ASPECTS OF IRON AND NITROGEN NUTRITION IN TWO RED TIDE
DINOFLAGELLATES, GYMNOCLIDINUM SANGUINEUM HIRASAKA AND
PROTOGONYAULAX TAMARENSIS (LEBOUR) TAYLOR

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Iron stress-mediated effects on growth, biochemical composition, iron and nitrogen uptake, and ultrastructure have been examined in the red tide dinoflagellates Gymnodinium sanguineum Hirasaka and Protogonyaulax tamarensis (Lebour) Taylor. The influence of nitrogen source (i.e. NO$_3$ or NH$_4$) on certain iron stress-mediated effects was studied, and some comparisons were made with nitrogen stress-mediated changes in biochemical composition. The half-saturation constant for iron-limited growth ($K_\mu = 1.7 \cdot 10^{-20}$ M) of G. sanguineum was estimated to be 10-1000 times greater than for other neritic species investigated previously. Also, the iron requirement of this dinoflagellate, in terms of Fe/C ratios, exceeded those of certain coastal diatoms by one to two orders of magnitude. Fe/N ratios demonstrated a larger (1.5-fold) minimum iron requirement for NO$_3$- than NH$_4$-grown cells, likely reflecting the iron content of NO$_3$ assimilatory enzymes. Acquisition of nitrogen by Fe-deplete, NO$_3$-grown cells was sufficiently inhibited to yield symptoms of N deficiency, revealed by decreased (ca. 1.4-fold) N quotas and free amino acid/protein ratios compared to Fe-deplete, NH$_4$-grown cells. Reductions in chlorophyll a (chl a) quotas ($Q_{chl}$) and photosynthetic electron transport (PET) efficiency (as measured by in vivo fluorescence indices) occurred under Fe depletion, and are consistent with the essential role of iron in chl a and PET component (i.e. cytochromes and Fe-S
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INTRODUCTION

Overview

Iron and nitrogen in marine systems. Iron is the fourth most abundant element in the earth's crust (Taylor 1964) and, of the trace elements essential for phytoplankton growth, it is quantitatively the most important (Huntsman and Sunda 1980). In natural waters, iron occurs in two oxidation states, Fe (II) and Fe (III). In oxic natural waters, the reduced and most soluble Fe (II) forms are converted to highly insoluble Fe (III) species, which predominate in oxygenated seawater (Byrne and Kester 1976, Sung and Morgan 1980). The distribution of these forms may be influenced by, among other factors, redox potential, pH, the presence of organic matter and photochemical reactions (Hong and Kester 1986). Iron has been hypothesized to be biologically available to phytoplankton as free ferrous or ferric ions (Anderson and Morel 1982), dissolved organic complexes (Trick et al. 1982, Allnutt and Bonner 1987) and recently-formed (i.e. thermodynamically unstable) colloids (Wells et al. 1983).

The concentration and distribution of iron in the ocean have been examined recently by several investigators (Gordon et al. 1982, Symes and Kester 1985, Landing and Bruland 1987, Martin and Gordon 1988). These studies, employing "ultra-clean" techniques, have minimized previous contamination problems, and yielded much lower values, and more defined and systematic profiles. Surface concentrations of dissolved
(i.e. < 0.4 μm) iron measured at inshore stations (6000 nM, San Francisco Bay) by Gordon et al. (1982) exceeded open ocean values (0.13 nM, N.E. Pacific) by over four orders of magnitude. However, in a surface transect across the continental shelf into the apex of the New York Bight, total iron ranged from ca. 3 to 540 nM (Symes and Kester 1985), a variation of slightly more than 100-fold. Further, dissolved iron concentrations as low as 0.05 nM have been measured in surface waters of the highly productive upwelling region off California (Martin and Gordon 1988). Vertical profiles of iron distributions generally show minimum surface concentrations which increase with depth and frequently show a maximum associated with the dissolved oxygen minimum (Symes and Kester 1985, Landing and Bruland 1987, Martin and Gordon 1988). The percentage of total iron in surface waters attributable to particulate (i.e. > 0.4 μm) forms appears to be highly variable, with values as disparate as ca. 10% (N. Atlantic slope water, Symes and Kester 1985) and 80-90% (nearshore waters off Peru, Hong and Kester 1986) having been reported.

Riverine input can be an important source of iron for nearshore environments, with average concentrations of dissolved iron in river water estimated at ca. 700 nM (Martin and Whitfield 1983). The fate of river-borne iron upon mixing with seawater is, however, quite complex. While certain data indicate that 95% of dissolved iron is converted to particulate form during estuarine mixing (Sholkovitz 1978),
other reports have demonstrated an 8-fold increase in dissolved iron in waters of intermediate salinity, relative to adjacent fresh or more saline water (Fletcher et al. 1983). Although little information is available on the temporal variation of iron concentrations, factors such as snowmelt and heavy rainfall would be expected to influence river-borne contributions in coastal regions (Ingle and Martin 1971, Fletcher et al. 1983).

Apart from carbon, hydrogen and oxygen, nitrogen is quantitatively the most important nutrient required by phytoplankton (Syrett 1981). Nitrogen is also generally considered to be the nutrient most often limiting marine phytoplankton reproductive rates (Parsons and Harrison 1983). While nitrogen exists in five principle oxidation states in seawater (-3(NH$_4^+$, NH$_3$), 0(N$_2$), +2(N$_2$O), +3(NO$_2^-$), +5(NO$_3^-$); Sharp 1983), two species, NH$_4^+$ and NO$_3^-$, are the predominant sources of inorganic nitrogen utilizable by eukaryotic algae. In the surface waters of central ocean gyres, nitrogen is consistently near analytical detection limits, with no apparent seasonal pattern (McCarthy 1980, Sharp 1983). Concentrations are invariably higher at depth. Nitrate, rather than ammonium, is the more abundant form of nitrogen in coastal regions (Sharp 1983), and exhibits distinct seasonal trends. Surface nitrate concentrations are elevated during the winter (> 10 $\mu$M) and, following density stratification during the spring and subsequent utilization by phytoplankton, are eventually depleted or reduced to near zero. Ammonium
levels show more variability, and although generally not exceeding 3 \( \mu \text{M} \) (Sharp 1983), are frequently below 0.5 \( \mu \text{M} \) (Paasche 1988). Exceptions occur in areas subject to considerable terrigenous input (e.g. sewage outfalls), where concentrations may range from 10 to 30 \( \mu \text{M} \) (Thomas et al. 1980).

Ecology of red tides: iron and nitrogen considerations. The detrimental aspects of both toxic and non-toxic red tides have been well documented (Steidinger and Haddad 1981, Ragelis 1984, Steidinger and Baden 1984, Taylor 1987). Others have recognized the importance of these blooms in their contribution to local primary production (Vargo et al. 1987). Irrespective of their perceived effects on the immediate or surrounding environment, red tides are of considerable ecological importance. Accordingly, the identification of factors contributing to or associated with the initiation and decline of these blooms has been of particular interest. In this regard, environmental cues and life history stage dynamics of the causative organisms have received perhaps the most attention.

It is frequently not possible to discern an obvious relationship between the timing of bloom formation and a particular environmental parameter(s) such as nutrient levels, salinity, light or photoperiod (e.g. Anderson and Morel 1979). Some work suggests that favorable temperatures and oxygen concentrations are critical to the successful development of
red tides (Anderson and Keafer 1985). Other reports have cited the potential importance of components associated with terrestrial runoff, including trace metals, humates and salinity/stratification (Prakash 1975, Anderson and Wall 1975, Provasoli 1979, Anderson et al. 1983). Among these factors, elevated levels of soluble iron, derived from river discharge, land runoff and also from sediment resuspension, have been frequently correlated with the development of red tides (Ingle and Martin 1971, Kim and Martin 1974, Glover 1978, see Iwasaki 1979, Yamochi 1984). While the stimulatory effects of other trace metals, humates or salinity reductions cannot be discounted without further investigation, this relationship may imply the alleviation of iron-limiting conditions by an increase in iron supply rate. Yet, there have been few studies on the iron nutrition of red tide dinoflagellates (or of dinoflagellates in general).

If the growth of these organisms may be at least initially iron-limited, as some evidence would suggest, the decline of a bloom could result from localized depletion of biologically available iron. A report by Anderson and Morel (1979), noting a sharp decrease in total iron concentration associated with the decline of a Gonyaulax tamarensis bloom, is consistent with this idea. Alternatively, nitrogen limitation has also been implicated in terminating blooms of the same species (Glibert et al. 1988). Although reduced ambient nutrient levels may contribute to the decline of red tide populations, other explanations, such as reproductive
rhythms or depletion of an internally-stored product can be inferred from certain data (Steidinger and Haddad 1981, Anderson et al. 1983).

The dynamics of life history stages, specifically cyst germination and formation, are closely linked with the initiation and decline, respectively, of red tides (Steidinger 1983, Anderson 1984, Taylor 1987). The induction of encystment and, to a lesser extent, cyst germination, have been investigated previously. Available data indicate that both nitrogen and phosphorus depletion can effect cyst formation in several species (see review by Pfiester and Anderson 1987). Anderson (1980) has demonstrated that excystment can be induced by temperature shifts. However, field observations suggest that the optimization of other factors, apart from temperature, is required for maximal cyst germination (Anderson et al. 1983). Given the potential importance of iron in regulating red tide population dynamics, it is of interest that no studies to date have examined the relationship between iron nutrition and life history stage. Red tide populations may be controlled by one or a suite of environmental cues, which, further, are likely to vary with species, location and perhaps stage of bloom development. The essential nutrients iron and nitrogen would appear to be among those factors potentially important in regulating bloom dynamics.
Metabolic relationships between iron and nitrogen. Both iron and nitrogen are elements essential to algal metabolism. Iron functions as a coenzyme in certain reactions of the tetrapyrrole biosynthetic pathway responsible for chlorophyll and cytochrome production (Marschner 1986). This micronutrient is an integral component of cytochromes and iron-sulfur proteins (Hipkins 1983), and of the reductive nitrate assimilatory enzymes, nitrate and nitrite reductase (NR and NiR, respectively) (Hewitt 1983). Some or all forms of several enzymes such as glutamate synthase, superoxide dismutase, catalase, peroxidase, hydrogenase and aconitase require iron to perform their catalytic functions (Hewitt 1983, Roessler and Lien 1984, Marschner 1986, Raven 1988).

The principle and ubiquitous role of nitrogen as a constituent of cellular proteins and nucleic acids is well understood. Chlorophyll pigment molecules of photosynthetic organisms contain ca. 6% nitrogen (by atoms). Also, it has been calculated that photolithothrophic (i.e. light-driven O₂ evolution with an inorganic carbon source) reproduction on nitrate-nitrogen requires 60% more iron than for growth on ammonium-nitrogen (Raven 1988).

Clearly, many aspects of iron and nitrogen nutrition are closely linked. Of particular interest to the current research are the metabolic interrelationships of these two nutrients as related to the metalloporphyrins chlorophyll a and cytochromes, the nitrate reducing enzymes NR and NiR, and also the non-heme iron sulfur proteins (e.g. ferredoxin).
Successful maintenance of the associations between iron and nitrogen are critical to the processes of photosynthesis, respiration and nitrogen (predominantly nitrate) assimilation, as well as the structural integrity of certain organelles. Because of the iron requirement associated with NR and NiR, growth on NO$_3$ should impart a greater degree of iron stress for a given subsaturating (for growth) iron concentration, relative to growth on NH$_4$. Apart from the work of Reuter and Ades (1987), and also the theoretical treatment by Raven (1988), no studies have examined this obvious interaction between iron and nitrogen nutrition in phytoplankton.

Objectives

This thesis addresses the following three objectives, which are considered in detail below:

1. To increase our knowledge of dinoflagellate iron nutrition, with emphasis on those species producing red tides.
2. To provide a better understanding of the interaction between algal iron and nitrogen nutrition.
3. To assess the susceptibility to iron-limited growth of red tide dinoflagellates, relative to other coastal phytoplankton.

Literature reviews in relation to these objectives are provided as background information prefacing each chapter.
The preceding remarks on iron and nitrogen have attempted to outline the importance of these nutrients at levels ranging from the marine environment to cellular nutrition, as well as some of their metabolic relationships. While the literature is replete with studies of nitrogen-based uptake, metabolic and growth processes of phytoplankton, comparatively little is known about phytoplankton iron nutrition. The latter situation clearly applies to dinoflagellates, frequently an important component of the phytoplankton community. The principle objective of the current research was to increase our knowledge of dinoflagellate iron nutrition, with emphasis on those species producing red tides. During this study, responses to iron stress as manifested through growth (Chapter 1), biochemical composition (Chapters 1 and 2), nutrient uptake (Chapter 3) and life history stage (Chapter 4) were examined in red tide-forming dinoflagellates. Several attempts were also made to verify the iron stress-mediated specificity of these observations by conducting analogous experiments utilizing nitrogen stress as a stimulus (Chapter 1).

The other main goal of this work was to provide a better understanding of the interaction between algal iron and nitrogen nutrition. As the algal requirement for iron varies depending on whether nitrogen is supplied as nitrate or ammonium, nitrogen source is likely to influence the nature of iron stress-mediated effects on these organisms. This possibility is addressed in two chapters herein. Measurements
obtained in Chapter 2 characterized the quantitative and qualitative response of several biochemical variables to iron depletion (relative to iron-replete conditions) for nitrate- and ammonium-grown cultures. Nutrient uptake experiments of Chapter 3 determined rates of iron, nitrate and ammonium uptake by iron-replete and iron-deplete cells grown on either nitrate or ammonium. Comparisons were further extended to include data collected during transitions between these two nitrogen sources. The latter results were useful in assessing the effect of nitrogen source and N source transitions on a cell's ability to successfully adapt to changes in iron availability.

In a more ecological sense, perhaps the most important objective of this thesis was to assess the susceptibility to iron-limited growth of red tide dinoflagellates, relative to other coastal phytoplankton. To this end, comparisons of iron nutritional characteristics were made throughout this work, between red tide and other neritic species for which similar data were available. Information pertaining to the question of growth limitation by iron has implications for the suggested role of this trace element in regulating red tide population dynamics. However, the actual balance between iron supply and phytoplankton demand (or acquisition capabilities) in coastal waters is the most critical, and as yet the most uncertain factor. Nevertheless, iron stress-mediated properties of growth, composition and uptake determined herein should indicate whether this coupling between iron supply and
demand is potentially tighter for red tide dinoflagellates than might be expected for other phytoplankton groups.

Experimental Organisms

Two photosynthetic dinoflagellates, *Gymnodinium sanguineum* Hirasaka and *Protogonyaulax tamarensis* (Lebour) Taylor, were the subjects of this investigation of red tide dinoflagellate iron and nitrogen nutrition. *G. sanguineum*, a non-toxic, athecate species, is taxonomically synonymous with *G. splendens* Lebour and *G. nelsoni* Martin (Tangen 1979). The culture used herein (#D354, North East Pacific Culture Collection (NEPCC), Dept. of Oceanography, University of British Columbia, Vancouver, Canada) was isolated from Esquimalt Lagoon (maximum depth 3.5 m, Watanabe and Robinson 1979), Vancouver Is., B.C. by A. Chan in September 1980. Strain #D354 is ca. $4.6-4.7 \times 10^4 \mu m^3$ in volume and measures ca. 40-50 $\mu m$ across the longest dimension (i.e. antero-postero).

*G. sanguineum* was employed in Chapters 1-3 to investigate various aspects of, and relationships between, iron and nitrogen nutrition. The decision to utilize this species as a test organism was based primarily on two important factors: its ability to form red tides and its widespread occurrence throughout the world's coastal waters. *G. sanguineum* was cited by Robinson and Brown (1983) as the causative organism of a recurrent red tide in Esquimalt Lagoon, B.C. for all but
one of six consecutive years documented, achieving peak cell densities as high as $1.14 \times 10^4$ cells·ml$^{-1}$. This species has also been identified as the numerical dominant in red tides off California (Kiefer and Lasker 1975), Virginia (Zubkoff 1979), Norway (Tangen 1979), Peru (Rojas de Mendiola 1979) and West Africa (Dandonneau 1970). Additional reports extend its distribution to include Japan and New Zealand (references cited by Watanabe and Robinson 1979).

While clearly of interest from an ecological point of view, *G. sanguineum* is somewhat problematic with regard to laboratory experimentation. The major hindrance lies in its extreme sensitivity to mechanical perturbation. Physical lability is a very important consideration when filtering or washing cells, procedures essential to studies of phytoplankton physiology and biochemical composition. Several such problems were encountered herein and acceptable alternative protocols have been developed where required. Despite these logistical drawbacks, the ubiquitous nature and numerical importance of *G. sanguineum* enhance the potential for ecologically relevant extrapolation of laboratory data to coastal dinoflagellate field populations.

The other organism used in this study, *P. tamarensis* (= *excavata*), is a toxic, thecate dinoflagellate responsible for numerous paralytic shellfish poisoning (PSP) outbreaks in many of the world’s temperate coastal waters (Taylor 1984). The culture employed was NEPCC #D255, which was isolated by R.
Waters off Lummi Is., Washington, U.S.A. in August 1976. The taxonomy of *P. tamarensis* at the generic level is the subject of an ongoing discussion in the literature. Although this species (and a group of closely related taxa - the "tamarensis group"), have been removed, by consensus, from the genus *Gonyaulax*, agreement on an alternative generic designation is yet to be reached. Several authors have assigned members of this group to one (or a combination) of three genera, *Protogonyaulax*, *Alexandrium* or *Gessnerium*, based on various criteria (Loeblich and Loeblich 1979, Taylor 1979, Balech 1985). It was of preeminent importance that the identity of the taxonomic entity employed herein, be unambiguously discernable to the reader. Also, while slight emendations to the generic diagnosis of *Protogonyaulax* may be necessary (Taylor 1985), its members are easily distinguished. Furthermore, this designation appears to be gaining relatively widespread acceptance. Based on these criteria, and in the absence of a unifying alternative, *Protogonyaulax tamarensis* was selected to identify the taxon used in this study.

*P. tamarensis* was utilized in Chapter 4 to examine the effects of iron stress on the life history stage of a red tide dinoflagellate. Of specific interest, was the possibility of iron stress-mediated cyst formation and the ultrastructure of any cysts thus produced. Apart from its reputation as an important red tide organism, cyst formation has been extensively documented for *P. tamarensis* both in laboratory cultures (Turpin et al. 1978, Anderson et al. 1984, Anderson
and Lindquist 1985) and in field populations (Anderson 1980, Anderson et al. 1983). This species was, therefore, considered an appropriate choice to accommodate the goals of the current research.
CHAPTER 1.

THE RED TIDE DINOFLAGELLATE GYMNODINIUM SANGUINEUM HIRASAKA: EFFECTS OF IRON STRESS ON GROWTH, CHLOROPHYLL A, IN VIVO FLUORESCENCE AND ULTRASTRUCTURE, INCLUDING SOME COMPARISONS WITH NITROGEN STRESS

BACKGROUND

Marine phytoplankton require a variety of trace metals (e.g. Fe, Mn, Zn, Co, Cu) for cell maintenance and growth. Depending on the element, concentrations in coastal regions can exceed those of oceanic waters by as much as four orders of magnitude (Brand et al. 1983). Recent work has established a distinction in trace metal-limited growth rates between neritic and oceanic phytoplankton species (Brand et al. 1983, Murphy et al. 1984). The clearest pattern for those metals compared (i.e. Fe, Mn, Zn) was observed under iron limitation. All neritic species exhibited significant reductions in growth rate below the same substrate concentration, whereas the reproduction of oceanic species was generally not (or only slightly) limited at any Fe level tested. Unfortunately, no dinoflagellates were among those coastal species considered.

Dinoflagellates account for about 30% of total productivity in the world's oceans (Yentsch et al. 1980). Included in this ecologically diverse group are species capable of producing red tides, virtually monospecific blooms in which cell densities often surpass $10^6$ cells·l$^{-1}$ (Taylor 1987). Red tide dinoflagellates can be either toxic or non-toxic. Because blooms of either type can result in the death
of marine organisms (Steidinger and Haddad 1981, Taylor 1987), these phenomena are of particular ecological significance. Although research continues into the nutritional factors which regulate the population dynamics of these organisms, some evidence suggests that iron bioavailability may be important in this regard (e.g. Ingle and Martin 1971, Glover 1978, Yamochi 1984). There is clearly a need for more information on the iron nutrition of dinoflagellates in general and, specifically, red tide species. Thus, a principle objective of this work was to investigate the growth of a red tide dinoflagellate, Gymnodinium sanguineum Hirasaka, under iron-limiting conditions. G. sanguineum is a non-toxic species and blooms frequently in a British Columbian lagoon, achieving concentrations greater than 10^7 cells·l^-1 (Robinson and Brown 1983).

Other effects of iron limitation on which few data exist for dinoflagellates are alterations to cellular compounds in which iron is either a constituent or required for biosynthesis. The role of iron in photosynthesis is essential. It is a component of photosynthetic electron transfer (PET) reactions in the form of cytochromes and iron-sulfur proteins (e.g. ferredoxin), and is necessary for the biosynthesis of chlorophyll pigments (see Reuter and Peterson 1987). Previous investigations of iron-mediated changes in algal pigments and characteristics associated with PET have employed diatoms and chrysophytes (Glover 1977, Sakshaug and Holm-Hansen 1977), cyanobacteria (Guikema and Sherman 1983,
Sandmann and Malkin 1983), and a chlorophyte (Verstreate et al. 1980). The present study examines the effect of iron limitation and depletion on chlorophyll a quota \(Q_{chl}\) and PET processes (as determined by in vivo fluorescence properties) in semi-continuous and batch cultures of a dinoflagellate. Further, to provide additional support for the specificity of iron stress-mediated changes in these variables, the same measurements were made on batch cultures grown into nitrogen depletion. As nitrogen is a constituent of both chlorophyll pigments and PET components, variations similar to those occurring under iron stress might be expected.

Alterations in the photosynthetic apparatus and general cell ultrastructure as a result of iron deficiency have been reported for several algal species (e.g. Meisch et al. 1980, Douglas et al. 1986, Hilt et al. 1987) including one dinoflagellate (Chapter 4). Such information allows a more direct structural interpretation of an organism's biochemical or physiological response to a given stimulus. As a final part of this study the ultrastructure of iron-replete and iron-deplete G. sanguineum cells was examined. Observations are compared with results from the literature, including those presented in Chapter 4 for another red tide dinoflagellate (Protogonyaulax tamarensis), and also considered with regard to chl a and in vivo fluorescence data.
MATERIALS AND METHODS

General culture maintenance. Stock cultures of Gymnodinium sanguineum (culture #D354, North East Pacific Culture Collection, Dept. of Oceanography, University of British Columbia) were maintained on filter-sterilized (Millipore 0.45 μm), ESAW-enriched artificial seawater (Harrison et al. 1980) with several adjustments to the original medium. Silicon was omitted while Na₂glycerophosphate and FeNH₄(SO₄)₂·6H₂O were replaced by equimolar concentrations of Na₂HPO₄ and FeCl₃·6H₂O, respectively. Na₂MoO₄·2H₂O was added at a concentration of 0.52 μM. Deionized distilled water (DDW) and reagent grade chemicals were used in preparing salt and nutrient enrichment solutions. Culture vessels employed throughout this research were soaked in freshly-made 10% HCl (v/v) for at least 2-3 days and rinsed thoroughly with DDW prior to use. All cultures (i.e. stock and experimental) were grown at 17°C without stirring, due to this species' sensitivity to physical perturbation. Continuous illumination was supplied by eight Vita-Lite® UHO fluorescent tubes (four on either side of culture vessels) filtered through 3 mm thick blue Plexiglas® (No. 2069, Rohm and Haas) at an irradiance of 145 μE·m⁻²·s⁻¹ (saturating for growth of G. sanguineum, Appendix 1).

Iron and nitrogen depletion. Media used to achieve iron (-Fe ESAW) or nitrogen (-N ESAW) depletion were modifications of stock maintenance ESAW with residual trace metal
contamination minimized by treatment with Chelex 100 ion exchange resin (Morel et al. 1979). Iron was omitted from -Fe ESAW. EDTA was combined with the remaining trace metals and its concentration reduced to provide an EDTA:trace metal ratio of 1.6. -N ESAW contained no added nitrogen. Media were sterilized by autoclaving after adjusting the pH to ca. 5.5 using Suprapur® HCl (Merck). After sterilization, pH was re-equilibrated to ca. 8.0-8.1 by bubbling with sterile (Millipore 0.22 μm) air. Chelexed, filter-sterilized (Millipore 0.22 μm) NaHCO₃ was added at a final concentration of 2 mM after autoclaving to avoid possible carbon limitation at high batch culture cell densities.

Iron (triplicate cultures) and nitrogen (single culture) depletion experiments were carried out in 2.8 l polycarbonate (PC) Fernbach flasks and initiated by inoculating with early stationary phase stock cultures to ca. 100 cells·ml⁻¹. Continuous bubbling with sterile (Millipore 0.22 μm) air or 1-2% CO₂ was required to control changes in pH (Appendix 2). Several variables, including cell density (CD), average cell volume (CV), chlorophyll a (chl a), and in vivo fluorescence (F) and DCMU-enhanced F (F_D), were monitored during the course of an experiment. Values for nutrient-deplete (-Fe or -N) cultures correspond to exhaustion of the growth-limiting nutrient as determined by no change or a decrease in CD on successive days. Depletion of the desired nutrient was confirmed for both -Fe (Appendix 2) and -N cultures by
bioassay and, in the case of the latter, by monitoring ambient NO₃ concentrations.

