-grade water (Sybron/Barnstead; >16.7 megaohms). The medium was passed through a cation exchange column (Chelex 100, Bio-rad Laboratories) to remove trace metal contaminants before metals were added. The complete medium was filter sterilized (gravity or vacuum-filtered at less than 5 psi) through acid leached 0.2 μ m Nucleopore filters and pH adjusted to 7.5 $\pm$ 0.1. All glassware and plasticware were soaked 24 hr in 5% HCl and rinsed in Nanopure water to minimize contamination.

TABLE I
MODIFIED FRAQUIL CULTURE MEDIUM

chemical component	concentration mol/L	chemical component	concentration mol/L

Ca+2	2.5×10^{-4}	CO ₃ -2	1.5×10^{-4}
Mg+2	1.5×10^{-4}	SO ₄ -	1.5×10^{-4}
K+	2.0×10^{-5}	Cl-	5.2×10^{-4}
Fe+3	1.0×10^{-6}	B(OH)3	1.0×10^{-7}
Mn+2	1.38×10^{-6}	MoO ₄ -2	1.5×10^{-9}
Co+2	1.25×10^{-7}	NO ₃ -	1.0×10^{-4}
Zn+2	1.2×10^{-7}	EDTA-4	1.0×10^{-6}
NH ₃	1.3×10^{-9}	Al+3	3.98×10^{-9}
H+	3.0×10^{-8}	Cu+2	6.0×10^{-8}

Scenedesmus quadricauda is a common freshwater chlorophyte frequently used in laboratory experiments. Taxonomists place Scenedesmus in its own family the Scenedesmaceae, of the order Chlorococcales (Prescott, 1978). S. quadricauda is usually found in a four-celled coenobium although single cells are frequently observed in natural and cultured populations.

Experimental cultures were started by transferring aliquots of log-phase cells from 7 to 10 day old maintenance cultures to fresh iron-deficient Fraquil medium. Iron concentrations of 1.0, 0.3, 0.1, 0.03, and 0.01 μM (log values of -6, -6.5, -7, -7.5, and -8 M, respectively) were obtained by adding appropriate volumes of 10^{-4}M FeEDTA stock into the medium. Prior to transfer, the algal aliquots were centrifuged 5 minutes at 1000 rpm, decanted, rinsed in Nanopure, and centrifuged again to minimize the transfer of exogenous nutrients. Experimental cultures were also frequently transferred to fresh medium in this manner to minimize the depletion of nutrients. Cell densities in stock and experimental cultures were monitored with a Coulter Counter electronic particle counter (model 2907 ZBI). One ml of culture was suspended in 9 ml of Isoton electrolyte solution and aspirated through a 100 μM orifice. Base channel threshold was adjusted to produce blank counts less than 100. Coulter Counter results were

occasionally verified with batch counting chamber results (Fuchs Rosenthal ultra plane counting chamber and Zeiss microscope).

Cellular fluorescence capacity (CFC; Vincent, 1980) was measured periodically in stock and experimental cultures. In vivo fluorescence was measured on 5 ml samples with a Turner Designs model 10 fluorometer calibrated to chlorophyll a. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-induced fluorescence was determined by adding 100 μ l of a saturated DCMU solution to the samples and remeasuring fluorescence. This provided information on the light-saturated photosynthetic capacity and photochemical capacity of each culture. Cellular fluorescence capacity, as a measure of photosystem efficiency, may be expressed with the following equation:

$$\text{CFC} = 1 - (\text{in vivo fluor.} / \text{DCMU-induced fluor.}).$$

Specifications of the Turner Designs model 10 fluorometer include:

light source-bulb F4T5
excitation filter-c/s 5-60
emission filter-c/s 2-64

EXPERIMENTATION

Experiments examining photosynthesis and nitrogen uptake were initiated when cultures grown in different iron

concentrations approached steady state and CFC values reflected the iron-nutritional status of the cultures. This usually occurred after 7 to 10 days of growth. CFC has been demonstrated to be very sensitive to nitrogen deficiency so it is important not to deplete nitrate concentrations in order to observe responses to iron nutrition.

Measurements of photosynthesis. Photosynthetic characteristics as a function of iron nutrition were determined by the photoassimilation of ^{14}C . Prior to addition of $\text{NaH}^{14}\text{CO}_3$, chlorophyll *a* samples were collected by filtration onto GF/A filters and preserved with 2 to 3 drops of MgCl_2 . The chlorophyll samples were stored frozen in darkness until extraction. Chlorophyll *a* concentrations were determined using the acetone extraction technique of Parsons et al. (1984) in which cells and filter are ground with a mortar and pestle, allowed to extract overnight in 90% acetone, and analyzed fluorometrically with the Turner Designs Model 10 fluorometer calibrated for chlorophyll *a*.

After time zero chlorophyll *a* samples were collected, cultures were equally divided in 250 ml erlenmeyer flasks and 50 μl $\text{NaH}^{14}\text{CO}_3$ with an activity of 5.0 $\mu\text{Ci}/\text{ml}$ was added for each 100 ml of culture. These subcultures were incubated for 2 hr in different light regimes before cell

samples were collected and analyzed for ^{14}C incorporation.

Light intensity, or photon flux density, was regulated by wrapping culture flasks in fiberglass screening and placing all flasks in a 22°C water bath with a $295 \mu\text{Einstein}/\text{m}^2/\text{sec}$ cool-white light source below. Three light intensities were selected, two at subsaturating intensities where photosynthesis is a linear function of light, and one at a saturating light intensity where the photosynthetic response to light is nonlinear. Each layer of screening reduced photon flux density by half without affecting light quality (spectrum). All cultures were removed from the light field upon completion of incubation. ^{14}C samples were collected by vacuum filtration (5 psi) onto GF/A filters and placed in 22 ml glass liquid scintillation (LS) vials where they were allowed to air dry overnight. Eight ml of Solvent Free liquid scintillation cocktail (Isolab, Inc.) were added to the LS vials and these samples were counted in a Beckman Liquid Scintillation Counter.

Measurement of nitrate and ammonium uptake rates.

When nitrate and ammonium uptake rates were examined as a function of iron concentrations in the medium, initial nitrate concentrations in the Fraquil medium were reduced by 95% to $10 \mu\text{M}$. These low-nitrate cultures were also grown with iron concentrations ranging from 0.01 to $1.0 \mu\text{M}$ for 7 to 10 days prior to experimentation. If nitrate uptake

rates were to be examined, each culture was enriched with a small amount of 0.1 M KNO_3 sufficient to produce ca 10-50 μM nitrate concentrations 24 hr before the experiment to induce nitrate reductase activity (Christenson, 1983). If ammonium uptake rates were to be determined, a small volume of 0.1M NH_4Cl was added so that all cultures had equal nitrogen concentrations before experimentation. Each culture was enriched again with KNO_3 or NH_4Cl approximately 30 minutes before the initiation of the experiment and placed in darkness. Time zero samples of nitrate, nitrite, and or ammonium as well as chlorophyll *a* were collected from cultures by vacuum filtration. Cultures were wrapped in fiberglass screening and incubated at 0, 27, 53, and 170 $\mu\text{Einstein/m}^2/\text{sec}$. Samples were collected again after the 2 hr incubation period. Nitrate, nitrite, and ammonium concentrations were determined by removing cells with filtration onto Whatman GF/A glass fiber filters and analyzing the filtrate colorimetrically. Rates of photosynthetic ^{14}C fixation were also determined from the filtered cells if cultures were also spiked with $\text{NaH}^{14}\text{CO}_3$ at time zero.