**Iron-limited growth.** Preparation of media was the same as described for iron depletion experiments except that following sterilization pH was allowed to re-equilibrate (ca. 8.0-8.1) during dark storage at 4°C, and ESAW NaHCO₃ concentrations were not supplemented. Also, the following changes in EDTA and trace metal enrichments were made. Six types of ESAW media, with EDTA and trace metals based on the formulation of Brand et al. (1983), were designed to achieve a range of iron-limited (as well as iron-replete) growth conditions by varying iron free ion activities. EDTA and iron (FeCl₃·6H₂O) were combined in single solutions with iron adjusted to provide final total concentrations (Fe_total) of 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ and 10⁻⁴ (= iron-replete) M. The remaining trace metals were prepared as one stock solution without EDTA. Iron and other trace metal free ion activities were buffered by maintaining the final EDTA concentration at 10⁻⁴ M. Transition metal free ion activities for each of the six media types (Table 1.1) were calculated using the chemical equilibrium program MINEQL (Westall et al. 1976) and the stability constants of Ringbom (1963) for an ionic strength of 0.7 at pH 8.1. Computations take into account all changes in original ESAW enrichments and thus provide reasonable estimates of pFe (i.e. negative log Fe free ion activity) for this system based on the conditions and stability constants invoked. Changes in pFe due to adsorption of free ions (Fe)
Table 1.1. Molar free ion activities of metals added to iron-limited growth media. Calculations were performed with MINEQL (Westall et al. 1976) and are based on the stability constants of Ringbom (1963). $10^{-4}$ M EDTA is present in all media.

<table>
<thead>
<tr>
<th>$pF_{e\text{total}}$</th>
<th>$pF_{e \text{**}}$</th>
<th>$pMn$</th>
<th>$pZn$</th>
<th>$pCu$</th>
<th>$pCo$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>16.0</td>
<td>7.6</td>
<td>9.6</td>
<td>12.9</td>
<td>10.4</td>
</tr>
<tr>
<td>5.0–9.0†</td>
<td>18.2–22.2‡</td>
<td>8.4</td>
<td>10.9</td>
<td>14.2</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* Negative log of total iron concentration.
** Negative log of iron free ion activity.
† Range includes individual values of 5.0, 6.0, 7.0, 8.0, 9.0.
onto polycarbonate culture vessels was assumed to be negligible. It should be recognized that the maximum error in pFe values will be associated with the lowest and highest iron additions due to the assumption of no iron contamination and to the addition of equimolar iron and EDTA, respectively. The latter, more poorly buffered system, exhibits a greater tendency for iron precipitation and higher free ion activities of other transition metals (see Table 1.1).

Experiments were run in duplicate as semi-continuous cultures (85 ml polycarbonate Oak Ridge tubes, Nalgene) established by transferring iron-deplete cells into each of the six iron concentrations. Cultures were maintained in early to mid-exponential phase (ca. 100-600 cells·ml⁻¹) by dilution with fresh medium, and specific growth rates calculated based on changes in cell density. Growth rates for each pFe represent an average of 6-12 sequential growth curves among which doubling times varied by less than ca. 15%. Kinetic parameters of maximum growth rate (\( \mu_{\text{max}} \)) and half-saturation constant for growth (\( K_{\mu} \)) were estimated from a Hanes-Woolf linear transformation given in Equation (1):

\[
\frac{S}{\mu} = \frac{K_{\mu}}{\mu_{\text{max}}} + \left(\frac{1}{\mu_{\text{max}}}\right) \cdot S,
\]

where \( S \) = molar substrate concentration, \( \mu \) = specific growth rate, \( K_{\mu} = S \) at half-maximal growth rate, and \( \mu_{\text{max}} \) = maximal growth rate. Approximate steady-state determinations of chl
a, F, and F_D were made twice on duplicate cultures of each pFe following an acclimation period of at least 6-7 generations.

Analytical methods. Cell counts were performed on a Coulter Counter model TAI1 (200 μm aperture, 44.2 μm calibration spheres), with data for average cell volume obtained simultaneously from a particle size distribution based on equivalent spherical diameter. Samples were homogenized (i.e. mixed) prior to counting by gentle inversion. Chl a concentration of filtered samples (gravity filtration, Whatman 934-AH) was determined fluorometrically (Holm-Hansen et al. 1965) in 90% acetone extracts (20 h, 4°C) using a Turner Designs model 10 fluorometer fitted with the following set of Corning filters: 3-66, reference; 5-60, excitation; 2-64, emission. It should be noted that grinding filters prior to extraction was unnecessary as ground extracts produced similar or slightly lower yields. In vivo F and F_D measurements of dark-equilibrated (20 min, see Loftus and Seliger 1975) culture aliquots employed the fluorometer and filters used for chl a determinations. Samples for F_D were treated with 10^-5 M (final concentration) 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU) prior to dark equilibration. Readings were taken following 30 s exposure to the fluorometer light source. Ambient NO_3 concentrations (NO_3 + NO_2) in -N batch culture were measured with a Technicon Autoanalyzer II according to the procedure of Wood et al. (1967).
Ultrastructure. Iron-replete and iron-deplete cultures were sampled for transmission electron microscopy (TEM) by collecting cells on 2 μm Millipore filters (type BS) and processing as follows: primary fixation with 1.5% glutaraldehyde in 0.1 M sodium cacodylate and 0.4 M sucrose (2 h, room temp.), and post-fixation with 1% osmium tetroxide in 0.1 M sodium cacodylate (1 h, room temp.). Samples were en bloc stained using 1% aqueous uranyl acetate, dehydrated in a graded ethanol/propylene oxide series, and embedded in Epon 812. A diamond knife was used to cut random and serial sections which were picked up on formvar-coated 50-mesh copper grids, stained with saturated uranyl acetate (in 50% methanol) and lead citrate, and examined in a Zeiss EM10C transmission electron microscope.

RESULTS

Iron-limited growth. Specific growth rates (μ) of G. sanguineum as a function of iron free ion activity are presented in linear (Fig. 1.1A) and semi-log (Fig. 1.1B) plots. The more conventional linear graph, including all iron concentrations examined (note scale break between two highest concentrations), demonstrates the hyperbolic relationship between μ and substrate concentration (S). A semi-log plot of these data shows more clearly that the most critical S interval occurs between pFe 20.2 (Fe_{total} = 10^{-7}) and 21.2 (Fe_{total} = 10^{-8}), over which μ declined from 0.27 to 0.13 d^{-1}. This difference in μ is at least twice that observed between
Figure 1.1. Linear (A) and semi-log (B) plots of growth rate as a function of iron free ion activity for G. sanguineum. Molar iron free ion activities in (A) may be determined by multiplying the plotted value by $6.3 \times 10^{-20}$. Error bars = ± 1 S.D. ($n = 6$ to 12), and are smaller than symbol where not apparent.
any other pair of adjacent $S$ values. The kinetic parameters for iron-limited growth were derived from a Hanes-Woolf linear transformation which plots $S/\mu$ against $S$ ($r^2 = 0.999$). The maximum growth rate ($\mu_{\text{max}}$) is $0.38 \text{ d}^{-1}$, while the estimated half-saturation constant ($K_\mu$) is $1.7 \cdot 10^{-20} \text{ M}$.

With the exception of the fluorescence index $1-F/F_D$, all variables and their ratios remained essentially constant above $pFe \ 20.2$ but changed rapidly as iron free ion activity decreased from $pFe \ 20.2$ to 21.2. While average cell volume (Fig. 1.2) declined by 35% over this interval, $Q_{\text{chl}}$ (Fig. 1.3) was reduced by half. Conversely, both $F$ and $F_D$ normalized per unit chl a (Fig. 1.4A) were ca. two-fold greater at $pFe \ 21.2$ than at 20.2. $1-F/F_D$ (Fig. 1.4B) showed a generally decreasing trend with increasing iron stress. Ratios for severely Fe-limited cells ($pFe \ 21.2$ and 22.2) were 20-30% below those exhibited under iron-replete conditions ($pFe \ 16.0$).

Iron and nitrogen depletion. Iron depletion (i.e. terminal point of a batch culture) caused all cellular characteristics monitored (Figs. 1.2-1.4) to change in the same relative direction as with increasingly Fe-limited growth (i.e. semi-continuous cultures) below $pFe \ 20.2$. Iron-deplete and the most Fe-limited cells ($pFe \ 22.2$) differed to the largest extent (ca. two-fold) in $Q_{\text{chl}}$ (1.6 and $2.9 \cdot 10^{-1}$ g·liter cell vol$^{-1}$, respectively; Fig. 1.3) and $F/$chl a (320 and 150, respectively; Fig. 1.4A).
Figure 1.2. Average cell volume of *G. sanguineum* as a function of iron free ion activity, and iron or nitrogen depletion. In Figs. 1.2-1.4: iron- (-Fe) and nitrogen- (-N) deplete cultures are plotted after the scale break; n = 2 for Fe-limited data, n = 3 for Fe-deplete point, n = 1 for N-deplete point; error bars = ±1 S.D. and are smaller than symbol where not apparent for n ≥ 2.
Figure 1.3. Chlorophyll a quota of G. sanguineum as a function of iron free ion activity, and iron or nitrogen depletion. Symbol labels, sample size and error bars as in Fig. 1.2.
Figure 1.4. In vivo fluorescence (F) and DCMU-enhanced fluorescence ($F_D$) expressed per unit chl a (A), and $1-F/F_D$ (B) of $G$. sanguineum as a function of iron free ion activity, and iron or nitrogen (◊,□,○) depletion. Symbol labels, sample size and error bars as in Fig. 1.2.
Comparison of iron- and nitrogen-deplete cells showed similar measurements of CV (Fig. 1.2) and $Q_{\text{chl}}$ (Fig. 1.3). The most notable distinction between these two nutrient-stressed conditions occurred in ratios of which F and, to a much lesser degree, $F_D$ were components. $F/\text{chl }a$ (Fig. 1.4A) and $F_D/\text{chl }a$ (Fig. 1.4A) exceeded N-deplete values by 2.5-fold (ca. 160%) and 1.6-fold (ca. 60%), respectively, while $1-F/F_D$ (Fig. 1.4B) was 40% lower for Fe-deplete cells. Furthermore, the $F/\text{chl }a$ ratio of N-deplete cells (125, Fig. 1.4A) was ca. 20% less than for the most Fe-limited condition (pFe 22.2), but still over 2.5-fold greater than for nutrient sufficiency (pFe 16.0). Nitrogen depletion resulted in a less than 10% decrease of $1-F/F_D$ below the nutrient-replete index (i.e. from 0.68 to 0.62) as compared to 25% (0.51) and 43% (0.39) for severe Fe limitation (avg. for pFe 21.2 and 22.2) and Fe depletion, respectively (Fig. 1.4B).

In the absence of available chl $a$ and in vivo fluorescence data for N-limited cells, Figs. 1.5 and 1.6 show changes in ratios of these variables during batch culture growth into nitrogen depletion (-N). Comparable data for iron depletion (-Fe) were also obtained (Figs. 1.5, 1.6). Ambient NO$_3$ was undetectable in the -N culture by Day 9 (Fig. 1.5). Thus, chl $a$ and in vivo fluorescence measurements on Day 12 are representative of moderately N-deficient conditions (i.e. continuing growth in the absence of ambient NO$_3$) while those of Day 14 are associated with either severe N deficiency or depletion (i.e. cessation of cell division). Under conditions
Figure 1.5. Changes in cell density over time for *G. sanguineum* batch cultures grown into iron (-Fe) or nitrogen (-N) depletion. Concurrent changes in ambient NO$_3$ concentration are shown for the -N culture.
Figure 1.6. Plots of in vivo fluorescence (F)/chl a (A), DCMU-enhanced F (F_D)/chl a (B), and 1-F/F_D (C) over time for G. sanguineum batch cultures (shown in Fig. 1.5) grown into iron (-Fe) or nitrogen (-N) depletion. Values plotted for each culture are mean ± 1 S.D. for duplicate determinations of each variable; error bars are smaller than symbol where not apparent.
of moderate N deficiency, \(F/\text{chl} \ a\) (Fig. 1.6A) and \(F_D/\text{chl} \ a\) (Fig. 1.6B) were only ca. 25% greater (Day 12: \(F/\text{chl} \ a\), 65; \(F_D/\text{chl} \ a\), 165) than for logarithmically growing cells (Day 4; lag phase still apparent on Day 2, Fig. 1.5). By Day 14 more notable increases in both ratios, to within ca. 15% of N-deplete values (Day 16: \(F/\text{chl} \ a\), 125; \(F_D/\text{chl} \ a\), 325), had occurred. Of particular interest were the minimal changes in \(1-F/F_D\) (Fig. 1.6C), which remained between 0.58 and 0.69 irrespective of culture nitrogen status. In contrast to N stress, \(F/\text{chl} \ a\) (Fig. 1.6A) and \(F_D/\text{chl} \ a\) (Fig. 1.6B) of the -Fe culture began to rise steadily during mid-exponential phase (Day 9), with both ratios exceeding N-deplete measurements prior to stationary phase (Day 15: \(F/\text{chl} \ a\), 155; \(F_D/\text{chl} \ a\), 355). \(1-F/F_D\) (Fig. 1.6C) also exhibited a similar (but directionally opposite, i.e. decreasing) trend (Day 15, 0.56) although its response lagged those of \(F/\text{chl} \ a\) and \(F_D/\text{chl} \ a\) by one sampling period (i.e. 3 days). Ambient iron concentrations, and thus estimates of iron status, were not determined during -Fe culture growth.

Ultrastructure. Iron-replete protoplasts (Fig. 1.7) were characterized by extensive vacuolar space interspersed with regions of cytoplasm. A predominant feature was the nucleus, which contained numerous permanently-condensed chromosomes and exhibited a granular layer associated with its double-membrane envelope (enlargement not shown herein; for morphological details see Stone and Vesk 1982). Another distinctive structure was the pusule system (Fig. 1.8). This reticulate
Figures 1.7-1.13. Iron-replete Gymnodinium sanguineum. NEPCC culture #D354. Fig. 1.7. Longitudinal section of protoplast showing the nucleus (N), an accumulation body (A), and cortical starch inclusions (S). Chloroplasts appear throughout the cell. Note vacuolate nature of cytoplasm. Scale = 10 μm. Fig. 1.8. Section exhibiting reticulate vacuole of pusule system (P) and peripheral regions of both flagellar openings (arrowheads). Note vesicle activity associated with flagellar canal (arrows). Scale = 2 μm. Fig. 1.9. Site of flagellar insertion with basal regions of both flagella (arrowheads) and one flagellar root (arrow) extending into adjacent cytoplasm. Note pusular vesicle (V) bordering on longitudinal flagellar canal (F). Scale = 1 μm. Fig. 1.10. Cross-section of flagellar canal (F) and associated pusular vesicles (V). Flagellar axoneme (arrowhead) and microtubule network (arrows) surrounding the canal are apparent. Scale = 0.5 μm. Fig. 1.11. Area containing extensive rough ER (ER), with several chloroplasts (C) and mitochondria (M) comprising characteristic close association of organelles. Note tubular cristae of mitochondria and similarity of matrix electron density to that of cytoplasm. Scale = 1 μm. Fig. 1.12. Portion of accumulation body with tightly packed contents showing "fingerprint"-like pattern. Scale = 1 μm. Fig. 1.13. High magnification of chloroplast lamellae (L) consisting of two or three closely appressed thylakoids. Lamellae are highly organized with no evidence of thylakoid degeneration (cf. Figs. 1.15,1.16). Scale = 0.2 μm.
vacuolar network surrounded the area of flagellar insertion (Fig. 1.9). Whether the pusule system comprised one or two pusules (see Taylor in press), could not be distinguished. The presence of vesicles was observed throughout the pusule system, but was concentrated more toward the cell surface (Figs. 1.8, 1.10) and around the longitudinal flagellar canal (Fig. 1.9). Numerous chloroplasts and mitochondria, as well as dense aggregates of endoplasmic reticulum (most notably rough ER), occurred throughout the cell, while starch and lipid reserves were restricted primarily to cortical regions (Figs. 1.7, 1.11). One or two accumulation bodies, often comprising tightly-packed material in a "fingerprint"-like pattern, were present in some cells (Figs. 1.7, 1.12). Organelles were generally grouped closely within small areas of cytoplasm due to the vacuolate nature of the protoplast (Figs. 1.7, 1.11). Chloroplasts exhibited lamellae consisting of two or three appressed thylakoids (Fig. 1.13). Orientation of lamellae was variable, ranging from closely parallel to severely undulating. Mitochondria contained tubular cristae within a granular matrix, similar in electron density to the surrounding cytoplasm (Fig. 1.11).

Iron-deplete protoplasts (Fig. 1.14) were considerably different from those growing under iron-replete conditions (Fig. 1.7), as demonstrated by comparing similar whole-cell longitudinal sections. Most obvious was a decline in the cytoplasm to vacuole ratio. Vacuolar regions occupied much of the cell periphery and appeared to be compartmentalized by the
Figures 1.14-1.17. Iron-deplete Gymnodinium sanguineum. NEPCC culture #D354. Fig. 1.14. Longitudinal section of protoplast containing nucleus (N) and highly vacuolate cytoplasm. Cytoplasmic area and general organelle abundance (e.g. chloroplasts) are sharply reduced from the iron-replete condition (cf. Fig. 1.7). Vacuolar regions appear divided by the tonoplast (arrows). Scale = 5 μm. Fig. 1.15. Association of organelles showing chloroplasts with "normal" (arrows; note thylakoids occurring only in pairs) and structurally disrupted (arrowheads) lamellae (cf. Fig. 1.13). Mitochondria (M) exhibit a reduction in matrix electron density relative to cytoplasm (cf. Fig. 1.11). Scale = 1 μm. Fig. 1.16. Enlargement of chloroplast containing few, dilated thylakoids. (arrowheads). Scale = 0.2 μm. Fig. 1.17. Accumulation body with loosely arranged contents exhibiting no apparent order (cf. Fig. 1.12). Scale = 1 μm.
tonoplast (Fig. 1.14). Organelles remained in close association within the available cytoplasm; however, the abundance of ER was much reduced (Fig. 1.15). Also characteristic of Fe-deplete cells was a notable (albeit unquantified) decrease in chloroplast number (Fig. 1.14). Thylakoids occurred in pairs or singly (cf. Figs. 1.11, 1.13). Degenerative structural changes were evident in the separation of adjacent thylakoids (Fig. 1.15) and the dilation of individual thylakoids (Fig. 1.16). Many chloroplast lamellae (and their constituent thylakoids) did, however, retain a "normal" appearance (Fig. 1.15), and no clear difference in the number of lamellae per chloroplast was discernable. Alterations in mitochondrial morphology associated with iron depletion appeared predominantly as reductions in electron density of the matrix (Fig. 1.15, cf. Fig. 1.11). Accumulation body contents of Fe-deplete cells (Fig. 1.17) were loosely arranged and showed no pattern or structural organization (cf. Fig. 1.12).

DISCUSSION

Iron-limited growth kinetics. Growth rates of several coastal diatom species and a neritic coccolithophorid, as a function of iron bioavailability, have been examined previously (Brand et al. 1983, Harrison and Morel 1986). The current work is the first to examine iron-limited growth in a coastal red tide dinoflagellate, *G. sanguineum*. Although meaningful comparisons are possible among these data, it is
first necessary to account for differences between pFe values given in Brand et al. (1983), and those provided herein and also by Harrison and Morel (1986). Briefly, equilibrium models in seawater employ a series of interdependent calculations to estimate concentrations of chemical species. These thermodynamic models are formulated sequentially according to the major ionic composition of the medium, interactions among these components, and chemical reactions involving trace constituents (Kester 1986). Metal free ion activities are determined largely by how a system is defined (e.g. ionic strength, pH, etc.) and the stability constants of chemical species present, and can vary considerably depending on the data input to satisfy these criteria. Media formulations used in the present study and by Brand et al. (1983) are very similar; however, estimates of iron free ion activities based on the stability constants of Ringbom (1963) and Sillen and Martell (1964), respectively, differ by an order of magnitude (higher in the former case). For purposes of this discussion pFe calculations of Brand et al. (1983) will be considered as ten-fold underestimates to allow direct comparison of kinetic constants for iron-limited growth. In other words, while erroneous values are not inferred for either study, pFe 20.2 of Brand et al. (1983), for example, is taken as equivalent to pFe 19.2 herein. Values of pFe given by Harrison and Morel (1986) are roughly equivalent to those determined herein, as free ion activities of both media were
calculated using MINEQL (Westall et al. 1976) with similarly defined systems and the stability constants of Ringbom (1963).

Iron-limited growth of *G. sanguineum* is similar to that reported for other neritic phytoplankton (Brand et al. 1983, Harrison and Morel 1986) in that all growth rates begin to decline rapidly below pFe 20.2. However, a more critical comparison of these data is possible based on the kinetic parameters of $\mu_{\text{max}}$ and $K_\mu$. These parameters were estimated herein and by Harrison and Morel for *Thalassiosira weissflogii* using a Hanes-Woolf linear transformation. Although Brand et al. (1983) did not calculate these growth constants for the nine coastal species examined, I determined values of $\mu_{\text{max}}$ and $K_\mu$ from $S/\mu$ vs. $S$ (i.e. Hanes-Woolf) plots of the numerical data provided by these authors. It was assumed that the maximum growth rate had been achieved for these species as $\mu$ varied by 10% or less between the two highest substrate concentrations in all but one case, which was not considered.

Estimated values of $\mu_{\text{max}}$ and $K_\mu$ for the iron-limited growth of ten neritic phytoplankton species are given in Table 1.2. Maximum growth rates range four-fold from 0.38 (*G. sanguineum*) to 1.54 (*T. weissflogii*) $\text{d}^{-1}$. However, the molar free ion concentration which limits growth by 50% (i.e. $K_\mu$) of the dinoflagellate is 10-1000 times greater than calculated for the other predominantly diatom species. This large disparity in $K_\mu$, although initially unexpected, may be better understood by examining minimum iron requirements of
Table 1.2. Estimates of $\mu_{\text{max}}$ (d$^{-1}$) and $K_\mu$ (M) for iron-limited growth of ten neritic phytoplankton species using a Hanes-Woolf linear transformation ($S/\mu$ vs. $S$).

<table>
<thead>
<tr>
<th>Species</th>
<th>$\mu_{\text{max}}$</th>
<th>$K_\mu$</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dinophyceae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gymnodinium sanguineum</td>
<td>0.38</td>
<td>$1.7 \cdot 10^{-20}$</td>
<td>1*</td>
</tr>
<tr>
<td><strong>Bacillariophyceae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asterionella glacialis</td>
<td>1.20</td>
<td>$1.7 \cdot 10^{-22}$</td>
<td>2**</td>
</tr>
<tr>
<td>Bacteriastrum hyalinum</td>
<td>0.90</td>
<td>$1.8 \cdot 10^{-22}$</td>
<td>2</td>
</tr>
<tr>
<td>Ditylum brightwellii</td>
<td>0.96</td>
<td>$1.0 \cdot 10^{-22}$</td>
<td>2</td>
</tr>
<tr>
<td>Lithodesmium undulatum</td>
<td>1.02</td>
<td>$6.2 \cdot 10^{-22}$</td>
<td>2</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>1.37</td>
<td>$1.2 \cdot 10^{-22}$</td>
<td>2</td>
</tr>
<tr>
<td>Streptotheca tamesis</td>
<td>1.00</td>
<td>$1.7 \cdot 10^{-22}$</td>
<td>2</td>
</tr>
<tr>
<td>Thalassiosira pseudonana</td>
<td>1.16</td>
<td>$3.7 \cdot 10^{-23}$</td>
<td>2</td>
</tr>
<tr>
<td>Thalassiosira weissflogii</td>
<td>1.54</td>
<td>$1.1 \cdot 10^{-21}$</td>
<td>3†</td>
</tr>
<tr>
<td><strong>Haptophyceae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hymenomona carterae</td>
<td>0.68</td>
<td>$9.0 \cdot 10^{-23}$</td>
<td>2</td>
</tr>
</tbody>
</table>

*1 - this study
**2 - Brand et al. (1983): pFe values given were recalculated using MINEQL (Westall et al. 1976) and the stability constants of Ringbom (1963) prior to linear transformation (see text for explanation)
†3 - Harrison and Morel (1986)
the two species for which such data are available, 
G. sanguineum (Chapter 2) and T. weissflogii (Harrison and 
Morel 1986).