Nitrate concentrations in the medium were measured by reduction to nitrite and analyzed colorimetrically (Jones, 1984). Nitrite was also measured in this manner excluding the reduction process. Ammonium was determined

colorimetrically using a modified phenol-hypochlorite method (Wetzel and Likens, 1979). Analysis was performed using a Baush and Lomb Spectronic 100 spectrophotometer and prepared standards.

Patterns in carbon metabolism. Patterns of photosynthetic carbon metabolism were examined by fractionation of the ^{14}C -labelled photosynthate using a slight modification of Morris' (1974) technique. The process involves the sequential separation of the hot ethanol-soluble, hot trichloroacetic acid-soluble, and insoluble fractions which respectively comprise the lipid-low molecular weight metabolite, polysaccharide, and protein-nucleic acid fractions.

^{14}C -labelled cells that were collected by filtration onto GF/A filters for photosynthate fractionation were placed in LS vials. Three ml of 80% ethanol were added to each vial and samples were stored in darkness at -14°C for further extraction. Stored samples were later placed upon a glass filtration apparatus (2.5 cm diameter Millipore) and 3 ml of boiling absolute ethanol were filtered through gravitationally. Two ml of a cold ethanol rinse were vacuumed through the filter and the entire ethanol soluble filtrate was added to the original 3 ml of 80% ethanol. This fraction was identified as the lipid-low molecular weight (LMW) metabolite component.

The filter was placed in a second LS vial and 3 ml of 5% (w/v) trichloroacetic acid (TCA) were added. The vial was loosely capped and placed in a water bath and boiled for 30 minutes. The hot TCA was poured through a second GF/A filter which retained any TCA insoluble material or portions of the original fiber filter. The original filter was rinsed with 2 ml of cold 5% TCA and the rinse was then filtered through the second filter. Both GF/A filters were air dried (which was facilitated with a 60W or 150W incandescent bulb) in LS vials before 5 ml of LS cocktail were added to each for counting.

One ml of ethanol and TCA solute was transferred to separate LS vials and 5 ml of LS cocktail were added. TCA and ethanol are not readily miscible in the LS cocktail and required 24 hr for phase breakdown and emulsification. The photosynthate fractionation and total ^{14}C uptake samples were analyzed with a Beckman liquid scintillation counter. Results were reported as counts per minute. A summary of the extraction process is shown in Figure 2.

Figure 2. The sequential extraction ^{14}C -labelled cellular components. LS-liquid scintillation; TCA trichloroacetic acid; lmw-low molecular weight cellular components.

CHAPTER III

THE INFLUENCE OF IRON AVAILABILITY ON PHOTOSYNTHESIS IN SCENEDESMUS QUADRICAUDA.

INTRODUCTION

Reductions in chlorophyll *a* and other light-harvesting pigments have been observed in iron-deficient algae (Oquist, 1971; Glover, 1977; Guikema and Sherman, 1983; and Mueller, 1985). Changes in thylakoid membrane composition which produce shifts in chlorophyll absorption maxima in blue-green algae have also been attributed to low-iron nutrition (Oquist, 1971; and Guikema and Sherman, 1983). Accompanying such changes in the light harvesting apparatus of these algae have been increases in chlorophyll fluorescence and reductions in light-harvesting capacity.

Reductions in the photosynthetic protein content of iron-stressed prokaryotic and eukaryotic algae have also been reported. Moderate reductions in iron nutrition were shown to decrease the iron-sulfur protein concentration in photosystem I of a blue-green alga without affecting growth rates. Severe iron deficiency, however, brought about a decrease in photosynthetic cytochromes which interfered with photosynthetic electron flow and growth rates (Sandmann

and Malkin, 1983). Cytochromes in the photosynthetic electron transport pathway were also sensitive to iron nutrition in a marine dinoflagellate, and this sensitivity was manifested in photosynthetic rates and efficiencies (Glover, 1977).

Similar reductions in light-harvesting pigments and photosynthetic proteins may also be expected in iron-deficient cultures of Scenedesmus quadricauda. This chapter will report the effects that iron nutrition has on chlorophyll a concentrations, chlorophyll fluorescence, and photosynthesis. The role light intensity has in accentuating or ameliorating iron deficiencies will also be examined.

METHODS

Subsamples of log growth phase cells of Scenedesmus quadricauda were transferred from complete Fraquil medium to medium in which iron concentrations ranged from 0.01 to 1.0 μM . EDTA-chelated iron was used for all experiments. Experimental cultures were incubated at 22°C with continuous light at 100 $\mu\text{Einsteins}/\text{m}^2/\text{sec}$ in erlenmeyer flasks for 7 to 10 days during which time cell densities and cellular fluorescence capacities were monitored.

Chlorophyll a samples were collected from each culture for later extraction with acetone and fluorometric analysis (Parsons, et al. 1984). Cultures were then equally divided

into subcultures and wrapped with fiberglass screening. These subcultures were then transferred to a second incubator with a 22°C water bath and a 275 μ Einsteins/m²/sec light field. Light intensity to each culture was regulated with the layers of screen wrapped around each flask. Light intensities of 27, 53, and 170 μ Einsteins/m²/sec were used for experimental incubations. Photosynthetic response is a linear function of light intensity for the two lower-light regimes of 27 and 53 μ Einsteins/m²/sec. Light is saturating at 170 μ Einsteins/m²/sec. Dark cultures were incubated in double-layered black plastic.

NaH¹⁴CO₃, with an activity of 5 μ Ci/ml, was added to each subculture at 50 μ l per 100 ml. These additions were staggered to allow for equal incubation periods. Labelled cells were vacuum-filtered at 5 psi onto Whatman GF/A filters and air-dried in LS vials. Solvent Free liquid scintillation solution (Isolab, Inc.) was added to these filters and net ¹⁴C uptake was determined with a Beckman scintillation counter.

RESULTS

Chlorophyll a. Chlorophyll a concentrations demonstrated a functional response to iron concentrations in the experimental culture medium; increasing as iron

concentration in the Fraquil medium increased from 0.01 μM to 1.0 μM (Figure 3). Response to greater iron availability is seen as a gradual increase in chlorophyll *a* concentrations. There was no evidence of a sudden jump in concentrations which might be expected if chlorophyll synthesis was completely repressed by iron deficiency. This same trend in chlorophyll *a* concentrations was seen in other experiments.

A slight depression in chlorophyll *a* concentration was observed in the 0.3 μM iron culture. This depression may be due to greater nutrient deficiency in this culture than in other experimental cultures. Culture density in the 0.3 μM Fe culture was 12% greater than other cultures. Since all cultures were initiated with an inoculum of equal cell density, greater growth rates and therefore greater nutrient depletion must have occurred in the 0.3 μM iron culture. This greater nutrient depletion depressed the chlorophyll concentration in this culture.

Cellular Fluorescence Capacity. CFC was monitored frequently during the incubation of cultures in experimental medium. Lower *in vivo* fluorescence relative to DCMU-induced fluorescence produced higher CFC values in iron-replete cultures (Figure 4). Lower CFC values in the iron-deficient cultures suggests that more photon energy captured in photosystem II is lost as fluorescence in these cultures than in

Figure 3. Chlorophyll a concentrations increase as a function of iron concentration in the media. The slight depression at $0.3 \mu\text{M}$ Fe (log value = -6.5) may be due to greater cell density and nitrogen depletion in this culture. The figure represents results from a single experiment which illustrates a general trend seen in other chlorophyll a extractions.

Figure 4. Cellular fluorescence capacity ($1 - \text{in vivo}$ fluorescence/DCMU-induced fluorescence) increases with greater iron sufficiency. Lower CFC values indicate greater *in vivo* chlorophyll fluorescence. Values are highly variable but maintain this pattern throughout the 7 to 10 day incubation period.

iron-sufficient cultures. CFC values less than 0.6, such as the low value seen in the $0.01 \mu\text{M}$ (10^{-8} M) iron culture, are generally found in senescent, or otherwise physiologically unfit cultures.

Fluorescence indices are especially sensitive to nitrogen deficiency and this is reflected in the variability of CFC values, which can change considerably during the incubation period as nutrients in the medium are depleted. Frequent dilution of the experimental cultures, or the transfer of cultures to fresh medium, may have decreased but did not eliminate this variability.

Photosynthetic Rates. The photoassimilation of inorganic carbon shows a strong dependence on iron and light availability (Figure 5). The optimal iron concentration for carbon fixation at all levels of photon flux density is observed at $0.3 \mu\text{M}$ while minimum rates are observed in the low-iron cultures ($0.03 \mu\text{M}$ and $0.01 \mu\text{M Fe}$). Cultures incubated with $0.3 \mu\text{M Fe}$ frequently support greatest culture densities, growth rates, and CFC values. For the high-iron cultures, total productivity is actually greater than Figure 5 suggests because chlorophyll *a* concentrations are also greater in these cultures. Low-iron cultures, on the other hand, result in low chlorophyll *a* concentrations, low growth rates, and depressions in other physiological responses.

Figure 5. ¹⁴C uptake per chlorophyll *a* demonstrates a functional response to iron concentrations at all photon flux densities. Photosynthesis is light saturated at 170 μEins/m²/sec. but is partially limited by light availability at 53 and 27 μEins/m²/sec. The influence of irradiance is most pronounced in iron-replete cultures.

The influence of irradiance on carbon fixation is best seen in iron-sufficient cultures where a large increase in photosynthesis is observed with each increase in photon flux density. As iron deficiency becomes more severe, the difference in carbon fixation at 27 and 53 $\mu\text{Einsteins}/\text{m}^2/\text{sec}$ decreases. Essentially no difference in ^{14}C uptake is observed among 0.03 μM and 0.01 μM Fe cultures incubated at these lower light intensities although large differences in photosynthetic rates are maintained at 170 $\mu\text{Einsteins}/\text{m}^2/\text{sec}$.

DISCUSSION

Chlorophyll a concentration. Chlorophyll a concentrations have been shown to be sensitive to iron availability in prokaryotic and eukaryotic photosynthetic algae (Oquist, 1971; Guikema and Sherman, 1983; Sandmann and Malkin, 1983; Glover, 1977). This sensitivity has been demonstrated although iron is not found in the chlorophyll molecule or its precursors. Chlorophyll synthesis is influenced by ferredoxin an iron-sulfur protein in photosystem I which functions as the primary electron acceptor in photosynthesis and regulates the distribution of photoreductant to metabolic pathways. Ferredoxin may activate the synthesis of delta amino-levulinic acid which is incorporated into the porphyrin ring of the chlorophyll molecule (Miller et al., 1984).

The relationship between chlorophyll *a* concentrations and iron suggests that the regulation of chlorophyll synthesis is altered. Ferredoxin, like other iron-sulfur proteins in photosystem I, is sensitive to iron deficiency (Sandmann and Malkin, 1983). Flavodoxin, an iron-free protein can carry out many of the reactions requiring ferredoxin, and may be substituted for ferredoxin during periods of iron deficiency. This substitution of flavodoxin for ferredoxin has been seen in cyanophytes and the green alga Chlorella (Zumft and Spiller, 1971). Whether chlorophyll synthesis is directly limited by ferredoxin concentrations, or if S. quadricauda has a substitution mechanism similar to that seen in Chlorella and how effectively that replacement mechanism may operate, is unknown.

Cellular Fluorescence Capacity. Fluorescence indices may reflect changes in the physiological state and photosynthetic capacity of an alga brought about by nutritional deficiencies (Prezlin, 1981). CFC is a measure of photosynthetic capacity relative to the structural and photochemical capacity of the cell (Vincent, 1980). Electron transport between the two photosystems has been identified as a rate limiting step in photosynthesis and is also sensitive to iron availability (Glover, 1977). Increased chlorophyll fluorescence in response to iron starvation has also been reported in cyanophytes by

Guikema and Sherman (1983). It is likely that iron deficiency decreases CFC by reducing the concentration or operating efficiency of iron-proteins in the electron transport system, creating an imbalance in the photosynthetic apparatus of an alga. Such an imbalance results in greater energy loss as fluorescence.

Photosynthetic Rates. The ability of an alga to maintain itself autotrophically is limited by environmental and physiological constraints. A reduction in photosynthesis brought about by a decrease in iron availability may be compensated for with an increase in irradiance. Similarly, increased iron availability may also compensate for decreased irradiance. Moderate reductions in two or more resources, however, can decrease photosynthesis to the point where cell maintenance requirements are not met. This may have been seen when experimental cultures were started with a small inoculum; growth rates in iron-deficient and nitrogen-deficient cultures were so low that cell densities sufficient for experimentation were never obtained.

Photosynthetic carbon fixation rates for S. guadricauda in Figure 5 are greatest in 0.3 μM iron cultures and decrease considerably at higher and lower iron concentrations. Inhibition of photosynthesis at high iron concentrations may be a toxic response to iron, or an

artifact of batch culturing. Although high iron concentrations may decrease algal productivity because of toxicity (Huntsman and Sunda, 1980) or complex chemical reactions which decrease the availability of some other essential nutrient such as phosphorus (Storch and Dunham, 1986), it is more likely that nitrogen was rapidly depleted resulting in lower carbon fixation rates.

Photosynthesis is most efficient when cells are well adapted to the prevailing irradiance. Chlorophyll to protein and photosystem reaction centers ratios (i.e., P680:P700) are optimized to capture and transfer photon energy. Iron deficiency has been demonstrated to bring about a reduction in photosystem I proteins, P700 reaction centers, and the heme-bearing cytochromes of the photosynthetic electron transport system in marine chlorophytes (Glover, 1977) and blue-green alga (Sandmann and Malkin, 1983), with a corresponding decrease in photosynthetic capacity (Oquist, 1971; and Terry, 1983).

Reports of iron-limited productivity, or increased productivity in iron-enriched waters are found for freshwater and oceanic systems (Goldman, 1973; Brand et al., 1983; Glover, 1977; and Miller et al., 1974). Responses to iron deficiency that increase in vivo chlorophyll fluorescence (decreased CFC) also diminish carbon fixation rates. Concomitant reductions in chlorophyll, cytochrome

and P700 proteins, may dramatically reduce photosynthetic efficiency and capacity, thereby providing little of the photochemical energy required for carbon fixation.