The carbon content per unit cell volume is inherently 
different between dinoflagellates and diatoms (Chan 1978, 
1980). Thus, minimum iron quotas ($Q_{Fe_{min}}$) expressed per unit 
cell carbon are likely the most valid means of comparing iron 
requirements. Because carbon quotas ($Q_C$) associated with 
$Q_{Fe_{min}}$ were not provided by Harrison and Morel (1986), $Q_C$ of 
log phase cells for the same T. weissflogii clone (Blasco et 
al. 1982) was used in the calculation. Further, if the iron 
stress resulting in $Q_{Fe_{min}}$ reduces $Q_C$ of T. weissflogii, as 
noted for G. sanguineum (16%, NO$_3$-grown cells; Chapter 2), 
Fe:C ratios may be slightly underestimated in the former 
species. Fe:C (by atoms) values of $1.1 \cdot 10^{-4}$ and $7.6 \cdot 10^{-7}$ 
obtained for G. sanguineum and T. weissflogii, respectively, 
indicate that the minimum cellular iron requirement of this 
red tide dinoflagellate may exceed that of a coastal diatom by 
over 100-fold. It should be noted that in an earlier study of 
T. weissflogii iron nutrition, Anderson and Morel (1982) 
obtained a $Q_{Fe_{min}}$ 4-9 times greater than reported by Harrison 
and Morel (1986). Nevertheless, it would appear that the 
order of magnitude difference in $K_\mu$ for Fe-limited growth 
between G. sanguineum and T. weissflogii may be related to the 
correspondingly large cellular iron requirement of the former 
species.
Cell volume, chl a quota, and in vivo fluorescence. CV, Qchl, and F and F_D/chl a determinations are consistent with those of growth rate in identifying pFe 20.2-21.2 as the most critical range of substrate concentrations. Changes in these variables were clearly associated with iron limitation. However, comparable trends (i.e. increases or decreases) have been observed with other forms of environmental stress and, in certain cases, the possibility of a more generalized response to lower growth rates must also be considered. For example, while iron depletion reduced CV by ca. 60%, nitrogen depletion or low irradiance levels (Appendix 1) can cause CV to decline by over 50% in G. sanguineum. While the response of cell characteristics derived from chl a and in vivo fluorescence measurements can also be similar under various types of nutrient deficiency (e.g. Sakshaug and Holm-Hansen 1977), physiological mechanisms specific to the limiting nutrient are likely responsible. By comparing iron and nitrogen stress-mediated variation in these characteristics for G. sanguineum and other species in the literature, the specific effects of Fe stress can be discerned with more confidence.

The current research shows that depletion of either iron or nitrogen in G. sanguineum lowers Qchl to 20-25% of nutrient-sufficient levels. Chl a is a magnesium-containing metalloporphyrin derived from the tetrapyrrrole biosynthetic pathway (Granick and Beale 1978). Iron and nitrogen participate in porphyrin biosynthesis as cofactors of certain enzymes (Fe only) and constituents of products (N only).
occurring in the pathway up to and including chl a (see Pushnik et al. 1984). Thus, it is not surprising that available data (e.g. Glover 1977, Guikema and Sherman 1983, Reuter and Ades 1987), including studies of Fe and N stress in a single species (Sakshaug and Holm-Hansen 1977, this study), demonstrate reduced $Q_{\text{chl}}$ under either Fe or N deficiency for representatives of five algal groups.

The active heme prosthetic group of photosynthetic electron transfer (PET) cytochromes is an iron metalloporphyrin and also a product of the tetrapyrrole biosynthetic pathway responsible for chl a synthesis. In addition, PET components include non-heme forms of Fe comprising iron-sulfur proteins such as ferredoxin (Jensen 1986). Given the involvement of Fe and N in the biosynthesis of PET cytochromes and Fe-S proteins, and the functional relationship between PET reactions and in vivo fluorescence (Lawlor 1987), depletion of either nutrient might be expected to effect similar changes in F/chl a. Indeed, previous laboratory results generally demonstrate a two- to four-fold increase in this ratio above nutrient-sufficient values (e.g. Kiefer 1973, Sakshaug and Holm-Hansen 1977, Guikema and Sherman 1983), irrespective of limiting nutrient (i.e. Fe or N). However, considering the extreme cases of nutrient depletion in the present work, F/chl a of Fe-deplete G. sanguineum increased seven times compared to less than three-fold for N-deplete cells. Even under conditions of severe nutrient limitation (as opposed to depletion, cf. Fig.
1.6) F/chl a was ca. two times greater for Fe than N stress. Sakshaug and Holm-Hansen (1977) also observed larger Fe stress-mediated increases in this ratio compared to those associated with N depletion for the diatom *Skeletonema costatum*. Differences in F/chl a, coupled with similar chl a quotas for Fe- and N-stressed *G. sanguineum*, indicate a greater specific Fe stress-mediated response of *in vivo* fluorescence and thus PET activity.

Among the factors which may contribute to increases in F/chl a are enhanced light energy capture per unit pigment and/or reduced efficiency of light energy transfer to photochemical reaction centers and of electron transport within the PET system. It is well documented that Fe-limited growth reduces both quantities of PET components (i.e. cytochromes and Fe-S proteins) and activity of the PET system in algae (Glover 1977, Sandmann and Malkin 1983, Sandmann 1985) and higher plants (Spiller and Terry 1980, Terry 1983). These specific detrimental effects on PET processes may be sufficient to account for the difference in F/chl a between Fe- and N-deplete *G. sanguineum*. Fluorescence may be further augmented by more efficient capture of light energy (i.e. increased specific absorption coefficient) by chl a through iron stress-mediated reductions in Q_{chl} and thus self-shading (cf. N-limited *Chaetoceros gracilis*, Cleveland and Perry 1987).
Another potentially important consideration is evidence indicating that iron deficiency preferentially reduces the electron and light energy transfer components and capacity of Photosystem I (PSI) relative to Photosystem II (PSII) (Öquist 1974, Nishio et al. 1985, Sandmann 1985). When PSII is reduced, fluorescence is emitted from chlorophyll in $10^{-9}$ s ($F$ is slower for oxidized PSII). By comparison, electron transport from the reaction centers of PSI occurs in $10^{-12}$ s (Lawlor 1987). Back reactions of photochemical events associated with PSI thus account for less than 10% of (room temperature) in vivo fluorescence measurements (Prézelin 1981). The enhanced effect of iron stress on PSI would likely lower both its energy and electron transfer efficiency, potentially increasing the contribution of PSI to the fluorescence signal. In this regard, work by Ley (1980) on the red alga *Porphyridium cruentum*, demonstrating that PSI normally receives about twice as much light energy as PSII (irrespective of wavelength), may also be of consequence. However, the ratio of photon flux into PSI and PSII is not constant and is thought to be optimized by organizational changes in the photochemical apparatus. Such alterations can occur on time scales ranging from seconds or minutes (e.g. State I-State II transitions) to days (e.g. changes in thylakoid morphology, see Ultrastructure below) (Barber 1985). While the possibility and significance of iron stress-mediated elevated PSI fluorescence remain unknown, its occurrence would
represent an additional in vivo F source comprising the large Fe-deficient F/chl a ratios of *G. sanguineum*.

In a recent study of the diatom *Ch. gracilis*, Cleveland and Perry (1987) suggested that increases in F/chl a associated with reduced nitrogen quotas resulted from both a rise in the specific absorption coefficient of chl a (due to reduced self-shading as chl a per cell decreased) and an uncoupling of photosynthesis (as reflected by lower quantum yields). Interestingly, the authors found no changes in the efficiency of energy transfer between the accessory pigment fucoxanthin and chl a, although fucoxanthin:chl a ratios increased. While similar mechanisms may help to explain the F/chl a increase of N-deficient *G. sanguineum*, changes associated with iron stress are clearly more effective mediators of elevated in vivo fluorescence in this species.

Measurements of F<sub>D</sub>/chl a, to the extent that F<sub>D</sub> approximates the potential maximum in vivo fluorescence (F<sub>max</sub>), provide further evidence that fluorescence properties of *G. sanguineum* are more sensitive to iron than nitrogen deficiency. Application of the herbicide DCMU blocks PET on the acceptor side of PSII, prohibiting further re-oxidation of PSII and thus electron flow between PSII and PSI. As a result, in vivo fluorescence increases to a larger (ca. maximum, Falkowski and Kiefer 1985) steady-state value (F<sub>D</sub>) considered to represent the energy otherwise available for PSII photochemistry (Prézelin 1981). If F<sub>max</sub> under Fe and N
stress were similar, little difference in $F_D/\text{chl a}$ would be expected. Slovacek and Hannan (1977) have demonstrated previously that fluctuations in $F/\text{chl a}$ induced by various forms of nutrient limitation can be eliminated by DCMU addition. However, exceptions to a constant fluorescence yield per unit chl a following DCMU application are not uncommon (e.g. Roy and Legendre 1979). In the case of *G. sanguineum*, variability between $F_D/\text{chl a}$ ratios was less than exhibited by those of $F/\text{chl a}$. Nevertheless, $F_D/\text{chl a}$ of Fe-deplete cells exceeded the N-deplete value by more than 1.5-fold, suggesting a greater $F_{\text{max}}$ under iron stress. Explanations similar to those proposed for large Fe-mediated $F/\text{chl a}$ ratios would also apply here.

Results of this study suggest that, while Fe and N are required for the production of both chl a and PET components, *G. sanguineum* can maintain biosynthesis of cytochromes and Fe-S proteins active in PET more successfully under nitrogen than iron stress. The biosynthetic pathways leading to chl a and heme are identical prior to metal insertion (chl a: Mg, heme: Fe) at which point they diverge (Castelfranco and Beale 1983). While data presented herein provide no biochemical evidence, it is interesting to speculate on the possibility of some N stress-mediated regulation operating at the point of divergence. Lower nitrogen quotas would favor the pathway to heme synthesis over that to chl a (under saturating irradiance), thereby reducing the relative effect of N stress on cytochrome production and thus PET activity. This branch
point in the tetrapyrrole biosynthetic pathway has been suggested as a potential regulatory site for the production of chlorophyll (see Granick and Beale 1978). In addition, Castelfranco and Jones (1975) have proposed that chlorophyll and heme biosynthesis share a common pool of precursor compounds. It is possible that iron stress more severely limits the supply of these intermediates (e.g. Marsh et al. 1963). This would explain why, that even if heme synthesis was also favored under Fe limitation, production of heme by N-stressed cells apparently remains greater. Decreased heme levels have also been induced by addition of Fe chelators (presumably reducing Fe availability) (Duggan and Gassman 1974), an effect thought to be mediated by rapid heme turnover rates (Castelfranco and Jones 1975).

In vivo fluorescence indices. In vivo fluorescence data (i.e. F and Fp/chl a) can be expressed as various indices which yield information largely about the operation of PSII. The following are three of these indices and the properties they are suggested to describe: 1) $F/F_D$, the proportion of captured light energy lost as fluorescence; 2) $1-F/F_D$, the proportion of absorbed light energy utilized by PSII (i.e. the efficiency of PSII); and 3) $F_D-F$, the relative output of PSII (i.e. the capacity of PSII) (Prézelin 1981, Droop 1985). It is assumed that PSI fluorescence is negligible. If this assumption were invalid (see above discussion), F would exhibit a proportionally greater increase than the larger $F_D$ signal. These indices would thus shift accordingly and their
applicability to strictly PSII processes would be questionable. The preceding discussion has demonstrated that F/chl a and F_D/chl a (upon which these indices are based) are quite variable depending on species, growth conditions, and physiological state. The above three fluorescence indices are therefore sensitive to similar effects, but would presumably be most useful in comparisons of different growth-limiting factors for one species under otherwise similar conditions (e.g. this study).

Data from the present work show increases in both F/chl a and F_D/chl a regardless of whether Fe or N limits growth. However, under conditions of Fe depletion, F increases to 60% of F_D (F/F_D = 0.61), while remaining below 40% of F_D for N depletion (F/F_D = 0.38). Conversely, the 1-F/F_D indices are 0.39 and 0.62 for Fe and N depletion, respectively. The F_D-F index (i.e. F_D/chl a-F/chl a) is equivalent for both Fe- and N-deplete cells (F_D-F = 200). Information provided by these in vivo fluorescence indices suggest that a greater proportion of harvested light energy is reemitted as fluorescence by iron-deplete G. sanguineum. Further, while the capacity of PSII is affected similarly by either Fe or N depletion, the former reduces the photochemical efficiency of PSII to ca. 60% of that maintained under the latter. Although such interpretations must be considered in light of the assumptions and limitations associated with these indices, they are indeed consistent with a more pronounced effect of iron over nitrogen stress on PET processes for this species.
Ultrastructure. The general ultrastructural features of iron-replete *G. sanguineum* are consistent with those described for other dinoflagellates (e.g. Dodge 1971). Of particular interest herein are changes in these characteristics associated with iron depletion and their relationship to the variation in chl a and *in vivo* fluorescence measurements discussed above. Perhaps the most obvious association is between the decline in $Q_{\text{chl}}$ and decreased chloroplast number. This observation eliminates the possibility that lower chl a quotas are strictly a result of smaller chloroplasts. The size of those remaining chloroplasts may also have been reduced, but quantitative comparisons of Fe-replete and -deplete chloroplast dimensions were not made. Fewer chloroplasts present in Fe-deplete cells supports the argument for an increase in the chl a specific absorption coefficient due to reduced self-shading.

Degeneration of the normally well-organized chloroplast lamellae and thylakoids did occur under iron stress in *G. sanguineum*, and similar effects have been reported for other algae (Meisch et al. 1980, Hardie et al. 1983, Chapter 4) and higher plants (Platt-Aloia et al. 1983). While functional interpretation of structural defects is generally lacking, the integrity of thylakoid membranes and their components clearly influences the efficiency of light harvesting as well as energy and electron transfer processes (Barber 1985). Thus, large *in vivo* fluorescence measurements of Fe-deplete cells may be at least partly explained by
changes observed in photosynthetic membranes. A characteristic of Fe-deplete chloroplasts, which may be an adaptation aimed at reducing the amount of harvested light energy lost as fluorescence, is a lesser degree of thylakoid appression as evidenced by fewer thylakoids (1-2) per lamella. Several investigators (see Baker and Webber 1987) have shown that decreased thylakoid stacking increases the efficiency of energy transfer between PSII and PSI (several mechanisms are currently postulated) thereby reducing fluorescence; however, other Fe-mediated changes effecting higher fluorescence yields apparently predominate in G. sanguineum. The fact that many lamellae appear "normal" (i.e. no structural disorders) suggests that alterations in photosynthetic components and processes are not always manifested as obvious ultrastructural deviations.

Other changes noted in Fe-stressed protoplasts, such as increased vacuolar area, decreased mitochondrial matrix density and ER abundance, and variation in accumulation body morphology, may be non-specific responses to reduced growth rates. Unfortunately, no N-deplete cells were examined for comparison, as in the case of chl a and in vivo fluorescence. Thus, even the iron stress-mediated specificity of chloroplast degeneration is not certain, although a study of Fe- and N-starved Agmenellum quadruplicatum (Cyanophyceae) (Hardie et al. 1983) demonstrated that altered thylakoid structure was restricted to the former. Apart from chloroplasts, mitochondria might also be expected to exhibit specific Fe-
mediated effects related to lower cytochrome production. Decreases in electron density of the matrix were encountered herein and for Fe-stressed Pro togonyaulax tamarensis (Chapter 4). Respiratory electron transport (RET) is associated with cristae membranes, which did not appear to change in either species. It is possible that reductions in RET components and activity, which remain to be demonstrated in Fe-deplete G. sanguineum, do not alter cristae structure. A considerable portion (over 70%) of RET subunit components extends beyond the plane of the inner mitochondrial membrane (Hackenbrock 1981), yet it seems unlikely that fewer RET cytochromes would affect the density of the entire matrix, as was observed. A more plausible explanation of lower matrix density in Fe-deplete mitochondria would be loss of TCA cycle constituents due to minimal energetic demands as growth rate declines.

Ecological considerations. Elucidation of nutritional factors affecting red tide population dynamics is essential to understanding the ecology of these natural phytoplankton blooms. To this end, indicators of nutrient limitation are of interest, as certain problems associated with their use may be minimized by the predominance of one or two species. Certain of these indicators exhibit considerable variation according to the nutrient and species in question. Indeed, a rigorous study of N and P limitation in five algal species (Healey and Hendzel 1979) revealed only two of fifteen compositional and metabolic variables to be generally useful indicators of limitation by either nutrient.
In addition to the implication of increased iron bioavailability in promoting bloom formation (see Background), a comparatively (cf. Brand et al. 1983, Harrison and Morel 1986) large iron requirement for two red tide dinoflagellates (Mueller 1985, this study, Chapter 4) further suggests the potential importance of this trace metal in red tide ecology. All characteristics of severely iron-limited cells monitored herein (pFe 21.2, 22.2) were easily distinguishable from those of nutrient-sufficient or N-limited G. sanguineum. Comparison of several variables for both Fe- and N-limited growth indicates that F/chl a is the most specific and sensitive indicator of iron limitation for this species. Perhaps employed in conjunction with other methods of assessing nutritional status (e.g. short-term nutrient enrichment, Healey 1979), the F/chl a ratio would be useful in detecting iron-stressed red tide populations. It must be recognized, however, that the present data reflect only Fe-limited cells of G. sanguineum and comparisons with those growing under N limitation. Thus, the generality of these findings in terms of both species and nutrient specificity requires further investigation.

**Summary.** Examination of iron-limited growth kinetics indicates that reproductive rates of G. sanguineum may be limited by Fe concentrations at least ten-fold greater than for other neritic (predominantly diatom) species. This disparity may be related to the comparatively large iron requirement per unit cell carbon of this red tide
dinoflagellate. While these data do not confirm a role of iron in affecting red tide population dynamics, they do suggest that this species may be more susceptible to reduced iron bioavailability than many other coastal phytoplankters. In vivo fluorescence properties clearly demonstrate the detrimental effects of Fe stress on the utilization of harvested light energy. The type and magnitude of observed changes, when compared with those for N-stressed cultures, provide evidence for the iron stress-mediated specificity of these effects on *G. sanguineum*. Because of the extent to which Fe limitation modifies F/chl a and the distinction from N-mediated changes, this ratio, in conjunction with other probes of nutritional status, may be a useful indicator of Fe-stressed red tide populations. Ultrastructural observations of Fe-deplete *G. sanguineum*, especially those revealing changes in chloroplast number and morphology, are consistent with and provide a structural interpretation of variation noted in chl a quotas and in vivo fluorescence.
CHAPTER 2.

ASPECTS OF IRON AND NITROGEN NUTRITION IN THE RED TIDE DINOFLAGELLATE GYMNO DinODINiUM SANGUINEUM HIRASAKA: EFFECTS OF IRON DEPLETION AND NITROGEN SOURCE ON BIOCHEMICAL COMPOSITION

BACKGROUND

Phytoplankton growth in marine systems is thought to be limited most frequently by macronutrients such as nitrogen or occasionally silicon (see Parsons and Harrison 1983). Several studies of offshore communities indicate that iron may also regulate primary production (Menzel and Ryther 1961, Entsch et al. 1983, Subba Rao and Yeats 1984, Martin and Fitzwater 1988). By comparison, it has been suggested that iron concentrations of inshore or neritic environments are less likely to limit the growth of phytoplankton (Brand et al. 1983, Murphy et al. 1984, Martin and Gordon 1988). Yet, episodic pulses of iron from land drainage or sediment resuspension have been implicated in the initiation of red tide dinoflagellate blooms (Glover 1978, see Iwasaki 1979). Although direct iron mediation of red tides remains equivocal, to suggest that iron controls these dinoflagellates' reproductive rate, while adequately supporting growth of other coastal species, implies a disparity in requirements for and/or abilities to acquire this trace metal.

Evaluation of iron supply versus demand by phytoplankton relies largely on extrapolation of laboratory results to natural species assemblages. However, the majority of work useful in this regard pertains almost exclusively to diatoms
(e.g. Anderson and Morel 1982, Brand et al. 1983, Murphy et al. 1984, Harrison and Morel 1986). A half-saturation constant for iron-limited growth of a red tide dinoflagellate 10-1000 times greater than calculated for several coastal, predominantly diatom, species was reported in Chapter 1. This finding provides some of the first evidence suggesting that these dinoflagellates may be more susceptible to reduced iron bioavailability than certain other neritic phytoplankton.

The principle goal of the present research was a better understanding of red tide dinoflagellate iron nutrition, as well as that of coastal dinoflagellates in general. Herein, the biochemical composition of iron-replete (i.e. nutrient-sufficient) and iron-deplete cells are compared, while the following chapter (Chapter 3) examines nutrient uptake rates under these same conditions. Biochemical constituents monitored for the present report included cellular quotas of carbon, nitrogen, iron, protein, free amino acids and chlorophyll a. Although not a compositional variable per se, in vivo fluorescence was also determined. Fluorescence properties provide an indirect measure of photosynthetic electron transport, a process involving (iron-containing) cytochromes and iron-sulfur proteins (see Chapter 1). The organism employed in this study was Gymnodinium sanguineum Hirasaka.

Iron bioavailability is largely a function of the relatively low solubility of hydrous ferric oxides, this
element's predominant form in oxygenated seawater (Byrne and Kester 1976). However, both biological and non-biological interactions of iron with other trace metals (Harrison and Morel 1986, and references therein) and/or macronutrients (see Huntsman and Sunda 1980) may also influence (and be influenced by) phytoplankton iron nutrition. Many aspects of cellular nitrogen metabolism, including certain assimilatory and biosynthetic pathways, exhibit an iron requirement (see Reuter and Peterson 1987). Of the two major sources of inorganic nitrogen available to eukaryotic algae, namely nitrate and ammonium, only NO$_3$ must be reduced prior to its incorporation into amino acids (Syrett 1981). Nitrate and nitrite reductase, both iron-containing metalloenzymes (see Galvan et al. 1986), are necessary for the reduction of NO$_3$ to NH$_4$. Nitrogen source would thus appear to be an important factor in determining phytoplankton iron requirements. Further, the nitrogen status (e.g. N quota) of iron-deficient cells might also be expected to vary depending on whether N is supplied as NO$_3$ or NH$_4$. An additional objective of the present work was to investigate the possibility that N source mediates the quantitative and/or qualitative effects of iron depletion. This was accomplished by extending determinations of compositional variables (this study) and nutrient uptake rates (following chapter) for iron-replete and -deplete cells to include both NO$_3$- and NH$_4$-grown cultures.
MATERIALS AND METHODS

General culture maintenance. Nitrate-grown stock cultures of Gymnodinium sanguineum (culture #D354, North East Pacific Culture Collection, Dept. of Oceanography, University of British Columbia) were maintained on filter-sterilized (Millipore 0.45 μm), ESAW-enriched artificial seawater (Harrison et al. 1980) prepared and modified as outlined in Chapter 1. Medium for ammonium-grown stock cultures was similar to this formulation, except that 80 μM NH₄Cl replaced 550 μM NaN₃ as the sole source of nitrogen. It should be noted here that G. sanguineum is extremely sensitive to elevated NH₄ concentrations, with toxic growth limitation occurring at ≥150 μM NH₄ (Appendix 3). Maximum growth rates (i.e. equivalent to those of NO₃-grown cultures) are supported by 80 μM NH₄. Containers used in culture maintenance and experiments were pre-treated with freshly-prepared 10% HCl (v/v) for 2-3 days, followed by several deionized distilled water rinses. All cultures employed herein (i.e. stock and experimental, both unstirred) were held in a circulating water bath at 17°C, and continuously illuminated with 145 μE·m⁻²·s⁻¹ of blue fluorescent light (further details in Chapter 1).

Iron-replete and iron-deplete cultures. The basal ESAW salt solution and individual enrichment stocks (except trace metals and vitamins) were passed over Chelex 100 ion exchange resin to reduce ambient trace metal contamination (Morel et al. 1979). For all experiments, 2.5 l of a given medium was
dispensed into a 2.8 l polycarbonate Fernbach flask, the pH lowered to ca. 5.5 with Suprapur HCl (Merck), and autoclaved. Sterile medium was bubbled with 0.22 μm (Millipore)-filtered air to re-equilibrate the pH at ca. 8.0-8.1 prior to initiating an experiment. Due to high cell densities achieved during iron depletion experiments, these media were supplemented with chelexed, filter-sterilized (Millipore 0.22 μm) NaHCO₃ to a final concentration of 2 mM after autoclaving to prevent carbon limitation.

Iron-replete culture medium contained either 80 μM NaNO₃ (designated as +Fe/NO₃ hereafter) or 80 μM NH₄Cl (designated as +Fe/NH₄ hereafter); otherwise, these two media were identical to general maintenance ESAW medium. The medium employed to deplete cultures of iron (-Fe) received no iron addition. EDTA and all other trace metals (TM) were added as one solution, with [EDTA] reduced to maintain an EDTA:TM ratio of 1.6. -Fe medium was supplied with either 550 μM NaNO₃ (designated as -Fe/NO₃ hereafter) or 80 μM NH₄Cl (designated as -Fe/NH₄ hereafter). As noted, an NH₄ concentration of ca. 80 μM is required to avoid growth limitation due to ammonium toxicity. However, at this concentration, NH₄-nitrogen was exhausted from cultures prior to achieving iron depletion. Thus, NH₄ levels were monitored on filtered (Whatman 934-AH) samples using a Technicon AutoAnalyzer (method of Slawyk and MacIsaac 1972) and maintained between ca. 5-80 μM, with additions made as needed to permit depletion of available iron. Iron depletion was verified for both -Fe/NO₃ and
-Fe/NH$_4$ cultures by bioassay (Appendix 2). Changes in the pH of these cultures with increasing cell density were controlled by bubbling with sterile (Millipore 0.22 μm) air or 1-2% CO$_2$ (Appendix 2).