Carbon fixation rates can also be examined relative to prevailing irradiance (Figure 6). Productivity, when examined as a function of photon flux density provides a carbon assimilation value per photon and is dependent on iron concentrations. This assimilation value is similar to photosynthetic efficiency and can be compared with CFC (Figure 7) to demonstrate the relationship between cellular fluorescence capacity and photosynthetic behavior. Strong relationships between photosynthetic efficiencies and fluorescence indices such as this have been reported elsewhere (Samuelsson and Oquist, 1977; and Prezlin and Ley 1980).

CONCLUSION

S. quadricauda responds to iron deficiency with a reduction in chlorophyll *a* concentrations, cellular fluorescence capacity and photosynthetic rates. Chlorophyll *a* concentrations are considerably greater when cells are grown at 1.0 μM iron concentrations than when grown at 0.01 μM . CFC is highly variable but maintains a positive relationship to iron concentrations throughout experimental incubation periods of 7 to 10 days. Short-term

Figure 6. ^{14}C uptake per chlorophyll *a* relative to photon flux density indicates that carbon assimilation or photosynthetic efficiency is controlled by iron nutrition.

Figure 7. Photosynthetic efficiency is linked to cellular fluorescence capacity. Reductions in chlorophyll fluorescence allow for greater carbon assimilation efficiencies. The data here were collected from cultures incubated at 27 μ Eins/ m^2 /sec.

photosynthetic carbon fixation rates also respond to iron concentrations in the culture medium. Photon flux density strongly affects the rate of carbon fixation and may compensate for moderate iron deficiencies. A strong relationship between photosynthetic efficiency and cellular fluorescence capacity is seen.

CHAPTER IV

THE EFFECTS OF IRON DEFICIENCY ON NITROGEN METABOLISM AND SOME PHYSIOLOGICAL RESPONSES

INTRODUCTION

Despite the continual influx of nitrogen from terrestrial and atmospheric sources and the internal recycling of nutrients within the aquatic ecosystem, the primary productivity of the oceans and some oligotrophic lakes is thought to be limited by nitrogen availability (Smith, 1984). Nitrogen is of major metabolic importance in algae; it is found in such fundamental components as amino acids, nucleotides, and the porphyrin containing pigments of chlorophylls and cytochromes. Cell quotas for nutrients can increase when an alga is grown with low nutrient or light availability (Rhee and Gotham, 1981). Simultaneous reductions in iron nutrition and irradiance may increase algal cell quotas for nitrogen while interfering with normal nitrogen metabolism, driving the algal population towards nitrogen starvation.

The path of inorganic nitrogen assimilation involves the movement of ions across cell membranes, reduction of nitrate and nitrite to ammonium, and the subsequent

formation of glutamate in enzyme mediated processes which require ATP and reductant. Light is tightly linked to this process although inorganic nitrogen uptake and assimilation can occur for some time in darkness if sufficient carbohydrate reserves exist to produce ATP and reductant via respiration (Ullrich, 1983).

Like light limitation, iron deficiency may hinder inorganic nitrogen metabolism by diminishing the supply of reductant and ATP from photosynthesis to the nitrogen uptake and assimilation pathways. Nitrogen metabolism may also be retarded if carbon-skeletons which accept nitrogen to form glutamate are unavailable. In addition, important iron-containing enzymes are also found in the pathways of nitrogen metabolism.

Algae grown in low-iron, low-light environments may be energetically stressed. Competition for photochemical energy among the various metabolic pathways will be intensified, decreasing rates of carbon assimilation, nitrogen metabolism and numerous other processes. Although ammonium is energetically more easily assimilated, algae readily assimilate both nitrate and ammonium, the two most important forms of inorganic nitrogen, (Dortch and Conway, 1984). This chapter will examine how iron deficiency and irradiance affect the rate of nitrate and ammonium uptake and how chlorophyll a concentrations and photosynthetic

behavior reflect the nitrogen nutrition of S. quadricauda.

METHODS

Experimental cultures of S. quadricauda were maintained in Fraquil medium with iron concentrations ranging from 1.0 μM to 0.01 μM . Cultures were started with subsamples from stocks grown in complete Fraquil. Initial nitrate concentrations in cultures used for nitrate and ammonium uptake experiments were reduced from 200 μM to 10 μM . Cultures were frequently centrifuged and transferred to fresh, iron-adjusted medium to prevent the depletion of other nutrients.

Nitrate, nitrite, and ammonium concentrations were measured in the the cultures during the 7 to 10 day incubation period and before the beginning of the uptake experiments. Nitrite and ammonium accumulations in the growth medium will indicate inefficient nitrate metabolism and the loss of intracellular nitrogen. All cultures, including those used for ammonium uptake experiments, were grown with nitrate as the nitrogen source.

Nitrate, ammonium, and ^{14}C uptake experiments were started 7 to 10 days after transfer from the original stock cultures. Nitrate reductase activity was induced with a small addition of 0.1M KNO_3 about 24 hr before experiments were conducted (Christenson, 1983). A similar volume of 0.1

M NH_4Cl was added to cultures used for ammonium uptake experiments to insure that nitrogen concentrations in all cultures were approximately 10 to 50 μM 24 hr prior to experimentation.

Time-staggered additions of small volumes of 0.1 M KNO_3 or NH_4Cl were again introduced into each culture 30 minutes before time zero samples were collected. Samples were swirled vigorously and immediately placed in darkness to allow for complete mixing of nitrate or ammonium before collecting time zero samples. Staggering nitrate and ammonium additions insured that incubation periods were equal for all cultures considering the time required to filter samples. Time zero samples of chlorophyll a, nitrate and nitrite, or ammonium were collected in the order cultures cultures received these additions.

Cultures were divided into equal subcultures, wrapped in fiberglass screening and spiked with 50 μl $\text{NaH}^{14}\text{CO}_3$ per 100 ml of culture after time zero sampling. The $\text{NaH}^{14}\text{CO}_3$ had an activity of 5.0 $\mu\text{Ci/ml}$. These subcultures were incubated at 22°C in a water bath with a 275 $\mu\text{Einsteins/m}^2/\text{sec}$ light source below. Layers of fiberglass screening produced light regimes of 170, 53, and 27 $\mu\text{Einsteins/m}^2/\text{sec}$. Cultures were incubated for 2 hr.

Nitrate, nitrite, ammonium, and ^{14}C samples were collected in the sequence they were spiked. ^{14}C -labelled

cells were vacuum-filtered (5 psi) onto Whatman GF/A filters and the filtrate saved in 50 ml polyethylene centrifuge tubes for nitrogen analysis. Eight ml of Solvent Free liquid scintillation fluid (Isolab, Inc.) were later added to liquid scintillation vials containing the air-dried filters. ^{14}C uptake rates were estimated with a Beckman liquid scintillation counter.

Nitrogen uptake rates were determined by measuring the depletion of nitrate or ammonium from the culture medium. Nitrate concentrations were determined colorimetrically after reduction to nitrite with cadmium and compared to a standard curve (Jones, 1984). Nitrite was also analyzed in this manner, excluding the cadmium reduction step. Ammonium was determined colorimetrically using a modified phenol hypochlorite method (Wetzel and Likens, 1979). All colorimetric analyses were performed with a Baush and Lomb Spectronic 100 spectrophotometer.