Late logarithmic phase stock cultures acclimated to the appropriate nitrogen source were used to inoculate experiments (i.e. NO$_3^-$ and NH$_4^+$-grown stocks used as inocula for +Fe/NO$_3$ and -Fe/NO$_3$, and +Fe/NH$_4$ and -Fe/NH$_4$ experiments, respectively) at a starting cell density of ca. 100 cells·ml$^{-1}$. All experiments were run in duplicate, with each flask initiated from a different stock culture to enhance the potential for variation between flasks. Iron-replete cultures (+Fe/NO$_3$ and +Fe/NH$_4$) were harvested in early to mid exponential phase for determination (duplicate measurements of all variables for each culture) of cell density (CD), average cell volume (CV), in vivo fluorescence (F) and DCMU-enhanced F ($F_D$), cellular quotas of carbon, nitrogen, iron, protein (Pr), free amino acids (AA) and chlorophyll a (chl a), and also several ratios comprising these variables. Iron-deplete cultures (-Fe/NO$_3$ and -Fe/NH$_4$) were sampled for these same variables upon depletion of biologically available iron, as indicated by no change or a decline in cell density on successive days. Measurements of iron and nitrogen (NO$_3^-$ and NH$_4^+$) uptake rates were also performed on all experimental cultures concurrent with sampling for biochemical constituents and are described in Chapter 3.
Cell density and volume, \( F, F_D \) and chl a. Culture cell density was monitored using a Coulter Counter\(^R\) model TAII (200 \( \mu \)m aperture, 44.2 \( \mu \)m calibration spheres), with the average volume of cells calculated based on equivalent spherical diameter of the particle size distribution. Details of methods for \( F, F_D \) and chl a determinations are given in Chapter 1. Briefly, in vivo fluorescence was measured on dark-equilibrated (20 min) samples after 30 s exposure to the fluorometer light source. The \( F_D \) tube received \( 10^{-5} \) M DCMU (final concentration) prior to dark treatment. Chl a was determined according to the fluorometric method of Holm-Hansen et al. (1965). As \( G. \) sanguineum is sensitive to physical perturbation, cells collected for all analyses were filtered by gravity whenever possible or using vacuum pressures of 25-50 mm Hg for larger volumes and/or higher cell densities (care was taken to avoid filtering to dryness).

\( C, N, \) protein and free amino acids. Particulate organic carbon and nitrogen concentrations were assessed on filtered samples (Whatman 934-AH, precombusted at 460\(^\circ\)C for 4 h) using a Carlo Erba Elemental Analyzer (model 1106) and standardized to acetonilide (Eastman Kodak Co.). Filters were stored at -15\(^\circ\)C between sampling and analysis times (ca. 1 month). Cellular protein and free amino acid measurements followed a slight modification (Q. Dortch pers. comm.) of the method outlined by Dortch et al. (1984). A technique very similar to that of Dortch et al. (1984) has been recommended recently, over several other phytoplankton extraction/analysis protocols.
(Clayton et al. 1988). Cells harvested on precombusted filters (Whatman 934-AH) were ground in 3% TCA (rather than 10% used by Dortch et al. 1984) and separated by centrifugation into pellet (Pr) and supernatant (AA) fractions, which were frozen individually and stored (-15°C) until analysis (ca. 2 months). Protein-N contained in the pellet was determined against a bovine serum albumin standard (using %N of BSA, Sigma Chemical Co.) according to Packard et al.'s (1972) modification of the Lowry method (Lowry et al. 1951). The supernatant was assayed fluorometrically (employing fluorescamine) for free amino acids utilizing glutamate standards (Undenfriend et al. 1972, Packard and Dortch 1975).

Iron quotas. Measurement of iron quotas requires that iron adsorbed to the cell through non-physiological mechanisms (e.g. ferric hydroxy colloids) be eliminated prior to analysis. Anderson and Morel (1982) demonstrated that for the diatom *Thalassiosira weissflogii*, 10⁻¹ M ascorbic acid dissolves surface-bound iron (by reduction of ferric to more soluble ferrous iron), thereby facilitating its removal by filtration. This technique proved unsuccessful in the present study due to cell lysis upon ascorbate addition. However the following experiments, employing ⁵⁵FeCl₃ (5 μM additions, New England Nuclear) as a label, suggest a fundamental difference in iron adsorption properties between *G. sanguineum* and *T. weissflogii*. Results indicate that non-biological,
filterable iron is a small percentage of total filterable iron associated with cells of *G. sanguineum*.

The formation of filterable iron retained by either 0.2 or 5.0 μm filters (polycarbonate, Nuclepore) for culture filtrate and suspensions of viable or glutaraldehyde (GTA, 1.5%)-killed cells (4000 cells·ml⁻¹) was followed over a time-course (note: GTA did not cause cell lysis). Experiments were conducted in 85 ml Oak Ridge tubes (polycarbonate, Nalgene) under culture conditions described above. As observed by Anderson and Morel (1982) in a similar experiment with *T. weissflogii* (formalin-killed cells, 0.1 and 3.0 μm filters), iron colloids appeared rapidly and were collected only on the 0.2 (or 0.1) μm filter (Fig. 2.1A). Label retained by dead *G. sanguineum* after 4 h represented a small fraction (ca. 20%) of that associated with viable cells (Fig. 2.1A), which is in sharp contrast to the equivalent labelling of *T. weissflogii* cells, irrespective of their viability (their Fig. 2.1). The predominantly physiological nature of iron associated with *G. sanguineum* cells and the negligible trapping of colloidal iron by 5.0 μm filters indicate that reasonable measurements of iron quotas for this species can be obtained without ascorbate treatment. In addition, removal of filterable (5.0 μm pore diam.) iron from viable and GTA-killed cells (ca. 2·10⁴ cells) by chelexed ESAW salt solution (CSS, 5 ml) was examined (5-40 ml CSS effects ca. equivalent removal of Fe from this number of cells, Appendix 4). Following 2-8 h incubations (Fig. 2.1B) dead cells retained only 15-20% of
Figure 2.1A. Formation of filterable iron ($^{55}$FeCl$_3$ label) by culture filtrate (0.2 μm filter: o; 5.0 μm filter: •) and suspensions of viable (□) or glutaraldehyde-killed (■) cells following 5 μM Fe addition. B. Acquisition of iron ($^{55}$FeCl$_3$, 5 μM addition) by unwashed (○) or washed (5 ml chelexed ESAW salt solution) (□) viable cells and unwashed (●) or washed (■) glutaraldehyde-killed cells. DPM = disintegrations·min$^{-1}$. 
iron associated with viable *G. sanguineum* (regardless of CSS washing), while a similar percentage of viable *T. weissflogii* iron incorporation was accounted for by formalin-killed cells after ascorbate treatment of each (i.e. viable and formalin-killed cells; Anderson and Morel 1982, Fig. 2.3A). CSS washing eliminated ca. 10% of total filterable iron (viable cells) herein (Fig. 2.1B), whereas ca. 70% of this iron fraction was removed by ascorbate from *T. weissflogii* (their Fig. 2.3A). Based on the above results, use of 5.0 μm filters and CSS washing appear to provide reliable determinations of *G. sanguineum* iron quota.

The concentration of cellular iron was measured spectrophotometrically based on methods described by Stookey (1970) and Mueller (1985), which detect the stable color complex of ferrozine (Sigma Chem. Co.) and ferrous iron. Cells collected on 5.0 μm filters (polycarbonate, Nuclepore) were washed with CSS and stored in a desiccator at room temperature until analysis. Iron determinations were performed by digesting cells with clean (single boiling, double sub-boiling distillation) ca. 3 N HNO₃ (20 h at room temp. followed by 1 h at 50°C), followed by quantitative separation of digestate from the intact filter, and addition of reagents (see Stookey 1970, note: HCl omitted from ferrozine-hydroxylamine hydrochloride reagent) to digestate. Absorbance along a 10 cm path length was read at 562 nm against a distilled water blank.
Data analysis. The effects of iron depletion and of nitrogen source on variables measured herein were determined from several comparisons. First, for cultures grown on either nitrate or ammonium, iron-replete and iron-deplete cells were compared within a given nitrogen source (e.g. +Fe/NO₃ vs. -Fe/NO₃). Second, for either Fe-replete or Fe-deplete cultures, NO₃⁻- and NH₄⁺-grown cells were compared within a given iron status (e.g. -Fe/NO₃ vs. -Fe/NH₄). Sampling design allowed comparison of variables by analysis of variance (ANOVA) employing a nested design, as each experiment included duplicate flasks with two measurements of a variable obtained and averaged per flask. The assumption of homogeneity of variances was verified prior to an ANOVA using an F-test. Ratios comprising variables (e.g. C/N, F/chl a, etc) are based on an n = 2 sample size (duplicate flasks) for each component of the quotient. Error terms for ratios take into consideration the propagation of error associated with combining two components and their respective variances into a single term. The appropriate formulae are given by Yates (1981). Comparisons between ratios were made by eye from bar graphs of their means ± 1 S.D.

RESULTS

Normalization of measured variables is an important consideration when describing chemical composition. The cell volume of *G. sanguineum* differed both as a result of iron depletion and as a function of nitrogen source (Table 2.1).
NH₄-grown cells were consistently larger than those grown on NO₃, with the disparity most evident under Fe depletion (-Fe/NH₄ ca. 35% > -Fe/NO₃). To eliminate the predominating effect of cell volume, all individual variables were expressed as concentrations (by atoms or weight) per liter of cell volume. Cell quotas of elements (C, N and Fe) and biochemical compounds (Pr, AA and chl a) are given in Table 2.1 and ANOVA probability levels of significance for comparisons are provided in Table 2.2.

**Elemental constituents and ratios.** Iron depletion significantly reduced the carbon quotas (Qc) of both NO₃- (16%) and NH₄- (28%) grown cells, while Qc was roughly equivalent between N sources for a given Fe status. The most notable difference in nitrogen quota was a function of N source under Fe depletion, with NH₄-grown cells containing almost 50% more N than those growing on NO₃. -Fe/NH₄ nitrogen quotas were even larger than for +Fe/NH₄ cultures (ca. 20%), although not statistically distinguishable. In terms of the more conventional C/N ratio (Fig. 2.2A), -Fe/NH₄ cultures averaged 3.8, while C/N values for all other conditions were between 6.2 and 7.0.

Variability among QFe determinations, particularly for NO₃-grown cultures, obscured any potential differences due to nitrogen source and further, prohibited certain statistical comparisons by ANOVA (Tables 2.1, 2.2). Regardless of this fact, iron depletion diminished iron quotas by ca. 1.5 orders
Table 2.1. Average cell volume and biochemical composition of iron-replete and iron-deplete cultures grown on nitrate or ammonium. See text for explanation of culture designations.

<table>
<thead>
<tr>
<th>CULTURE</th>
<th>CV</th>
<th>C³</th>
<th>N³</th>
<th>Fe⁴</th>
<th>Pr³</th>
<th>AA³</th>
<th>CHL a⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Fe/NO₃</td>
<td>4.63</td>
<td>10500</td>
<td>1510</td>
<td>56200</td>
<td>1300</td>
<td>207</td>
<td>634</td>
</tr>
<tr>
<td></td>
<td>(0.20)</td>
<td>(400)</td>
<td>(360)</td>
<td>(18200)</td>
<td>(50)</td>
<td>(34)</td>
<td>(102)</td>
</tr>
<tr>
<td>+Fe/NH₄</td>
<td>4.84</td>
<td>11000</td>
<td>1780</td>
<td>50500</td>
<td>1360</td>
<td>153</td>
<td>585</td>
</tr>
<tr>
<td></td>
<td>(0.08)</td>
<td>(630)</td>
<td>(190)</td>
<td>(8500)</td>
<td>(60)</td>
<td>(8)</td>
<td>(44)</td>
</tr>
<tr>
<td>-Fe/NO₃</td>
<td>2.24</td>
<td>8810</td>
<td>1400</td>
<td>938</td>
<td>1160</td>
<td>106</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>(0.10)</td>
<td>(80)</td>
<td>(40)</td>
<td>(261)</td>
<td>(40)</td>
<td>(8)</td>
<td>(7)</td>
</tr>
<tr>
<td>-Fe/NH₄</td>
<td>3.05</td>
<td>7920</td>
<td>2070</td>
<td>923</td>
<td>1330</td>
<td>159</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>(0.23)</td>
<td>(500)</td>
<td>(250)</td>
<td>(56)</td>
<td>(130)</td>
<td>(15)</td>
<td>(63)</td>
</tr>
</tbody>
</table>

1. Values for all experiments are means (n = 2) with one S.D. given in parentheses; duplicate samples were analyzed for all variables in each of n = 2 cultures per experiment.
2. μm³·10⁴
3. mg-at·liter cell volume⁻¹
4. μg-at·liter cell volume⁻¹
5. mg·liter cell volume⁻¹
Table 2.2. Probability levels of significance for differences between experiments compared (as indicated) using an analysis of variance (nested design). Asterisks (*) identify comparisons precluded by heterogeneous sample variances as determined with an F-test. N.S. = not significantly different at the 95% confidence level.

<table>
<thead>
<tr>
<th>COMPARISON</th>
<th>CV</th>
<th>C</th>
<th>N</th>
<th>Fe</th>
<th>Pr</th>
<th>AA</th>
<th>CHL a</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃-GRWN, +Fe vs. -Fe</td>
<td>$&lt;$0.001</td>
<td>$&lt;$0.01</td>
<td>N.S.</td>
<td>*</td>
<td>$&lt;$0.02</td>
<td>$&lt;$0.01</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>NH₄-GRWN, +Fe vs. -Fe</td>
<td>$&lt;$0.001</td>
<td>$&lt;$0.01</td>
<td>N.S.</td>
<td>*</td>
<td>N.S.</td>
<td>N.S.</td>
<td>$&lt;$0.001</td>
</tr>
<tr>
<td>Fe-RPLT, NO₃- vs. NH₄-GRWN</td>
<td>$&lt;$0.01</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>$&lt;$0.01</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fe-DPLT, NO₃- vs. NH₄-GRWN</td>
<td>$&lt;$0.001</td>
<td>N.S.</td>
<td>$&lt;$0.05</td>
<td>N.S.</td>
<td>N.S.</td>
<td>$&lt;$0.005</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
of magnitude under NO$_3$ or NH$_4$ growth, yielding a minimum $Q_{Fe}$ ($Q_{Fe,min}$) of 0.9 mg-at•liter $CV^{-1}$. When normalized to N (Fig. 2.2B) or C (Fig. 2.2C), the cellular iron content in iron-replete cultures was similar for either N source, although the mean Fe/N ratio under NO$_3$ supply was 31% greater than for growth on NH$_4$. This discrepancy in Fe/N increases further to 50% in the case of iron depletion and is likely a real difference. Fe/C values are the same for -Fe/NO$_3$ and -Fe/NH$_4$ cultures (avg. ca. $1.1 \cdot 10^{-4}$).

**Biochemical compounds and ratios.** The protein and free amino acid content of NO$_3$-grown cells declined significantly (11% and 49%, respectively) with iron-depletion, while in cultures grown on NH$_4$, these constituents appeared unaffected by iron stress (Table 2.1, 2.2). Compared to cellular protein ($Q_{Pr}$), free amino acid quotas ($Q_{AA}$) were influenced more by N source. $Q_{AA}$ of +Fe/NO$_3$ cultures exceeded those of Fe-replete, NH$_4$-grown cells by 35%. Conversely, under Fe-deplete conditions cellular AA concentrations were 50% greater for NH$_4$- than NO$_3$-grown cultures. Ratios of AA/Pr (Fig. 2.3) predominantly reflected variation in $Q_{AA}$, as iron stress-mediated changes in this index occurred only in cells grown on NO$_3$ (42% decrease). As a result, -Fe/NO$_3$ values (avg. = 9.2) were significantly (23%) less than for the -Fe/NH$_4$ experiment (avg. = 12.0).

Similar to $Q_{Fe}$ above, variability in chl a quota measurements for certain cultures (e.g. -Fe/NH$_4$, coefficient
Figure 2.2. Elemental ratios by atoms of carbon:nitrogen (A), iron:nitrogen (B) and iron:carbon (C) for iron-replete and iron-deplete cultures grown on nitrate or ammonium. Values are mean (n = 2) ± 1 S.D. Duplicate samples were analyzed for all individual ratio components in each of n = 2 cultures per experiment. Note different scales for Fe-replete and Fe-deplete cultures in (B) and (C).
Figure 2.3. Free amino acid (AA):protein (Pr) ratios, expressed as mole percent, of iron-replete and iron-deplete cultures grown on nitrate or ammonium. Average values and error terms presented are as described in Fig. 2.2.
of variation = 41%) precluded any distinction between NO$_3$- and NH$_4$-grown cultures (Tables 2.1, 2.2). Although an effect of N source was not discernable, iron-deplete cells (-Fe/NO$_3$ and -Fe/NH$_4$) contained only 26% of chl a present under nutrient sufficiency.

To further characterize cell nitrogen status, the percent of Q$_N$ accounted for by these N-requiring compounds (Pr, AA and chl a; internal NO$_3$ pools from Chapter 3) was estimated (Table 2.3). Calculations indicate that in both NO$_3$- and NH$_4$-grown cultures, percentages of Q$_N$ accounted for, were reduced by ca. 10% under iron depletion. However, those N-containing constituents monitored, invariably contributed to ca. 15% less of the total nitrogen in cells grown on NH$_4$, irrespective of iron status.

In vivo fluorescence ratios and indices. A trend of increasing in vivo fluorescence normalized either per unit chl a (Fig. 2.4A) or per unit iron (Fig. 2.4B) occurred in response to iron depletion, but changes in F/Fe were over an order of magnitude larger than for F/chl a. Although +Fe/NO$_3$ and +Fe/NH$_4$ cultures exhibited identical F/chl a and F/Fe measurements, iron-deplete values of both ratios for cultures grown on NO$_3$ were ca. two-fold greater than for those grown on NH$_4$. The in vivo fluorescence index of F$_D$-F remained unaltered for all experimental treatments (avg. ca. 150, Fig. 2.5A). By comparison, 1-F/F$_D$ (Fig. 2.5B), while equivalent for both iron-replete experiments (+Fe/NO$_3$ and +Fe/NH$_4$),
Table 2.3. Individual and total percent of cell nitrogen quota accounted for by various nitrogen-containing biochemical compounds. See text for explanation of culture designations.

<table>
<thead>
<tr>
<th>CULTURE</th>
<th>Pr(^1,3)</th>
<th>AA(^1,3)</th>
<th>NO(_3)(^2,3)</th>
<th>CHL a(^1,3)</th>
<th>% TOTAL N(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Fe/NO(_3)</td>
<td>86.0</td>
<td>13.7</td>
<td>0.9</td>
<td>0.19</td>
<td>100.8</td>
</tr>
<tr>
<td>+Fe/NH(_4)</td>
<td>76.3</td>
<td>8.6</td>
<td>0.8</td>
<td>0.15</td>
<td>85.9</td>
</tr>
<tr>
<td>-Fe/NO(_3)</td>
<td>82.7</td>
<td>7.6</td>
<td>1.0</td>
<td>0.05</td>
<td>91.4</td>
</tr>
<tr>
<td>-Fe/NH(_4)</td>
<td>64.2</td>
<td>7.7</td>
<td>0.5</td>
<td>0.03</td>
<td>72.4</td>
</tr>
</tbody>
</table>

1. Values for protein, amino acids and chl a from Table 2.1.
2. Data from Chapter 3 for intracellular nitrate pools immediately following 50 μM NO\(_3\) addition to cultures for uptake experiments (i.e. obtained at time zero); values are percent of total cell nitrogen given in Table 2.1.
3. Percent of total cell nitrogen (Table 2.1).
4. Percent of total cell nitrogen accounted for by all compounds considered.
Figure 2.4. In vivo fluorescence normalized per unit chl a (A) and per unit iron (B) of nitrate- or ammonium-grown, iron-replete and iron-deplete cultures. Average values and error terms presented are as described in Fig. 2.2. Note different scales for Fe-replete and Fe-deplete cultures in (B).
Figure 2.5. *In vivo* fluorescence (F) and DCMU-enhanced fluorescence (F_D) indices of F_D-F (A, relative units normalized to chl a, and representative of PSII capacity) and 1-F/F_D (B, unitless, and representative of PSII efficiency) for iron-replete and iron-deplete cultures grown on nitrate or ammonium. Average values and error terms presented are as described in Fig. 2.2.
declined significantly under iron stress; however, the reduction in -Fe/NO$_3$ cultures (56%) was more than 1.5 times that of NH$_4$-grown cultures (34%).

DISCUSSION

The current research provides some of the first quantitative information on dinoflagellate iron nutrition, with particular reference to red tide species. Results demonstrate that not only does iron stress cause significant changes in the biochemical composition of G. sanguineum, but the extent and nature of this variation is often a function of nitrogen source. Effects of iron depletion common to both NO$_3$- and NH$_4$-grown cultures are discussed first. Subsequently, alterations unique to either NO$_3$- or NH$_4$-grown cultures will be considered.

Effects of iron depletion. The compositional variable most directly relevant to the iron nutrition of G. sanguineum (and comparisons with other phytoplankton species) is cellular iron quota, and also those ratios it comprises. Minimum iron quotas ($Q_{Fe,min}$) of three coastal phytoplankters examined previously (i.e. Dunaliella tertiolecta (Chlorophyceae) Davies 1970, Pavlova lutheri (Chrysophyceae) Droop 1973, Thalassiosira weissflogii (Bacillariophyceae) Anderson and Morel 1982, Harrison and Morel 1986) have been summarized by Harrison and Morel (1986, see their Table 2.2). Values range from a minimum of $2.1 \cdot 10^{-5}$ mol Fe·liter cell vol$^{-1}$ for
T. weissflogii (data of Harrison and Morel 1986) to a maximum of 3.3·10^{-4} mol Fe·liter cell vol^{-1} for D. tertiolecta (Davies 1970). Of the available Q_{Fe}^{min} data, perhaps the most accurate estimate is that of Harrison and Morel (1986) as concurrent measurements of iron quota (with removal of surface-bound Fe) and cell volume were employed. The Q_{Fe}^{min} of G. sanguineum (this study) was calculated to be 9.3 ± 0.1·10^{-4} mol Fe·liter cell vol^{-1} (avg. for NO_3- and NH_4-grown cells). Thus, on a per unit cell volume basis, the minimum iron requirement of this dinoflagellate is ca. 3 and 45 fold greater than those of a chlorophyte and a diatom, respectively.

Such comparisons of Q_{Fe} normalized to cell volume may be biased by variation in the amount of actual biomass per unit cell volume among species and groups of phytoplankton (see Hitchcock 1982). Conventionally, this problem is minimized by expressing biochemical constituents based on cell carbon. The T. weissflogii Q_{Fe}^{min} of Harrison and Morel (1986) has been normalized previously to Q_C (Chapter 1), employing carbon quotas of the same clone reported by Blasco et al. (1982). Fe/C ratios meeting minimum and optimal growth requirements of phytoplankton have also been estimated by Anderson and Morel (1982) and Morel and Hudson (1985), based on earlier studies of T. weissflogii. Calculated minimum Fe/C values range from 7.6·10^{-7} to 7.5·10^{-6} depending on the data set utilized, while that allowing maximal growth is ca. 9.4·10^{-5}. Empirically determined minimum and maximum Fe/C ratios of G. sanguineum
(avg. for NO\textsubscript{3}- and NH\textsubscript{4}-grown cells) are 1.1 \pm 0.1 \cdot 10^{-4} and 5.0 \pm 0.5 \cdot 10^{-3}, respectively. These results suggest that per unit cell carbon, the iron quota of this dinoflagellate exceeds that of the diatom \textit{T. weissflogii} by one to two orders of magnitude. This disparity in cellular iron requirement was considered in Chapter 1 as a principle factor contributing to the 10-fold greater half-saturation constant for iron-limited growth of \textit{G. sanguineum} over \textit{T. weissflogii}.

While iron quotas of these two algal taxa may be considerably different, their molar iron requirements for photosynthetic and respiratory electron transport components (PET and RET, respectively), as well as for other constituents either containing or functionally dependent on iron (e.g. nitrate and nitrate reductase, glutamate synthase), are assumed to be equivalent. Raven (1988) has proposed a minimum mol Fe:mol C ratio of 2.33 \cdot 10^{-5} for a plant growing photolithotrophically (i.e. light-driven \textsubscript{O}_2 evolution with an inorganic carbon source) at a maximum specific rate of \textsubscript{3} \cdot 10^{-5} s^{-1} at 20°C. His calculations take into consideration those catalytic Fe-containing components of both PET and RET systems (i.e. cytochromes and iron-sulfur proteins). As the iron requirements of nitrate and nitrite reductase are not included, this Fe/C ratio is more directly applicable to NH\textsubscript{4}-grown plants. One can thus determine the proportion of a cell's critical iron quota (i.e. that required to just sustain maximal growth, \( \mu_{\text{max}} \)) accounted for by this catalytic iron while growing at \( \mu_{\text{max}} \) (20°C). Raven has performed such
calculations using two of the data sets available for T. weissflogii, and determined that from 16% (data of Morel and Hudson 1985) to 78% (data of Harrison and Morel 1986) of its critical $Q_{Fe}$ is comprised by catalytic iron. These percentages may be underestimates, as the catalytic Fe requirement was based on NH$_4$ growth, while data employed were for NO$_3$-grown T. weissflogii.

Although a true "critical" $Q_{Fe}$ was not obtained herein, similar calculations can be made using the $Q_{Fe\text{ max}}$ for G. sanguineum ($4.58 \cdot 10^{-3}$ mol Fe:mol C, +Fe/NH$_4^+$, Fig. 2.2A). In marked contrast to the values of T. weissflogii, only about 0.1% of $Q_{Fe\text{ max}}$ is accounted for by catalytic iron in NH$_4^+$-grown G. sanguineum. For cells grown on NO$_3^-$, the percentage is closer to 0.2%. The critical $Q_{Fe}$ of T. weissflogii is 45% of the maximum iron quota determined by Harrison and Morel (1986). If the same were true for G. sanguineum (purely speculative), the Fe accounted for would remain below 0.4%. While these values are small relative to those of the diatom, percentages of ca. 1% have been suggested previously for higher plants (Smith 1984). Elevated levels of divalent cations, including iron, associated with dinoflagellate chromatin (ca. 6 atoms Fe/ca. 100 nucleotides, Sigee 1983,1984) have not been demonstrated for other algae, and would represent at least one non-catalytic iron requirement unique to dinoflagellates. Nevertheless, more information on the nature and metabolic role of a considerable portion of the
iron quota of *G. sanguineum* and other dinoflagellates is clearly needed.