RESULTS

Inorganic Nitrogen Uptake. Nitrate uptake rates in iron-sufficient cultures were much greater than rates observed in iron-deficient cultures (Figure 8). Light also clearly enhanced nitrate uptake, with greater uptake rates seen in cultures incubated at $170 \mu\text{Einsteins}/\text{m}^2/\text{sec}$ at all iron concentrations. As with carbon uptake in the

Figure 8. Nitrate uptake rates per chlorophyll *a* increase with greater iron nutrition and irradiance. Differences in total nitrate uptake are partially hidden by normalization to chlorophyll *a*.

preceeding chapter, the much greater nitrate uptake rates in iron-replete cultures are partially hidden by normalization to chlorophyll a.

Ammonium uptake rates were also examined as a function of iron concentration and irradiance, however it soon became apparent that ammonium uptake occurs so rapidly that concentrations were below levels of detection at the end of the 2 hr incubation period. Consequently, a second series of experiments examined nitrate and ammonium uptake rates in cultures incubated for 30 minutes in complete darkness (Figure 9).

Nitrate depletion in the absence of light was generally greatest in iron-sufficient cultures with maximum removal of nitrate seen in $0.3 \mu\text{M}$ ($\log -6.5\text{M}$) iron cultures. Uptake rates were nearly the same in cultures of moderate and high iron deficiency.

Ammonium depletion from the medium is slightly greater in iron-sufficient, or moderately-deficient cultures than in iron-deficient cultures. Maximum uptake capacity is unknown in these cultures because ammonium concentrations were typically below the level of detection by the end of the 0.5 hr dark incubation. It is possible that greater uptake rates may have been demonstrated by the iron-replete cultures if additional ammonium had been available. The sharp decline in ammonium depletion rates seen in the $0.03 \mu\text{M}$ Fe ($\log$

Figure 9. A comparison of nitrate and ammonium depletion from the culture media during a 30 minute dark incubation. Greatest depletion occurs in the 0.5 μM ($\log = -6.5$ M) iron culture. Values are not normalized to chlorophyll *a* and are the mean value of duplicate samples from a single experiment.

value $-7.5M$) culture may not be attributed to factors such as cell density or growth rates because a similar response was not also seen in nitrate depletion rates at this iron concentration and is probably an experimental artifact.

Large differences in nitrate and ammonium uptake rates are also seen when iron-deficient cultures ($0.01 \mu M$) are incubated at different photon flux densities (Figure 10). Ammonium uptake rates are greater than nitrate rates at all levels of irradiance and appear to be independent of light intensity. Light has been reported to be strongly linked to nitrate uptake and this is seen at low photon flux density. At high irradiances nitrate uptake is light saturated and independent of photon flux density.

Accumulations of nitrite in the culture medium were not seen in at any level of iron nutrition. Nitrite was below the level of detection throughout the 7 to 10 day incubation period and after experimentation with low light incubations. Similarly, no accumulations of ammonium were detected in any of the iron-treatment cultures grown on nitrate.

Chlorophyll a. Chlorophyll a concentrations were examined in cultures grown nitrate-sufficient and nitrate-deficient ($200 \mu M$ and $10 \mu M$ respectively) with iron concentrations that ranged over two orders of magnitude (10^{-6} to $10^{-8} M$ Fe). A functional threshold exists which

Figure 10. Nitrate and ammonium uptake rates as a function of irradiance or photon flux density. Nitrate uptake is light-saturated at 53 μ Eins/m²/sec and becomes a nonlinear function of light. Ammonium uptake is independent of irradiance.

partitions the expression of each nutrient deficiency (Figure 11). Iron deficiency is the predominant factor regulating chlorophyll *a* concentrations in cultures grown with iron concentrations of $0.1 \mu\text{M}$ (10^{-7} M) or less. Chlorophyll *a* concentrations were essentially the same for both nitrate-sufficient and nitrate-deficient cultures grown at these lower iron concentrations.

Chlorophyll *a* concentrations in iron-sufficient cultures were a function of nitrate availability. Concentrations in iron-replete, nitrate-replete cultures were several times greater than concentrations in iron-replete, nitrate-deplete cultures. A distinct decline in chlorophyll *a* concentrations is observed in both nitrate deficient and sufficient cultures at the highest iron concentration ($1.0 \mu\text{M}$, 10^{-6} M Fe).

Photosynthetic rates. Photosynthetic carbon fixation rates per chlorophyll *a* were examined in nitrate-sufficient and deficient cultures incubated over a range of photon flux densities (Figure 12). Iron was not a factor in this experiment and all cultures were grown in iron-replete (10^{-6} M) medium. As seen in chlorophyll *a* concentrations, a physiological threshold exists; high photon flux density compensates for nutrient deficiency and carbon fixation rates in nitrogen-deficient and nitrogen-sufficient cultures are nearly equal. Again however, because of the greater

Figure 11. Chlorophyll a concentrations examined as a function of iron and nitrate nutrition indicates that iron nutrition more strongly controls chlorophyll concentrations than nitrate. Only in iron-replete cultures does nitrate nutrition determine chlorophyll a concentrations.

Figure 12. The influence of light intensity and nitrate nutrition on ^{14}C uptake. Nitrate-replete cultures have greater carbon assimilation efficiencies than nitrate-deplete cultures. This is seen as a steeper slope in the region where carbon uptake is a linear function of irradiance. High light intensity mitigates for nitrate deficiency.

chlorophyll *a* concentrations, net productivity is actually much greater in the nitrate-replete cultures. Nitrogen deficiency is accentuated at low-light intensity where carbon fixation is a linear function of irradiance. Carbon fixation in nitrogen-deficient cultures is considerably less than rates exhibited by nitrogen-sufficient cultures in these low-light regimes.

Carbon fixation rates were also examined in cultures enriched with either nitrate or ammonium shortly before incubation with $\text{NaH}^{14}\text{CO}_3$ at different photon flux densities (Figure 13). Cultures with ammonium as a nitrogen source demonstrated considerably greater carbon fixation rates than cultures with a nitrate-nitrogen source at all photon flux densities. This greater photosynthetic activity is also reflected in the linear slope of the productivity curve (Figure 13).

DISCUSSION

Nitrate uptake and assimilation is energetically costly to an alga requiring nearly half as much of the cell's metabolic energy as carbon fixation (Syrett, 1981). Ions must be actively transported against a concentration gradient into the cell and reduced to ammonium in a series of processes consuming ATP and reductant. Nitrate is reduced to nitrite in the cytoplasm of the cell using reductant supplied by NADPH. Nitrite is then reduced to

Figure 13. A comparison of the influence of nitrate and ammonium-nitrogen sources on photosynthesis. Cultures incubated with ammonium demonstrate greater ^{14}C uptake rates at all light intensities. Saturating light intensity ($170 \mu\text{Eins/m}^2/\text{sec}$) does not compensate for the difference in nitrogen sources.

ammonium in the chloroplast where ferredoxin serves as the electron donor. The glutamine synthetase/ glutamate synthetase (GS/GOGAT) pathway, converts inorganic ammonium into an organic nitrogen compound (glutamate) which is available for the synthesis of nitrogenous organic molecules such as amino acids, porphyrins, and other essential cellular components (Ullrich, 1983; see Figure 1).