Ratios of Fe/N declined significantly under iron stress irrespective of nitrogen source, influenced predominantly by large reductions in \( Q_{\text{Fe}} \) (ca. 1.5 orders of magnitude, -Fe/NO\(_3\) and -Fe/NH\(_4\) cultures). However, in contrast to Fe/C ratios, -Fe/NO\(_3\) values of Fe/N were 1.5 times greater than for Fe-deplete, NH\(_4\)-grown cells (Fig. 2.2B). This disparity is likely attributable to the iron requirement of NO\(_3\) assimilatory enzymes. By considering both iron and nitrogen as a single quotient, Fe/N quite accurately reflects the 1.6-fold larger Fe requirement calculated by Raven (1988) for cells growing on NO\(_3\) over those supplied with NH\(_4\). Thus, while measurements of \( Q_{\text{Fe}} \) alone are insufficient to distinguish this inherent difference in iron demand, normalizing to \( Q_N \) apparently provides the required resolution.

Reductions in chl a associated with iron depletion, and the resulting chl a quotas, were similar for both NO\(_3\)- and NH\(_4\)-grown cultures (Tables 2.1,2.2). The chlorotic nature of iron-deplete cells observed in this and other reports on iron-stressed phytoplankton (e.g. Sakshaug and Holm-Hansen 1977, Reuter and Ades 1987) reflects iron's essential role in chlorophyll biosynthesis (Pushnik et al. 1984). *G. sanguineum* \( Q_{\text{chl} \ a} \) and its response to iron depletion, including ultrastructural manifestations, were considered previously (Chapter 1).
Cytochromes of both PET and RET chains not only share similar biosynthetic Fe requirements with chlorophyll, but they also contain a heme prosthetic group (Marschner 1986). Data reported herein and by other investigators (e.g. Glover 1977, Terry 1983, Sandmann 1985) show that a decline in Fe availability translates into fewer PET components and lower PET capacity, as indicated in this study by marked increases in F/chl a (Fig. 2.4A). The iron stress-mediated specificity of, and additional factors contributing to, elevated F/chl a ratios in G. sanguineum are discussed in Chapter 1. Of particular interest to the present research is the variation in in vivo fluorescence ratios and indices between -Fe/NO₃ and -Fe/NH₄ cultures.

Ratios of F/chl a (Fig. 2.4A) and F/Fe (Fig. 2.4B) increased dramatically under iron depletion. However, values for both ratios were 40-50% less in -Fe/NH₄ than -Fe/NO₃ cultures. Perhaps the most intuitive explanation of these results concerns the difference in Fe requirement for growth on NO₃ versus NH₄. The additional iron needed by NO₃-grown cells for N assimilation may be supplied partly at the expense of PET components, thereby lowering PET efficiency. As QFe was similar for -Fe/NO₃ and -Fe/NH₄ cultures (Table 2.1), this disparity in apportionment of cellular Fe (more directly interpreted as F/Fe) implies that for an equivalent reduction in available Fe, PET activity would decline most in NO₃-grown cells (accompanied by a higher F yield). Additional support for the enhanced effect of iron stress on PET in cells grown
on NO$_3$ was provided by $F_D^{-}$F (Fig. 2.5A) and 1-F/F$_D$ (Fig. 2.5B). These indices are considered to be indicative of Photosystem II (PSII) capacity and efficiency, respectively (see Chapter 1). Values obtained herein suggest that while PSII capacity is similar among all experiments, the efficiency of PSII under Fe depletion is significantly less (ca. 35%) in NO$_3$- than NH$_4$-grown cells. Measurements of Fe-containing components involved in PET and NO$_3$/NO$_2$ reduction are required to empirically verify this or an alternative interpretation.

**Effects of nitrogen source.** Certain ratios were altered by iron depletion only in either NO$_3$- or NH$_4$-grown cultures (but not both), indicating an overriding influence of nitrogen source on the response of some variables to iron stress. A departure of C/N from the average iron-replete value of 6.6 ± 0.5 occurred only in -Fe/NH$_4$ cultures (C/N = 3.8 ± 0.5, Fig. 2.2A). Few comparisons of C/N ratios between NO$_3$- and NH$_4$-grown phytoplankton are available, while still fewer exist for Fe-deficient algae. Reports by several investigators (Conover 1975, Larsson et al. 1985, Thompson et al. in press) demonstrate a negligible effect of N source on the C/N ratios of nutrient-replete cultures. Results obtained herein for +Fe/NO$_3$ and +Fe/NH$_4$ *G. sanguineum* are consistent with these data. In the only previous study to examine C/N as a function of Fe depletion, Sakshaug and Holm-Hansen (1977) were unable to detect a clear change in this ratio (range: 7-11, with considerable variation about the mean) for either a diatom (*Skeletonema costatum*) or a chrysophyte (*Pavlova lutheri*),
each grown on NO₃-N. Similarly, the present experiments revealed no difference in C/N between +Fe/NO₃ and -Fe/NO₃ cultures (avg. = 6.6 ± 0.5), yet this ratio declined by ca. 40% in NH₄-grown cultures under Fe depletion.

The C/N ratio of nutrient-sufficient phytoplankton is generally considered to approximate the Redfield ratio of 106 C:16 N (i.e. 6.6), while allowing for differences between taxonomic groups. For example, Parsons et al. (1961) report an average C/N of 6.2 ± 1.8 for 11 phytoplankton species distributed among five algal classes. C/N values invariably rise under nitrogen limitation or depletion (e.g. Laws and Bannister 1980, Dortch et al. 1984), and are thus a reliable indicator of N deficiency. Conversely, it follows that a decrease in C/N should represent a surplus of nitrogen relative to carbon, and presumably to the growth requirement. Iron depletion significantly reduced carbon quotas of both NO₃- and NH₄-grown cultures, but only the latter exhibited a lower C/N ratio. These data suggest a concomitant drop in carbon and nitrogen acquisition by -Fe/NO₃ cells, whereas N assimilation is maintained relative to declining C fixation in Fe-deplete cells grown on NH₄. This discrepancy likely reflects a reduced capacity of Fe-deplete G. sanguineum to provide iron needed for nitrate assimilation, while ammonium-N is incorporated in excess of the growth requirement.

A comparative inability of NO₃-grown cells to assimilate nitrogen under iron stress is further supported by AA/Pr
ratios, which decreased markedly (ca. 40\%) and exclusively in cultures receiving NO\textsubscript{3}-N (Fig. 2.3). This reduction was largely due to a 50\% decline in free amino acids, overshadowing a small (10\%) loss of cellular protein. In contrast, both $Q_{AA}$ and $Q_{Pr}$ of NH\textsubscript{4}-grown cells were unaffected by Fe depletion. The incorporation of N into free amino acids is a required step in the assimilation of inorganic N in protein and other nitrogenous cellular compounds (Wheeler 1983). It has been suggested that the free AA pool functions as a nitrogen buffer, and is utilized when cells are deprived of N (Admiraal et al. 1986). Although -Fe/NO\textsubscript{3} cultures contained saturating ambient NO\textsubscript{3} concentrations, the lower NO\textsubscript{3} assimilatory capacity (a result of Fe stress) caused cells to become effectively N-limited in relation to their growth requirement. Thus, upon cessation of cell division (i.e. time of sampling herein), free amino acid pools were greatly reduced from previous iron-replete levels.

Dortch et al. (1984) proposed that AA/Pr ratios (highest for nutrient sufficiency) are a more universal indicator of N deficiency than C/N, AA/N or Pr/N, with a greater range of values over similar N-replete and -deplete conditions. Recall that C/N ratios for -Fe/NO\textsubscript{3} and -Fe/NH\textsubscript{4} cultures did not indicate a nitrogen shortage in the former, but rather an N surplus in the latter. By comparison AA/Pr ratios, while equivalent for both +Fe/NH\textsubscript{4} and -Fe/NH\textsubscript{4} cultures, reveal symptoms of N deficiency in -Fe/NO\textsubscript{3} cells. According to AA/Pr and C/N ratios, the current research suggests that relative to
their respective cellular requirements, -Fe/NO₃ G. sanguineum cells are N deficient, while Fe-deplete, NH₄-grown cells contain an excess of nitrogen.

**Qualitative nitrogen composition.** Phytoplankton nitrogen quotas comprise a variety of N-containing compounds such as those considered herein (i.e. Pr, AA, chl a, internal NO₃) and others, including NH₄ pools, RNA and DNA. Calculations presented in Table 2.3 indicate that the nitrogenous constituents measured account for 15-20% less of Qₙ in NH₄- than NO₃-grown cells. For the most extreme case (i.e. -Fe/NH₄) ca. 30% of Qₙ was not included in a summation of Pr, AA, chl a and internal NO₃. Values exceeding 60% Qₙ unaccounted for, have been reported previously in more detailed analyses of nitrogenous compounds (Conover 1975, Dortch et al. 1984). Failure to measure all N components in the AA fraction and loss of N with a lipid fraction were suggested as possible explanations. In the present study, however, there appears to be a qualitative difference in N composition unique to NH₄-grown G. sanguineum and attributable to a constituent(s) not determined or underestimated.

Variations in internal NH₄, RNA and DNA percentages (of Qₙ) between NO₃- and NH₄-grown cultures of *Amphidinium carterae* (Dinophyceae) and also of *Thalassiosira nordenskioldii* (Bacillariophyceae) were observed by Dortch et al. (1984), but did not exceed 2% for any single component. Plants supplied with ammonium can exhibit distinctly different
free amino acid profiles compared to those receiving nitrate (Harada et al. 1968). Pertinent to these observations, Clayton (1985) has demonstrated that fluorescamine-based free AA analyses standardized to glutamate (as herein), considerably (by a factor of 2-3) underestimate concentrations of certain amino acids (e.g. aspartate). The AA composition of phytoplankton is reported to vary widely among species and as a function of nutritional status (e.g. Admiraal et al. 1986). Nevertheless, the disparity between NH$_4^-$ and NO$_3^-$-grown *G. sanguineum* may represent amino acids such as aspartate, likely augmented by additional factors including those mentioned above. A less equivocal explanation awaits a comprehensive investigation of nitrogenous cell constituents.

*Ecological considerations.* Several reports noted at the outset of this paper have identified iron as a potentially important factor in the initiation of red tides. However, the belief that coastal iron concentrations are unlikely to limit reproductive rates of phytoplankton results in an apparent paradox. It was also noted above that most evidence supporting the latter contention relies on studies of diatom iron nutrition. For example, calculations used by Martin and Gordon (1988) to suggest an adequate iron supply for neritic phytoplankton growth, employ optimum Fe/C ratios of the diatom *Thalassiosira weissflogii* (Anderson and Morel 1982, Morel and Hudson 1985). Data obtained herein provide values between one and two orders of magnitude greater for the dinoflagellate *G. sanguineum*. Use of a critical Fe/C (discussed above) ratio
may reduce this discrepancy, but differences would still approach a factor of ten. It is difficult to determine the extent to which Fe supply exceeds its demand by phytoplankton. However, estimates utilizing *G. sanguineum* Fe/C ratios tighten the relationship considerably and indicate that red tide dinoflagellates may be more susceptible to iron-limited growth than coastal diatoms. A similar conclusion was reached in Chapter 1, based on the comparatively large half-saturation constant for iron-limited growth of *G. sanguineum*.

Raven (1988) has estimated that assimilation of nitrate increases a plant's iron requirement by 60% relative to growth on ammonium. Determinations of biochemical composition reported in this study (e.g. Fe/N) provide empirical, albeit indirect, evidence consistent with these calculations. Thus, the quantity of cellular iron yielding maximum growth rates (i.e. critical $Q_{Fe}$) would ultimately be a function of nitrogen source. By determining the critical $Q_{Fe}$ for a species, nitrogen source also regulates the degree of growth limitation for a given subsaturating (for growth) iron concentration. The ecological implications of these effects are uncertain, as both predominating N source and blooms of coastal dinoflagellates exhibit temporal variation (e.g. Anderson et al. 1983, Robinson and Brown 1983). However, in the event that iron supply and demand are closely balanced, the influence of nitrogen source on phytoplankton iron requirements may be important in determining growth rate.
A final comment should be made concerning the use of indices based on biochemical constituents to identify a limiting nutrient. As demonstrated herein and in Chapter 1, F/chl a ratios of NO₃-growen G. sanguineum increase considerably (5-7 fold) in response to iron depletion. Yet, for cells grown on NH₄, the magnitude of these changes is only a factor of three, approaching that observed under nitrogen depletion (see Chapter 1). It is also apparent that iron stress effects symptoms of N deficiency (e.g. reduced AA/Pr) in -Fe/NO₃ cultures. In the present case of iron depletion, the potential for conflicting identification of the limiting nutrient as a function of N source and the index employed is clear. Such complications reinforce the need to consider multiple indicators of nutrient deficiency in the field, and for careful interpretation of results.

Summary. The first measurements of iron quota for G. sanguineum, a red tide dinoflagellate, have been obtained. Its iron requirement exceeds those of certain neritic diatoms by one to two orders of magnitude. G. sanguineum iron quotas, and a comparably large half-saturation constant for Fe-limited growth (Chapter 1), support the contention that iron may regulate the growth of certain dinoflagellates without similarly affecting diatom reproductive rates. Ratios of biochemical constituents (e.g. Fe/N) reveal a greater Fe requirement for NO₃- than NH₄-grown cells, likely attributable to the essential role of iron in the reductive NO₃ assimilatory enzymes nitrate and nitrite reductase. The
magnitude of changes in F/chl a and 1-F/F_D exhibited by -Fe/NO_3 cultures suggests that under iron stress, supply of Fe for NO_3 assimilation is partly at the expense of PET components. Nevertheless, acquisition of nitrogen by cells grown on NO_3 is sufficiently inhibited by iron depletion to yield symptoms of N deficiency. Perhaps the most important effect mediated by N source would be the determination of critical Q_{Fe}, thereby regulating the degree of growth limitation for a given subsaturating iron concentration.
CHAPTER 3.

ASPECTS OF IRON AND NITROGEN NUTRITION IN THE RED TIDE DINOFLAGELLATE GYMNOdinium Sanguineum HIRASAKA: EFFECTS OF IRON DEPLETION AND NITROGEN SOURCE ON IRON AND NITROGEN UPTAKE

BACKGROUND

Iron is quantitatively the most important of all trace elements known to be essential for phytoplankton growth (Huntsman and Sunda 1980). Nitrogen, the macronutrient required in greatest abundance after carbon, hydrogen and oxygen, can account for as much as 10% of algal cell dry weight (Syrett 1981). Among the biochemical roles of iron in algae (and plants in general), several are closely linked with various aspects of inorganic (e.g. assimilatory) and organic (e.g. biosynthetic) nitrogen metabolism. Iron is a constituent of the reductive nitrate assimilatory enzymes nitrate and nitrite reductase (Guerrero et al. 1981), and also of cytochromes and iron-sulfur proteins (Marschner 1986). The biosynthesis of chlorophyll (ca. 6% N, by atoms) and the catalytic activity of some or all forms of several enzymes, including glutamate synthase, superoxide dismutase, catalase, peroxidase and hydrogenase (Roessler and Lien 1984, Raven 1988) also require iron. The iron nutrition of algal cells may thus influence not only the incorporation of certain inorganic N species into amino acids, but also some biosynthetic pathways utilizing these compounds and ultimately, the metabolic processes dependent upon their end products (e.g. photosynthesis and respiration).
In aquatic environments inorganic nitrogen is acquired by eukaryotic algae primarily as either nitrate or ammonium. Assimilation of the former requires two iron-dependent reduction steps, while the latter may be incorporated directly into amino acids (Wheeler 1983). Raven (1988) calculated that for photolithotrophic (i.e. light-driven $O_2$ evolution with an inorganic carbon source) reproduction on nitrate, plants require 60% more iron than when growing at the same rate on ammonium. Thus, the physiological status of phytoplankton under iron-limiting conditions would likely be a function of nitrogen source (e.g. $NO_3$ or $NH_4$) as well as iron bioavailability.

Iron has been implicated in the initiation of red tides (see Chapters 1,2). Consistent with this suggestion are data (Chapter 1) showing a half-saturation constant for Fe-limited growth of a red tide species at least ten-fold greater than reported for some neritic diatoms (e.g. Harrison and Morel 1986). These dinoflagellate blooms are of ecological importance due not only to their apparent detrimental aspects, including toxin production and high BOD resulting in anoxia (Yentsch et al. 1980, Taylor 1987), but also their potential contribution to local primary production (Vargo et al. 1987). The predominant species of inorganic nitrogen in coastal waters, varies both temporally and spatially according to local conditions (Sharp 1983), as do red tides themselves. There are few data presently available on the iron nutrition of red tide dinoflagellates and coastal dinoflagellates in
general. Furthermore, it is also of interest to at least address the possibility that nitrogen source may influence the effects of iron deficiency on these organisms.

From the preceding chapter (2), it is apparent that iron depletion alters the biochemical composition of Gymnodinium sanguineum, a red tide species. However, both the extent and nature of these changes are, in fact, strongly influenced by nitrogen source (i.e. NO\textsubscript{3} or NH\textsubscript{4}). In addition, Reuter and Ades (1987) have observed a greater effect (i.e. reduction) of iron limitation on the uptake of NO\textsubscript{3} than NH\textsubscript{4} (enhanced under light limitation) by Scenedesmus quadricauda (Chlorophyceae). It was the aim of this report to further define both the consequences of iron stress and the relationship between iron and nitrogen nutrition for G. sanguineum. This assessment was based on Fe, NO\textsubscript{3} and NH\textsubscript{4} uptake rates of iron-replete (= nutrient-sufficient) and iron-deplete cultures growing on either NO\textsubscript{3} or NH\textsubscript{4}. Internal nitrate pools and rates of nitrite excretion during NO\textsubscript{3} uptake experiments were measured to provide additional information on nitrate assimilation under these conditions.

MATERIALS AND METHODS

General culture maintenance. Nitrate- and ammonium-grown stock cultures of Gymnodinium sanguineum (culture #D354, North East Pacific Culture Collection, Dept. of Oceanography, University of British Columbia) were maintained on modified
ESAW-enriched artificial seawater (Harrison et al. 1980). Media preparation and culture conditions have been described in Chapter 2.

**Iron-replete and iron-deplete cultures.** All experimental data acquired herein were obtained from aliquots of those cultures utilized in Chapter 2 for assessment of biochemical composition. Samples employed were harvested from cultures immediately after those used to examine compositional variables. Measurements made in the present study (and described in detail in the following sections) include iron and nitrogen (NO$_3$ and NH$_4$) uptake rates, nitrite excretion rates and internal NO$_3$ pool formation for nitrate- or ammonium-grown, iron-replete (i.e. +Fe/NO$_3$ and +Fe/NH$_4$) and iron-deplete (i.e. -Fe/NO$_3$ and -Fe/NH$_4$) cultures.

**Cell pretreatment for uptake experiments.** Determination of iron uptake by $^{55}$FeCl$_3$ requires that cells be placed in iron-free medium before labelling to allow calculation of tracer specific activity (i.e. µCi·µmol ambient Fe$^{-1}$), and thus transport and specific uptake rates. Conventional washing/resuspension methods for phytoplankton employ a standard filtration apparatus (e.g. Anderson and Morel 1982). As *G. sanguineum* is sensitive to physical stress (see Chapter 2), this technique was first tested by comparing carbon uptake (NaH$^{14}$CO$_3$, New England Nuclear) of unwashed (UW) and washed (W) samples (all samples from one culture; n = 3 for UW,W). UW experiments involved simply the addition of $^{14}$C (0.1
pCi·ml\(^{-1}\)) to an aliquot of untreated culture. For W incubations, cells were removed from their original medium by filtration until the meniscus was just above the filter (5.0 μm filter, Nuclepore; 25-50 mm Hg), washed by gently adding culture filtrate (Whatman GF/C filtered) and refiltering (as before), and resuspended in culture filtrate prior to \(^{14}\)C addition. Cell densities of UW and W incubations differed by < 5%. Uptake experiments were conducted in 85 ml Oak Ridge tubes (polycarbonate, Nalgene) and incubated (unbubbled, unstirred) for 6 h under culture conditions described earlier (Chapter 2). Results clearly demonstrate an adverse effect of the conventional washing technique, with W cells showing negligible \(^{14}\)C uptake even after 6 h (Fig. 3.1A). As a physically more gentle alternative, washing by reverse-flow filtration (RFF, see Holm-Hansen et al. 1970) was tested. Briefly, RFF involved allowing a tube (T1, 3 cm I.D.), with its bottom occluded by screening (10 μm Nitex\(^R\)), to settle by gravity through a slightly larger diameter tube (T2, 4.5 cm I.D.) containing the culture. Culture medium moves up through the screening into T1, where the filtrate is collected and continuously removed, leaving a concentrated cell suspension (ca. 3-5 ml) in T2. Filtration pressure is determined by the difference between the height of the water in T1 and T2. Cells were washed by resuspension in culture filtrate and repeating this procedure. The uptake of \(^{14}\)C (0.4 pCi·ml\(^{-1}\)) (all samples from one culture; n = 3 for UW,W) by RFF-washed cells (two resuspensions; ca. 10-15 min) was equivalent to
Figure 3.1A. Uptake of carbon (0.1 μCi·ml⁻¹ NaH¹⁴CO₃) by unwashed (○, n = 3) and washed (●, n = 3) cultures. Cells were washed by filtration and resuspension using standard filtration apparatus. B. Uptake of carbon (0.4 μCi·ml⁻¹ NaH¹⁴CO₃) by unwashed (○, n = 3) and washed (●, n = 3) cultures. Cells were washed by reverse-flow filtration (RFF) (details in text). C. Uptake of iron (0.08 μCi·ml⁻¹ ⁵⁵FeCl₃, 5 μM addition) by unwashed (○, n = 1) and RFF-washed (●, n = 1) cultures. Error bars for (A-C) represent ± 1 S.D. (or only 1 S.D. to prevent overlap) and are smaller than symbol where not apparent for experiments in which n > 1. DPM = disintegrations·min⁻¹.
that of UW cells (Fig. 3.1B), indicating the suitability of this technique for washing *G. sanguineum*. The effect of RFF on iron uptake was also examined by following incorporation of $^{55}\text{FeCl}_3$ (5 μM additions, 0.08 μCi·ml$^{-1}$, New England Nuclear) into UW and W (RFF procedure) cells over a 6 h period (all samples from one culture; n = 1 for UW,W). Samples collected for $^{55}\text{Fe}$ counts were filtered and washed of filter- and surface-bound iron as outlined below (see *Iron uptake*). Accumulation of label was expressed as DPM·cell$^{-1}$ due to the uncertainty of ambient iron concentrations in the medium, and hence the inability to calculate specific activity. Iron specific activity would be lowest in UW cultures. Both UW and W cultures exhibited similar rates of saturated iron uptake (Fig. 3.1C). Thus, cells employed in iron uptake experiments were washed by the RFF procedure prior to resuspension in uptake medium. To maintain consistency in treatment of samples, the RFF procedure was also followed for all nitrogen uptake incubations.

*Iron and nitrogen uptake, nitrite excretion and internal nitrate pools.* Iron and nitrogen uptake experiments were conducted in either 85 ml Oak Ridge tubes or 250 ml Erlenmeyer flasks (both containers polycarbonate, Nalgene) and incubated (unbubbled, unstirred) under 145 μE·m$^{-2}·$s$^{-1}$ of continuous illumination at 17°C. Saturated rates of Fe, NO$_3$ and NH$_4$ uptake by +Fe/NO$_3$, -Fe/NO$_3$, +Fe/NH$_4$ and -Fe/NH$_4$ cultures were measured following RFF washing of cells with unenriched (i.e. no nutrient additions), chelexed ESAW salt solution (CSS, see
Chapter 2), and resuspension in the respective uptake media at a final cell density of ca. 3-5 $\cdot 10^2$ cells $\cdot ml^{-1}$ (all incubations). The chelexing protocol (Morel et al. 1979) minimized residual iron contamination, thereby facilitating more complete removal of iron by washing. Ambient iron is thus assumed to be negligible following RFF washing, which allows iron added only subsequently (i.e. after washing) to be considered in calculations of iron specific activity for $^{55}$Fe uptake experiments. Iron uptake rates for all cultures included determinations made in the presence of either added NO$_3$ or NH$_4$ (i.e. cultures grown on NO$_3$ or NH$_4$ and resuspended in saturating Fe plus either NO$_3$ or NH$_4$). These uptake experiments employed chelexed ESAW medium (full enrichment except 50 $\mu$M NO$_3$ or 50 $\mu$M NH$_4$) spiked to a final (full ESAW) concentration of 6.56 $\mu$M $^{55}$FeCl$_3$ (48 $\mu$Ci $\cdot$ mol Fe$^{-1}$, New England Nuclear)/14.86 $\mu$M EDTA, 20 h prior to their initiation by adding RFF-washed cells. ESAW trace metal enrichment was added immediately following Fe/EDTA. This 20 h delay ensured pre-equilibration of EDTA-trace metal complexes. Incubations of Fe-replete cells were conducted over 24-33 h, while those utilizing Fe-deplete cultures were extended to 72-82 h, ensuring full recovery from iron depletion and achievement of nutrient satiety. Labelled cells were collected on 5.0 $\mu$m filters (Nuclepore, 25-50 mm Hg), washed with CSS to remove cell surface- and filter-bound (i.e. non-biological) iron (see Chapter 2), and counted by liquid scintillation employing techniques similar to those used for $^{14}$C ($^{55}$Fe is a low energy
As neither culture nor incubation conditions were axenic, it was desirable to estimate the bacterial contribution to iron uptake rates. *G. sanguineum* cultures (CULT, n = 2) and 0.2 μm-filtered filtrate from these cultures (FILT, n = 2) were spiked with $^{55}\text{FeCl}_3$ (5 μM additions, New England Nuclear) and incubated for 4 h. Samples from CULT incubations were size-fractioned using either 5.0 or 0.2 μm filters (Nuclepore), which retained primarily cells (CULT/5.0 μm) or cells, bacteria and colloidal iron (CULT/0.2 μm), respectively. FILT samples collected on 0.2 μm filters contained predominantly iron colloids (FILT/0.2 μm). All samples were washed with CSS (see above) after filtration. Results (Table 3.1) indicate the rate of colloidal iron label incorporation (FILT/0.2 μm) is equivalent to that of bacteria plus colloidal iron (approximated by subtracting the cellular contribution to uptake rate: $= \text{CULT}/0.2 \mu\text{m} - \text{CULT}/5.0 \mu\text{m}$). Thus, the proportion of *G. sanguineum* iron uptake rates attributable to bacteria appears minimal.

Nitrogen uptake media were identical to that utilized in iron uptake experiments except for the addition of unlabeled iron. Also, incubation times for Fe-replete and -deplete cultures were as for Fe uptake experiments (i.e. 24-33 h and 72-82 h, respectively). Disappearance of $\text{NO}_3^-$ and $\text{NH}_4^+$ was monitored on gravity-filtered (Whatman 934-AH) samples with a β-emitter, 0.232 MeV). Counts for all samples were $\geq 3 \cdot 10^3$ CPM.
Table 3.1. Size-fractioned rates of $^{55}$FeCl$_3$ incorporation (5 µM additions) as collected on 5.0 µm (CULT/5.0 µm, n = 2) or 0.2 µm (CULT/0.2 µm, n = 2) filters, and formation of colloidal iron in 0.2 µm filtrate (same cultures) retained by 0.2 µm filters (FILT/0.2 µm). See text for functional definition of size fractions.

<table>
<thead>
<tr>
<th>CULT/5.0 µm$^1$</th>
<th>CULT/0.2 µm$^1$</th>
<th>CULT/0.2 µm - CULT/5.0 µm</th>
<th>FILT/0.2 µm$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8 ± 0.1</td>
<td>9.4 ± 0.4</td>
<td>6.6</td>
<td>6.4 ± 1.5</td>
</tr>
</tbody>
</table>

1. Values are mean (n = 2) DPM·h$^{-1}$ ($\cdot 10^4$) ± 1 S.D. over 4 h incubations.
Technicon AutoAnalyzer\textsuperscript{R} II (methods of Wood et al. 1967 and of Slawyk and MacIsaac 1972, respectively) and used to calculate uptake rates. Fe, NO\textsubscript{3} and NH\textsubscript{4} uptake are expressed as transport rates per liter of cell volume (i.e. mol Fe or mol N\cdot liter cell vol\textsuperscript{-1} \cdot h\textsuperscript{-1}) and/or specific uptake rates (d\textsuperscript{-1}, Fe uptake only), the latter based on particulate iron concentrations determined in Chapter 2 for cultures employed both in the preceding paper and herein.

During NO\textsubscript{3} uptake experiments, rates of nitrite excretion (i.e. NO\textsubscript{2}\textsuperscript{-} appearance rate in the culture medium detected by AutoAnalyzer\textsuperscript{R}, Wood et al. 1967) and internal NO\textsubscript{3} pool formation were also followed. NO\textsubscript{3} pools were extracted into 20 ml boiling distilled deionized water according to the C-2 protocol of Thoresen et al. (1982), and normalized per liter of cell volume. The average cell volume of a culture was derived from particle size distributions (Coulter Counter\textsuperscript{R} model TAII, 200 \textmu m aperture, 44.2 \textmu m calibration spheres) based on equivalent spherical diameter.

RESULTS

Data for iron and nitrogen uptake are presented in Figs. 3.2-3.5. All values plotted are midpoint determinations, approximating the average rate between two adjacent measurements.

Iron uptake. Iron uptake rates are presented for +Fe/NO\textsubscript{3}, +Fe/NH\textsubscript{4}, -Fe/NO\textsubscript{3} and -Fe/NH\textsubscript{4} cells resuspended in
Figure 3.2. Time course of iron uptake (A,B) and specific iron uptake (C), following resuspension in NO$_3$, for iron-replete (A) and iron-deplete (B,C) cultures grown on nitrate (o) or ammonium (●). Rates represent midpoint determinations for two adjacent measurements and are given as the mean ± 1 S.D. for two incubations, one from each of n = 2 cultures per experiment. In certain cases only 1 S.D. (either + or -) is shown to avoid overlapping and/or to allow use of more expanded scales. Note differences in time scale between Fe-replete and Fe-deplete experiments.
Figure 3.3. Time course of iron uptake (A, B) specific iron uptake rates (C), following resuspension in NH₄⁺, for iron-replete (A) and iron-deplete (B, C) cultures grown on nitrate (○) or ammonium (●). Values at 12 h in (B) are offset for clarity. Values at 60 h in (B), (C) were ≤ 0 and are not shown (see text for further explanation). Data points and error terms plotted are as described in Fig. 3.2. Note differences in time scale between Fe-replete and Fe-deplete experiments.
either NO₃ (Fig. 3.2) or NH₄ (Fig. 3.3). Maximum saturated iron uptake (i.e. transport) rates by -Fe/NO₃ cultures exceeded those measured for +Fe/NO₃ cultures by ca. 1.5-fold, irrespective of nitrogen species available during incubations (i.e. whether cells were resuspended in 50 μM NO₃ or NH₄) (Figs. 3.2A,B and 3.3A,B). Maximum rates of Fe uptake by -Fe/NH₄ cells were greater than for +Fe/NH₄ cells (by a factor of 2.5), only when resuspended in NH₄ (Figs. 3.3A,B). Conversely, following resuspension in NO₃-nitrogen, +Fe/NH₄ cells exhibited the highest Fe uptake rates, with values twice those determined for -Fe/NH₄ cells (Figs. 3.2A,B). Initial specific rates of Fe uptake (invariably the maximum rates) for all iron-deplete cultures were roughly equivalent (avg. = 0.075 ± 0.003·h⁻¹), and exceeded the maximum growth rate of 0.0158 h⁻¹ by a factor of ca. five (Figs. 3.2C,3.3C).

The most notable distinction in iron uptake rates between +Fe/NO₃ and +Fe/NH₄ cultures occurred with nitrogen available only as NO₃ (Fig. 3.2A). Initially (4 h point), +Fe/NH₄ cells acquired iron at 3.5 times the rate of +Fe/NO₃ cells, following resuspension in NO₃. The error associated with the +Fe/NH₄ cultures' average 4 h rate is large (Fig. 3.2A), yet this point remains distinctly above the comparable determinations for +Fe/NO₃ cultures. It should be noted that at 60 h the iron uptake rates of -Fe/NO₃ and -Fe/NH₄ cultures provided with NH₄ (Fig. 3.3B,C) were ≤ 0, and thus not included on the graph. All Fe-deplete cultures, regardless of N source accompanying iron additions, exhibited no further
accumulation of label at 72 h. Uptake rates ≤ 0 likely represent label equilibration due to extended incubation periods, coupled with enhanced initial uptake of iron.

Nitrate uptake. Nitrate uptake rates are presented for +Fe/NO$_3$, +Fe/NH$_4$, -Fe/NO$_3$ and -Fe/NH$_4$ cells resuspended in 50 μM NO$_3$ and 6.56 μM Fe (plus EDTA) (Fig. 3.4). The uptake of NO$_3$ was markedly affected by iron depletion for both NO$_3$- and NH$_4$-grown cells (Fig. 3.4). No change in ambient [NO$_3$] occurred over the first 24 h of all -Fe/NO$_3$ and -Fe/NH$_4$ incubations after iron resupply (Fig. 3.4B), while maximal uptake rates were attained in all +Fe/NO$_3$ and +Fe/NH$_4$ experiments within ≤ 18 h (Fig. 3.4A). The initial lag in NO$_3$ uptake under Fe depletion was further exacerbated for NH$_4$-grown cultures. These cells showed 36 h NO$_3$ uptake rates only 30% of those measured in -Fe/NO$_3$ cultures. An acclimation period (ca. 18 h) was also evident for +Fe/NH$_4$ cells (Fig. 3.4A); however, some NO$_3$ was taken up immediately (1.5 h resolution) with rates increasing steadily to a sustained maximum at 18 h. Maximal rates of NO$_3$ uptake were expected for +Fe/NO$_3$ cells, yet an early, albeit short, delay (1.5 h interval) was observed (Fig. 3.4A). This lag may have resulted from brief (ca. 0.5 h) exposure to unenriched ESAW salt solution during RFF washing and prior to NO$_3$ addition. Upon termination of all experiments (i.e. nutrient sufficiency), NO$_3$ uptake rates were essentially indistinguishable between NO$_3$- and NH$_4$-grown cells, irrespective of previous iron status (Figs. 3.4A,B).
Figure 3.4. Time course of nitrate uptake rates for nitrate- (○) or ammonium-grown (■), iron-replete (A) and iron-deplete (B) cultures resuspended in 50 μM NO$_3$. Iron-deplete cultures also received saturating iron additions (6.56 μM Fe/14.86 μM EDTA). Data points and error terms plotted are as described in Fig. 3.2. Note differences in time scale between Fe-replete and Fe-deplete experiments.
Figure 3.5. Time course of ammonium uptake rates for nitrate- (o) or ammonium-grown (●), iron-replete (A) and iron-deplete (B) cultures resuspended in 50 μM NH₄. Iron-deplete cultures also received saturating iron additions (6.56 μM Fe/14.86 μM EDTA). Data points and error terms plotted are as described in Fig. 3.2. Note differences in time scale between Fe-replete and Fe-deplete experiments.
**Ammonium uptake.** Ammonium uptake rates are presented for +Fe/NO$_3$, +Fe/NH$_4$, -Fe/NO$_3$ and -Fe/NH$_4$ cells resuspended in 50 µM NH$_4$ and 6.56 µM Fe (plus EDTA) (Fig. 3.5). Iron depletion inhibited the initial uptake of NH$_4$ (Fig. 3.5), although not to the same degree noted for NO$_3$ uptake. A lag in rates of NH$_4$ uptake following saturating iron additions occurred in NO$_3$- and NH$_4$-grown cells, with the highest values for both obtained at 60 h (Fig. 3.5B). Similarly, maximum NO$_3$ uptake rates for -Fe/NO$_3$ and -Fe/NH$_4$ cultures were not achieved until ca. 60 h (Fig. 3.4B). Rates of NH$_4$ uptake by -Fe/NO$_3$ and -Fe/NH$_4$ cultures converged toward the end of incubations, yet for initial samples, NO$_3$-grown cells showed ca. three-fold higher rates (Fig. 3.5B).

**Nitrite excretion and internal nitrate pools.** The initial excretion of nitrite, as a percentage of nitrate being taken up, was negligible (Fig. 3.6, note: as percentages comprise rate measurements, NO$_2^-$ uptake, rather than excretion, yields negative values) due to partial or complete suppression of NO$_3$ uptake in Fe-replete (Fig. 3.4A) and Fe-deplete (Fig. 3.4B) cells, respectively. As incubations progressed, +Fe/NO$_3$ and -Fe/NO$_3$ cultures exhibited either minimal NO$_2^-$ excretion or a removal of ambient NO$_2^-$ (Fig. 3.6). In contrast, as NH$_4$-grown cells adjusted to the presence of NO$_3$, progressively higher NO$_3$ uptake rates (Fig. 3.4) were accompanied by elevated rates of NO$_2^-$ excretion, especially in -Fe cultures (e.g. 36 h, Fig. 3.6B). NO$_2^-$
Figure 3.6. Rates of nitrite excretion expressed as a percent of nitrate uptake rate following resuspension in 50 µM NO₃ for iron-replete (A) and iron-deplete (B) cultures grown on NO₃ (○) or NH₄ (●). Negative values result from NO₂⁻ uptake, rather than excretion. Experiments are those presented in Fig. 3.4. Data points and error terms plotted are as described in Fig. 3.2. Note differences in time scale between Fe-replete and Fe-deplete experiments.
Figure 3.7. Time course of changes in internal nitrate pools during NO$_3$ uptake experiments involving iron-replete (A) and iron-deplete (B) cultures grown on NO$_3$ (○) or NH$_4$ (●). Measurements were made during the experiment shown in Fig. 3.4. Data points and error terms plotted are as described in Fig. 3.2, except that values correspond to actual sample times rather than to midpoint determinations. Note differences in time scale between Fe-replete and Fe-deplete experiments.
excretion became negligible (or NO$_2^-$ was taken up) when NO$_3^-$ uptake rates reached a stable maximum past 60 h.

Internal nitrate was detected in all cultures immediately following NO$_3^-$ additions (Fig. 3.7), the smallest initial (0 h) pools occurring in -Fe/NH$_4^+$ experiments (Fig. 3.7B). After a sharp initial decrease in both +Fe/NO$_3^-$ and +Fe/NH$_4^+$ cultures, NH$_4^+$-grown cells began to accumulate nitrate over a 9 h period (i.e. from 3-12 h), while pools of those cells previously grown on NO$_3^-$ continued to decline steadily (Fig. 3.7A). A trend similar to the latter was also evident in -Fe/NO$_3^-$ cultures (Fig. 3.7B). Internal NO$_3^-$ concentrations of -Fe/NH$_4^+$ cells changed little during the 82 h incubations.

DISCUSSION

*Iron uptake.* -Fe/NO$_3^-$ and -Fe/NH$_4^+$ *G. sanguineum* cells exhibited elevated (cf. +Fe/NO$_3^-$ and +Fe/NH$_4^+$ cells) iron uptake rates ($\rho = \text{mol Fe} \cdot \text{liter cell vol}^{-1} \cdot \text{h}^{-1}$) when nitrogen was available as ammonium. However, in the presence of NO$_3^-$-nitrogen, a capacity for elevated Fe uptake was restricted to those Fe-deplete cells previously adapted to this N source (i.e. -Fe/NO$_3^-$ cells). These results are the first to demonstrate an enhancement of Fe uptake for an iron-stressed dinoflagellate. Specific rates of iron uptake ($V = \text{h}^{-1}$) by -Fe/NO$_3^-$ and -Fe/NH$_4^+$ cells, initially exceeded maximum growth rates ($\mu_{\text{max}}$) of this species by a factor of five. However, unlike measurements of $\rho$ above, no change in actual uptake
(i.e. transport) rate can be inferred based on \( V \). In other words, \( p \) can remain equal, while specific uptake \( (V) \) increases as a result of normalizing rates to the lower, Fe-deplete cell quotas. Enhancement of iron uptake has been reported for Fe-limited cells of the diatom *T. weissflogii* (Harrison and Morel 1986). This response is also characteristic of manganese, another trace element essential for phytoplankton growth (Sunda and Huntsman 1986).

The 12 h (cf. \( \approx 1.5 \) h for +Fe cultures) resolution for iron-deplete cultures was chosen based on the slow recovery of these cultures to their maximum growth rate (ca. 48-72 h, data not shown). While recognizing that such rates likely represent net rather than gross uptake, all values will be considered as short term measurements in the ensuing discussion. As the potential for reductions in maximum uptake rates \( (p_{\text{max}} = \text{the maximum transport rate measured during an experiment}) \) due to back reactions and feedback inhibition increases with decreasing initial resolution (i.e. increasing incubation time), \( p_{\text{max}} \) of Fe-deplete cultures (i.e. \( p_{-\text{Fe}}^{\text{max}} \)) may be underestimated. As a result, differences between \( p_{-\text{Fe}}^{\text{max}} \) and \( p_{\text{max}} \) for Fe-replete cultures (i.e. \( p_{+\text{Fe}}^{\text{max}} \)) may, similarly, be conservative estimates. For purposes of discussion, the quantitative difference between \( p_{-\text{Fe}}^{\text{max}} \) and \( p_{+\text{Fe}}^{\text{max}} \) for an experiment will be represented hereafter as the quotient \( p_{-\text{Fe}}^{\text{max}}/p_{+\text{Fe}}^{\text{max}} \). The extreme \( p_{-\text{Fe}}^{\text{max}}/p_{+\text{Fe}}^{\text{max}} \) values (both low and high) were observed in NH\(_4\)-grown cultures. A minimum of 0.5 was noted for NH\(_4\)-grown cultures resuspended in
NO₃ (Figs. 3.2A,B), while the maximum value of 2.5 occurred in these same cultures (i.e. NH₄-grown), but resuspended, instead, in NH₄ during Fe uptake experiments (Figs. 3.3A,B). This observation is important in that \( \rho^{-}\text{Fe}_{\text{max}}/\rho^{+}\text{Fe}_{\text{max}} \) of G. sanguineum can apparently vary by a factor of five, depending on both pre-conditioning N source and the N source supplied while iron is being taken up. An explanation will first be proposed to account for the effects of N source, followed by possible implications for the ability of cells to acclimate over a range of iron concentrations, as influenced by N source.

The difference in iron requirements between NO₃- and NH₄-grown cells is attributable to the iron content of nitrate and nitrite reductase enzymes (NR and NiR, respectively), both essential for NO₃ assimilation. Once reduced to NH₄ via NR- and NiR-catalyzed reactions, incorporation of NO₃-N into amino acids likely proceeds by the same major pathway as does nitrogen originally acquired as NH₄ (i.e. the GS-GOGAT pathway in most phytoplankton, Wheeler 1983). Thus, apart from the demand associated with NO₃ assimilation, additional iron requirements (e.g. chlorophyll and cytochrome biosynthesis, iron-sulfur proteins, glutamate synthase, etc.) are presumably common to both NO₃- and NH₄-grown cells of the same species. Calculations performed by Raven (1988) indicate that a plant reproducing photolithotrophically while supplied with NO₃, requires 1.6 times as much iron to sustain the same growth rate on NH₄. Consistent with this suggestion are the 1.5-fold
greater minimum Fe/N ratios of NO₃⁻ versus NH₄⁻-grown G. sanguineum reported in Chapter 2.

Consider now the two extreme values of $P_{\text{Fe max}}^{-}/P_{\text{Fe max}}^{+}$ noted above. Both estimates were obtained from cultures previously grown on NH₄. Thus, the five-fold difference in these ratios is solely a function of N source assimilated (i.e. NO₃ or NH₄) during iron uptake incubations. Prior to Fe uptake experiments, NH₄-grown cultures required the least amount of iron for growth (i.e. 40% less than NO₃-grown cultures, Raven 1988). As N source is unaltered by resuspending +Fe/NH₄ cells in NH₄, the Fe demand of these cells, and consequently $P_{\text{Fe max}}^{+}$, remains the lowest encountered (0.47 mol Fe·liter cell vol⁻¹·h⁻¹·10⁻³, Fig. 3.3A). In contrast, $P_{\text{Fe max}}^{-}$ of -Fe/NH₄ cells resuspended in NH₄ (1.2 mol Fe·liter cell vol⁻¹·h⁻¹·10⁻³, Fig. 3.3B) is > rates determined for any other experiment. Because -Fe/NH₄ cultures exhibit an excess of cellular N (C/N = 3.8, Chapter 2), and with Fe not required for N (i.e. NH₄) assimilation nor still limiting growth, recovery toward nutrient satiety (e.g. maximum Fe quota) in the presence of saturating Fe levels proceeds unimpeded. The resulting large $P_{\text{Fe max}}^{-}$ (1.2 mol Fe·liter cell vol⁻¹·h⁻¹·10⁻³), coupled with a minimum $P_{\text{Fe max}}^{+}$ (0.47 mol Fe·liter cell vol⁻¹·h⁻¹·10⁻³), yielded the highest $P_{\text{Fe max}}^{-}/P_{\text{Fe max}}^{+}$ ratio observed (i.e. 2.5).

Precisely the opposite trends in $P_{\text{Fe max}}^{+}$ and $P_{\text{Fe max}}^{-}$ are apparent when NH₄-grown cells are resuspended in NO₃-N. This
change in nitrogen source requires the maximum possible increase in iron needed to support growth and, under Fe-replete conditions, translates into the largest (by 2.5-fold) $p^{+Fe}_{max}$ measured during this study (1.1 mol Fe·liter cell vol$^{-1}·h^{-1}·10^{-3}$, Fig. 3.2A). However, for Fe-deplete cells, such a compensatory enhancement of Fe uptake does not occur. The resulting low $p^{-Fe}_{max}$ (5.2 mol Fe·liter cell vol$^{-1}·h^{-1}·10^{-3}$, Fig. 3.2B) likely reflects continued physiological stress due, however, to N rather than Fe deficiency, in the absence of a functional assimilatory NO$_3^-$ reducing system (see Nitrogen uptake below). The $p^{-Fe}_{max}/p^{+Fe}_{max}$ value for NH$_4^+$-grown cells resuspended in NO$_3^-$ during iron uptake incubations thus represents the minimum for this ratio (i.e. 0.5). These findings suggest that the qualitative nature of a transition between N sources (e.g. NH$_4^+$ to NO$_3^-$) may cause adjustment of uptake rates, as required to facilitate any inherent changes in the cell's demand for iron. Such transitions might also be expected to influence the range of iron concentrations over which a cell, through short term acclimation, can continue growing at maximum rates.

The preceding statement may be understood at a more quantitative level within the conceptual framework outlined recently by Morel (1987). His interpretation of experimental data, in the context of proposed hyperbolic relationships for growth and uptake rates, identifies the range of substrate concentrations ($S$) over which algae can maintain growth rates near $\mu_{max}$ as being bounded by $K_{\mu}$ (i.e. the half-saturation
constant for steady state growth) and $K_p$ (i.e. the half-saturation constant for short term uptake), where $K_\mu << K_p$. The derivation of $p$ versus $S$ curves for *T. weissflogii* provided an average $K_p$ of $3.6 \pm 1.3 \times 10^{-19}$ M between iron-limited and non-limited cells (Table 2, Harrison and Morel 1986). Thus, from the quotient $K_p/K_\mu$ ($K_\mu = 1.1 \times 10^{-21}$ M, Table 2, Harrison and Morel 1986) a greater than 300-fold acclimation range of iron free ion activities can be estimated for this diatom. Values of $K_p$ could not be determined from the saturating iron additions employed exclusively herein.

However, through algebraic manipulation of fundamental equations describing growth and uptake, Morel (1987) provides a means by which $K_p/K_\mu$ can be estimated based on $p^{+Fe}_{max}$, $p^{-Fe}_{max}$, minimum cellular quota ($Q_{min}$) and $Q_{max}$:

$$K_p/K_\mu = p^{-Fe}_{max}/p^{+Fe}_{max} \cdot Q_{max}/Q_{min}$$

(inverse of his equation (9)). It should be noted that in Morel's terminology, $p^{+Fe}_{max}$ is given as $p^{10}_{Fe}$_{max} (i.e. maximum short term uptake rate achieved under nutrient satiety) and $p^{-Fe}_{max}$ as $p^{hi}_{Fe}$_{max} (i.e. maximum short term uptake rate achieved under severe nutrient stress). Minimum and maximum iron quotas (per cell volume) of NO$_3$- and NH$_4$-grown *G. sanguineum* were obtained previously in Chapter 2, and varied little as a function of N source (Table 2.1).

Values for intermediate quotients (i.e. $p^{-Fe}_{max}/p^{+Fe}_{max}$, $Q_{max}/Q_{min}$) and $K_p/K_\mu$ for all experiments herein are presented in Table 3.2. These calculations suggest that the pFe (i.e. negative log iron free ion activity) acclimation range of
Table 3.2. Calculated values of $K_0/K_\mu$ for iron-limited growth of *G. sanguineum* grown on either nitrate or ammonium. The equation employed is $K_0/K_\mu = \rho^{-\text{Fe}_{\text{max}}}/\rho^{+\text{Fe}_{\text{max}}} \cdot Q_{\text{max}}/Q_{\text{min}}$, the inverse form of equation (9) given by Morel (1987). See text for further details.