Iron nutrition and irradiance influence nitrate metabolism in a similar manner. Iron sufficiency is required for an uninterrupted supply of photochemical energy to the nitrate reduction and assimilation enzymes. Irradiance is linked to nitrate metabolism for many of these same reasons. The light reaction of photosynthesis reduces ferredoxin providing reductant for nitrate and nitrite reduction and the conversion of glutamine to glutamate. ATP, in addition to driving the active transport mechanism of nitrate uptake, is required by glutamine synthetase to convert ammonium to glutamine. Irradiance is also required to insure that sufficient carbon skeletons are available to accept ammonium in the formation of glutamine (Syrett, 1981).

The energetic limitation of nitrate uptake was shown here to be brought about by the simultaneous reduction in iron and light availability (Figure 9). Ammonium uptake was also examined and was found to be largely independent of

irradiance (Figure 2). Unlike nitrate, ammonium can enter the cell via nonmediated diffusion or by a cotransport mechanism driven by a charge gradient (Raven, 1980), and since it is completely reduced and available for conversion to glutamate, requires little reductant or ATP. These energetic considerations may account for the rapid uptake of ammonium in cultures incubated in total darkness.

Ammonium is preferred over nitrate as a nitrogen source by freshwater and marine microalgae when available in adequate supply. Ammonium uptake and assimilation has been reported to be dependent on irradiance, however these reports are from studies conducted in the field with indigenous algae incubated in different light regimes (Prisco, 1984). I have examined the short-term uptake of ammonium in cells that may have accumulated considerable reserves of carbohydrates prior to experimental incubation, which may for a limited time, provide reductant and ATP from respiration to drive ammonium metabolism. As these cells were held in darkness these carbon reserves would diminish and it is likely that a reduction in ammonium uptake rates and a dependence on light intensity would emerge.

Iron deficiency may decrease nitrogen metabolism because of energetic limitations or reductions in the synthesis or activity of enzymes involved in the metabolic pathway. Iron starvation has been reported to decrease

nitrite reductase and glutamine synthetase activity in Scenedesmus (Storch and Dunham, 1986). Accumulations of nitrite in the Fraquil medium of low-iron cultures might be expected because of these energetic and nutritional deficiencies. However the lack of detectable nitrite and ammonium concentrations in all nitrate-grown cultures indicates that iron deficiency was not severe enough to bring about such responses. The rapid removal of ammonium from the Fraquil during the ammonium uptake experiments also suggests that the lowest iron concentration ($0.01 \mu\text{M}$, 10^{-8} M) used here did not hinder glutamine synthetase activity.

Chlorosis induced by nitrogen deficiency is common in plants and has been demonstrated to reflect the ambient nitrogen concentration in the sea (Vaccaro and Ryther, 1960). Nitrogen as ammonium is incorporated into delta amino levulinic acid (ALA) during the synthesis of the heme portion of the chlorophyll molecule. Chlorophyll synthesis, however appears to be more sensitive to iron availability, which is required as ferredoxin to activate the synthesis of ALA, rather than nitrogen deficiency. Nitrogen appears to exert greater influence on chlorophyll synthesis in iron-replete cultures iron-deplete cultures (Figure 11).

The depression in chlorophyll a concentrations in iron-replete cultures ($1.0 \mu\text{M}$) is similar to patterns

occasionally seen in other physiological responses.

Greater growth rates and more rapid depletion of nutrients in these iron-sufficient batch cultures may have produced this decline. A second possibility is that 1.0 μM iron concentrations provides excessive iron which may somehow interfere with normal metabolic processes.

Nitrogen sufficiency, as with iron and other nutrients is required for maximum photosynthetic efficiency. Nitrogen deficiency influences fluorescence indices such as CFC more strongly than other nutrient deficiencies (Vincent, 1980). Moderate reductions in nutrient availability may be ameliorated with an increase in irradiance or other nutrients. This was seen when increased photon flux density decreased the reduction in photosynthetic carbon fixation in iron-deficient cultures as described in Chapter III and was also seen here with nitrogen deficiency.

Carbon and nitrogen uptake and assimilation pathways compete for metabolic energy (Ullrich, 1983). This competition for reductant and ATP is intensified if photosynthetic capacity is reduced by nutrient deficiency or decreased irradiance. Ammonium, which requires less energy and reductant than nitrate to be fully metabolized, permits greater carbon fixation to occur at a given photon flux density. Short-term ammonium metabolism, as demonstrated, is largely independent of light. Ammonium is taken up by

the cell very rapidly and nitrogen-deficient cells can quickly become nitrogen sufficient enhancing photosynthetic efficiency and decreasing the competition for photoreductant with the Calvin cycle permitting greater carbon fixation.

Nitrate metabolism is more dependent on light because it consumes greater quantities of ATP and reductant than ammonium uptake and assimilation. A greater portion of chemical energy produced in photosynthesis is directed to the nitrate uptake and assimilation pathway and this is reflected in decreased carbon fixation rates. This is also seen in the slope when photosynthetic carbon fixation is compared to irradiance in Figure 13; carbon assimilation increases more rapidly with irradiance for cultures incubated with ammonium than nitrate.

CONCLUSION

Iron deficiency and reduced irradiance diminish photosynthetic capacity in S. quadricauda sufficiently to energetically limit nitrate uptake rates. Moderate reductions in one parameter may be partially compensated for by an increase in the availability of the other. Ammonium depletion from the culture medium was found to be independent of irradiance and exceeded nitrate uptake in all iron concentrations and photon flux densities. Iron nutrition had no effect on maximum ammonium uptake rates.

Chlorophyll a concentrations and carbon fixation rates in nitrogen-sufficient and nitrogen-deficient cultures were strongly influenced by the availability of iron. Only in iron-replete cultures do chlorophyll a concentrations reflect nitrogen deficiency. Likewise, high photon flux density is seen to compensate for nitrogen deficiency in carbon fixation rates. The competition for photochemical energy (ATP and reductant) between the nitrogen and carbon metabolism pathways is most pronounced in cultures using nitrate as a nitrogen source rather than ammonium. Ammonium, being completely reduced, requires less reductant and ATP to be assimilated by S. quadricauda and supports greater rates of carbon fixation than completely oxidized nitrate. The nutrient and energetic interactions of iron, nitrogen, and photon flux density in regulating patterns of photosynthesis and carbon assimilation will be examined further in the following chapter.

CHAPTER V

REGULATION OF PATTERNS IN SHORT-TERM CARBON METABOLISM BY IRON NUTRITION

INTRODUCTION

Evaluation of photosynthetic carbon fixation rates with ^{14}C offers valuable insight into algal photosynthetic responses to changes in the physical or chemical environment. The relative synthesis of photosynthetic end products may be related to the nutritional and physiological status of an algal culture. These end product polymers may be grouped into 3 classes; 1) proteins, 2) polysaccharides, and 3) lipids and low molecular weight (LMW) metabolites. The latter group consists primarily of membranes and metabolic intermediates. The polysaccharide fraction includes structural carbohydrates such as cellulose, and starch, which functions as a fixed carbon pool. The protein component also includes the nucleic acids but these reportedly account for less than 5% of the total fraction (Morris et al., 1974).