<table>
<thead>
<tr>
<th></th>
<th>NO$_3$-GROWN/NO$_3$ RESUSP.</th>
<th>NO$_3$-GROWN/NH$_4$ RESUSP.</th>
<th>NH$_4$-GROWN/NO$_3$ RESUSP.</th>
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<td>101.46</td>
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1. Nitrogen source culture grown on (i.e. NO$_3$ or NH$_4$)/nitrogen source cells resuspended in for iron uptake experiment (i.e. NO$_3$ or NH$_4$).
2. Data from Figs. 3.2 and 3.3 (this study).
3. Average value from Chapter 2; $Q_{\text{Fe}}$ (per cell volume) did not vary as a function of nitrogen source (Tables 2.1 and 2.2).
G. sanguineum may be lower by as much as five-fold if reduced Fe bioavailability is accompanied by a transition from \( \text{NH}_4^+ \) to \( \text{NO}_3^- \) nitrogen nutrition (cf. continuous supply of \( \text{NH}_4^+ \)). Variations in \( \rho_{\text{max}} \) and \( Q_{\text{Fe}} \) made approximately equivalent contributions to defining the \( \rho_{\text{Fe}} \) acclimation range for T. weissflogii (Morel 1987), while reductions in \( Q_{\text{Fe}} \) account for 80-100% of this range herein (Table 3.2). The contribution of \( \rho^{-\text{Fe}}_{\text{max}}/\rho^{+\text{Fe}}_{\text{max}} \) in the current study may, however, be underestimated, as discussed above. In general terms, the magnitude of \( \rho_{\text{Fe}} \) ranges over which G. sanguineum can maintain near maximal growth rates (a factor of ca. 30-150) is 2-10 times less than for T. weissflogii (a factor of ca. 300, Morel 1987). Further, the absolute \( \rho_{\text{Fe}} \) bounds as delimited by \( K_\mu \) and \( K_\rho \) may be ten-fold higher for the dinoflagellate based on its \( K_\mu \) for iron-limited growth (Chapter 1). As in Chapters 1 and 2, results obtained herein appear to support the contention that this, and perhaps other, coastal dinoflagellate species may be more susceptible to iron-limited growth than are diatoms from neritic waters.

**Nitrogen uptake.** The most extreme consequences of iron depletion were apparent in nitrate uptake experiments. Following saturating Fe resupply, immediate acquisition of nitrate was completely inhibited for both \( \text{NO}_3^- \) and \( \text{NH}_4^+ \)-grown G. sanguineum, exhibiting a 24 h delay relative to Fe uptake by the same Fe-deplete cultures. Maximum \( \text{NO}_3^- \) uptake rates were achieved by both -Fe/\( \text{NO}_3^- \) and -Fe/\( \text{NH}_4^+ \) cultures at 60 h. However, 36 h rates of \( \text{NO}_3^- \) uptake in -Fe/\( \text{NH}_4^+ \) cultures were 3-
fold less than for -Fe/NO₃ cultures and further, were accompanied by the greatest rates of nitrite excretion (as a % of concurrent NO₃ uptake) measured during this study. These data suggest a somewhat more rapid development of maximum NO₃ uptake and assimilatory capacity following iron depletion in NO₃- than NH₄+-grown cells. Iron is not required for NO₃ uptake, and the Fe-dependent form of glutamate synthase (GOGAT), an NH₄ assimilatory enzyme, would be unaffected by N source. Thus, the suppression of NO₃ uptake and the temporal difference between NO₃- and NH₄+-grown cultures is likely related to enzymes essential for the assimilation of NO₃.

Nitrate assimilation is facilitated by two iron-containing enzymes, NR and NiR. Although there is clearly a shortage of cellular iron for continued enzyme synthesis, the precise mechanism(s) by which NO₃ assimilation is affected are uncertain. Possibilities include complete breakdown of NR and/or NiR, or perhaps partial degradation of these enzymes, allowing retention of precursor protein molecules. Extensive efforts to measure NR activity in nutrient-sufficient G. sanguineum cells have proved unsuccessful (W. Cochlan unpubl. results). While the implications with regard to the biochemical nature (or the existence) of NR remain to be elucidated, elevated Fe/N ratios for -Fe/NO₃ over -Fe/NH₄ cells (1.5-fold, Chapter 2) are consistent with a greater iron requirement associated with NO₃ assimilation. Further, symptoms of N deficiency observed in -Fe/NO₃ cultures (Chapter 2) indicate an iron stress-mediated functional impairment of
the assimilatory \( \text{NO}_3 \) reducing system. An inactive demolybdo form of nitrate reductase has been reported in the green alga *Chlorella* (Funkhouser et al. 1983); however, there is no evidence to suggest the existence of an inactive NR specifically devoid of a heme subunit(s). Reversible inactivation of NR, such as that often effected by ammonium uptake and assimilation, occurs over short time scales and by mechanism unlikely (i.e. specific to \( \text{NH}_4 \) or a product of its assimilation) herein (see Losada and Guerrero 1979).

Based on the more rapid elevation of \( \text{NO}_3 \) uptake (and presumably assimilation) upon Fe addition for \( \text{Fe}/\text{NO}_3 \) *G. sanguineum*, it would appear that de novo synthesis of NR and NiR proteins, and regeneration from inactive precursor protein molecules (a product of partial enzyme degradation), are likely of greater importance to \( \text{NH}_4^- \) and \( \text{NO}_3^- \)-grown cells, respectively. This is supported by reports of \( \text{NH}_4 \), or more correctly a product of its metabolism (e.g. glutamine), repression of NR synthesis (see Guerrero et al. 1981). A delay in achieving maximum \( \text{NO}_3 \) uptake and assimilation rates, exhibited by Fe-replete, \( \text{NH}_4^- \)-grown *G. sanguineum*, has been demonstrated for other algal species (see Collos 1983). However, opinions as to the relative contributions of de novo protein synthesis and enzyme activation to maximizing \( \text{NO}_3 \) assimilatory capacity (and thus uptake rates) in \( \text{NH}_4^- \)-grown cells (i.e. following transition to \( \text{NO}_3 \)), even under nutrient sufficiency, remain divided (see reviews by Guerrero et al. 1981, McCarthy 1981, Syrett 1981).
The iron required to assimilate NH$_4^+$ is equivalent for NO$_3^-$ and NH$_4^+$-grown cells, as was their rate of NH$_4^+$ uptake under Fe-replete conditions. However, NH$_4^+$ uptake for -Fe/NO$_3^-$ cultures exceeded that of -Fe/NH$_4^+$ cultures (after Fe resupply) by a factor of ca. three. In this case, the nitrogen rather than iron status of cells prior to experiments is best invoked as an explanation. -Fe/NO$_3^-$ cultures exhibit symptoms of N deficiency, while -Fe/NH$_4^+$ cultures are characterized by an excess of intracellular N (Chapter 2). It is well documented that saturated N (particularly NH$_4^+$) uptake rates of N-limited phytoplankton are greater than if organisms are N replete (see Goldman and Glibert 1983). Thus, as immediate acquisition and assimilation of NH$_4^+$ is not precluded by the need to synthesize/regenerate Fe-containing enzymes (cf. NO$_3^-$ assimilation), NH$_4^+$ uptake proceeds at an initially higher rate in NO$_3^-$-grown cells than for "nitrogen-sufficient" -Fe/NH$_4^+$ cells.

Conclusions. The current research indicates that Fe-stressed G. sanguineum develops an enhanced capacity for the uptake (i.e. transport) of iron. However, in the event Fe-stressed cells are required to switch from ammonium to nitrate nutrition, elevated iron uptake rates fail to be manifested. Presumably, the inability to immediately assimilate this oxidized N source (i.e. NO$_3^-$) maintains cells under a certain degree of nutritional (specifically N) stress. The initial delay in NO$_3^-$ uptake observed in these experiments would be consistent with this idea. Intuitively, it follows that a
continuous NH$_4^+$ supply to NH$_4^+$-grown cells should confer an adaptive advantage with regard to short term adjustments to iron stress. Data reported herein support this argument, insofar as the magnitude of the pFe range over which a cell can successfully acclimate on short time scales is approximated by $K_p/K_{\mu}$ (as elaborated by Morel 1987).

The importance of these findings as they relate to the ecology of red tide species, and coastal dinoflagellates in general, is uncertain. Based on the $K_{\mu}$ for Fe-limited growth (Chapter 1), the optimum Fe/C ratio (Chapter 2) and the magnitude of the pFe acclimation range (this chapter) of G. sanguineum as compared to the neritic diatom T. weissflogii, the former species is apparently more susceptible to growth limitation by iron. Nitrogen source and N source transitions may further affect a cell's ability to rapidly acclimate to iron stress. However, it is difficult to quantify the balance between Fe supply and phytoplankton demand or acquisition capabilities, and thus the actual potential for Fe-limited growth in coastal waters.
CHAPTER 4.
THE ULTRASTRUCTURE OF IRON STRESS-MEDIATED CYSTS IN THE TOXIC RED TIDE DINOFLAGELLATE PROTOGONYAULAX TAMARENSIS (LEBOUR) TAYLOR

BACKGROUND

Attempts to explain the occurrence of red tide dinoflagellate blooms have considered among other possibilities the potential importance of factors associated with terrestrial runoff such as humates, trace metals and salinity/stratification (Prakash 1975, Anderson and Wall 1978, Provasoli 1979, Anderson et al. 1983). Several authors (e.g. Kim and Martin 1974, Glover 1978) have noted an apparent correlation between elevated levels of soluble iron derived from river discharge and land runoff, and the development of red tides. Such a relationship may imply the alleviation of iron-limiting conditions by an increase in iron supply rate. Thus, if growth is at least initially iron-limited, the decline of a bloom may result from localized depletion of biologically available iron.

An important factor known to be associated with bloom decline is the initiation of sexual reproduction leading to the formation of dormant resting cysts (Anderson 1984). As natural cyst production is triggered by an as yet undefined set of environmental cues, and iron bioavailability may influence bloom population dynamics, the effects of iron stress on red tide dinoflagellate life histories are of interest. Further, if we are to consider the possibility of
iron stress-mediated population decline, the question of whether iron stress can induce cyst formation in a bloom species must also be addressed. The present study thus includes a description of changes in life history stage of a red tide dinoflagellate subjected to increasing iron stress.

Protogonyaulax (= Gonyaulax) tamarensis (Lebour) Taylor is a red tide dinoflagellate responsible for numerous paralytic shellfish poisoning (PSP) outbreaks in many of the world's temperate coastal waters (Taylor 1984). Cyst formation has been well documented for this species both in laboratory cultures (Turpin et al. 1978, Anderson et al. 1984, Anderson and Lindquist 1985) and in field populations (Anderson 1980, Anderson et al. 1983). Evidence for the importance of cysts in the population dynamics of P. tamarensis blooms has also been reported (Anderson and Morel 1979, Anderson 1984, Anderson and Keafer 1985). Owing to its ecological importance and the availability of data regarding cyst formation by this species P. tamarensis was selected as a test organism.

Several distinct types of dinoflagellate cysts are presently recognized (Taylor in press). Of these, only temporary (= pellicle) and resting (= hypnozygote) cysts are known to form under conditions of nutrient stress. In P. tamarensis, the former result from an asexual process (i.e. ecdysis), while the latter are products of sexual reproduction. Both cyst types have been described previously
for this species (Anderson and Wall 1978, Fritz 1986). There is a growing body of literature describing many aspects of dinoflagellate cysts, including cyst morphology (see reviews by Dale 1983, Loeblich and Loeblich 1984), the process of sexual reproduction (see review by Pfiester 1984) and the environmental control of sexual cycles (see review by Pfiester and Anderson 1987). While recent work by Fritz (1986) has produced a detailed ultrastructural account of the *P. tamarensis* life cycle, including encysted stages from laboratory (induced by nitrogen deficiency) and field populations, few data exist on the ultrastructure of cyst protoplasts (Bibby and Dodge 1972, Dürr 1979, see also Pfiester 1984). Therefore, in addition to some insights concerning life history response to iron stress, this study provides an ultrastructural description of iron stress-mediated *P. tamarensis* cysts and a comparison with nitrogen stress-mediated cysts examined by Fritz (1986).

MATERIALS AND METHODS

*General culture maintenance.* *Protoctogonyaulax tamarensis* (culture #D255, North East Pacific Culture Collection, Dept. of Oceanography, University of British Columbia) was maintained on modified ESNW-enriched natural seawater medium (Harrison et al. 1980). The original formulation was adjusted to contain 1 µM iron (as FeCl₃·6H₂O) and 0.5 µM molybdenum (as Na₂MoO₄), and designated +Fe ESNW. Medium used to grow cells into iron depletion (-Fe ESNW) was prepared with natural
seawater cleaned of organics by treatment with activated charcoal, and of residual iron by passage over Chelex 100 ion exchange resin (Morel et al. 1979). This chelexed seawater was enriched using modified ESNW nutrients (as described above) with the omission of iron and EDTA. All batch cultures (unstirred) were grown at 16°C with an irradiance of 120 \( \mu E\cdot m^{-2}\cdot s^{-1} \) supplied on a 14:10 L/D cycle by Sylvania\textsuperscript{R} VHO Daylight fluorescent tubes.

Iron depletion. *P. tamarensis* was subjected to conditions of increasing iron stress by inoculating 10 l of -Fe ESNW with 1 l of late exponential phase cells maintained on +Fe ESNW (Day 0), and allowing the culture to grow until depleted of iron (Day 24). On each of thirteen sampling dates, 1 l of culture was removed for cell counts, determination of life history stage (i.e. vegetative cell, pellicular or zygotic cyst) and detection of dissolved Fe-binding siderophores (G. Boyer unpubl. data). For the first six samplings, 1 l of fresh -Fe ESNW was added back to the culture in order to reduce sampling limitations imposed by culture volume restrictions, and to dilute residual iron in the medium. Data on life history stages are reported as percent of total cells\cdot ml\(^{-1}\). A qualitative assessment of culture iron status was performed twice during the course of the experiment (Day 17, Day 24) as follows: one part culture was combined with two parts -Fe ESNW and split into two equal volumes; 1 \( \mu M \) FeCl\(_3\)\cdot6H\(_2\)O (freshly prepared, without EDTA) was added to one half, while nothing was added to the other. The
growth response of these batch cultures, based on light microscope cell counts, was monitored for 25 days.

Ultrastructure. Samples for transmission electron microscopy (TEM) were taken just prior to (control, Day 0) and following inoculation into -Fe ESNW (Days 1, 7, 13 and 20). Cells were harvested on 0.45 μm Millipore filters (type HA). Samples prepared for TEM were fixed on filters with 1.5% glutaraldehyde in 0.1 M sodium cacodylate and 0.4 M sucrose (2 h, room temp.), post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate (1 h, room temp.), en bloc stained with 1% aqueous uranyl acetate, and dehydrated in a graded ethanol/propylene oxide series. Samples were embedded in a 1-2 mm thick disk of Epon 812 to facilitate microscopic identification and selection of individual specimens for sectioning. Random and serial sections were cut using a diamond knife, picked up on uncoated 200- or formvar-coated 50-mesh copper grids, stained with saturated uranyl acetate (in 50% methanol) and lead citrate, and examined in a Zeiss EM10 transmission electron microscope. Light microscopy (LM) of living and 2% formalin-preserved material was performed with a Zeiss Standard microscope.

Ultrastructural descriptions of vegetative cells are based exclusively on control samples (Day 0), while treatment of cysts includes material from Days 1, 7, 13, and 20. Terminology used to describe the amphiesma is that of Morrill and Loeblich (1983).
RESULTS

Iron bioassays. Inoculation of iron-replete *P. tamarensis* cells into -Fe ESNW resulted in iron depletion by Day 24. In the present context, iron depletion implies cessation of growth due to the absence of biologically available iron, at which time it is assumed that the iron cell quota reaches a minimum value. Bioassays (Day 24) confirmed that cells were, in fact, depleted of iron and not of another nutrient (Figs. 4.1A,B). Cells harvested on Day 17 were not iron-deplete, as a growth response was observed irrespective of iron addition. Conversely, for the Day 24 bioassay, growth occurred only with the addition of iron, indicating that cellular iron reserves were exhausted between Day 17 and 24. The first bioassay (Day 17) began to show a decrease in cell density for the -Fe culture after seven days. Assuming minimal iron contamination of -Fe ESNW added in bioassays, biologically available iron was likely depleted in the primary culture vessel (i.e. source of bioassay inoculum) on or just prior to Day 24.

Life history dynamics. Culture composition based on life history stage (i.e., vegetative cells, pellicular and zygotic cysts) is shown in Fig. 4.2. Pellicular cyst numbers were below detection limits of LM counts for the control sample (Day 0). Although this cyst type was encountered on Day 1 in TEM material, they were first observed in LM counts on Day 5. Values were initially below 1% and did not exceed 5% until Day
Figure 4.1. Iron bioassays performed on Day 17 (A) and Day 24 (B) showing changes in cell density of -Fe (○) and +Fe (●) cultures.
Figure 4.2. Changes in life history stage over a time course of increasing iron stress. Values for vegetative cells (▲), pellicular (○), and zygotic (●) cysts are expressed as percent of total cells·ml⁻¹.
24, when pellicular cysts accounted for over 25% of cells in the culture. Large (ca. 50-60 μm), heavily pigmented cells comprised about 1% of the control culture, and were identified as products of sexual fusion (i.e. zygotes). Zygote percentages fluctuated near control levels for almost two weeks, increased to 11% (8% hypnozygotes, 3% planozygotes) by Day 13, and averaged about 5% for the remainder of the experiment. Quadriflagellate planozygotes (precursors to hypnozygotes), first noted on Day 13, were always less than 3% of the total cell number. Vegetative cells exceeded 85% until Day 24 when increasing pellicular cyst percentages reduced them to 72%. A decrease in vegetative cell size (from ca. 35 μm to ca. 25 μm, diam.) and reduced pigmentation were associated with increasing iron stress.

Vegetative cells. Vegetative cells were surrounded by an amphiesma consisting of five components (Fig. 4.3). The three most distal elements included the outer membrane (OM), the outer plate membrane (OPM) and a system of thecal plates. It did not appear that each plate was completely enclosed within an individual vesicle. Directly below the plates was a continuous pellicle. The pellicular layer showed a trilaminar structure and was associated with a thin matrix of 'fuzzy' material on both proximal and distal sides. The innermost component of the amphiesma was the cytoplasmic membrane (CM). A network of microtubules occurred immediately beneath the CM.
Figures 4.3-4.8. *Protothecomyxa tamarensis* vegetative cells. NEPCC culture #D255. Fig. 4.3. Amphiesma comprised of outer membrane (O), outer plate membrane (P), thecal plates (Pl), pellicle (Pe), and cytoplasmic membrane (C). Scale = 0.2 μm. Fig. 4.4. Longitudinal section of protoplast. C-shaped nucleus (N) contains nucleolus (Nu) around inner curvature. Crystalline material (arrowhead) is present in hypothecal vacuolar region. Scale = 5 μm. Fig. 4.5. Chloroplast with lamellae exhibiting closely paired thylakoids (arrows). Scale = 0.5 μm. Fig. 4.6. Grana-like stacks of tightly appressed thylakoid membranes. Scale = 0.5 μm. Fig. 4.7. Mitochondrion with tubular cristae and characteristic matrix density. Scale = 0.2 μm. Fig. 4.8. Aggregate of crystalline material showing details of crystal morphology. Vesicle (V) containing concentric membrane swirls is apparent. Scale = 0.2 μm.
The vegetative protoplast (Fig. 4.4) was dominated by a large C-shaped nucleus with a single nucleolus contained around its inner curvature. Chromosomes were densely packed and exhibited a periodic transverse banding pattern in longitudinal section. Dictyosomes and concentrations of endoplasmic reticulum were partially encircled by the C-shape of the nucleus. Chloroplast lamellae appeared roughly parallel to each other and generally consisted of two closely appressed thylakoids (Fig. 4.5). Grana-like stacks of as many as eight thylakoids were common (Fig. 4.6). Mitochondria possessed tubular cristae embedded in a relatively dense matrix material (Fig. 4.7).

Vacuolar areas were extensive and exhibited a variety of membranous inclusions (e.g. Fig. 4.8) and prominent aggregates of a crystalline material (Figs. 4.4,4.8). Crystals showed an electron-dense outline and were approximately 30 nm wide and variable in length. The occurrence of starch and lipid storage reserves was negligible. A small accumulation body was occasionally present.

Pellicular cysts. The most consistent feature characterizing pellicular cysts, irrespective of culture iron status, was the amphiesma. Of the five vegetative wall components, these cysts retained only the pellicle and CM (Fig. 4.9), yielding a rounded cell of irregular outline (Fig. 4.10). The trilaminar structure of the cyst pellicle was subtended by a layer of granular material not present in
Figures 4.9-4.12. *Protogonyaulax tamarensis* pellicular cysts. NEPCC culture #D255. N = nucleus. Fig. 4.9. Amphiesma consisting of pellicle (Pe) subtended by thick layer of granular material (G) possibly originating from vesicles (arrowhead) derived from the cytoplasmic membrane (C). Scale = 0.1 μm. Fig. 4.10. Cyst with proplast resembling vegetative cell. Note lack of storage products and presence of crystalline material (arrowheads). Scale = 5 μm. Fig. 4.11. Cyst showing densely aggregated cytoplasm with minimal vacuolar space. Several starch granules (S) and accumulation bodies (A) are present. Scale = 5 μm. Fig. 4.12. Cyst containing numerous starch (S) and lipid (L) storage inclusions. Crystalline material (arrowhead) is apparent. Scale = 5 μm.
vegetative cells. This layer varied in thickness but was generally about 100 nm wide. Material appeared to be delivered by vesicles derived from the CM (Fig. 4.9).

Pellicular cysts were similar in size to vegetative cells and were also smaller under increased iron stress. Intracellular organization varied considerably among samples collected on Day 1. Ultrastructure of these cysts ranged from that closely resembling vegetative cells (Fig. 4.10) to a dense aggregate of cytoplasmic constituents, including accumulation bodies and starch inclusions (Fig. 4.11). However, throughout the experiment pellicular cysts generally contained a moderate amount of cytoplasm and at least several starch and lipid reserves (Fig. 4.12). Aggregates of the crystalline material occurring in vegetative cells were evident in most cysts (Figs. 4.10,4.12).

The cyst nucleus and chromosomes were indistinguishable from those of vegetative cells until Day 20, when changes in chromatin structure became evident. Regular banding (Fig. 4.13) was disrupted to varying degrees, resulting in clumping of chromatin into electron-dense aggregates (Fig. 4.14). Differences between cyst and vegetative chloroplasts were apparent on Day 1, but were considerably more prevalent later in the time course. Alterations ranged from slight swelling of thylakoids (Fig. 4.15) to extensive tubular dilation of membranes (Fig. 4.16). While lamellae of Day 20 cysts frequently exhibited single rather than paired thylakoids,
Figures 4.13-4.17. *Protogonyaulax tamarensis* pellicular cysts. NEPCC culture #D255. Fig. 4.13. Chromosomes similar to those of vegetative cells exhibiting highly organized transverse banding of chromatin (arrows). Scale = 0.5 µm. Fig. 4.14. Chromosomes with disrupted chromatin structure. Chromatin is clumped into electron-dense aggregates (arrows). Nucleolus (Nu) is apparent. Scale = 1 µm. Fig. 4.15. Chloroplast showing single thylakoids and grana-like stacks containing slightly swollen membranes (arrows). Scale = 0.5 µm. Fig. 4.16. Chloroplast exhibiting extensive tubular dilation of thylakoid membranes seen in cross- (arrowheads) and longitudinal- (arrows) section. Scale = 0.2 µm. Fig. 4.17. Mitochondrion with matrix of reduced density and possibly fewer cristae relative to vegetative cells. Note 'filled-in' appearance of cristae (arrows) Scale = 0.5 µm.
grana-like stacks containing partially dilated membranes also occurred in many chloroplasts (Fig. 4.15; cf. Fig. 4.6). Due to the inherent morphological variability of mitochondria, deviations from the vegetative state were difficult to assess. The most obvious alterations were reductions in the density of the matrix, and possibly in the number of cristae profiles (Fig. 4.17). Also, cristae appeared to be filled with electron-dense material. This condition was primarily associated with cysts exhibiting aberrant chromosome ultrastructure.

Hypnozygotes. Hypnozygotes averaged 50-60 μm in the longest dimension and were generally heavily pigmented. Unlike pellicular cysts, degenerative ultrastructural alterations in response to increasing iron stress were minimal. Among hypnozygotes harvested at each sampling time, two morphologies (Stages 1 (S1) and 2 (S2)) could be distinguished based on shape, surface outline, and nuclear fine structure. S1 cysts (Fig. 4.18) were rounded or slightly elongate with an irregular outline while S2 cysts (Fig. 4.19) were more elongate and generally smoother. Chromosome profiles in S1 nuclei were densely-packed, and often ill-defined and similar to the nucleoplasm in electron density (Fig. 4.18). Nuclei of most S2 cysts showed very discrete chromosomes of near electron-opaque density (Fig. 4.19). Many chromosomes contained one or two small electron-lucent areas. Clusters of cables or filaments (15-25 nm diam.) were
Figures 4.18-4.23. Protogonyaulax tamarensis hypnozygotes. NEPCC culture #D255. Fig. 4.18. Stage 1 cyst with irregular outline and nucleus containing densely-packed, somewhat ill-defined chromosomes. Crystalline material (arrowhead) and accumulation bodies (A) are apparent. Scale = 10 μm. Fig. 4.19. Smooth-walled, elongate Stage 2 cyst. Nucleus (N) shows discrete, electron-dense chromosomes. Note presence of crystalline material (arrowhead), cortical lipid inclusions (L), and an accumulation body (A). Scale = 10 μm. Fig. 4.20. S2 nucleolus (Nu) and associated filaments (arrowhead). Note cross-section of filament penetrating nucleolus (arrow) and presence of large nucleolar organizing chromosome (No). Scale = 1 μm. Fig. 4.21. Amphiesma exhibiting pellicle (Pe), granular layer (G), and amorphous, electron-dense material (A) immediately adjacent to cytoplasmic membrane (C). Membrane fragments distal to cytoplasmic membrane are likely remains of vesicles depositing amorphous material. Scale = 0.1 μm. Fig. 4.22. Annulate nuclear vesicles formed as invaginations of nuclear envelope (Ne). Nuclear pores (arrows) are apparent. Scale = 0.5 μm. Fig. 4.23. Aggregation of mitochondria showing reduced matrix density. Note dividing mitochondrion (arrowhead). Scale = 1 μm.
associated with and penetrated the nucleolus of certain S2 nuclei (Fig. 4.20).