Iron deficiency has been shown to increase rates of protein synthesis relative to other polymers in a marine dinoflagellate (Glover, 1977). This pattern is similar to

photosynthate production in energetically-deficient, light-limited culture of marine diatoms (Morris et al., 1974). Cultures of Scenedesmus obliquus grown with low nitrogen nutrition were found to have high (45% of biomass) lipid concentrations (Piorreck et al., 1984). Nitrogen deficiency was apparently severe enough to decrease protein synthesis; however it is important to note that these workers were not looking at relative rates of polymer synthesis. In Chapter III it was shown that iron nutrition strongly influences the photosynthetic behavior of Scenedesmus quadricauda; cellular fluorescence capacity and carbon fixation rates are reduced in iron-deficient cultures. In Chapter IV it was shown that iron deficiency inhibited nitrate uptake rates. This chapter examines patterns of short-term carbon incorporation into cellular components in S. quadricauda to compare the relative effects of iron and light on carbon and nitrogen metabolism. These patterns of incorporation were determined by the sequential extraction of the lipid-LMW metabolite, polysaccharide, and protein fractions.

METHODS

Semi-continuous cultures of S. quadricauda were incubated 7 to 10 days in Fraquil medium (Morel et al., 1979; as modified by Petersen, 1982). Iron concentrations of 1.0, 0.3, 0.1, 0.03, and 0.01 μM (10^{-6} to 10^{-8} M) were

achieved with the addition of FeEDTA. Nitrate concentrations were either 200 μM for nitrate-replete cultures, or 10 μM for nitrate-deplete cultures. Light and temperature were continuous at 100 $\mu\text{Einsteins}/\text{m}^2/\text{sec}$ and 22°C respectively.

Patterns of inorganic carbon metabolism were examined using the method of Morris et al. (1974). Cultures were subdivided and spiked with 50 μl $\text{NaH}^{14}\text{CO}_3$ (activity ca 5.0 $\mu\text{Ci}/\text{ml}$) per 100 ml of culture. These subcultures were wrapped with fiberglass screening and incubated at 27, 53, and 170 $\mu\text{Einsteins}/\text{m}^2/\text{sec}$ for 2 hr. Temperature was held at 22°C with a water bath. Dark bottles were wrapped in black plastic.

Cultures were removed from the light field after incubation and 25 ml subsamples were vacuum-filtered onto GF/C glass fiber filters. Filters used for fractionation were placed in 3 ml of 80% ethanol and frozen until extraction. Reference filters were allowed to air dry and placed in liquid scintillation vial for total ^{14}C uptake values.

After extraction, 1 ml of each ethanol and TCA fraction was added to 5 ml of Solvent Free liquid scintillation cocktail and allowed to emulsify 12 to 24 hr. The filter with the protein fraction was placed directly in a LS vial. ^{14}C uptake was determined using a Beckman liquid scintillation counter. Each fraction was corrected for

dilution and presented as a percentage of total ^{14}C incorporated in reference samples.

RESULTS

Patterns of ^{14}C incorporation indicate that a greater portion of the photoassimilated carbon is allocated to protein synthesis as iron deficiency becomes more pronounced (Figure 14). Protein accounts for over 50% of the photosynthate in low iron cultures incubated at 53 or 27 $\mu\text{Einsteins}/\text{m}^2/\text{sec}$. This fraction steadily declines as iron concentrations in the growth medium increase and more fixed carbon is incorporated into the polysaccharide fraction. Synthesis of the ethanol-soluble lipid and LMW metabolite fraction remains largely unaffected by iron nutrition. No apparent pattern in photosynthate partitioning was observed in cultures incubated at 170 $\mu\text{Einsteins}/\text{m}^2/\text{sec}$ suggesting that high light may compensate for iron deficiency.

The partitioning pattern seen in Figure 14 is similar to results reported for nitrogen-deficient or light-limited cultures by Morris et al. (1974). To insure that iron nutrition rather than nitrogen nutrition was responsible for the results in Figure 14, experiments were conducted with 200 μM and 10 μM nitrate concentrations in the culture medium. Although some variability in the results

Figure 14. Patterns of ¹⁴C incorporation into cellular components indicate that greater than 50% of the photo-assimilated carbon is allocated to the protein component in iron-deficient cultures. This percentage decreases with increasing iron nutrition as more ¹⁴C is allocated to other cellular components.

were obtained, patterns of protein synthesis were the same.

Relative rates of protein synthesis appears to be a physiological marker in iron-deficient cultures of S. quadricauda incubated at subsaturating light intensities. Glover (1977) reported similar trends in carbon metabolism in iron-starved cultures of marine algae. These cultures also demonstrated depressed carbon fixation rates and assimilation efficiencies. Total protein synthesis, although declining with increasing iron deficiency, accounted for a greater percentage of the total ^{14}C incorporated.

Much of the assimilated carbon is allocated to protein synthesis when photosynthetic rates are restricted by iron deficiency (Figure 15). Protein accounts for much less of the total photosynthate in cultures with moderate to high carbon fixation rates. Total protein synthesis, however, is much higher in the iron-replete cultures than in the other cultures. For example in Figure 15, total protein production in the intermediate and high-iron cultures is approximately 75% greater than in the low-iron cultures but this difference is hidden by the greater synthesis of other polymers in the iron-replete cultures.

DISCUSSION

Protein synthesis is conserved in algal metabolism

Figure 15. A comparison of photosynthetic rates and ^{14}C incorporation into protein reveals that protein accounts for a relatively constant percentage of incorporated ^{14}C in cultures with moderate to high iron nutrition.

and is affected less in iron-deficient cultures. Iron deficiency, in addition to hindering photosynthetic efficiency and functioning as an energetic limitation to carbon fixation, may also increase protein turnover rates. Continued protein synthesis is essential if the metabolic machinery in the cell is to remain operational. The synthesis of the lipid-LMW metabolite and polysaccharide fractions is also important to maintain cellular fitness, however the synthesis of these polymers is much more variable as they may also function as fixed-carbon reserves. The relative increase in polysaccharide synthesis in iron-replete cultures may indicate an increase in starch synthesis which might be anticipated if these cells can afford the energetic luxury of producing fixed-carbon reserves.

Similar patterns of carbon metabolism have been demonstrated in iron, light, and nitrogen-deficient cultures (Glover, 1977; and Morris et al., 1974, respectively). Iron and light deficiencies, as discussed in Chapter III, are both expressed as energetic limitations which decrease the rate of carbon fixation. Protein synthesis may be enhanced by the release from nitrogen deficiency with the nitrate or ammonium addition that occurs prior to ^{14}C incubation when nitrogen uptake rates are also examined. The sudden availability of nitrogen for amino acid synthesis could result in the rapid catabolism of other polymers to produce

protein. However this response would reduce the amount of ^{14}C incorporated into the new protein fraction since amino groups would be added to previously synthesized carbon skeletons.

Protein synthesis in cultures recently released from nitrogen starvation is linked to nitrogen uptake (Syrett, 1981). In Chapter IV it was demonstrated that nitrate uptake rates are greatest in iron-replete cultures. These cultures also have the greatest growth rates and total protein production. Proportional rates of protein synthesis, however were not related to nitrate (or ammonium) uptake rates.

Carbon metabolism may be strongly affected by diurnal and temporal growth patterns. Morris et al., (1974) reported that polysaccharide and lipid-LMW metabolite synthesis was dependent on the stage of the algal life cycle whereas protein production was not. Rivkin (1985) showed that the ethanol-soluble fraction is catabolized for protein synthesis during the dark phase of a diurnal cycle. These problems have been eliminated by incubating cultures under continuous light.