S1 and S2 cysts had several characteristics in common. Although hypnozygote and pellicular cyst amphiesmae were similar, the former had an additional layer of amorphous electron-dense material between the thicker granular region and CM (Fig. 4.21). Hypnozygote nuclei exhibited invaginations of the nuclear envelope which appeared as annulate vesicles in the nucleoplasm (Fig. 4.22). Invaginations enclosed a central core of electron-dense material surrounded by a less electron-dense fibrous region. Storage products, including lipid reserves in the cortical cytoplasm and accumulation bodies (as many as ten per cell seen), were present in all zygotic cysts examined (Figs. 4.18, 4.19). Starch bodies were notably absent from most of these cysts. Chloroplasts were generally similar to those of vegetative cells, while hypnozygote mitochondria exhibited a reduction in the density of the matrix and were frequently grouped in large clusters (Fig. 4.23). The crystalline material noted in both vegetative cells and pellicular cysts was evident in most zygotic cysts.

DISCUSSION

*Life history dynamics.* Life history stages of *P. tamarensis* were monitored under conditions of increasing iron stress. Results provide the first evidence of iron
stress-mediated sexuality in a red tide dinoflagellate. The response of *P. tamarensis* to iron stress, as determined by zygote production (ca. 10%, planozygotes + hypnozygotes, this study), appears weaker than for depletion of nitrogen (ca. 20%, hypnozygotes only, Anderson et al. 1984) or phosphorus (ca. 25%, planozygotes + hypnozygotes, Anderson and Lindquist 1985). However, zygote percentages near 20% have been measured for *P. tamarensis* during iron depletion experiments not reported here (A. Cembella unpubl. data). Additional work is required to quantify zygote production of this species under iron stress.

Nutrient depletion has frequently been employed to induce dinoflagellate sexuality (see review by Pfiester and Anderson 1987). Nevertheless, apart from several exceptions (e.g. Anderson et al. 1985, Anderson and Lindquist 1985), the temporal relationship between sexuality and ambient or internal nutrient levels has received little attention. Although iron concentrations (ambient or internal) were not measured, bioassays confirmed depletion of biologically available iron by termination of the present experiment. Observation of maximum zygote percentages more than one week prior to this indicates that sexuality is associated with conditions of iron limitation rather than depletion. Anderson and Lindquist (1985) have suggested that phosphorus stress-mediated sexuality in *P. tamarensis* may occur in response to nutrient limitation at detectable phosphorus concentrations.
Their results could indicate a high cellular requirement and a low uptake affinity for this element.

Although no kinetics of iron uptake are available for *P. tamarensis*, Mueller (1985) has determined cellular iron concentrations ($Q_{Fe}$) in iron-deficient cells of the same clone used in the present study. His value of $1.3 \times 10^{-18}$ mol Fe $\mu$m$^{-3}$ represents essentially an over estimate of minimum iron cell quota. It is likely that Mueller's cultures were not truly depleted of iron. Further, no attempt was made to remove surface-bound (i.e. non-biological) iron. This fraction may be a considerable portion of iron associated with diatom cells (Anderson and Morel 1982), but it appears to be of less significance for certain dinoflagellates (see Chapter 2). Overlooking these uncertainties, the "minimum" $Q_{Fe}$ of this species appears to exceed ($\geq 10$-fold) $Q_{Fe,min}$ measured for several other phytoplankton species, including *Thalassiosira weissflogii*, *Dunalieilla tertiolecta* and *Pavlova lutheri* (see Harrison and Morel 1986, Table 2). These data suggest a high iron requirement for *P. tamarensis* and may thus explain the occurrence of iron stress effects (e.g. sexual induction) prior to the depletion of biologically available iron. Also consistent with this idea is a study by Anderson and Morel (1979) of red tides caused by *P. tamarensis*, where a sharp decrease in total iron concentration was associated with the early stages of population decline. Although total iron was never less than 1 $\mu$M, it is now accepted that free ion activity rather than total concentration largely regulates the
bioavailability of a metal in seawater (Morel and Morel-Laurens 1983). Iron stress-mediated growth limitation thus remains a possible explanation of declining cell densities observed by Anderson and Morel (1979); however, the contribution of sexuality was not quantified.

_Hypnozygotes._ Sexuality in _P. tamarensis_ involves the fusion of two gametes (morphologically similar to vegetative cells) to produce a thecate, quadriflagellate planozygote which, following loss of plates and flagella, yields a hypnozygote. The hypnozygote cell wall is initially indistinguishable from the temporary cyst amphiesma but develops small (ca. 0.5 µm) outfoldings of the pellicle (= papillae), and upon maturation is smooth and consists of three layers (Fritz 1986). This process is accompanied by an elongation of the hypnozygote. Two classes of hypnozygote (S1 and S2) were encountered in this study and appear to be early phases in the development of sexual cysts. Although no papillae were observed, S1 cysts are likely intermediate between planozygotes and S2 cysts, based on their irregular outline and absence of plates. S2 cysts probably represent the next stage in hypnozygote development characterized by elongate shape, smooth surface and also the presence of filaments of unknown function associated with the nucleolus (Fritz 1986).

Hypnozygotes from culture and field populations examined by Fritz exhibited several notable differences from S2 cysts.
Cell wall thickness was ca. five times greater than for S2 cysts. In addition, S2 cysts contain several, rather than one, accumulation bodies and rarely show starch reserves. These discrepancies are probably related to maturation period, and possibly differences in induction mechanism (i.e. iron vs. nitrogen stress-mediated) and other culture conditions. It should be noted that the implications of enhanced chromosome condensation in S2 over S1 zygotic cysts are uncertain, but may be associated with dormancy and reduced nuclear activity.

Annulate nuclear vesicles encountered in hypnozygotes during the present study, have been observed previously in the nuclei of fusing *P. tamarensis* gametes (L. Fritz pers. comm.). Although the vesicles' function may be associated with sexuality, unequivocal confirmation of their absence from vegetative cells is still required. The occurrence of intranuclear bacteria and their association with toxin production in *P. tamarensis* has been suggested (Kodama et al. 1988). The *P. tamarensis* strain employed in the present study is among the more toxic isolates of this species (Boyer et al. 1986, Cembella et al. 1987). Aside from the vesicles mentioned above (whose function remains to be elucidated), nuclei contained no other unusual inclusions.

**Pellicular cysts.** In the present study, the percentage of pellicular cysts (less than 5%) was lower than expected, as similar experiments with this species have yielded numbers exceeding 30% upon transfer into -Fe ESNW (A. Cembella unpubl.)
data). The sharp increase to over 25% on Day 24 appears related to exhaustion of biologically available iron. Temporary cysts represent a very dynamic stage in the life history of *P. tamarensis*. Formed under a variety of environmental stimuli (see Fritz and Triemer 1985 and this study), these cysts can germinate to release vegetative cells within 8-24 h of return to favorable growth conditions (Fritz and Triemer 1985). In the present study, however, a relatively constant percentage of pellicular cysts (excluding Day 24) and decreasing iron supply suggest that extensive germination was unlikely.

Available evidence indicates that pellicular cysts and hypnozygotes function in the maintenance of viability over brief (days-weeks) and extended (months-years) exposure to adverse environmental factors, respectively. Upon termination of this experiment, degenerative changes in organelle morphology were largely restricted to pellicular cysts. These data are consistent with the above ecological roles and support the contention that temporary cysts are more susceptible to short-term stress than are hypnozygotes.

Anderson and Wall (1978) demonstrated that the duration of pellicular cyst viability is regulated, in part, by the induction mechanism. Cysts induced by undefined nutritional stress (medium deficient in N, P, Si and vitamins) exhibited viability (50%) superior to those formed under copper toxicity (15%) or low temperature (0%) following 25 days of encystment.
The present results suggest further that viability might also be affected by physiologically-mediated changes in ultrastructure specific to the type of nutrient deficiency.

Iron is involved in chlorophyll biosynthesis and is a component of photosynthetic and respiratory electron transport cytochromes (Marschner 1986). Irregularities in chloroplasts and mitochondria could therefore result from insufficient iron supply. These effects, as well as accumulation of storage products, are also associated with other forms of nutrient stress (e.g. Shifrin and Chisholm 1981, Doucette et al. 1987) and may instead represent a general response to poor growth conditions. As there are no previous reports of nutrient stress-mediated alterations in chromatin structure (to the extent encountered during this work), a similar explanation of aberrant cyst chromosomes seems less likely.

Dinoflagellate chromosomes contain elevated (relative to the nucleoplasm and cytoplasm) levels of transition metal cations, including iron (see Sigee 1984,1986), thought to aid in the stabilization of chromatin structure. Sigee and Kearns (1981) have shown that reduced ambient ion concentrations (10% of original medium) lowered the relative proportion of divalent metal cations and increased the degree of chromatin condensation in chromosomes of Glenodinium foliacium. Depletion of biologically available iron could thus alter the spatial organization of DNA and result in clumping of chromatin fibers exhibited by cysts late in the experiment.
The specificity and influence on viability of various iron stress-mediated ultrastructural modifications is difficult to assess; however, disruption of chromatin arrangement may be directly related to iron depletion and is likely to reduce the probability of germination.

**Vegetative cells.** The ultrastructure of the *P. tamarensis* vegetative amphiesma and protoplast is generally consistent with that of other thecate dinoflagellates (see reviews by Netzel and Dürr 1984, Dodge and Greuet 1987). A feature also observed in pellicular and zygotic cysts is the crystalline material present in certain vacuolar regions (Figs. 4.4,4.10,4.12,4.18,4.19). A variety of crystalline inclusions have been reported from other dinoflagellates (e.g. Bibby and Dodge 1972, Pokorny and Gold 1973, Wedemeyer and Wilcox 1984). In comparison to those described previously, crystals of *P. tamarensis* are narrower, not closely associated with a limiting membrane, and are largely different in their location, general morphology and tendency to form dense, discrete aggregates. Speculation as to crystal composition has included guanine (Hastings et al. 1966) and calcium oxalate (Taylor 1968). Energy dispersive x-ray analysis of *P. tamarensis* crystals failed to discern the primary constitutive element(s); however, sample preparation was not designed to optimize this technique. Some evidence indicates that crystals occurring in peripheral vacuoles may be involved in cell wall formation (J. Chesnick pers. comm.). Although this is unlikely in *P. tamarensis* due to the crystals'
location, the possibility of a storage (cf. Gold and Pokorny 1973) or excretory role (cf. Schmitter 1971) warrants further investigation.

Summary. Iron stress can induce both pellicular and zygotic cyst formation in the toxic red tide dinoflagellate P. tamarensis. Ultrastructural data indicate that temporary cysts are more susceptible than hypnozygotes to short-term stress effects and are thus consistent with their hypothesized ecological roles. It is clear that a causal relationship between iron bioavailability and red tides cannot be established in the laboratory. Nevertheless, these results demonstrate that iron deficiency has the potential to effect changes in life history stage and thus population dynamics of this red tide species.
GENERAL CONCLUSIONS

Various aspects of red tide dinoflagellate iron and nitrogen nutrition have been considered in this thesis. Certain findings represent new, and much needed, information on the iron nutrition of these ecologically important organisms. The major conclusions and contributions of this work are summarized below.

1. The first iron-limited growth kinetics for a coastal dinoflagellate (G. sanguineum) have been determined. The half-saturation constant \( K_\mu \) was estimated to be 10-1000 times greater than for other neritic species examined previously. This disparity in \( K_\mu \) was explained, in part, by this species' comparatively large iron requirement (as measured by Fe/C ratios), which exceeded those of certain coastal diatoms by one to two orders of magnitude.

2. Reductions in G. sanguineum chlorophyll a (chl a) quotas \( Q_{\text{chl}} \) and photosynthetic electron transport (PET) efficiency (as measured by the index \( 1-F/F_D \), where \( F = \text{in vivo fluorescence} \) and \( F_D = \text{DCMU-enhanced } F \)) occurred under iron limitation and depletion. These alterations translated into a 7-fold increase in \( F/\text{chl a} \) ratios for iron-deplete cells. These observation are consistent with, and were discussed in relation to, the essential role of iron in chl a and PET component (i.e. cytochromes and Fe-S proteins) biosynthesis.
3. Nitrogen depletion effected a decline in
G. sanguineum Q_{chl} of a magnitude similar to that caused by
iron depletion. Conversely, N stress did not reduce PET
efficiency nor enhance F/chl a ratios to the same extent as Fe
stress. These results provided evidence for the specificity
of iron stress-mediated effects on certain PET components.

4. F/chl a, in conjunction with other probes of
nutritional status, was suggested as a potentially useful
indicator of iron stress in field populations, owing to the
magnitude and apparent specificity of changes in this ratio.
However, as F/chl a was elevated to a lesser degree in iron-
deplete, NH\textsubscript{4}-grown than NO\textsubscript{3}-grown cells, and the latter cells
exhibited symptoms of N deficiency, consideration of multiple
indices of nutrient deficiency and careful interpretation of
results were also stressed.

5. Fe/N ratios demonstrated a larger (1.5-fold) minimum
iron requirement for NO\textsubscript{3}- than NH\textsubscript{4}-grown G. sanguineum cells.
This difference was considered to be a reflection of the iron
requirement of the reductive NO\textsubscript{3} assimilatory enzymes, nitrate
and nitrite reductase.

6. Acquisition of nitrogen by iron-deplete, NO\textsubscript{3}-grown
G. sanguineum cells was sufficiently inhibited to yield
symptoms of nitrogen deficiency. Supporting evidence was
provided by decreased (ca. 1.4-fold) nitrogen quotas and free
amino acid/protein ratios.
7. The first demonstration of enhanced iron transport rates ($\rho$) in an iron-stressed dinoflagellate (*G. sanguineum*), has been presented. It was noted, however, that an elevated $\rho$ failed to be manifested in iron-deplete cells following a transition from $\text{NH}_4^+$ to $\text{NO}_3^-$ nutrition. This suppression was believed to result from concurrent iron and nitrogen stress, due to the inability of $\text{NH}_4^+$-grown cells to rapidly assimilate $\text{NO}_3^-$. The complete initial inhibition of $\text{NO}_3^-$ uptake when Fe-deplete, $\text{NH}_4^+$-grown cells were given saturating iron additions was consistent with this idea. In contrast, $\text{NH}_4^+$ was taken up immediately by these, and iron-deplete, $\text{NO}_3^-$-grown cells.

8. Based on the $K_\mu$ for iron-limited growth, the comparatively large Fe/C ratio and the relatively narrow range of iron concentrations over which maximum or near maximum growth rates can be maintained by short term adaptation (as estimated theoretically by $K_\rho/K_\mu$), it was suggested that *G. sanguineum* is more susceptible to iron-limited growth than the neritic diatom *T. weissflogii*.

9. Iron stress caused reductions in chloroplast number and some degeneration of lamellar organization in *G. sanguineum* and provided a structural basis for changes noted in $Q_{\text{chl}}$ and fluorescence properties. For *P. tamarensis*, iron limitation induced the formation of temporary (= pellicle) and resting (= hypnozygotes) cysts. Degenerative changes in organelle (i.e. chloroplasts, mitochondria and chromosomes) ultrastructure were largely restricted to
pellicular cysts, consistent with their hypothesized role of maintaining viability over brief, rather than extended (cf. hypnozygotes), exposure to adverse conditions.


Murphy, L.S., Guillard, R.R.L. & Brown, J.F. 1984. The effects of iron and manganese on copper sensitivity in diatoms:


nitrogen from marine phytoplankton. J. Plankton Res. 4:695-704.


APPENDIX 1

Growth-irradiance curve of G. sanguineum, with associated changes in cell volume

Objective: This experiment was performed to obtain a growth ($\mu$)-irradiance (I) curve for G. sanguineum and to observe any concurrent changes in cell volume as a function of I.

Methods: Cultures of G. sanguineum were grown according to the conditions and procedures outlined in the General culture maintenance section of Chapter 1, except that neutral density screening (without blue Plexiglas$^R$) was used to achieve irradiance levels of 208, 92, 52, 30 and 12 $\mu$E·m$^{-2}$·s$^{-1}$ (one culture for each irradiance). Following stabilization of $\mu$, cell division rates and volumes were obtained from three growth curves at each irradiance. Cell counts and average cell volumes based on equivalent spherical diameter were determined with an electronic particle counter (Coulter Electronics; see Chapter 1, Materials and Methods for additional details). Values plotted are mean ± 1 S.D. for data derived from the three growth curves.

Results & Conclusions: Results presented in Graph A indicate that irradiances saturating for growth are ca. $\geq$ 90 $\mu$E·m$^{-2}$·s$^{-1}$, with no photoinhibition apparent at the highest irradiance examined (208 $\mu$E·m$^{-2}$·s$^{-1}$). It was concluded that an irradiance of 145 $\mu$E·m$^{-2}$·s$^{-1}$, which could be achieved with
eight Vita-Lite® UHO fluorescent tubes filtered through blue Plexiglas® (No. 2069, Rohm and Haas), was well within the saturated portion of the μ vs. I curve. As this lighting configuration does not alter the maximum growth rate from that obtained with unfiltered light, and more closely approximates an underwater light environment than white light, it was employed exclusively in the current research. Average cell volume (Graph B) decreased with irradiance to a minimum of 2.2·10^4 μm^3 (ca. 50% of the cell volume at μ_{max}) at the lowest irradiance (12 μE·m^{-2}·s^{-1}).

Graph A. Growth rates of G. sanguineum at various irradiance levels. Graph B. Average cell volumes of G. sanguineum at various irradiance levels. Values for Graphs A and B represent means ± 1 S.D. for n = 3 growth curves per irradiance level. Error bars are smaller than symbols where not apparent.
APPENDIX 2

Iron depletion of *G. sanguineum*: pH regulation and bioassays

**Objective:** This series of experiments was performed to demonstrate pH regulation of *G. sanguineum* batch culture cell yield and thus, of the depletion of biologically available iron from the culture medium.

**Methods:** Cultures of *G. sanguineum* were grown according to the conditions and procedures outlined for iron depletion in the *Iron and nitrogen depletion* section of Chapter 1 (Materials and Methods), except that one culture was not bubbled and the other was bubbled with air or 1-2% CO₂ (50-100 ml·min⁻¹) as required to minimize changes in pH. Upon cessation of growth, aliquots of culture were transferred to 125 ml Erlenmeyer flasks (polycarbonate, Nalgene) for bioassay experiments (same culture conditions, no bubbling).

Three bioassay treatments were employed to confirm the pH (rather than iron) control of cell yield in the unbubbled culture: 1. addition of ESAW concentrations of all enrichments except iron (other trace metals were chelated with EDTA at an EDTA:trace metal ratio of 1.6); 2. addition of iron/EDTA at ESAW concentrations; 3. addition of Suprapur™ HC1 (Merck) to lower pH to 8.2. On Day 6 the pH of treatment flasks 1 and 2 was lowered to 8.2 with Suprapur™ HC1. In the case of the bubbled culture, three bioassay treatments were run to demonstrate the depletion of
biologically available iron from the culture medium: 1. control (i.e. no additions); 2. addition of iron/EDTA at ESAW concentrations; 3. addition of ESAW concentrations of all enrichments except iron (other trace metals were chelated with EDTA at an EDTA:trace metal ratio of 1.6). Cell counts were performed with an electronic particle counter (Coulter Electronics; see Chapter 1, Materials and Methods for additional details).

Results & Conclusions: The cell yield of the bubbled culture (BC) exceeded that of the unbubbled culture (UC) by three-fold (Graph A). Upon cessation of growth, the pH of UC and BC was 9.3 and 8.3, respectively (Graph B). During the middle to late exponential growth phase of BC, pH did rise to a maximum of 8.8, but remained at this level only for a brief period (3 days). Bubbling with 1-2% CO₂ was insufficient to prevent any elevation in pH. Although increasing the percentage of CO₂ may have avoided pH changes, this action was considered undesirable due to alterations in carbon assimilatory enzymes (e.g. carbonic anhydrase) induced by high CO₂ concentrations (Lawlor 1987). Furthermore, the temporary rise in pH did not prohibit the depletion of biologically available iron from BC (see bioassay data below).

Results of the pH bioassay for UC (Graph C) clearly demonstrate pH control of batch culture yield, as cell division commenced immediately upon the return of pH to 8.2
(treatment 3, HCl addition). The regulatory role of pH was supported further by the fact that no growth response was noted for treatments 1 (-Fe ESAW) and 2 (Fe/EDTA) until after their pH was lowered to 8.2 on Day 6. The iron bioassay conducted on BC (Graph D) confirmed the depletion of biologically available iron, as only the treatment receiving iron showed a resumption of growth (treatment 2, Fe/EDTA). While these iron bioassay data apply to NO3-grown cultures, similar results were obtained from identical treatments with NH4-grown cultures.

It was concluded that bubbling was necessary for iron depletion experiments and that termination of cell division in -Fe ESAW did result from the depletion of biologically available iron from the culture medium.
Graph A. Growth curves of unbubbled (●) and bubbled (○) G. sanguineum batch cultures. B. pH changes in unbubbled (●) and bubbled (○) batch cultures shown in Graph A. C. Bioassay demonstrating pH control of unbubbled batch culture (Graph A) cell yield. Bioassay treatments included additions of ESAW enrichments without Fe (○), ESAW Fe/EDTA enrichments (●), or Suprapur® HCl to a pH of 8.2 (□). On Day 6 the pH of the first two treatments was lowered to 8.2 using HCl. D. Bioassay demonstrating iron depletion of bubbled culture (Graph A). Bioassay treatments included a control (i.e. no addition) (○), additions of ESAW Fe/EDTA enrichments (●), or ESAW enrichments without Fe (□).
APPENDIX 3

Effect of ammonium concentration on G. sanguineum growth rate

Objective: This experiment was performed to determine if elevated ammonium concentrations effect growth rate reductions in G. sanguineum.

Methods: Cultures of G. sanguineum were grown according to the conditions and procedures outlined in the General culture maintenance section of Chapter 1, except for the addition of 50 μM rather than 550 μM nitrate. Cells were allowed to exhaust the ambient nitrogen, after which they were transferred into one of six types of media containing full ESAW nutrients and the following sources/concentrations of nitrogen. Two control cultures were provided with either ESAW nitrate concentrations (i.e. 550 μM) or 80 μM NO₃. Four experimental cultures contained either 80, 150, 350 or 450 μM ammonium. Duplicate trials were conducted for each nitrogen source/concentration. Growth rates (d⁻¹) were determined by cell counts with an electronic particle counter (Coulter Electronics) and are given as the mean (n = 2) ± 1 S.D. (in parentheses).
Results & Conclusions:

GROWTH RATES (d⁻¹)

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<th>NO₃ 550 μM</th>
<th>NO₃ 80 μM</th>
<th>NH₄ 80 μM</th>
<th>NH₄ 150 μM</th>
<th>NH₄ 350 μM</th>
<th>NH₄ 450 μM</th>
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<tr>
<td>Rate</td>
<td>0.35</td>
<td>0.35</td>
<td>0.34</td>
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<td>(0.01)</td>
<td>(0.03)</td>
<td>(0.02)</td>
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Results indicate a reduction in growth rate with ≥ 150 μM NH₄. Similar findings have been reported previously by Thomas et al. (1980) for this species. Growth rates of 80 μM NH₄ and both NO₃ control cultures appear equivalent. It was concluded that for all cases in which *G. sanguineum* would be supplied with ammonium nitrogen (i.e. growth or uptake experiments), concentrations should not exceed 80 μM.
APPENDIX 4

Removal of non-biological iron from G. sanguineum: a test of wash volume

Objective: This experiment was performed to assess the effect of increasing wash volume on the removal of surface-bound iron from G. sanguineum.

Methods: Cultures of G. sanguineum were grown according to the conditions and procedures outlined in the General culture maintenance section of Chapter 1, except that unlabeled ESAW FeCl₃ was replaced by an equimolar concentration of ⁵⁵FeCl₃. At a cell density of ca. 4·10³ cells·ml⁻¹, 5 ml aliquots were collected on 5 µm (pore diameter) filters (polycarbonate, Nuclepore), washed with 5, 10, 20 or 40 ml of chelexed ESAW salt solution (CSS, see Chapter 2, Materials and Methods), and counted by liquid scintillation. The experiment was conducted in duplicate (n = 2). Values (mean ± 1 S.D.) are expressed as a percent of the ⁵⁵Fe label (i.e. disintegrations·min⁻¹, DPM) removed by the smallest (5 ml) wash volume.

Results & Conclusions:

<table>
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<th></th>
<th>5 ml</th>
<th>10 ml</th>
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<th>40 ml</th>
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<tr>
<td>100</td>
<td>94 ± 2</td>
<td>102 ± 18</td>
<td>92 ± 16</td>
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Results demonstrate that the quantity of non-biological iron removed from ca. $2 \cdot 10^4$ cells by 5, 10, 20 or 40 ml is equivalent. As no advantage is provided by use of greater volumes and it is desirable to minimize total filtration times, 5 ml wash volumes were employed in the Fe adsorption experiments in Chapter 2.


AWARDS (continued from previous page):

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