CONCLUSION

Protein synthesis accounts for the largest fraction of the carbon fixed in iron-deficient cultures. Poly-

saccharides replace protein as the predominant polymer as iron concentrations in the culture medium increase. Total protein synthesis, however remains greater in iron-replete cultures which are photosynthesizing and growing most rapidly. No clear pattern in carbon metabolism was seen in high-light cultures, possibly due to high light compensation for iron deficiencies. Nitrogen nutrition may also affect patterns of carbon metabolism; total protein synthesis was greatest in iron-replete cultures which, in other experiments also had greatest nitrate uptake rates.

Conservation of protein synthesis reflects the metabolic priority of that fraction. Polysaccharides, lipids and LMW metabolites are also critical for cell maintenance but are much more variable. These fractions may also serve as fixed-carbon pools which may be catabolized to produce protein. Greater polysaccharide synthesis in iron-replete cultures may also be attributed to greater growth rates and the increased demand for cellulose and perhaps the synthesis of starch as a storage compound for extra fixed-carbon pools. The lipid-LMW fraction remains relatively unchanged by the iron nutrition of the culture.

Chapter VI

A GENERAL MODEL FOR IRON LIMITATION AND ENVIRONMENTAL SIGNIFICANCE

Iron nutrition strongly regulates photosynthetic behavior in batch cultures of Scenedesmus quadricauda. Carbon uptake per ug chlorophyll a and total productivity is greatest in iron-replete cultures. Similar results have been reported in laboratory and field experiments with freshwater and marine algae (Oquist, 1971; Miller et al., 1974; Glover, 1977; and Mueller, 1985). Cellular fluorescence capacity also reflects the iron nutrition of each culture, suggesting that iron deficiency may impede electron flow through the algal photosynthetic apparatus. This energetic hinderance is strongly linked to the observed reductions in ¹⁴C photoassimilation and photosynthetic efficiency.

Iron deficiency decreases the chlorophyll a concentration in algae, possibly due to reductions in ferredoxin concentrations. Fluorescence indices, such as cellular fluorescence capacity, and chlorophyll a concentrations are good markers for possible iron deficiency in algal populations. These physiological markers were more sensitive to iron deficiency than growth or culture biomass

in the marine diatom Thalassiosira pseudonana (Mueller, 1985). Reductions in the primary light harvesting pigment of the cell create energetic hinderances which are possibly exacerbated by decreased photosynthetic efficiencies.

Fluorescence indices have been linked to nutritionally influenced patterns of photosynthesis in natural populations of marine phytoplankton (Vincent, 1980). The demonstrated relationship between CFC and photosynthetic efficiency in cultures of S. quadricauda incubated at subsaturating light intensities may also occur in natural populations. Further development and refinement of fluorescence indices may provide researchers with a valuable tool for investigating photosynthesis in natural phytoplankton populations.

Energetic limitations brought about by iron deficiency affect nitrogen metabolism as well as carbon fixation. The uptake, reduction, and assimilation of nitrate requires reductant and ATP, each a product of photosynthesis. Nitrate uptake and reduction occurs independently of nitrite reduction. Nitrate reduction to nitrite is less energetically demanding than the reduction of nitrite to ammonium. The occurrence of a deep-water nitrite maximum may be due to energetically limited nitrate metabolism in phytoplankton populations (Kiefer et al., 1976). Phytoplankton growing in low-light environments might continue to take up and reduce nitrate to nitrite

while rates of nitrite reduction decline, allowing nitrite to accumulate within the cell and eventually leak out into the surrounding environment. Nitrite production, which might be anticipated in the most energetically stressed cultures (the $0.01 \mu\text{M}$ iron cultures incubated at $53 \mu\text{Einstein/m}^2/\text{sec}$), was not observed, indicating that light and iron availability was adequate to support nitrite reduction to ammonium at rates which prevented the accumulation of nitrite in the culture medium.

Inorganic nitrogen and carbon uptake and assimilation compete with other metabolic pathways for photochemical energy. Ammonium, a nitrogen source which requires less reductant and ATP than nitrate to be completely assimilated, is taken up more rapidly and supports greater photosynthetic activity than does a nitrate-nitrogen source. As expected, nitrate-replete cultures contained more chlorophyll *a* and were more productive than nitrate-deplete cultures of *S. quadricauda*. However productivity, independent of nitrate nutrition is strongly influenced by iron nutrition. Chlorophyll *a* concentrations were greater in nitrate-replete cultures only if iron concentrations in the Fraquil were also high, thereby determining total productivity of the culture. Light also mitigates for nitrogen deficiency, reducing differences in photosynthetic rates among nitrate-sufficient and deficient cultures at higher intensities.

The iron-nutritional status of experimental cultures is also reflected in patterns of carbon metabolism in experimental cultures at subsaturating light intensities. Patterns of ^{14}C allocation into cellular components, as with patterns of nitrogen metabolism, are less defined when cultures are incubated at high light intensity. Predictions of carbon allocation in energetically or nutritionally-limited cells can be made using a growth model developed by Shuter (1979). The Shuter model predicts that the synthesis of cellular components will be optimized in such a manner as to maintain maximum feasible growth rates under existing environmental conditions. Figure 16 illustrates the strategy of the Shuter model.

Structural cell components in the Shuter model are a fixed metabolic cost and must be maintained. Reductions in carbon assimilation due to energetic restrictions mobilize carbon storage reserves which are catabolized to support structural and metabolic component synthesis. This preferential synthesis of certain cellular components is seen in iron deficient cultures which continue protein synthesis at the expense of the polysaccharide and lipid-low molecular weight metabolite fractions. These other fractions also continue to be synthesized for the maintenance of structural and metabolic components, but protein synthesis accounts for an increasingly larger

Figure 16. The effects of iron supply on cellular composition demonstrated with the Shuter model approach (Shuter, 1979). The relative width of each fraction indicates the percentage of total carbon allocated to each component. The structural component remains constant at all levels of nutrition. Metabolic machinery such as photosynthetic and respiratory proteins, account for an increasing amount of total cellular carbon as iron availability decreases at the expense of fixed-carbon reserves.

portion of the total carbon assimilated as photosynthetic rates decline with increasing iron deficiency.

Maintenance costs are proportional to culture growth rates and cell synthesis. Cultures with moderate to high rates of carbon fixation allocate a relatively constant fraction of the fixed ^{14}C to protein synthesis. This consistency was also seen in the lipid-LMW metabolite fraction at all iron concentrations. The proportional increase in the polysaccharide fraction from cell maintenance levels implies that iron-replete cultures are synthesizing starch as an energy storage pool in addition to the continued production of cellulose and other structural carbohydrates.

The goal of this research effort was to examine the physiological response of a common freshwater alga, *S. quadricauda* to changes in iron nutrition. Responses to iron deficiency were often similar to responses in light-limiting conditions reported in the literature. Energetic restrictions are seen in carbon assimilation, nitrate metabolism, and photosynthate partitioning. Therefore, primary productivity in iron-limited natural waters would be partially restricted by greater inefficiencies in the algal photosynthetic apparatus, lower chlorophyll synthesis, and decreased nitrogen metabolism.

Iron limitation in a natural system will be less

pronounced if easily assimilated nutrients are available for algal use. Ammonium can support greater photosynthetic rates at lower light intensities and iron concentrations than nitrate-nourished cultures. Co-limitation of primary productivity is more likely to be observed than iron limitation alone. A system with low nitrogen and iron concentrations might be such an example; nitrogen would probably be identified as the limiting resource although iron deficiency may energetically hinder the use of available nitrogen.

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